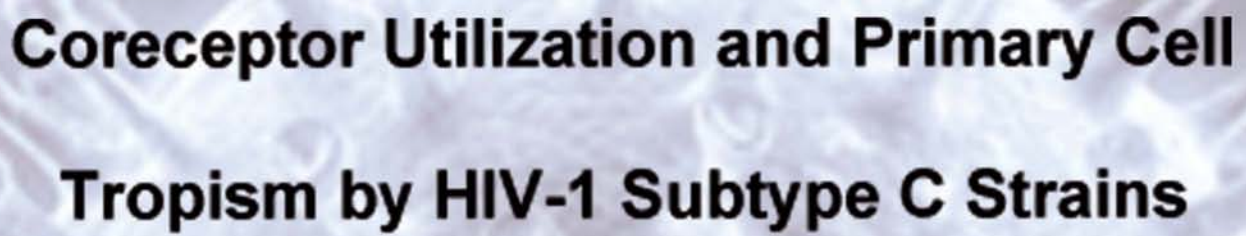

Coreceptor Utilization and Primary Cell Tropism by HIV-1 Subtype C Strains

Ashika Singh

2010

Coreceptor Utilization and Primary Cell Tropism by HIV-1 Subtype C Strains

by

Ashika Singh

Submitted in fulfilment for the requirements for the degree of a Doctor of Philosophy in Medicine in the HIV Pathogenesis Programme, Doris Duke Medical Research Institute, Department of Paediatrics and Child Health, Faculty of Medicine, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal.

2010

Declaration

I Ashika Singh declare that

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Publications and Presentations

Peer Reviewed Publications:

- Singh A, Page T, Moore PL, Allgaier RL, Hiramen K, Coovadia HM, Walker BD, Morris L and Ndung'u T. 2009. Functional and genetic analysis of coreceptor usage by dualtropic HIV-1 subtype C isolates. Virology. 393(1):56-67.

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List of Figures

	Page
Figure 1.1: Global distribution of adults and children estimated to be living with HIV in 2007.	2
Figure 1.2: Global distribution of estimated number of adults and children infected with HIV during 2007.	3
Figure 1.3: Global distribution of estimated adult and child deaths from AIDS in 2007.	4
Figure 1.4: Schematic representation of the HIV-1 genome.	6
Figure 1.5: Schematic representation of gp160.	8
Figure 1.6: Three dimensional structure of gp120 indicating inner and outer domains, bridging sheet and V3 regions.	9
Figure 1.7: Interaction of the V3 base and bridging sheet with coreceptor N terminus is critical for R5 tropism and interaction with distal regions of V3 and coreceptor extracellular loops is critical for X4 tropism.	13
Figure 1.8: Schematic representation illustrating virus variants and tropism states.	18
Figure 2.1: Coreceptor usage using the DEAE-dextran method of infection.	39

Figure 2.2: Viral infection of PBMCs. 41

Figure 2.3: Neighbour-joining phylogenetic tree constructed from the *env* gene sequences. 48

Figure 2.4: Alignment of V3 sequences of clones of primary viral isolates. 50-51

Figure2.5: Box plots of predicted N-linked glycosylation sites (PNLGS) and *env* variable loop lengths. 55-56

Figure 3.1: Coreceptor usage using the spinnoculation method of infection. 71

Figure 3.2: Coreceptor usage in transformed cell lines. 75-77

Figure 3.3: Coreceptor usage in CD4 + T cells. 79-81

Figure 3.4: Coreceptor usage in monocyte derived macrophages. 82-84

Figure 3.5: Viral infection of U87.CD4.CXCR4 in the presence of AMD3100 and U87.CD4.CCR5 in the presence of maraviroc. 89

Figure 3.6: Genotypic analysis. 91-93

Figure 4.1: Frequency of drug resistance mutations and thymidine analog mutations (TAMs). 111-112

Figure 4.2: Relationship of viral tropism of ARV-naive and ARV-failing patients. 115-116

Figure 4.3: Neighbour-joining trees. 121-122

Figure 4.4: Alignment of V3 sequences of clones of ARV-failing and ARV-naive patients. 124-125

Figure 4.5: Box plots of predicted N-linked glycosylation sites (PNLGS). 131

Figure 4.6: Box plots of *env* variable loop lengths. 132-133

Figure 5.1: Viral evolution of HIV-1 subtypes B and C. 150

List of Tables

Page

Table 1.1: Classes of antiretroviral drugs. 20

Table 2.1: Coreceptor usage characterization of primary viral isolates. 43

Table 2.2: Coreceptor usage characterization of HIV-1C *env* clones. 44-45

Table 2.3: Summary table of V3 characteristics of clones of primary viral isolates. 52

Table 3.1: Comparison of the DEAE-dextran method and spinnoculation method of infection. 73-74

Table 4.1: Patient information. 104-105

Table 4.2: Functional coreceptor utilization. 117-118

Table 4.3: Summary table of V3 characteristics of clones of ARV-failing and ARV-naive patients. 126-127

Table 4.4: V3 loop-based methods for coreceptor usage prediction. 135

Abbreviations

A (Ala)	Alanine
AA	Amino acid
ABC	Abacavir
ACD	Acid Citrate Dextrose
AIDS	Acquired Immune Deficiency Syndrome
ART	Antiretroviral treatment
ARV	Antiretroviral
ATCC	American Type Culture Collection
bp	Base pair
Ca++	Calcium
cDNA	Complementary deoxyribonucleic acid
CO_2	Carbon Dioxide
D (Asp)	Aspartic Acid
d4T	Stavudine
ddI	Didanosine
DLV	Delavirdine
DMEM	Dulbecco's Minimal Essential Media
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
DM	Dual/mixed
EDTA	Ethylenediaminetetraacetic acid
EFV	Efavirenz
ELISA	Enzyme Linked Immunosorbent Assay
env	Envelope
ETR	Etravirine
FBS	Fetal Bovine Serum
FDA	Food and drug Administration
FTC	Emtricitabine
g	Gram
G (Gly)	Glysine
Gp	Glycoprotein
gag	Group-specific antigen
H (His)	Histidine
HAART	Highly Active Antiretroviral Therapy
HCl	Hydrochloride acid
HIV-1 C	Human Immunodeficiency Virus Subtype C
H_2O	Water

I (Iso)	Isoleucine
IL-2	Interleukin-2
K (Lys)	Lysine
kb	Kilo base
l	Litre
L (Leu)	Leucine
LB	Luria Bertani
LTR	Long terminal repeats
M	Molar
M (Met)	Methionine
M-CSF	Macrophage colony stimulating factor
MDM	Monocyte derived macrophage
Mg++	Magnesium
$MgCl_2$	Magnesium Chloride
mg	Milligram
MIP	Macrophage Inhibitory Protein
ml	Millilitre
mM	Millimolar
N	Amino
N (Asn)	Asparagine
NaCl	Sodium Chloride
Ng	Nanogram
NNRTI	Non Nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NSI	Non Syncytium-Inducing
NVP	Nevirapine
P (Pro)	Proline
PBMCS	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHA	Phytohaemagglutinin
PI	Protease Inhibitor
PNLGS	Predicted N-Linked Glycosylation Site
Pol	Polymerase
PSSM	Position Specific Scoring Matrix
Q (Glu)	Glutamine
R (Arg)	Arginine
RIT	Ritonavir
RLU	Relative Light Unit
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RT	Reverse Transcriptase

S (Ser)	Serine
SSIII	SuperScript III (Invitrogen Co.)
SI	Syncytium-Inducing
T (Thr)	Threonine
TAM	Thymidine Analog Mutation
TBE	Tris-Borate-EDTA
$TCID_{50}$	50% tissue culture infective dose
TDF	Tenofovir
T-Tropic	T Cell Line-Tropic
U	Unit
V (Val)	Valine
UNAIDS	Joint United Nations Programme on HIV/AIDS
WHO	World Health Organization
ZDV/AZT	Zidovudine
3TC	Lamivudine
µg	Microgram
µl	Microlitre
°C	Degree celcius

Abstract

Human immunodeficiency virus type 1 (HIV-1) isolates can be differentiated based on their ability to use particular coreceptors – R5 viruses use CCR5, X4 viruses use CXCR4 and R5X4 (dual tropic) viruses use both CCR5 and CXCR4. It is widely reported that HIV-1 subtype C (HIV-1C) has a unique viral coreceptor evolution pattern in that a complete switch from the predominant CCR5 (R5) to CXCR4 (X4) phenotype is less common for this subtype compared to other subtypes. However, dual tropic HIV-1C isolates have occasionally been described. Furthermore, it has been reported that certain highly active antiretroviral drugs (HAART) may select for X4 viral variants. Therefore, this thesis study was undertaken to better understand the functional and genotypic characteristics of dual tropic HIV-1C isolates, and to characterize drug resistance and coreceptor usage patterns in HAART-naïve versus HAART-failing HIV-1C infected patients.

Thirty-five functional HIV-1 *env* clones derived from seven dual tropic HIV-1C strains were generated and their coreceptor usage characterized in transformed cell lines. All 35 *env* clones efficiently infected transformed cells expressing CXCR4. Twenty of 35 clones (57%) also utilized the CCR5 receptor. No R5-only clones were detected. Functional coreceptor usage data was correlated to *env* gene sequence data.

The ability of the HIV-1C *env* clones to facilitate infection of primary lymphocytes and monocyte-derived macrophages was next investigated. The majority of clones characterized as X4 or R5X4 on cell lines used either CXCR4 alone or CXCR4 and CCR5, respectively, in primary cells. A few viruses displayed comparable CCR5 and CXCR4 usage and clones from one virus preferred CCR5 usage in macrophages. Thus in a few cases coreceptor phenotyping in transformed cell lines does not predict usage in primary cells. Genetic determinants for coreceptor usage in primary cells require further investigation.

Finally the patterns of drug resistance mutations were studied and coreceptor usage among 45 HAART-naïve and 45 HAART-failing HIV-1C infected patients analyzed. Ninety-five percent of HAART-failing patients had viruses with at least one drug resistance mutation. Thymidine analog resistance mutations (TAMs) were present in 55% of patients. HAART-failing patients had significantly higher prevalence (59%) of X4/R5X4-utilizing viruses compared to HAART-naïve patients (30%) ($p<0.02$) using the Trofile Co-receptor Tropism Assay while 41% of HAART-failing patients used CCR5 and 70% of HAART-naïve patients used CCR5. Functional results correlated with predictive algorithm methods.

This study enhances our understanding of HIV-1 pathogenesis and the results have important implications for the use of coreceptor antagonists for the clinical management of HIV-1C infection.

Ethical Approval

Full ethical approval was obtained from the Biomedical Research Ethics Committee (BREC), UKZN (H108/06 and BF088/07). Informed consent was obtained from all patients that participated in this study and guidelines for human experimentation were followed in the conduct of the clinical research.

TABLE OF CONTENTS

	Page
Declaration	ii
Publications and Presentations	iii
Acknowledgements	iv
List of Figures	vii
List of Tables	x
Abbreviations	xi
Abstract	xiv
Ethical Approval	xvi
Chapter 1: Literature Review	1
1.1. Introduction	1
1.2. Viral Genome and Structure	5
1.3. Viral Life Cycle	6
1.4. Viral entry into Host Cell	7
1.4.1. The HIV-1 Envelope Glycoprotein	8
1.4.1.1. Structure of the Envelope protein	8
1.4.1.2. Genetic Diversity and the Envelope Glycoprotein	10
1.4.1.3. The V3 region and Coreceptor Usage	10
1.4.1.4. Other regions contributing to Coreceptor Usage	13
1.4.2. HIV-1 Coreceptors	14
1.4.2.1. Major Coreceptors	14
1.4.2.2. Alternate Coreceptors	15
1.4.3. Phenotype and Tropism	16

1.4.3.1. V3 region and Phenotype Prediction 18

1.5. Antiretroviral Therapy 20

1.6. Drug Resistance 22

1.7. Drug Resistance and Coreceptor Usage 24

1.8. Coreceptor Usage and Primary Cells 25

1.9. Coreceptor Usage in South Africa 27

1.10. Outline of Thesis 28

Chapter 2: Functional and Genetic Analysis of Coreceptor Usage by Dualtropic HIV-1 subtype C isolates 30

2.1. Introduction 30

2.2. Materials and Methods 33

2.2.1. Viral isolation 33

2.2.2. Cells and Cell Lines 33

2.2.3. Viral Isolate Propagation and DNA Extraction 34

2.2.4. Confirmation of Dual Tropism of Primary Viral Isolates 35

2.2.5. Amplification of Envelope (*env*) gene 36

2.2.6. Cloning 36

2.2.7. Limiting end point dilution PCR 37

2.2.8. Coreceptor Usage assays 38

2.2.9. Sequencing and Sequence Analysis 39

2.3. Results 41

2.3.1. Viral Infection of Stimulated PBMCs 41

2.3.2. Confirmation of Dualtropism of Primary Viral Isolates 42

2.3.3. Determination of Coreceptor Usage by *env* clones 43

2.3.4. Limiting end point dilution PCR 46

2.3.5. Genotypic Analysis of the *env* gene 46

2.4. Discussion 58

Chapter 3: Distinct Patterns of Coreceptor Use by R5X4 HIV-1 Subtype C Clones in Primary CD4+ Lymphocytes and Macrophages versus Transformed Cell Lines 65

3.1. Introduction 65

3.2. Materials and Methods 67

3.2.1. Cells and Cell Lines 67

3.2.2. Transfections 68

3.2.3. Infections 69

3.2.4. Genotypic Analysis 71

3.3. Results 72

3.3.1. Transformed Cell Line Coreceptor Usage 72

3.3.2. Coreceptor Usage in CD4+ T Lymphocytes and Monocyte Derived Macrophages 78

3.3.3. Genotypic Analysis 90

3.4. Discussion 95

Chapter 4: Drug Resistance and Viral Tropism in HIV-1 Subtype C-Infected Patients in KwaZulu Natal, South Africa: Implications for future Treatment Options 100

4.1. Introduction 100

4.2. Materials and Methods 103

4.2.1. Study Participants 103

4.2.2. Sample Collection, Viral Load and CD4 Management 105

4.2.3. Genotype Resistance Testing 105

4.2.4. Phenotype Coreceptor Analysis 106

4.2.5. cDNA Synthesis, Envelope Amplification and Cloning 106

4.2.6. Envelope sequencing and Sequencing Analysis 108

4.2.7. Statistical Analysis 108

4.3. Results 108

4.3.1. Patient Demographics 108

4.3.2. Genotypic Drug Resistance Typing 109

4.3.3. Coreceptor Utilization 114

4.3.3.1. Phenotypic Coreceptor Analysis 114

4.3.3.2. Genotypic Analysis of the *env* gene 119

4.4. Discussion 136

Chapter 5: Discussion and Conclusion 141

References 151

Appendices 177

CHAPTER 1

Literature Review

1.1. Introduction

Acquired immune deficiency syndrome (AIDS) is caused by the human immunodeficiency virus (HIV) and is characterized by a serious disorder of the immune system in which the body's protective defenses against infection cannot function leaving the body vulnerable to severe infections. According to the UNAIDS (Joint United Nations Programme on HIV/AIDS) and WHO (World Health Organization) 2007 AIDS epidemic update report the estimated number of people living with HIV worldwide was 33.2 million (Figure 1.1). This was a reduction of 16% when compared to the published estimate in 2006 (UNAIDS 2007; UNAIDS 2008).

HIV is the most serious infectious disease challenging the public health sector with more than 6,800 persons becoming infected with the virus and approximately 5,700 deaths daily (UNAIDS 2006). The most seriously affected region in the world is Sub-Saharan Africa and HIV/AIDS is the leading cause of death in this region. In 2007, the estimated number of deaths as a result of HIV/AIDS was 2.1 million (Figure 1.2) and of these 75% were in Sub-Saharan Africa. Of the 2.5 million new infections in 2007, 68% occurred in Sub-Saharan Africa (Figure 1.3) (UNAIDS 2007; UNAIDS 2008). South Africa accounts for the highest number of HIV infections world-wide (UNAIDS 2007).

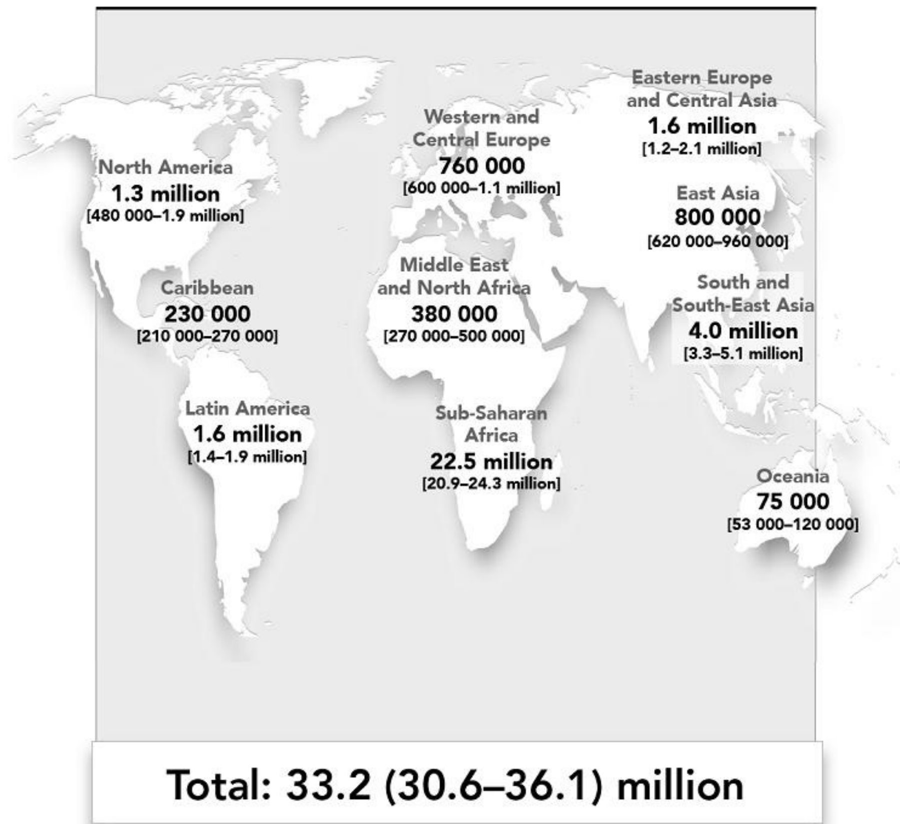


Figure 1.1: Global distribution of adults and children estimated to be living with HIV in 2007

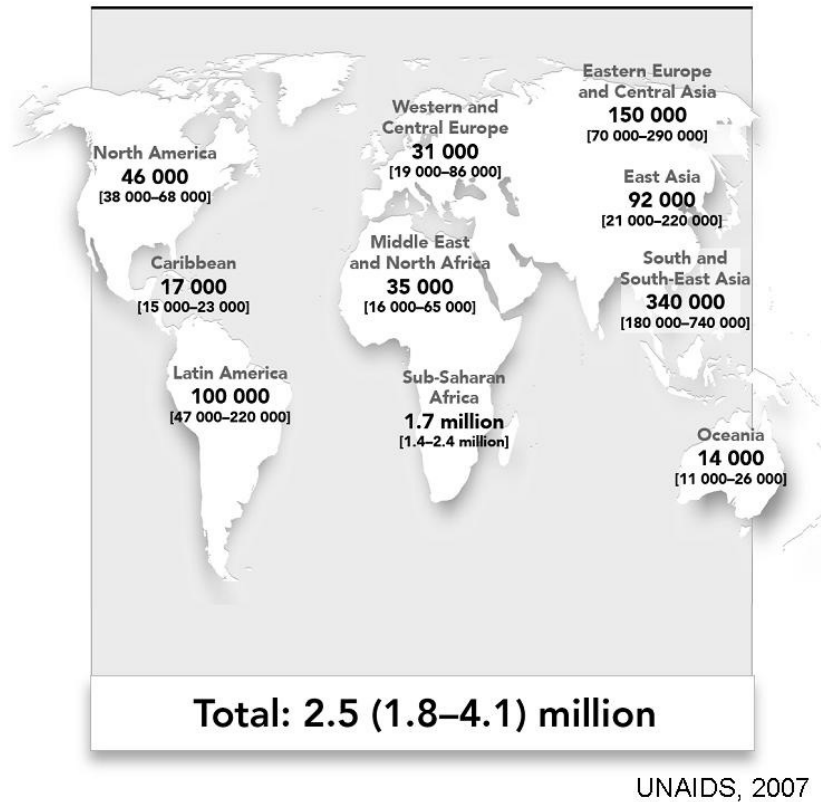


Figure 1.2: Global distribution of the estimated number of adults and children newly infected with HIV during 2007

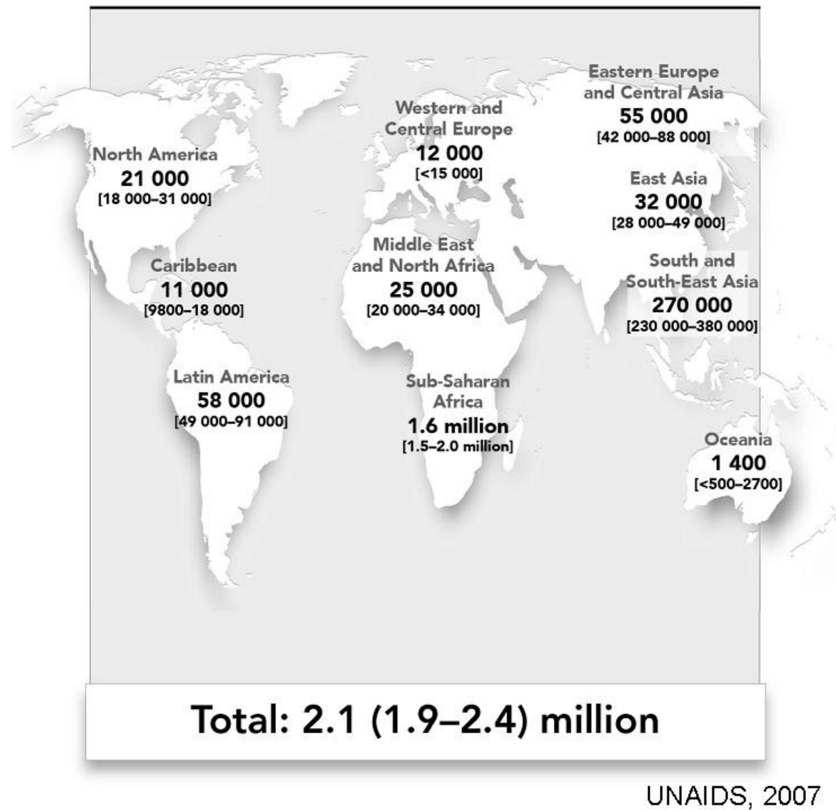


Figure 1.3: Global distribution of estimated adult and child deaths from AIDS during 2007

HIV-1 is divided into 3 groups, HIV-1 major group (HIV1-M), Outlier (HIV1-O) and HIV1-N group. This is Non-M or Non-O or may be also be referred to as "New". The strains of HIV-1 isolated from people in U.S.A. and Europe are genetically diverse from strains isolated in Africa and Asia. HIV-1 major group can be further classified into subtypes or clades designated A through K. Such subtypes have envelope gene sequences that vary by 20% or more between subtypes. In addition other circulating recombinant forms (CRF) exist, and subtypes E and I are are now defined as being CRFs (http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html).

In South Africa, the most prevalent circulating HIV subtype is HIV-1 subtype C (HIV-1 C). This subtype accounts for approximately 56% of infections worldwide (Esparza 2005; Hemelaar et al. 2006; Visawapoka et al. 2006). Subtype C is unique from all other subtypes as it is the only subtype that has been reported to retain predominant CCR5 coreceptor usage throughout infection whereas other subtypes show a switch in coreceptor usage from CCR5 to CXCR4 (Bjorndal and Sonnerborg 1999; Cecilia et al. 2000; Cilliers et al. 2003; Ndung'u et al. 2006; Tscherning et al. 1998). Subtypes differ based on sequence variations within all gene and non-coding regions, but it is the envelope gene (*env*) that accounts for the most dramatic differences of over 20% amino acid differences between subtypes (Gao et al. 1998). The *env* gene product, the envelope glycoprotein (Env) plays an important role in viral transmission by determining which coreceptor the virus binds to upon entry into the body (Rizzuto et al. 1998; Wyatt 1998).

1.2. Viral Genome and Structure

The HIV-1 viral genome is approximately 9.8 kb and encodes several major structural genes and several non structural genes. The structural and enzymatic proteins include Gag, Pol and Env. The *gag* (group-specific antigen) gene codes for the protein that constitutes the physical infrastructure of the virus encoding the components of the inner capsid protein . The *pol* gene codes for viral enzymes integrase, reverse transcriptase, HIV protease and RNAse H. The *env* (envelope) gene codes for the glycoprotein precursor gp160, which consists of gp 120 and gp 41 which are proteins that are embedded in the viral envelope and enable the virus to attach to and fuse with target cells. The RNA-binding regulatory proteins include transactivators Tat and Rev; and the accessory proteins include other regulators Vif, Vpr, Vpu and Nef. All these assist the virus in entering the host cell and enhance reproduction. The long terminal repeats (LTR) are positioned on either end of the genome. These LTRs are used by the integrase

enzyme to insert the HIV genome into the host DNA. Once they are integrated into the host genome they act as enhancers/promoters which influence the cell components which transcribe DNA (McCance and Huether 2006) (Figure 1.4). Transactivation of the LTR by the Tat protein is essential for both viral gene expression and virus replication. Transactivation involves the binding of the HIV-1 LTR to the TAR sequence along with other cellular factors, resulting in increased viral transcription initiation and elongation (Boykins et al. 1999).

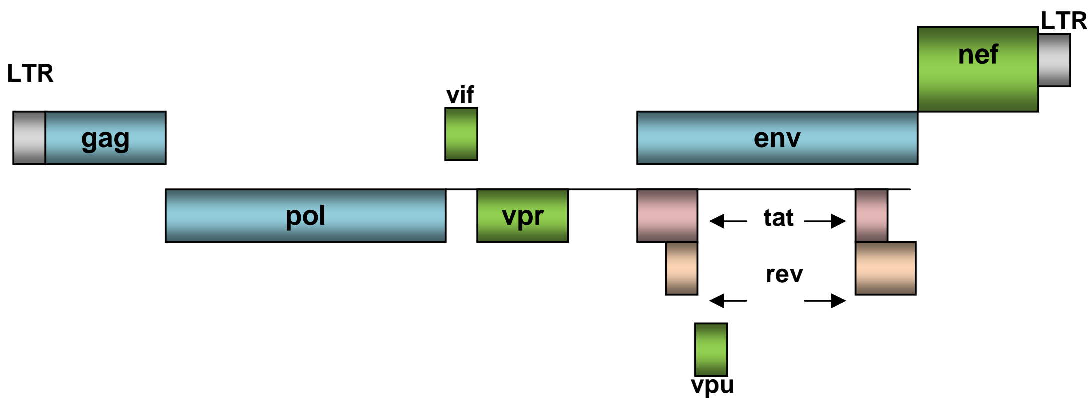


Figure 1.4: Schematic representation of the HIV-1 genome

gag codes for the protein that constitutes the physical infrastructure of the virus encoding the components of the inner capsid protein; *pol* codes for viral enzymes integrase, reverse transcriptase and HIV protease and *env* codes for the glycoprotein precursor gp160, which consists of gp 120 and gp 41. The RNA-binding regulatory proteins include transactivators Tat and Rev; and the accessory proteins include other regulators Vif, Vpr, Vpu and Nef.

1.3. Viral Life Cycle

The first step in the viral life cycle is binding and fusion of the virus to the host cell. The virus attaches to the surface of a CD4 + T lymphocyte cell by binding to a CD4 receptor and one of two cellular coreceptors. The viral envelope undergoes a conformational change allowing the virus to fuse with the host cell (Rizzuto et al. 1998; Wyatt et al. 1995). After

fusion, the virus releases RNA into the host cell cytoplasm. Reverse transcription then occurs as the HIV enzyme reverse transcriptase converts the single-stranded HIV RNA to a double-stranded HIV DNA provirus. Integration then follows as the HIV DNA provirus migrates to and enters the nucleus of the host cell and the HIV enzyme integrase incorporates the HIV DNA into the host cell DNA. The provirus may remain latent and upon activation of the infected cell (e.g. by cytokines) the provirus is transcribed and translated into viral protein precursors. These precursor proteins are modified by viral and cellular proteases (Gag and Env proteins respectively) into smaller proteins i.e. the HIV enzyme protease cleaves the HIV proteins into functional proteins which are used to package the viral RNA into new virions i.e. assembling into a new virus particle. The newly assembled particle buds from the host cell taking with it a part of the cell outer envelope containing the HIV glycoproteins which are necessary for the virus to bind CD4 and coreceptors continuing the cycle. The virus buds as an immature particle and only once full cleavage is completed is it a mature infectious particle (McCance and Huether 2006; Zhang and Moore 1999).

1.4.Viral Entry into Host Cell

The entry of HIV requires the interaction of the viral Env glycoprotein (gp120) with two host surface proteins, the CD4 glycoprotein and a chemokine receptor located on the cell surface resulting in the fusion of the viral and cellular membranes. When the viral Env binds to the CD4 glycoprotein which serves as the primary receptor, a conformational change occurs in the gp120 glycoprotein resulting in the exposure and/or formation of a specific binding site for a chemokine receptor. These chemokine receptors are secondary receptors for viral entry and are most often CCR5 and CXCR4 (Alkhatib et al. 1996a; Choe et al. 1996; Deng and Liu 1996; Dragic and Litwen 1996; Feng et al. 1996; Kwong et al. 1998; Lusso 2006; Rizzuto et

al. 1998; Wyatt et al. 1995). Thereafter, additional structural changes occur allowing the gp41 glycoprotein to initiate the fusion process (Trkola et al. 1996; Wu et al. 1996).

1.4.1. The HIV-1 Envelope Glycoprotein

1.4.1.1. Structure of the Envelope protein

The Env glycoprotein is a single chain glycoprotein precursor, gp160 consisting of two functional subunit glycoproteins gp120 and gp41 which assemble non-covalently on the virion surface as trimers (Cilliers and Morris 2002; Wyatt 1998). Located within gp120 are five hypervariable regions interspaced between 5 constant regions (Figure 1.5).

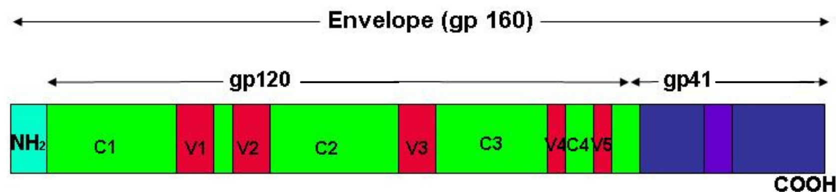


Figure 1.5: Schematic representation of gp160

The five variable regions (V1-V5) are interspaced between the conserved regions (C1-C3).

The CD4 binding site within gp120 is located within a cavity at the interface of the outer domain, inner domain and bridging sheet and is fairly well conserved (Figure 1.6). For the formation of the gp120-CD4 structure, 22 CD4 amino acid residues and 26 gp120 amino acid residues are required to interact. Three CD4 molecules are required to bind to gp120 for the resultant conformational changes to occur, causing the virus to then bind to the chemokine

receptor. This leads to the formation of a ninety degree angle between the CD4-binding site and the chemokine receptor-binding site consisting of the bridging sheet and the base of the first two variable loops (V1/V2) (Rizzuto et al. 1998). Upon binding, of gp120 to CD4, a displacement of V1/V2 and the third variable loop (V3) occurs allowing chemokine receptor binding. Further conformational changes are induced that activate the fusion machinery of gp41. Fusion of the viral Env and cellular membrane occurs by insertion of the gp41 subunit into the lipid bilayer of the target cell. The ribonucleic acid (RNA) and enzymes required for viral replication contained within the viral core is released into the host cell cytoplasm where reverse transcription and incorporation into the host genome takes place. A series of events then occur allowing the virus to bud out of the cell enabling the infection of new cells (Cilliers and Morris 2002; Kwong et al. 1998; Masciotra et al. 2002; Rizzuto et al. 1998).

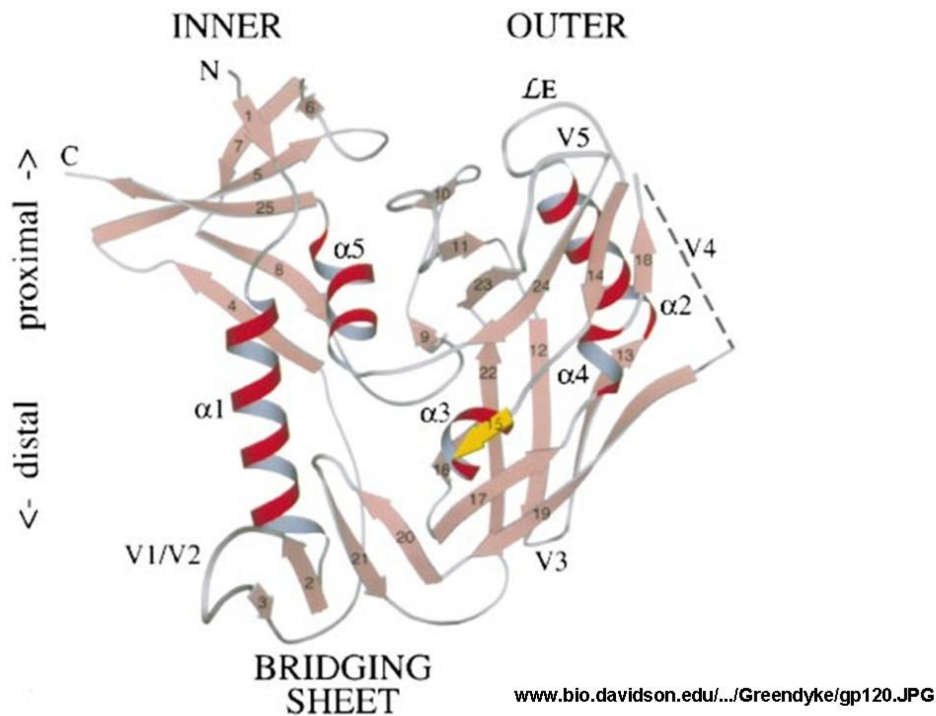


Figure 1.6: Three dimensional structure of gp120 indicating the inner and outer domains, bridging sheet and variable regions (accessed 11 April 2009)

N is the amino-terminus and C is the carboxyl terminus.

1.4.1.2. Genetic Diversity and the Envelope Glycoprotein

The Env is an important target of humoral immune responses and is a crucial determinant of overall viral fitness (Ball et al. 2003; Marozsan et al. 2005). This protein plays an important role in viral transmission by determining which coreceptor the virus binds to upon entry of the cells. During transmission and subsequent to infection, viral fitness and target cell tropism properties are thought to be important determinants of infectivity and the rate of disease progression. The importance of the Env as a major target of humoral immunity and as a major contributor to overall fitness during transmission and in the rate of disease progression make it a particularly attractive target for vaccine and drug development, although progress towards these goals has been greatly hampered by the extreme genetic variability of the *env* gene. This is because viral populations within an individual are evolving continually and can differ by as much as 10% in sequence by the end stage of disease (Shankarappa et al. 1999). Genetic variation is largely due to the escape from host selective pressures such as neutralizing antibodies. Genetic variation is also facilitated by recombination, high viral replication rate and an error prone reverse transcriptase. Env changes include various parameters – variable loop length, the number and position of predicted N-linked glycosylation sites as well as amino acid substitutions causing a change in the net charge (Coetzer et al. 2007).

1.4.1.3. The V3 Region and Coreceptor Usage

The V3 region is critical for coreceptor binding and is a major determinant of whether CXCR4 or CCR5 will be the accessory protein used by the virus for membrane fusion (Cilliers et al. 2003; Coetzer et al. 2007; Coetzer et al. 2006; Fouchier et al. 1992; Huang et al. 2005; Morris et al. 2007; Ndung'u et al. 2006). The V3 region typically consists of approximately 35 amino acids in CCR5-tropic viruses (Coetzer et al. 2006; Mefford et al.

2008). Variations within this region such as length, the amino acids at positions 11 and 25, changes within the crown motif and the overall net charge may all contribute to coreceptor usage (Coetzer et al. 2007; Coetzer et al. 2006; Jensen et al. 2006; Jensen et al. 2003; Renjifo et al. 1999). X4 variants among different subtypes appear to select basic amino acid substitutions in the V3 loop as a strategy for evolving to use CXCR4 as a coreceptor. They are more variable than R5 viruses in this region particularly at positions 11 and 25 which frequently have a positively charged amino acid most often arginine (R), lysine (K) or histidine (H) situated here. Insertions in the V3 region particularly between positions 13 and 14 are indicative of CXCR4-usage. This contributes to an increased length of the V3 loop in such viruses. X4 variants may also be distinguished from R5 viruses as they usually have an increased net V3 charge and a more diverse V3 loop sequence (Cilliers et al. 2003; Coetzer et al. 2007; Coetzer et al. 2006; Morris et al. 2007; Ndung'u et al. 2006). Additionally, changes in the crown motif may also determine coreceptor usage. The crown motif is a conserved region in the tip of the V3 loop. In subtype C R5 viruses, where a switch in coreceptor usage is rarely seen, the crown motif sequence reads GPGQ. In X4 viruses where a switch in coreceptor usage is seen, GPGQ changes to GPGX where X may be any other amino acid. Therefore, the crown motif in X4 viruses show variation and frequently display positively charged amino acids. A predicted N-linked glycosylation site is generally present at position 6-8 of R5 viruses. A loss of this glycosylation site has been correlated with X4-usage (Coetzer et al. 2006; Hartley et al. 2005; Morris et al. 2007). Further mutations have been described. For example, a recent study by Polzer and colleagues showed that R5-tropic viruses exhibited higher infection rates when N-linked glycosylation site (g15) was present. Infection was impaired by mutations deleting this site. X4-tropic viruses lacking this site showed higher infection rates resulting in these viruses becoming highly sensitive to neutralization (Polzer et al. 2009). Another study where a single mutation at position 13 of

V3 was introduced resulted in the Env being selectively resistant to one group of anti-V3 monoclonal antibodies (Patel et al. 2008).

Other additional biological properties of the V3 region have been proposed. Huang *et al.*, (2005) have proposed that V3 acts as a molecular hook having two uses. It may be used for “trapping” the coreceptor as well as for modulating subunit associations within the viral spike. Crystallization structures show three regions of the V3 loop. These include a conserved base which is closely associated with the bridging sheet on the gp120 core, a flexible stem extending away from the core and a conserved β-hairpin tip. The bridging sheet and the V3 base comprise a surface that interacts with the coreceptor amino (N) terminus, while the more distal V3 regions engage the coreceptor extracellular loops (Dragic and Litwen 1996; Hartley et al. 2005; Huang et al. 2005). Another study in 2008 has shown that although Env interactions with both the N terminus and the extracellular loops of the coreceptor occur, the interaction of the bridging sheet and the base of the V3 with the coreceptor N terminus is critical for R5 tropism while the interaction between the more distal part of the V3 and the coreceptor extracellular loops is critical for X4 tropism (Nolan et al. 2008) (Figure 1.7). Considering these, the amino acids within the tip and the base of the V3 loop are important in coreceptor usage.

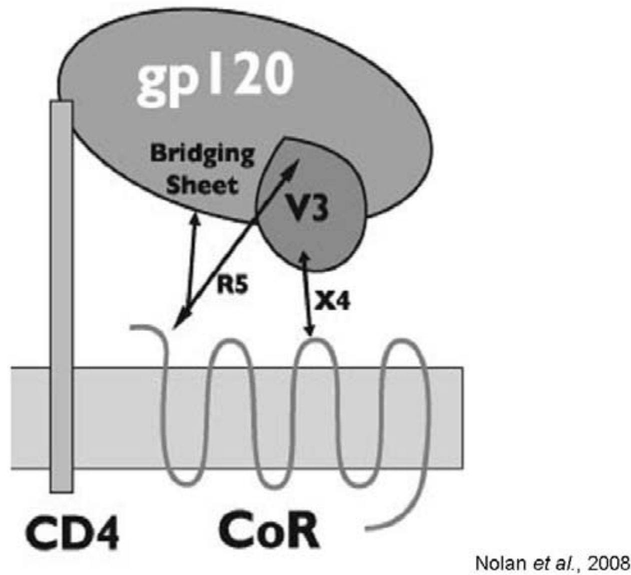


Figure 1.7: Interaction of V3 base and bridging sheet with coreceptor N terminus is critical for R5 tropism and interaction with the distal regions of V3 and coreceptor extracellular loops is critical for X4 tropism

1.4.1.4. Other Regions Contributing to Coreceptor Usage

Coreceptor utilization is genetically determined by sequence characteristics within the *env* gene, primarily specific amino acid changes within three of the five hypervariable regions namely the V1/V2 and V3 loops, as well as the number and pattern of predicted N-linked glycosylation sites. The HIV Env glycoprotein gp120 is among the most heavily glycosylated proteins in nature containing approximately 24 N-linked glycosylation sites and alterations within these sites may affect protein folding and distant parts of the protein through masking or conformational alterations. Changes in the glycosylation pattern therefore affect and influence receptor binding and phenotypic properties of the virus (Fouchier et al. 1992; Nabatov et al. 2004; Pastore and Nedellec 2006; Pollakis et al. 2001). Pollakis *et al.*, have shown that the V1/V2 region was significant in providing the virus with dual tropic properties. They also demonstrated that the loss of an N-linked glycosylation site within the

V3 loop was significant causing the virus to switch from an R5 to X4 phenotype. Masciotra and colleagues (2002) have shown that an elongated V2 region is correlated with consistent CCR5 usage. Another study by Coetzer *et al*., in 2008 has shown that the V4/V5 region is involved in coreceptor usage. Additional sequence changes within the *env* gene have also been implicated in coreceptor determination or the coreceptor switching process (Aasa-Chapman et al. 2006a; Coetzer et al. 2008).

1.4.2. HIV-1 Coreceptors

1.4.2.1. Major Coreceptors

The two main coreceptors involved in HIV-1 replication *in vivo* are CCR5 and CXCR4 (Alkhatib et al. 1996b; Deng and Liu 1996; Dragic and Litwen 1996; Feng et al. 1996). These coreceptors are seven-transmembrane molecules belonging to the G-protein-coupled receptor family and are activated by chemotactic cytokines (chemokines) (Platt et al. 2001). These receptors consist of a single polypeptide chain containing an extracellular N terminal domain and three extracellular loops on the cell surface. Four cysteine residues are contained within the extracellular domain. These form disulfide bonds between the N terminus and the third extracellular loop as well as between the first and second extracellular loops. Both coreceptors share approximately 32% amino acid identity but this is reduced to 20% when the amino acids located on the extracellular surface are compared. CCR5 and CXCR4 both consist of 352 amino acids (Cilliers and Morris 2002). CCR5 and CXCR4 are natural chemokine receptors. They belong to one of two major subfamilies: the CXC and CC subfamilies, based on the arrangement of the first two of the 4 cysteine residues. The 2 cysteines are separated by a single amino acid in CXC chemokines while the 2 cysteines are adjacent in CC chemokines (Vila-Coro et al. 2000). The natural ligands for CC chemokines i.e. for the CCR5 receptor are RANTES (now known as CCL5), macrophage inflammatory

proteins, MIP-α (now known as CCL3) and MIP-β (now known as CCL4). Stromal cell-derived factor, SDF-1α is the natural ligand for CXCR4 (Chen et al. 1997; Nabatov et al. 2004; Pollakis et al. 2004; Shalekoff and Tiemessen 2001). The CCR5 coreceptor plays a vital role in the entry of the virus as it is this coreceptor that is initially used by the virus during transmission. Its importance in HIV-1 infection has been illustrated by the effect of a 32-base pair (bp) deletion in the CCR5 gene. The resultant mutant gene Δ32CCR5, encodes a truncated CCR5 molecule which is not expressed at the cell surface. Individuals heterozygous for this gene progress slowly in infection and homozygotes are usually resistant to infection (Arien et al. 2006; Carrington et al. 1997; Ditzel et al. 1998; Lin et al. 2002; Taylor et al. 2001).

1.4.2.2. Alternate Coreceptors

Although the two major coreceptors involved in HIV-1 infection are CCR5 and CXCR4, other coreceptors may also play a role. Previous studies have shown alternate coreceptor use on coreceptor-transfected cell lines. These included CCR1, CCR2b, CCR3, GPR1, CX3CR1 (V28), Bob/GPR15, CXCR6 (Bonzo/STRL33/THYMSTR), APJ, Chem R23, RDC-1, US28 and CCR8 (Chan et al. 1999; Cilliers et al. 2003; Cilliers et al. 2005; Deng et al. 1997; Edinger et al. 1998; McKnight et al. 1998; Ohagen et al. 2003; Pohlmann et al. 1999; Xiao et al. 1998; Zhang et al. 1998a; Zhang and Moore 1999). Although the use of alternate coreceptors in primary cells is rare (Cilliers et al. 2003), some of these receptors such as CXCR6 and CCR8 have been demonstrated to facilitate entry in primary cell lines (Lee et al. 2000; Sharron et al. 2000; Zhang et al. 2000; Zhang et al. 2001). Some studies have suggested that CCR3 may possibly play a larger role in primary cell viral tropism (Aasa-Chapman et al. 2006b; Zhang et al. 1998a). It has been reported that CCR3 tropism depends on sequences located within the V1/V2 region but does require the presence of a CCR5-tropic

V3 sequence in cis (Ross and Cullen 1998). The use of alternate coreceptors has also been illustrated in studies where the major coreceptors were either absent or blocked and viral entry was still observed (Zhang et al. 2000). Agents that target CCR5 preventing entry and infection *in vitro* include the natural ligand, RANTES; PRO 140, a mouse monoclonal antibody; TAK-779, a small molecule that binds to the pocket within transmembrane helices 1, 2, 3 and 7; and SCH-C, a small molecule which acts as a CCR5 antagonist (Baba et al. 1999; Dragic et al. 2000; Olson et al. 1999; Strizki et al. 2001; Zhang et al. 1998b). CXCR4 antagonist AMD3100 is a small molecule binding to CXCR4 coreceptors (Donzella et al. 1998). Further, these studies also made use of peripheral blood mononuclear cells (PBMCs) containing the mutant gene Δ32CCR5 in the presence of a CXCR4 inhibitor. No viral entry was observed (Zhang et al. 2000). However, their significance for HIV-1 replication *in vivo* and disease progression is unclear (Aasa-Chapman et al. 2006b; Cilliers et al. 2005; Dash et al. 2008).

1.4.3. Phenotype and Tropism

HIV-1 isolates are differentiated based on the coreceptors they use. Viruses that use CCR5 are termed R5 viruses and viruses that make use of CXCR4 are termed X4 viruses. Dual tropic viruses use both coreceptors and are termed R5X4 viruses (Ohagen et al. 2003; Pontow and Ratner 2001; Verhofstede et al. 2009). It has been argued that these isolates replicate in cells containing CCR5 and CXCR4 but probably do so because they contain a mixture of CCR5 and CXCR4-tropic viruses (Moore et al. 2004). There have been studies however, that have characterized viruses proving them to be truly dual tropic (Coetzer et al. 2006). Those viruses that primarily use CCR5 are macrophage-tropic (M-tropic) viruses and grow in primary macrophages and primary lymphocytes. Those that use CXCR4 are T cell line-tropic (T-tropic) viruses and grow in T cell lines and primary lymphocytes. T-tropic viruses are able

to cause syncytia in cell lines and were formerly referred to as syncytium-inducing (SI) viruses. M-tropic viruses do not induce syncytia and were referred to as non syncytium-inducing (NSI). SI viruses are called rapid/high viruses as they cause rapid progression and occur late in infection. NSI viruses are slow/low viruses and occur early in infection and during the acute and asymptomatic phase (Figure 1.8) (Baribaud et al. 2001; Cilliers and Morris 2002; Ohagen et al. 2003; Ping et al. 1999; Platt et al. 2001; Zhang and Moore 1999). It has been documented that it is the CCR5 tropic (R5) viruses that play a critical role in viral entry as these viruses are associated with viral transmission. CXCR4 tropic (X4) viruses are found later in infection and are associated with a decline in CD4 cells and disease progression. There have been a number of studies in subtype B-infected individuals as well as other major non-B subtypes that have shown that in 50% of infected individuals a switch in coreceptor usage was seen where the viruses initially used CCR5 and as the disease progressed, the more pathogenic X4 variant became dominant (Aasa-Chapman et al. 2006a; Arien et al. 2006; Ping et al. 1999; Pollakis et al. 2001). However, HIV-1 subtype C-based studies have not reported similar observations. HIV-1C is associated with an epidemic that is rapidly expanding. There have been numerous studies showing that the use of CXCR4 by isolates of this subtype is rare and that R5 variants dominate (Bjorndal and Sonnerborg 1999; Cilliers and Morris 2002; Coetzer et al. 2006; Johnston et al. 2003; Ndung'u et al. 2001; Ndung'u et al. 2006; Ping et al. 1999). This suggests that there may be some constraining factors that are limiting the development of X4 viruses in this subtype and it is not clear whether these are host immune or Virologyogical constraints (Coetzer et al. 2006).

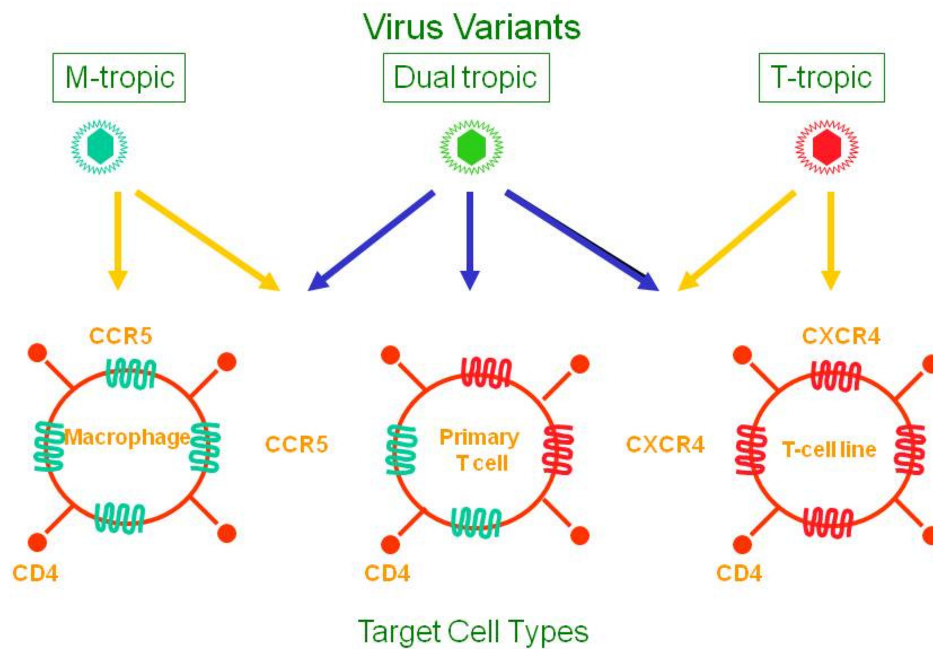


Figure 1.8: Schematic representation illustrating virus variants and tropism states

1.4.3.1. V3 Region and Phenotype Prediction

Since the V3 is a major determinant of coreceptor usage and viral tropism this region has been used to predict viral phenotype (Jensen and van't Wout 2003). Phenotype prediction is important in studies requiring coreceptor usage information for a patient who for example is receiving antiretroviral (ARV) therapy. Phenotypic assays are expensive and laborious and a prediction method based on sequence information would provide rapid results and would be a less expensive process (Garrido et al. 2008). There are a number of factors used to examine the V3 region that provide an indicator of phenotype prediction.

The “11/25 charge rule” assists in determining viral phenotype. This method is based on the presence of basic amino acids at positions 11 and/or 25. If basic (positively charged) amino acids are present, this is indicative of virus with the X4 phenotype. If there are neutral or acidic (negatively charged) amino acids present this indicates R5 viruses (Fouchier et al. 1992; Low et al. 2007). This method has shown >90% sensitivity and specificity for predicting the SI phenotype in clonal sequences but the sensitivity is reduced to <60% for R5X4 clonal sequences. This percentage is reduced further in bulk (population-based) sequences from clinical sequences (Jensen and van't Wout 2003). The overall net charge is also an indicator of biological phenotype. A V3 region having an overall net charge of >+4.5 is indicative of X4 usage. Charges lower than this indicates R5 usage (Coetzer et al. 2006). In addition, the crown motif, a conserved region at the tip of the V3 region provides an indicator of coreceptor usage. In subtype C R5 viruses, the crown motif sequence reads GPGQ. X4 viruses usually display a crown motif with the sequence GPGX where X represents a basic amino acid (Coetzer et al. 2006).

Insertions and deletions are also indicators of coreceptor usage in the V3 region. Previous studies have shown that insertions particularly at positions 13-14 occur in X4 viruses and a deletion such as a deletion of an N-linked glycosylation site at position 15 will drive the virus to use CXCR4. The presence of this site will restore R5 usage. A loss of predicted N-linked glycosylation site at positions 6-8 also indicates X4 usage (Coetzer et al. 2006; Polzer et al. 2009).

A web- based programme, Position-Specific Scoring Matrix (PSSM) (http://indra.mullins.microbiol.washington.edu/pssm/) also assists in determining viral phenotype. PSSM is a bioinformatic tool used for predicting HIV-1 coreceptor usage from

the amino acid sequence of the V3 loop. This programme detects a non-random distribution of sequences at a specific site within an alignment. It then compares the target sequence to a group of sequences with known phenotypes (Jensen et al. 2003). Higher PSSM scores indicate X4 usage.

1.5. Antiretroviral Therapy

Antiretroviral therapy (ART) has substantially decreased the rates of morbidity and mortality (Palella et al. 1998). There are six classes of HIV-1 drugs. To date, there are approximately 25 ARV drugs that have been licensed for HIV-1 treatment. These consist of nine nucleoside reverse transcriptase inhibitors (NRTI), four non-nucleoside reverse transcriptase inhibitors (NNRTI), nine protease inhibitors (PI), one fusion inhibitor, one CCR5 inhibitor and one integrase inhibitor (Shafer and Schapiro 2008) (Table 1.1).

Table 1.1: Classes of Antiretroviral Drugs

NRTIs	NNRTIs	PIs	Fusion Inhibitor	CCR5 Inhibitor	Integrase Inhibitor
3TC (lamivudine)	DLV (delavirdine)	ATV/r (atazanavir/r)	(T20) enfurvirtide	Maraviroc	RAL (raltegravir)
FTC (emtricitabine)	EFV (efavirenze)	TPV/r (tipranavir/r)			
ABC (abacavir)	ETR (etravirine)	IDV/r (indinavir/r)			
ddI (didanosine)	NVP (nevirapine)	SQV/r (saquinavir/r)			
TDF (tenofovir)		LPV/r (lopinavir/r)			
d4T (stavudine)		FPV/r (fosamprenavir/r)			
ZDV(ZDV/AZT) (zidovudine)		DRV/r (duranavir)			
ddC (zalcitabine)		NFV (nelfinavir)			
ZDV/3TC (combivir, duovir)		RTV (ritonavir)			

/r indicates boosting with ritonavir (Division of Clinical Pharmacology 2006; http://hivdb.stanford.edu ; Shafer and Schapiro 2008).

ARV drugs function at various stages of the HIV life cycle. These drugs target the virus and interfere with the virus' replication process by attaching to one of the enzymes or proteins required for replication of the virus and effectively stop viral replication. NRTI and NNRTI target the reverse transcriptase enzyme required for the transcription of single stranded RNA into double stranded DNA. Integrase inhibitors bind to the integrase enzyme and prevent the integration of viral DNA into the host cell DNA which enables the virus to make use of the host cell machinery to produce more viral particles. PI bind and inhibit HIV protease resulting in deformed viral particles reducing infectious capacity. Currently, entry inhibitors are used by those individuals who have had previous treatment experience, developed multi-drug resistance and are intolerant to other ARV drugs. This class of drugs functions by preventing the attachment of gp120 to CD4, preventing the interaction of this complex with a coreceptor and by preventing the gp41 mediated membrane fusion process. Fusion inhibitors target gp41 and prevent the fusion of the viral envelope with the target cell membrane (Morris et al. 2007; Weston et al. 2006; Yeni 2006). As these drugs do not completely eradicate HIV infection their goal is to decrease the viral load to undetectable levels and to maintain this reduction (Yeni et al. 2004). It has been shown that undetectable viral loads coupled with an increase in CD4+ T cell count toward the normal range has been the most effective in controlling viral replication and hence the duration of antiretroviral therapy is lifelong and may change according to the drugs currently available (Yeni 2006).

1.6. Drug Resistance

According to surveillance data obtained from antenatal clinics in South Africa, there is some hope that HIV infection levels might be reaching a plateau. There was a 1% reduction in infection from 2005 to 2006. A decrease in HIV prevalence has been observed among young pregnant women between the ages of 15 to 24 years suggesting a decline in new infections

(UNAIDS 2007). One factor contributing to this decline may be the widespread implementation of antiretroviral (ARV) therapy (Yeni 2006). However, although ARV therapy has been extremely effective and greatly valuable in assisting in the control of HIV/AIDS, the emergence of drug resistance has posed a major problem. There are several factors contributing to drug resistance. These include intolerance to certain drug regimens, treatment adherence and interruptions in the supply of drugs (Marconi et al. 2008). Resistance to ARV therapy is divided into two categories. These include primary resistance, which is the acquisition of a drug resistant strain of HIV by a newly infected individual and secondary resistance. Secondary resistance is acquired resistance and only develops after a period of HIV treatment (Taylor et al. 2008).

As it is the *pol* gene which is responsible for the mechanism by which the virus reproduces and because it encodes for viral enzymes integrase, reverse transcriptase, HIV protease and RNAse H, this is a region of interest for drug resistance mutations. Resistance mutations may vary between populations (Brenner et al. 2003; Morris et al. 2003) but some important resistance mutations within the reverse transcriptase regions include M184V, thymidine analog mutations (TAMs), L74V, K65R, Q151M, T69ins, K103N, Y181C, Y188C and others. ZDV and d4T select for TAMs which decrease susceptibility to these NRTIs and to ABC, ddI and TDF to a lesser extent (Whitcomb et al. 2003). These mutations are common in economically disadvantaged countries where thymidine analogs are frequently used, as well as in viruses from individuals who began treatment with incompletely suppressive thymidine analog-containing regimens prior to the introduction of HAART. There are 2 distinct pathways for the accumulation of TAMs. The TAM1 pathway includes the mutations M41L, L210W, and T215 and the TAM2 pathway includes D67N, K70R, T215F, and K219Q/E

(Shafer and Schapiro 2008). There are also a number of protease inhibitor mutations in the protease region (Meintjies 2005).

Currently, the patterns of drug resistance are only partially understood largely due to the fact that most of the drug resistance data that has been generated is based on subtype B infections whereas the circulating subtypes in developing countries are in most part subtypes other than subtype B (Wallis et al. 2009). Contradictory data suggests that drug resistance patterns may differ among various subtypes after failure of first-line treatment. An early study by in Zimbabwe showed that drug resistance mutations in subtype C were similar to those in subtype B (Kantor et al. 2002) whereas other studies identified subtype C-specific mutations (Brenner et al. 2003; Morris et al. 2003).

1.7. Drug Resistance and Coreceptor Usage

During the early stages of infection, R5 viruses are usually seen to predominate whereas X4 and dual tropic variants, which are associated with rapid disease progression, emerge in the late chronic phase of disease in a significant proportion of patients (Connor et al. 1997; Scarlatti et al. 1997). Several studies have shown that all subtypes can undergo the switch from the utilization of CCR5 to CXCR4 but this is less frequently observed in HIV-1 C infections even in late stages of disease (Bjorndal and Sonnerborg 1999; Cecilia et al. 2000; Cilliers et al. 2003; Ndung'u et al. 2006; Tscherning et al. 1998). This switch in coreceptor usage was initially identified in untreated patients but X4 viral variants have also been identified in a significant number of patients receiving highly active antiretroviral therapy (HAART). A study in Zimbabwe found that a high frequency (50%) of X4-tropic viruses were observed in patients failing ARV therapy while no such viruses were found in ARV-

naïve patients although their CD4 cell counts were comparable suggesting that ARV therapy may be selecting for the more virulent CXCR4-tropic strains in subtype C infection (Johnston et al. 2003). According to a study in 2006, a higher proportion (41%) of dual/mixed or X4-tropic viruses was also encountered in treated individuals as compared to 18% of treatment-naïve individuals (Hunt et al. 2006). Several reasons have suggested that ARVs may alter the prevalence of X4 viruses. An increase in HIV-specific T cell responses may be a result of partially suppressive therapy and because X4-virus variants are more susceptible to cytotoxic T cell responses, an increase in these HIV-specific T cell responses during viral suppression may select against X4 viruses (Deeks et al. 2004). Antiretroviral therapy also decreases the expression of CCR5 on T cells thereby selecting for CXCR4-using viruses (Andersson et al. 1998; Giovannetti et al. 1999). In addition, certain drugs such as enfurvirtide may select for one virus population because of enhanced activity against X4 viruses (Yuan et al. 2004). Other drugs such as zidovudine may select for one virus population due to poor drug metabolism in the cellular reservoirs for X4 viruses (Boucher et al. 1992). Therefore, coreceptor tropism among individuals initiating or failing ARV therapy particularly in HIV-1 subtype C infections needs to be understood as this may impact on the use of CCR5 antagonists as first line or salvage therapy.

1.8. Coreceptor Usage and Primary Cells

Macrophage-tropic, NSI, R5 HIV-1 virus variants replicate in primary lymphocytes and macrophages but not in transformed cell lines. They are poorly cytopathic *in vitro* and use CCR5 as their major coreceptor of entry. T cell line-tropic, SI, X4 virus variants replicate in lymphocytes and CD4+ transformed cell lines but not in macrophages. These viruses are highly cytopathic *in vitro* and use CXCR4 as their principle coreceptor of entry. Dual tropic

viruses, which may be intermediate viruses in the evolution from R5 to X4 variants infect both macrophages and CD4+ T-cell lines as well as primary lymphocytes (Carrington et al. 1997; Follis et al. 1998; Li et al. 1999; Naif et al. 2002; Simmons et al. 1998; Yi et al. 1999; Zerhouni et al. 2004). Alternatively, there have been reports that late-stage X4 variants were able to retain their capacity to infect macrophages suggesting that these variants are more similar to dual tropic isolates than to T-tropic isolates (Connor et al. 1997). Other reports have shown that minor coreceptors may also be used by some viruses in transfected cell lines (Chan et al. 1999; Cilliers 2005; Cilliers et al. 2003; Deng et al. 1997; Edinger et al. 1998; McKnight et al. 1998; Ohagen et al. 2003; Pohlmann et al. 1999; Xiao et al. 1998; Yi et al. 1999; Zhang et al. 1998a; Zhang and Moore 1999), but are rarely used by primary cells (Cilliers et al. 2003). Some of these receptors however, have exhibited entry in primary cell lines (Lee et al. 2000; Sharron et al. 2000; Zhang et al. 2000; Zhang et al. 2001). It is therefore uncertain if coreceptor usage in transformed cell lines is indicative of coreceptor usage *in vivo* i.e. in the infection of primary cells. There have been a number of interesting studies in primary cells. Contrary to earlier beliefs, CXCR4 is also present on macrophages and studies have shown that some viral isolates use this coreceptor for entry even though macrophages do not permit the entry of prototype T-tropic X4 strains (Yi et al. 1999). Other studies have used dual tropic strains to investigate coreceptor usage in primary lymphocytes. CCR5 usage in transformed cell lines was correlated with coreceptor usage in primary lymphocytes and the entry pathways for infection of primary lymphocytes by dual tropic strains were investigated. Results showed that several dual tropic primary and prototype strains used CXCR4 for entry and infection in primary lymphocytes even though CCR5 was present. In macrophages however, these strains used both CCR5 and CXCR4. From these observations, it was concluded that although HIV-1 strains were phenotypically characterized as dual tropic viruses in transformed cell lines this does not necessarily mean that they will

have dual coreceptor use in primary cells (Yi et al. 2005). These findings that coreceptor usage differs between transformed cell lines and primary cells were observed in subtype B infections and there is a need to determine if these findings are reproducible in subtype C infections.

1.9. Coreceptor Usage in South Africa

Significant differences between HIV-1 subtype C and other HIV-1 subtypes exist. A cross-sectional study in Kenya showed that women infected with subtype C had significantly lower CD4 cell counts and higher plasma RNA levels than those infected with subtypes A or D (Neilson et al. 1999). Another study reported that subtype C appears to have been introduced after subtypes A and D were already established in Tanzania. HIV-1 C quickly became one of the major subtypes while subtype D appears to have declined (Renjifo et al. 1998). A further study in a mother-to-child transmission cohort, observed that mothers infected with subtype C and intersubtype recombinants were more likely to transmit the virus to their children compared to those infected with subtype D viruses (Blackard et al. 2001). These studies indicate that subtype-specific differences exist, and suggest that HIV-1 C in particular may be epidemiologically unique. The utilization of coreceptors other than CCR5 is rare for HIV-1 C, even in isolates obtained from late-stage AIDS patients (Bjorndal and Sonnerborg 1999; Cecilia et al. 2000; Tscherning et al. 1998). The observation that HIV-1 C viruses predominantly use CCR5 as the principle coreceptor for entry into cells even in late stages of disease is intriguing, because in approximately 50% of HIV-1 subtype B infections, progression to AIDS is associated with development of viral variants that utilize CXCR4 as coreceptor (Aasa-Chapman et al. 2006a; Arien et al. 2006; Ping et al. 1999; Pollakis et al. 2004). This suggests that HIV-1 C is unique in maintaining its predominant CCR5 tropism

throughout infection and these observations may indicate that infection with subtype C may have a different outcome from other subtypes.

1.10. Outline of Thesis

This study examines the coreceptor usage in HIV-1C strains in South Africa. Firstly, we investigated whether dual tropic HIV-1C strains represented truly dual tropic viruses at the clonal level, or mixed R5 and X4 clones. In view of the fact that variation within the *env* gene, particularly the V3 region is associated with cellular tropism and coreceptor affinity, we analyzed the *env* sequences of 5 clones each from 7 dual tropic HIV-1C primary viral isolates and correlated the genotype of each clone to its respective phenotype in transformed cell lines that express either CCR5 or CXCR4. Secondly, since there have been studies in subtype B infections showing that coreceptor usage in transformed cell lines does not necessarily predict coreceptor usage in primary cells, we investigated coreceptor utilization in primary cells for the HIV-1 subtype C clones. Thirdly, we investigated the emerging patterns of drug resistance mutations among persons failing highly active antiretroviral therapy in Durban, KwaZulu-Natal, South Africa. Furthermore, since some studies in both HIV-1 subtypes B and C infections have suggested that ARV therapy (especially partially suppressive therapy) may be selecting for the more virulent X4 stains, we investigated and coreceptor usage in our cohort of ARV treated patients failing therapy and ARV-naïve patients. The aims of this thesis were to answer the following questions:

- Are dual tropic viruses truly dual tropic at the clonal level or are they a mixture of dual tropic, X4 and R5 viruses?
- What pathways do dual tropic and CXCR4-tropic subtype C viruses use to enter primary cells?

- Are there differences in chemokine receptor utilization between transformed cell lines and primary cells?
- What drug resistance mutations and patterns of resistance are emerging as HAART is rolled out widely in South Africa?
- What is the prevalence of X4 variants in HIV-1 infected individuals requiring or receiving ARV therapy?
- Is Virologyogical failure among HIV-1 subtype C infected persons receiving ARV therapy associated with higher proportion of the more virulent X4 tropic strains in HIV-1C infection?

The results of this thesis study have important implications both for HIV pathogenesis in HIV-1 subtype C infected individuals, for monitoring of persons on therapy and for future ARV treatment options for individuals failing HAART in resource-poor settings.

CHAPTER 2

Functional and Genetic Analysis of Coreceptor Usage by Dual tropic HIV-1 Subtype C Isolates

2.1. Introduction

HIV/AIDS remains the leading cause of death in the Sub-Saharan region and in 2007 alone, 76% of deaths were as a result of HIV/AIDS, with 68% of all new HIV infections occurring in this region. Southern Africa is the most seriously affected sub-region and in 2007 accounted for 32% of all new infections and AIDS-related deaths world-wide. South Africa constitutes the highest number of HIV infections globally (UNAIDS 2007). The main circulating HIV-1 subtype in South Africa is HIV-1 subtype C (HIV-1C), which accounts for approximately 56% of infections worldwide (Esparza 2005; Hemelaar et al. 2006; Visawapoka et al. 2006).

Subtype groupings are based on sequence variations that occur within all gene and non-protein coding regulatory regions, but the most dramatic differences are found in the envelope (*env*) gene (Gao et al. 1998). The envelope is an important target of humoral immune responses and is a crucial determinant of overall viral fitness (Ball et al. 2003; Marozsan et al. 2005). The *env* gene plays an important role in viral transmission by determining which coreceptor the virus uses to mediate entry. During transmission and subsequent to infection, viral fitness and target cell tropism properties are thought to be important determinants of infectivity and the rate of disease progression (Troyer et al. 2005). The importance of the envelope as a major target of humoral immunity, its contribution to

overall fitness during transmission, and its role in the rate of disease progression make it a particularly attractive target for vaccine and drug development. However, progress towards these goals has been greatly hindered by the extreme genetic variability of the *env* gene.

HIV-1 utilize members of the seven transmembrane chemokine receptor family as coreceptors for entry into target cells (de Roda Husman et al. 1999; Oppermann 2004; Ross and Cullen 1998; Vila-Coro et al. 2000; Xiao et al. 1999). The virus gp120 envelope glycoprotein first binds to the primary CD4 receptor on target cells, which induces conformational changes on the envelope exposing the coreceptor binding site (Rizzuto et al. 1998; Wyatt et al. 1995). The two main coreceptors that the HIV-1 envelope binds to subsequent to the conformational change are CCR5 or CXCR4 (Alkhatib et al. 1996a; Choe et al. 1996; Deng and Liu 1996; Doranz et al. 1996; Dragic and Litwen 1996; Feng et al. 1996). HIV-1 strains can be classified based on their coreceptor utilization, with CCR5 tropic viruses termed R5, CXCR4 tropic viruses termed X4 and viruses that use both coreceptors (dual tropic viruses) termed R5X4 (Berger 1998; Berger et al. 1999). R5 viruses predominate in the early stages of HIV-1 infection, whereas dual tropic and X4 variants, which are associated with rapid disease progression, emerge in the late chronic phase of disease in a significant proportion of patients (Connor et al. 1997; Scarlatti et al. 1997). It is well established that while all subtypes are capable of undergoing coreceptor utilization switch from CCR5 to CXCR4, this is less frequently encountered in HIV-1 subtype C infections, even in late stages of disease (Bjorndal and Sonnerborg 1999; Cecilia et al. 2000; Cilliers et al. 2003; Ndung'u et al. 2006; Tscherning et al. 1998). Furthermore, expanded coreceptor usage beyond CCR5 and CXCR4 has also been occasionally reported but its significance for HIV-1 replication in vivo and disease progression is unclear (Aasa-Chapman et al. 2006b; Cilliers et al. 2005; Dash et al. 2008).

Coreceptor utilization is genetically determined by sequence characteristics within the *env* gene, primarily specific amino acid changes within three of the five hypervariable regions namely the V1/V2 and V3 loops, as well as the number and pattern of predicted N-linked glycosylation sites (Fouchier et al. 1992; Pastore and Nedellec 2006; Pollakis et al. 2001). Additional sequence changes within the *env* gene have also been implicated in coreceptor determination or the coreceptor switching process (Aasa-Chapman et al. 2006b; Coetzer et al. 2008).

In several instances where HIV-1 subtype C isolates able to mediate cell entry via CXCR4 have been described, dual tropic (R5X4) strains that utilize both CCR5 and CXCR4 have been more frequently encountered compared to X4 monotropic viruses (Cilliers et al. 2003; Coetzer et al. 2006; Dash et al. 2008; Johnston et al. 2003; Ndung'u et al. 2006; van Rensburg et al. 2002). However, despite the occasional isolation of dual tropic HIV-1C viruses, such viruses have rarely been extensively characterized at both the functional and genetic clonal level. It is therefore largely unknown whether dual tropic HIV-1C strains represent a mixture of R5 and X4 viruses or truly dual tropic strains (or both) at the clonal level. Furthermore, genetic determinants associated with change in coreceptor usage have rarely been described for HIV-1 subtype C, particularly those that may reside outside of the V3 loop region. This study investigated whether dual tropic HIV-1C primary isolates represented truly dual tropic viruses at the clonal level, or mixed R5 and X4 clones. The generation of functional envelope clones from dual tropic HIV-1C isolates and the sequence characteristics in the HIV-1C *env* gene, both within and outside of the V3 region that are associated with coreceptor utilization phenotype are described.

2.2. Materials and methods

2.2.1. Viral isolates

Seven primary viral isolates were analyzed in this study. 96BW17 is a dual tropic HIV-1C virus isolated in 1996 from an infected person with acquired immunodeficiency syndrome (AIDS) in Botswana (Ndung'u et al. 2006). 99ZATM1B; RP1; 99ZASW20; 99ZASW30; 99ZACM9 and 01ZADu36_5 were obtained from the AIDS Virus Research Unit, National Institute for Communicable Diseases, Johannesburg, South Africa and were from patients at various disease stages: acute infection (01ZADU36_5), slow progressor (99ZATM1B), rapid progressor (RP1) and AIDS (99ZASW20; 99ZASW30; 99ZACM9) (Choge et al. 2006; Cilliers et al. 2003; Coetzer et al. 2006). Previously well-characterized dual tropic (Du179) and CCR5-tropic (96BWM01_5) primary isolates were used as positive controls. CXCR4-tropic 96BW17#10 was also used as a positive control.

2.2.2. Cells and Cell lines

U87.CD4 cells with or without the expression of the chemokine receptors CCR5 or CXCR4 were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD). U87.CD4 cells without chemokine receptors were cultured in Dulbecco's modified eagles medium (DMEM) containing L-glutamine (Gibco, NY, USA) supplemented with 15% heat inactivated fetal bovine serum (FBS) (Gibco, NY, USA), 300 µg/ml G418 (Sigma, Germany) and 50 µg/ml penicillin-streptomycin (Gibco, NY, USA). U87.CD4 cells expressing CCR5 or CXCR4 were propagated in the same medium but additionally supplemented with 1 µg/ml puromycin (Sigma,

Germany). 0.5 x 10^6 cells were cultured in 6-well flat-bottomed plates in a total of 2 ml culture medium at 37 C and 5% CO_2.

293T cells were obtained from the American Type Culture Collection (ATCC) and were cultured in DMEM containing L-glutamine supplemented with 10% heat inactivated FBS and 50 μg/ml gentamicin (Sigma, Germany). 50,000 293T cells per well were seeded in a total volume of 0.3 ml per well of a 48-well flat-bottomed plate. These cells were incubated at 37 °C in 5% CO_2 overnight before transfection.

2.2.3. Viral isolate Propagation and DNA extraction

PBMCs from anonymous low risk HIV negative volunteers were separated by density-gradient centrifugation on Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden). The vacutainer tubes containing approximately 24 ml blood were gently inverted to ensure that the blood was properly mixed. The blood was then pipetted into a 50 ml tube containing Ficoll-Paque and centrifuged for 15 minutes at 2200 rpm at room temperature. The plasma was removed. The PBMC layer was then carefully transferred to a 50 ml tube and was diluted to 50 ml by adding 1% PBS and mixed by inverting the tube several tubes. The cells were then pelleted by centrifugation at 1200 rpm for 10 minutes and the supernatant decanted. Cells were resuspended in 10 ml 1% PBS and mixed well. Cell counts were performed using *Guava technology* (as per manufacturer's instructions) and approximately 15 million cells were stored in the required volume of freezing solution which consisted of 10% Dimethylsulfoxide (DMSO) in FBS.

Samples were confirmed to be HIV-negative by performing HIV RNA testing on the plasma (Ampliscreen v1.5, Roche Diagnostics, Rotkreuz, Switzerland). 5 x 10^6 PBMCs

from 2 donors were combined and stimulated by culturing in RPMI 1640 with penicillin/streptomycin (50 µg/ml and 50 U/ml), 10% heat inactivated FBS, 5 µg/ml phytohaemagglutinin (PHA) (Sigma, Germany) and 20 U/ml interleukin-2 (IL-2) (Roche Applied Science, Germany) at 37 C and 5% CO_2 for 72 hours in a T-25 flask. For infection of the stimulated PBMCs, 5 ng p24 antigen equivalent of virus was used. On days 1, 4, 7, and 10, 50% of the media was removed and replaced with fresh medium. Aliquotted supernatant was retained for quantification of p24 antigen using the Vironostika HIV-1 Antigen Microelisa System (Biomerieux, Boxtel, Netherlands) as per manufacturers instructions. On day 14, supernatant was removed and preserved for p24 antigen quantification. Cells were harvested and resuspended in 200 µl PBS. DNA was then extracted using the QiaAmp DNA Blood Mini kit (Qiagen, Germany) as per manufacturers instructions.

2.2.4. Confirmation of dualtropism of primary viral isolates

PBMC-grown virus corresponding to 2 ng of p24 HIV-1 antigen was used for infection of U87.CD4 cells expressing either CCR5 or CXCR4. On days 0, 4, 7 and 10 half of the media was removed and replaced with fresh medium. The removed supernatant was retained for quantification of p24 antigen using the Vironostika HIV-1 Antigen Microelisa system.

2.2.5. Amplification of envelope (*env*) gene

The 3 kb *env* gene was amplified by polymerase chain reaction (PCR) using Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes, Finland) with the following primers: Env1A 5’-CACCGGCTTAGGCATCTCCTATGGCAGGAAGAA-3’ and EnvM 5’-TAGCCCTTCCAGTCCCCCCTTTTCTTTTA-3’. The forward primer Env1A was designed to include the 4 base pair sequence (CACC) necessary for directional cloning on the 5’ end. 1 µl of template was used in the reaction. Cycling conditions were as follows: a 5 minute denaturation at 94 °C, followed by 35 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C, and 4 minutes at 72 °C. The final extension was at 72 °C for 10 minutes. The amplified product was then run on a 1% agarose gel in 1 x Tris-Borate-EDTA (TBE) buffer and visualised using the Syngene gel documentation system. The amplified product was then gel purified using the Qiaquick gel extraction kit (Qiagen, Germany).

2.2.6. Cloning

Once the DNA was purified, the *env* of each primary viral isolate was cloned into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen, Carlsbad, CA). The reaction mixture consisted of the following – 10 ng fresh PCR product, 1 l salt solution, 1 l TOPO vector and distilled water adjusted to a total volume of 6 l. For the transformation procedure, Stratagene XL-10 Gold Ultracompetent cells (Stratagene, USA) were used. Molecular clones were screened as follows. A colony PCR was performed after the colony was incubated for 1.5 hours at 37 °C with continuous shaking (225 rpm) in a 96-well plate containing 100 µl Luria Bertani (LB) media (Sigma, Germany) and ampicillin (100 g/ml) (Calibiochem, Darmstadt, Germany) to determine positivity of the cloned

insert. This is a directional *env* insert-specific PCR as it uses the forward primer T7 (5' TAATACGACTCACTATAGGG 3') found on the vector and reverse primer Env M which is *env* specific. SuperTherm Taq Polymerase (Southern Cross Biotechnology, Cape Town, South Africa) was used and cycling conditions were as follows: denaturation for 5 minutes at 94 °C, followed by 35 cycles of 30 seconds at 94 °C, 30 seconds at 55 °C, and 4 minutes at 68 °C. The final extension was for 10 minutes at 68 °C. The amplified products were then run on a 1% agarose gel. Clones were considered positive if they yielded a 3 kb band on an agarose gel. These clones were then grown up at 37 °C overnight with shaking in 3 ml LB broth containing 100 g/ml ampicillin. Plasmid DNA was then isolated using Qiaprep Spin Miniprep kit by following the manufacturer's instructions (Qiagen, Germany). The first five functional *env* clones identified from each isolate were selected for further analysis.

2.2.7. Limiting endpoint dilution PCR

A limiting endpoint dilution PCR from DNA was performed on one primary viral isolate, 01ZADU36_5 in order to determine whether the bulk PCR resampling bias resulted in clones biased towards either CCR5 or CXCR4 coreceptor usage. Single genome amplification was undertaken as previously described (Salazar-Gonzalez et al. 2008). Primers and cycle conditions were the same as used in the bulk PCR reactions. Once confirmed by agarose gel electrophoresis, 30 PCR products were purified and cloned and coreceptor usage was tested for the 26 clones that were functional.

2.2.8. Coreceptor usage assays

Cotransfection was carried out by first combining 50 µl serum free DMEM and 2.5 µl Fugene reagent (Roche Applied Science, Germany) and incubating for 5 minutes at room temperature. This was then incubated at room temperature together with 0.6 µg gp160 *env* DNA (i.e. cloned product) and 0.3 µg pNL4-3.Luc.R-E- (Connor et al. 1995; He et al. 1995). pNL4-3.Luc.R-E- is a full-length HIV plasmid with two frameshifts that render the clone *env* and *vpr* deleted. The reporter firefly luciferase gene has been inserted into the *nef* gene. The transfection mixture was incubated for 30 minutes, and then added to assigned wells of the plate seeded with 293T and incubated at 37 °C and 5% CO_2 for 48 hours. The supernatant together with 7.5 µg/ml DEAE-Dextran (Sigma, Germany) was added to U87.CD4 cells as well as U87.CD4 cells expressing the coreceptors CXCR4 or CCR5. This was incubated at 37 °C and 5% CO_2 for 48 hours. The cells were lysed using Glo Lysis buffer (Promega, Madison, WI, USA) and incubated with Bright-Glo Assay reagent (Promega, Madison, WI, USA). The luciferase activity was then determined using the Turner-Biosystems Modulus Microplate instrument (Promega, Madison, WI, USA) (Figure 2.1). A negative control consisting of the plasmid pNL4-3.Luc.R-E- and various positive controls were used in each coreceptor expressing cell line. The positive controls were previously characterized *env* clones Du179 (dual tropic), 96BWM01_5 (R5), and 96BW17#10 (X4). In addition, for each assay plate, 100 nM of CCR5 inhibitor RANTES and 500 nM of CXCR4 inhibitor AMD3100 were used with the respective controls to confirm specificity of entry into target cells. Experiments were done in duplicate and the average relative luminescence units (RLUs) for each clone was calculated. A positive result was considered to be twice the average of the negative control plus standard deviation.

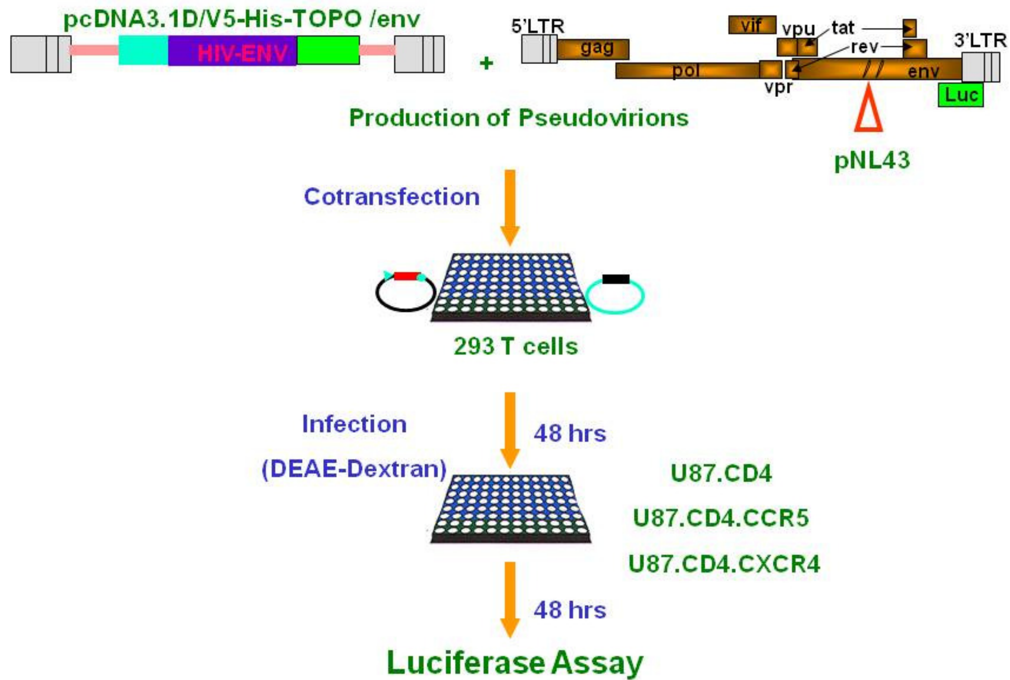


Figure 2.1: Coreceptor usage using the DEAE-dextran method of infection

48 hours post transfection, infection of U87.CD4 cells with/without coreceptors is performed using DEAE-Dextran.

2.2.9. Sequencing and sequence analysis

The *env* gene was sequenced after cloning using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit version 3.1 (Applied Biosystems, CA, USA). The primers used for sequencing are listed below:

envM 5'-TAGCCCTTCCAGTCCCCCCTTTTCTTTTA-3'

ES7 5'-CTGTTAAATGGCAGTCTAGC-3'

ES8 5'-CACTTCTCCAATTGTCCCTCA-3'

SQ5.5RC 5'-CTAGGAGCTGTTGATCCTTTAGGTAT-3'

SQ6RC(2) 5'-GAATTGGGTCAAAAGAGACCTTTGGA-3'

SQ13F(2)C 5'-TATATAAATATAAAGTGGTAGAAATTAAGC-3'

SQ14FC 5'-ACTCACGGTCTGGGGCATTA-3'

SQ3R(2)C 5'-GCTATGGTATCAAGCAGACTAATAGCACTC-3'

EF00 5'-AAAGAGCAGAAGACAGTGGCAATGA-3'

The sequence reaction consisted of 0.4 l terminator ready reaction mix, 2 l 5 x sequencing buffer, 3.2 pmol primer, 100 ng DNA template and distilled water adjusted to a total volume of 10 l. Cycle conditions were as follows: the initial denaturation at 96°C for 1 minute followed by 35 cycles of 96°C, 10 seconds; 50°C, 5 seconds and a final extension of 60°C for 4 minutes. Sequences were assembled and edited using Sequencher 4.8. They were then aligned with Mega 4. Phylogenetic trees were constructed in Paup 4.0 to evaluate the clustering of these sequences with each other and with subtype references. Phylogenetic trees were then visualized using Treeview 1.6.6. The consensus sequence for clones from each isolate was generated using BioEdit Sequence Alignment Editor Software (Tom Hall, North Carolina State University). Coreceptor utilization was predicted using the web-based subtype C-specific position specific scoring matrix (C-PSSM) programme (http://indra.mullins.microbiol.washington.edu/pssm/), a bioinformatics tool that reliably predicts coreceptor phenotype using V3 loop sequences (Jensen et al. 2006). Predicted N-linked glycosylation sites were examined using the web-based programme N-GLYCOSITE (www.hiv.lanl.gov). All reference sequences were obtained from the Los-Alamos database (www.hiv.lanl.gov).

2.3. Results

2.3.1. Viral infection of stimulated PBMCs

Seven HIV-1 subtype C dual tropic isolates from individuals in South Africa (Cilliers et al. 2003) and Botswana (Ndung'u et al. 2006) were selected for this study. In addition, a well characterized CCR5-only utilizing HIV-1 subtype C isolate, 96BWM01_5 was used as a positive control (Ndung'u et al. 2006). Infection of the stimulated PBMCs was assessed by HIV-1 p24 antigen ELISA over a 14-day culture period. As shown in Figure 2.2, p24 antigen concentration increased in culture supernatant for all the isolates. The isolates replicated to different levels and with different replication kinetics. On day 14, culture supernatants were removed and genomic DNA extracted from the cells for *env* gene amplification.

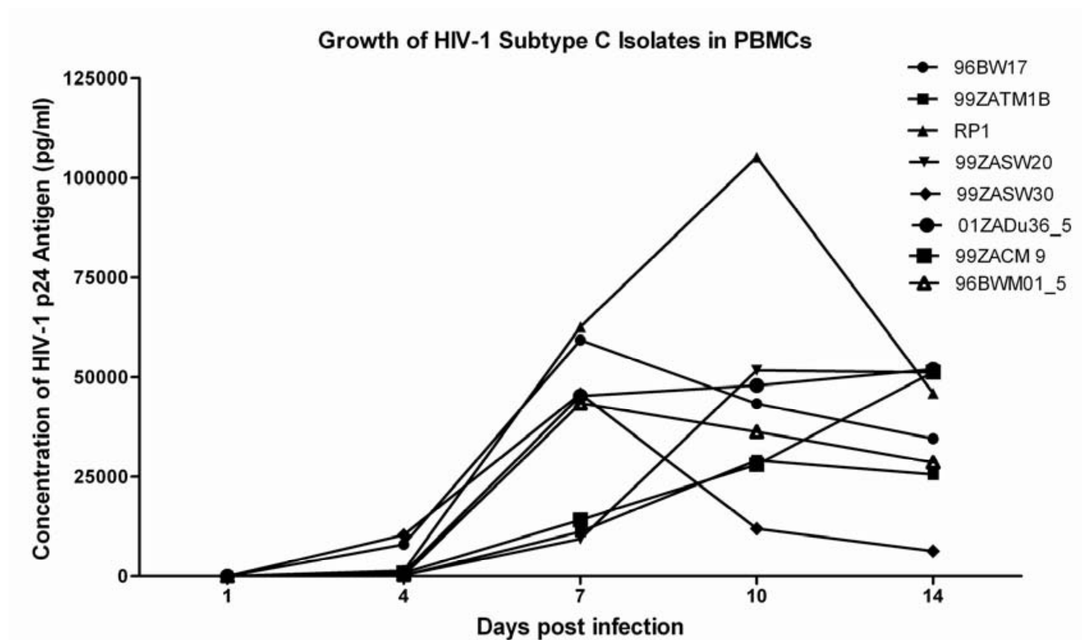


Figure 2.2: Viral infection of PBMCs

5000 pg p24 antigen equivalent of virus was used to infect stimulated PBMCs. Virus growth was monitored by p24 antigen concentration increased over a 14-day period. 96BWM01_5 is an R5 monotropic viral isolate and was used as a positive control.

2.3.2. Confirmation of dualtropism of primary viral isolates

Seven primary viral isolates propagated in PBMCs were first analyzed for their ability to use multiple coreceptors on cell lines. Specifically, the ability to mediate cell entry via CCR5 or CXCR4 was analyzed because these are the main coreceptors previously described for a significant proportion of HIV-1 primary isolates. In order to assess the ability of the isolates to utilize these coreceptors, virus equivalent to 2 ng of p24 antigen each was used to infect U87.CD4 glioma cell lines with or without the co-expression of the coreceptors. Table 2.1 shows the highest amounts of p24 antigen reached by the primary isolates over a 10-day period in culture. All 7 primary viral isolates replicated in cells expressing CXCR4 and CCR5. It was noteworthy that while all the isolates replicated efficiently in CXCR4 expressing cells, 3 isolates (RP1, 99ZASW30 and 99ZACM9) replicated to relatively low titers in cells expressing CCR5. Isolates 99ZACM9 and 99ZASW30 were previously shown to replicate efficiently in both CCR5 and CXCR4 expressing cell lines (Cilliers et al. 2003) and therefore our results could indicate that *in vitro* passages of the isolates is selecting against CCR5 utilization.

Table 2.1: Coreceptor usage characterization of primary viral isolates

Isolate	CD4 Count (cells/µl)	Viral Load (copies/ml)	Highest p24 antigen (pg/ml) reached by primary isolates over 10-day period in culture		
			U87.CD4	U87.CD4.CCR5	U87.CD4.CXCR4
96BW17	NA	NA	*(19)* 630	*(25)* 707,297	*(10)* 209,119
99ZATM1B	NA	190,000	*(13)* 610	*(151)* 32,012	*(126)* 98,654
RP1	7	178,830	*(0.9)* 610	*(1.2)* 2,120	*(646)* 961,835
99ZASW20	2	43,595	*(0.2)* 0	*(0.2)* 160,683	*(1.5)* 47,787
99ZASW30	2	73,860	*(0.8)* 1	*(0.8)* 2,141	*(1.0)* 158,623
99ZACM9	24	NA	*(1.1)* 2	*(1.2)* 2,201	*(1.1)* 11,979
01ZADu36_5	25	54,944	*(1.3)* 1	*(2.1)* 1,658 728	*(1.0)* 46,525
96BWM01_5	NA	NA	-	-	-
Du179	279	2,640	-	-	-

Note: NA- not available. All patients were ART naïve. 2000 pg of p24 viral supernatants from PBMC co-cultures were used to infect U87.CD4 cells expressing different coreceptors i.e. CCR5 or CXCR4 coreceptors. The U87.CD4 cell line was used as a control cell line. 2000 pg of HIV-1 p24 equivalent of virus was used for infection and cells were washed 3 times after overnight incubation with infecting stock. Virus growth above 2000 pg (amount used to infect) was considered productive infection of target cells. All italicized figures in brackets indicate background level i.e. the p24 antigen (pg/ml) on day 0.

2.3.3. Determination of coreceptor usage by *env* clones

The *env* gene (approximately 3kb) was then amplified from PBMC genomic DNA by PCR, gel purified and cloned into a mammalian cell expression plasmid vector (pcDNA3.1D/V5-His-TOPO). Five clones for each patient isolate were generated. Pseudoviruses were prepared by cotransfecting 293T cells with each of the 35 *env* plasmid clones with the pNL4-3.Luc.R-E- construct (Connor et al. 1995; He et al. 1995). This construct contains the infectious NL4-3 provirus backbone but is *env* and *vpr* deleted and *nef* has been replaced by the firefly luciferase gene. Virus supernatant from the transfected 293T cells equivalent to 2 ng p24 antigen was then used to infect U87 cells expressing the chemokine receptors CCR5 or CXCR4. A previously characterized

dual tropic *env* clone Du179 (Coetzer et al. 2006) was used in parallel as a positive control. Productive entry of target cells was determined by measurement of firefly luciferase activity in cell lysates of infected U87 cells. All 35 *env* clones were able to efficiently infect cells expressing CXCR4 (Table 2.2). Five of 35 (14.3%) clones tested were also able to mediate entry via the CCR5 receptor, indicating that they were dual tropic. Two of the clones able to utilize CCR5 were from isolate RP1 while the other three were from isolate 99ZACM9. Surprisingly, there were no CCR5 monotropic clones detected from the bulk PCR envelope analysis of dual tropic isolates in this study.

Table 2.2: Coreceptor usage characterization of the HIV-1C *env* clones

Clone	U87.CD4.CCR5	U87.CD4.CXCR4
96BW17 #2	-	+++
96BW17 #3	-	+++
96BW17 #6	-	+++
96BW17 #7	-	+++
96BW17 #15	-	+++
99ZATM1B #3	-	+++
99ZATM1B #5	-	+++
99ZATM1B #6	-	++
99ZATM1B #8	-	++
99ZATM1B #13	-	++
RP1 #5	-	++
RP1 #6	-	++
RP1 #8	-	++
RP1 #10	++	++
RP1 #13	++	++
99ZASW20 # 2	-	++
99ZASW20 #3	-	++
99ZASW20 #11	-	++
99ZASW20 #14	-	+++

99ZASW20 #15	-	++
99ZASW30 #1	-	++
99ZASW30 #2	-	+++
99ZASW30 #3	-	+++
99ZASW30 #6	-	+++
99ZASW30 #9	-	+++
01ZADu36_5 #2	-	+++
01ZADu36_5 #7	-	+++
01ZADu36_5 #8	-	++
01ZADu36_5 #9	-	+++
01ZADu36_5 #10	-	++
99ZACM9 #1	++	+++
99ZACM9 #2	++	+++
99ZACM9 #16	-	+++
99ZACM9 #18	-	+++
99ZACM9 #21	+	+++
96BWM01_5*	+++	-
96BW17#10*	-	+++
Du179*	+++	+++
pNL4-3.Luc.R-E-*	-	-

Note: For each experiment, a positive result was considered to be 2 x the average relative luminescence units (RLUs) of the negative control wells + standard deviation. RLUs above this but less than 3 x this cut off value are indicated by "+" values 3 x to 10 x above cut off are shown as "++" and values above 10 x the cut off are indicated as "+++". *96BWM01_5 is an R5-only control, 96BW17#10 is an X4-only control and Du179 is a dual tropic control. pNL4-3.Luc.R-E- is the negative control.

These results could be explained by two possibilities; one is that CCR5 monotropic envelopes are present at very low frequencies and therefore are virtually undetectable as clones from amongst the primary isolate viral quasispecies *or* that the CXCR4 viruses may be more fit and outcompeting CCR5 clones when propagated in PBMCs. In order to differentiate between these two possibilities, we infected U87.CD4.CCR5 cells with each

of the primary viral isolates, with the objective of amplifying or favouring the CCR5-tropic clones in CCR5-only expressing cells. RNA was then extracted from the viral supernatant from U87.CD4.CCR5 cells, functional *env* clones generated and coreceptor usage determined as described for the PBMC-derived clones. All clones generated from U87.CD4.CCR5 cell supernatants showed dualtropism. This result suggests that *env* clones using CCR5 only were virtually absent or present at very low frequencies within the quasispecies of the 7 primary isolates analyzed here.

2.3.4. Limiting endpoint dilution PCR

There is also the possibility that bulk PCR *env* amplification and cloning could result in resampling bias and explain the absence of CCR5-only *env* clones. A single genome amplification approach to generate diverse clones from isolate 01ZADU36_5 was used. This clone was selected for this analysis because it showed a bias towards CCR5 utilization and yet we had failed to identify CCR5-only tropic clones from this isolate. Thirty clones of 01ZADU36_5 were amplified and cloned by this limiting endpoint dilution PCR approach. These clones were then tested for coreceptor usage in the U87.CD4.CCR5 and U87.CD4.CXCR4 cell lines. Of the 26 functional clones, 24 were dual tropic, one clone used CXCR4 exclusively and one clone showed exclusive R5-usage.

2.3.5. Genotypic Analysis of the *env* gene

All 35 *env* full-length clones generated in this study by bulk PCR were sequenced to investigate phylogenetic relationships and to correlate coreceptor usage phenotype to

genotype data. Phylogenetic analysis showed that all clones clustered with subtype C references with a high degree of confidence (Figure 2.3). Furthermore, the clones from each primary viral isolate clustered together. As described above, sequences were also generated from U87.CD4.CCR5 and U87.CD4.CXCR4 cells infected with each of the primary isolates. These clones utilized both CCR5 and CXCR4, and their sequences were virtually phylogenetically indistinguishable from those obtained from PBMC cultures. Results obtained by using position-specific scoring matrix for HIV-1 subtype C (C-PSSM), a phenotype predictive tool based on HIV-1 subtype C sequences (Jensen et al. 2006) indicated CXCR4 or dual tropic phenotype and high net V3 charges.

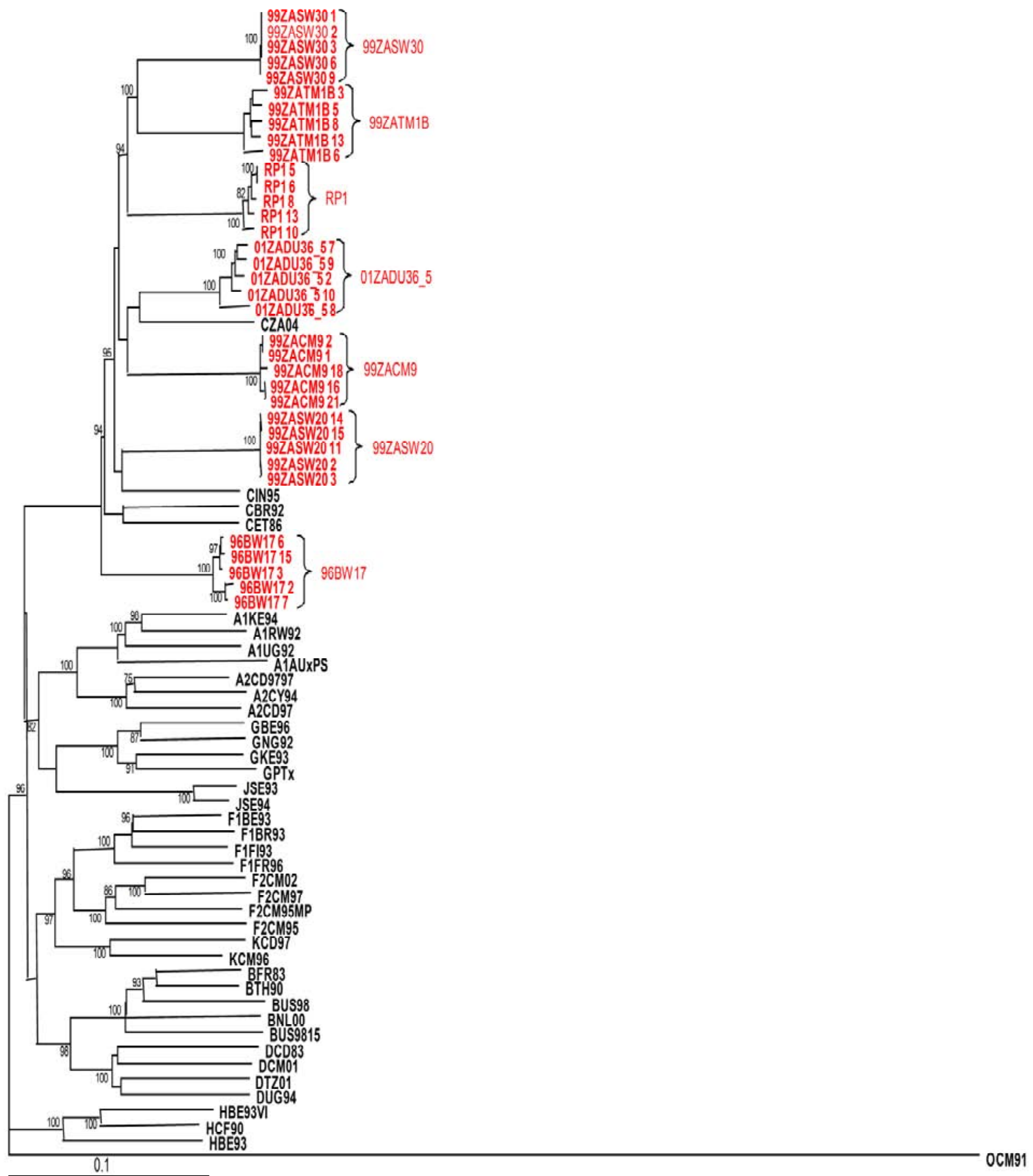


Figure 2.3: Neighbour-Joining phylogenetic tree constructed from the *env* gene sequences

All clones (represented in red) of a particular viral isolate cluster closely together. Furthermore, all clones cluster with the subtype C reference with a high degree of confidence.

The envelope V3 loop is an important determinant of coreceptor utilization (Briggs et al. 2000; Cann et al. 1992; Fouchier et al. 1992; Rizzuto et al. 1998; Wu et al. 2006). Therefore, the V3 loop of the functionally characterized *env* clones was further analyzed in order to identify and describe sequences associated with dualtropism and CXCR4 utilization (Figure 2.4 and Table 2.3). Of particular interest was the crown motif, a conserved tetrapeptide located at the tip of the V3 loop. Changes within this region may influence coreceptor usage. The consensus crown motif for clones from isolate RP1 was GPGQ, which is the conventional V3 loop crown sequence observed in CCR5-tropic subtype C sequences. The crown motifs for clones generated from 01ZADu36_5 and 99ZACM9 were GPGR and GPRY respectively, sequence substitutions that are indicative of CXCR4 tropism (Coetzer et al. 2006). Clones from 96BW17, 99ZATM1B and 99ZASW20 each displayed consensus crown motif sequences that read GRGQ. The consensus crown motifs of 99ZASW30, 01ZADu36_5 and 99ZACM9 read GRGH, GPGR and GPRY respectively. Thus CXCR4 utilization in HIV-1 subtype C is commonly associated with a basic amino acid substitution in the V3 tetrapeptide although this is not an absolute requirement.

Consensus C	CTRPNNNTRKSIRI..GPGQTFYATGDIIGDIRQAHC
96BW17 #2	-----------M--GIGRGQ----M-R----------
96BW17 #3	-----------M--GIGRGQ----M-R----------
96BW17 #6	-----------M--GIGRGQ----M-R----------
96BW17 #7	-----------M--GIGRGQ----M-R----------
96BW17 #15	-----------M--GIGRGQ----M-R----------
Consensus	-----------M--GIGRGQ----M-R----------
99ZATM1B #3	----------NV--GIGRGQ----N-----N------
99ZATM1B #5	----------NV--GIGRGQ----N-----N------
99ZATM1B #6	----------NV--GIGRGQ----M-R---N------
99ZATM1B #8	----------NV--GIGRGQ----N-----N------
99ZATM1B #13	----------NV--GIGRGQ----N-----N------
RP1 #5	-I--G-----RV-LGIGPGQ------RV-R-------
RP1 #6	-I--G-----RV-LGIGPGQ------RV-R-------
RP1 #8	-I--G-----RV-LGIGPGQ------RV-R-------
RP1 #10	-I--G-----RV-LGIGPGQ------RV-R-------
RP1 #13	-I--G-----RV-LGIGPGQ------RV-R-------
Consensus	-I--G-----RV-LGIGPGQ------RV-R-------
99ZASW20 #2	-------------TGIGRGQ------Q---V-----
99ZASW20 #3	-------------TGIGRGQ------Q---V-----
99ZASW20 #11	-------------TGIGRGQ------Q---V-----
99ZASW20 #14	-------------TGIGRGQ------Q---V-----
99ZASW20 #15	-----------V-IGIGRGHA--T—KV--N-------
Consensus	-------------TGIGRGQ------Q---V-----
99ZASW30 #1	-----------V--GIGRGHA--T-GKV--N------
99ZASW30 #2	-----------V--GIGRGHA--T-GKV--N------
99ZASW30 #3	-----------V--GIGRGHA--T-GKV--N------
99ZASW30 #6	-----------M--GIGRGHA--T-GKV--N------

```
99ZASW30 #9          -----------V--GIGRGHA--T-GKV--N------

Consensus            -----------V--GIGRGHA--T-GKV--N------


01ZADu36_5 #2        ----D-KINMKRIKI.GPGRA-V--KG-R---R--Y-

01ZADu36_5 #7        ----D-KISMKRIKI.GPGRA-V--KG-K---R--Y-

01ZADu36_5 #8        ----D-KINMKRIKI.GPGRA-V--KG-K---R--Y-

01ZADu36_5 #9        ----D-KINMKRIKI.GPGRA-V--KG-R---R--Y-
01ZADu36_5 #10       ----D-KISMKRIKI.GPGRA-V--KG-K---R--Y-
Consensus            ----D-KINMKRIKI.GPGRA-V--KG-K---R--Y-

99ZACM9 #1           -A--G---I-R---..GPRYA---KET----------

99ZACM9 #2           -A--G---I-R---..GPRYA---KET----------
99ZACM9 #16          -A--G---I-R---..GPRYA---KET----------
99ZACM9 #18          -A--G---I-R---..GPRYA---KET----------
99ZACM9 #21          -A--G---I-R---..GPRYA---KET----------

Consensus            -A--G---I-R---..GPRYA---KET----------
```

Figure 2.4: Alignment of V3 sequences of clones of primary viral isolates

The crown motif for each sequence is indicated in red and dual tropic clone sequences are indicated in green.

Table 2.3: Summary table of V3 characteristics of clones of primary viral isolates

Clone	A.A Length	Calc V3 net charge	PSSM V3 net charge	Amino Acid (11/25)	Crown Motif	PSSM Coreceptor usage	Phenotype Prediction
R-5 only virus 96BWM01_5	35	4	4	Ser (S)/Asp (D)	GPGQ	CCR5-using only	CCR5-using
96BW17 # 2; 3; 6; 7; 15	37	7	7	Ser (S)/Arg(R)	GRGQ	CXCR4-using	CXCR4-using or dt
99ZATM1B # 3; 5 ;6; 8; 13	37	8	8	Asp(N)/Arg(R)	GRGQ	CXCR4-using	CXCR4-using or dt
RP1 # 5; 6; 8; 10; 13	37	8	8	Arg(R)/Arg(R)	GPGQ	CXCR4-using	CXCR4-using or dt
99ZASW20 # 2; 3; 11; 14; 15	37	6	6	Ser(S)/Glut(Q)	GRGQ	CXCR4-using	CXCR4-using or dt
99ZASW30 # 1; 2; 3; 6; 9	37	9	8	Ser(S)/Lys(K)	GRGH	CXCR4-using	CXCR4-using or dt
01ZADu36_5# 2; 9	36	8	8	Arg(R)/Gly(G)	GPGR	CXCR4-using	CXCR4-using or dt
01ZADu36_5# 7; 8; 10	36	7	7	Arg(R)/Gly(G)	GPGR	CXCR4-using	CXCR4-uing or dt
99ZACM9 # 1; 2; 16; 18; 21	35	6	6	Thr(T)/Arg(R)	GPRY	CXCR4-using	CXCR4-using or dt

Another feature of the *env* V3 loop associated with tropism determination is the property of amino acids at positions 11 and/or 25 (Fouchier et al. 1992). The consensus sequences for all isolates with the exception of 99ZASW20 showed a positively charged amino acid substitution at one or both of these positions. 96BW17 has serine (S) (neutral charge) and arginine (R) (positively charged); 99ZATM1B has asparagine (N) (neutral) and arginine,

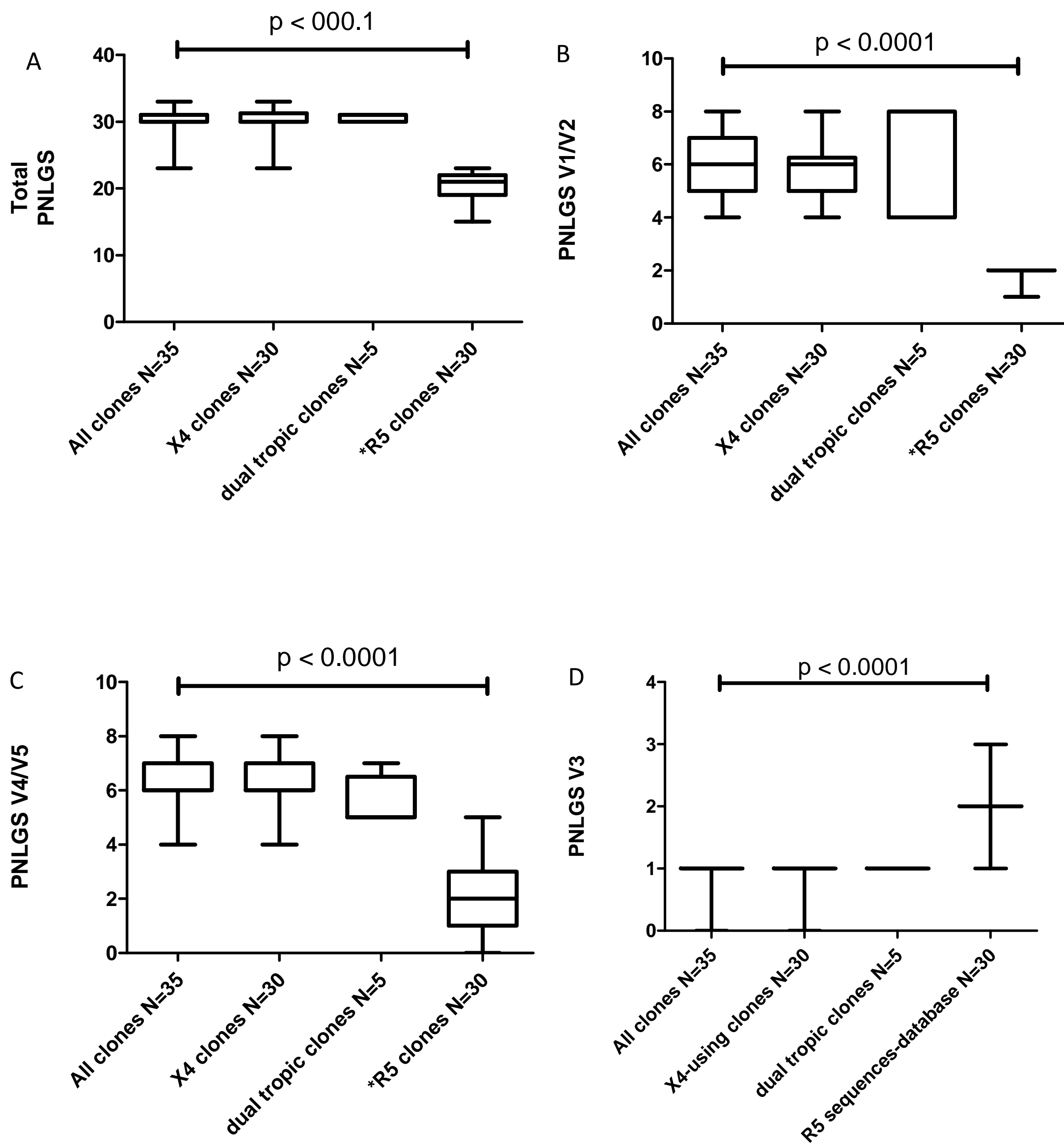
99ZASW20 has serine and glutamine (Q) both of which carry neutral charges, 99ZASW30 has serine and lysine (K), 01ZADu36_5 has arginine and glycine (G) and 99ZACM9 has arginine and threonine (T) at positions 11 and 25 respectively. RP1 has arginine at both positions. The number of amino acids in the V3 loop can also be indicative of coreceptor usage. The typical V3 loop from CCR5 tropic viruses has 35 amino acids. Clones from 99ZACM9 were 35 amino acids long in the V3 loop, whereas clones from 01ZADu36_5 were 36 amino acids long. Clones from isolates 99ZATM1B, 99ZASW30 and 96BW17 had 2-amino acid insertions, increasing the length of the V3 loop to 37 amino acids. The insertions occurred at positions 13 and 14 of the V3 loop for clones from isolates RP1 and 99ZASW20 and at positions 6 and 7 for clones from 01ZADu36_5. Clones from 99ZATM1B, 99ZASW30 and 96BW17 had insertions between positions 15 and 16. Amino acid insertions in the V3 loop, particularly at positions 13 and 14 are features consistent with CXCR4 utilization as previously described (Coetzer et al. 2006). None of the insertions observed in the V3 loop of the clones from this study were noted in HIV-1 subtype C R5 sequences downloaded from the Los Alamos database (www.hiv.lanl.gov). The V3 region was also analyzed by manually calculating the overall net amino acid charge, another indicator of *env* coreceptor tropism (Table 2.3). C-PSSM, a web-based bioinformatic tool used for predicting HIV-1C coreceptor usage from the amino acid sequences of the V3 loop (Jensen et al. 2006) was also used. Both manual and C-PSSM calculations were comparable except for the clones from 99ZASW30 where calculated scores were slightly higher than C-PSSM generated scores. Higher overall net V3 charges are associated with X4-usage. A charge less than +4.5 is regarded as R5-using and charges above +4.5 are regarded as X4-using (Coetzer et al. 2006; Fouchier et al. 1995; Fouchier et al. 1992; Kuiken et al. 1992). Therefore, based on the multiple V3 loop sequence based algorithms

available for phenotype prediction, all clones generated in this study were either only CXCR4-using or dual tropic, consistent with the functional data.

The V1/V2 and V4/V5 regions of the *env* gene were also analyzed as these regions have also been implicated in playing a role in viral tropism. Sequence features in these regions that may influence coreceptor utilization are the amino acid length and the number of predicted N-linked glycosylation sites (Chohan et al. 2005; Coetzer et al. 2007; Coetzer et al. 2008; Masciotra et al. 2002; Pollakis et al. 2001). The number of predicted N-linked glycosylation sites in clones from this study varied from 23 to 33. Clones for RP1, 99ZASW20 and 99ZASW30 all had 30 predicted N-linked glycosylation sites. Within the V1/V2 and V3 regions, the N-linked glycosylation sites varied between isolates but occurred at the same positions for all clones of the same isolate irrespective of whether they were X4-using or dual tropic except for one clone from 99ZATM1B which had 2 predicted N-linked glycosylation sites in the V2 region whereas the other 4 clones of this isolate had 3. The sites within the V4/V5 regions for all clones of all isolates showed slight variations in position. However, all clones from 01ZADu36_5 exhibited CXCR4-usage and showed variation in the positions of the sites in all five hypervariable regions. The positions of N-linked glycosylation sites varied from clone to clone and based on these positions no pattern emerged that could distinguish CXCR4-using clones from those that used both CCR5 and CXCR4.

When the total number of predicted N-linked glycosylation sites within the *env* as well as within the V1/V2 and V4/V5 regions were analysed, no significant difference was

observed between the CXCR4-using clones and dual tropic clones. However, the median number of N-linked glycosylation sites for X4/R5X4 clones from this study was significantly higher at (30) compared to (21) for R5 clones (30 sequences downloaded from the Los Alamos HIV-1 database) ($p < 0.0001$) (Figures 2.5A-C). R5 sequences showed a lower number of predicted N-linked glycosylation sites within the entire *env* as well as within the V1/V2 and V4/V5 regions when compared to R5X4/X4 clonal sequences.



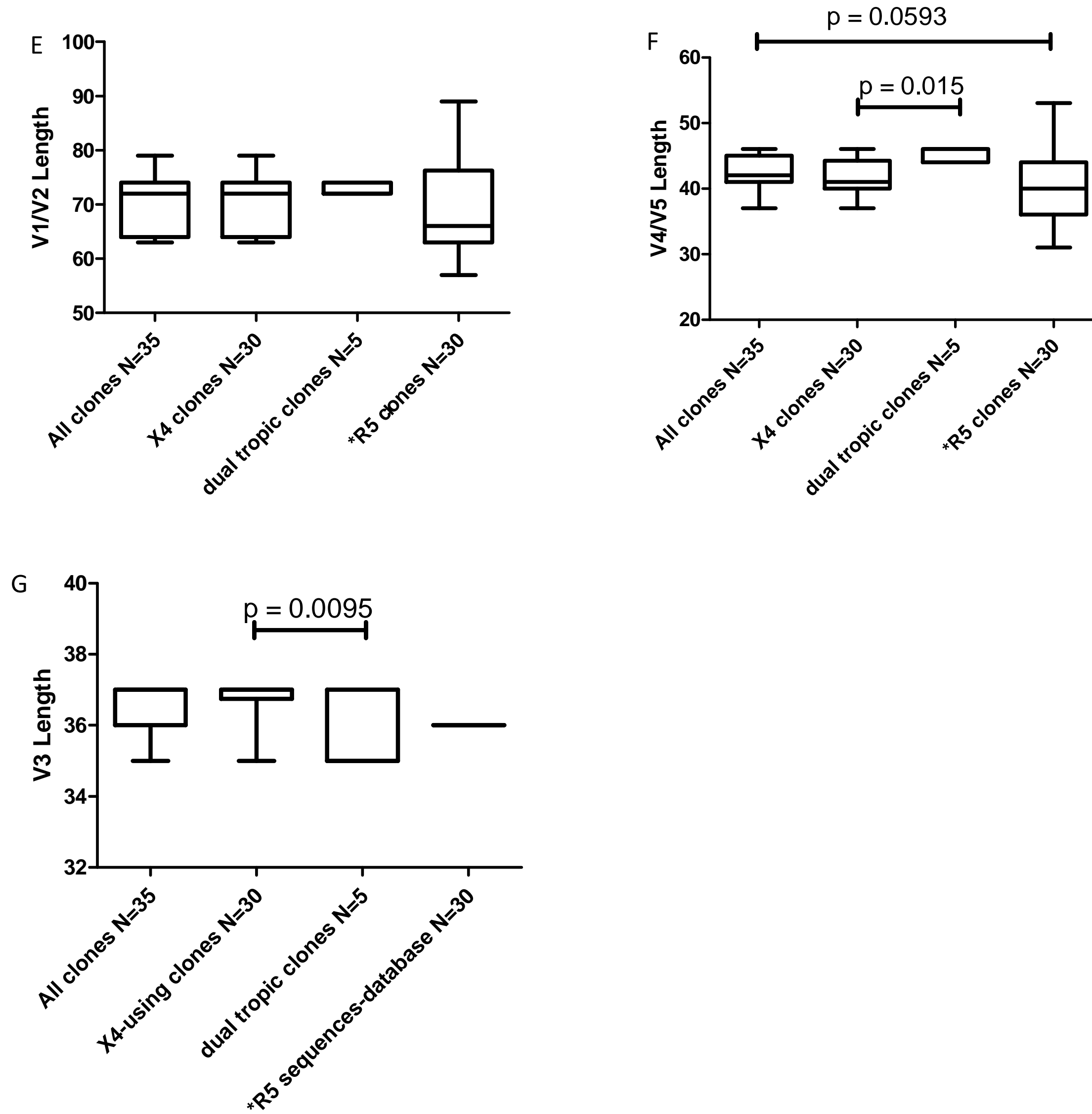


Figure 2.5: Box plots of predicted N-linked glycosylation sites (PNLGS) and *env* variable loop lengths

A) shows the total number of PNLGS within the *env*. B) the number of PNGLS within the V1/V2 region C) the number of PNGLS within the V4/V5 region D) the number of PNLGS within the V3 region E) the V1/V2 loop length. F) the V4/V5 loop length G) the V3 loop length. The line within each box represents the median value for each group. * indicates R5 sequences downloaded from the Los Alamos database (www.hiv.lanl.gov).

Previous reports have suggested that a lack of predicted N-linked glycosylation sites at positions 6-8 of the V3 loop may be indicative of CXCR4-usage (Coetzer et al. 2006). This site was conserved in the clones analyzed in this study, despite the utilization of CXCR4 by all the clones. All clones (except those from isolate 01ZADu36_5) contained a predicted N-linked glycosylation site at position 6 although they were CXCR4-using. This was also observed in a previous study by Johnston and colleagues (Johnston et al. 2003) where all but one X4 sequence maintained this site. A significant reduction in the number of predicted N-linked glycosylation sites within the V3 region of clones generated in this study was observed as compared to the R5 sequences from the database (Figure 2.5D).

The entire *env* sequence i.e. gp160 of all CXCR4- and CCR5/CXCR4-using clones were compared to determine if any distinguishing features could be identified. Specifically, analysis for unique signature patterns such as conversation of amino acids with a specific charge or physical property at a particular position, putative N-linked glycosylation sites, deletions, insertions or number of amino acids was performed. The 2 clones of RP1 displaying dualtropism (i.e. clone # 10 and 13) had leucine (L) at position 373 whereas the CXCR4-using clones of this isolate (clone # 5; 6; 8) had proline (P) at this position. The other isolate that produced clones exhibiting dualtropism was 99ZACM9. No distinguishing signature sequences were noted that could differentiate between X4 and R5X4 sequences.

Differences in the loop lengths between X4, R5X4 and R5 sequences were next analyzed. Most variation was seen in V1 which ranged from 16-27 amino acids. V2 had a relatively constant loop length (40-45). The combined V4/V5 loop length ranged from 37-46. The V1/V2 and V4/V5 loop lengths of the clones produced in this study were plotted against R5 sequences from the Los-Alamos database. No significant differences were observed between the V1/V2 sequences of clones generated in this study and the R5 sequences from the Los Alamos database (Figure 2.5E). However, for the V4/V5 region, there was a significant difference between the generated clones using the CXCR4 coreceptor for viral entry and the dual tropic clones ($p = 0.015$) (Figure 2.5F), with the dual tropic clones having an increased V4/V5 loop length. For the V3 loop, all analyzed R5 sequences had a loop length of 36 amino acids whereas X4 and R5X4 clones from this study showed variability with a range from 35 to 37 amino acids (Figure 2.5G).

2.4. Discussion

The requirement by HIV-1 for specific cellular interacting factors during the entry step offers an opportunity for the development of vaccines and drugs that target this crucial step in the virus replication cycle (Dhami et al. 2009; Hunt and Romanelli 2009; Pantophlet and Burton 2006; Phogat et al. 2007). Coreceptors play an important role in initiating infection at the cellular level. Additionally, coreceptor utilization is an important determinant of the rate of disease progression. The emerging availability of entry inhibitors such as the CCR5 antagonists underlines the importance of better characterization of coreceptor utilization and cellular tropism by HIV-1 isolates particularly in heavily burdened countries where the drugs are likely to be required on a large scale for the clinical management of HIV/AIDS. In this study, 35 full-length *env* clones from seven dual tropic isolates of HIV-1 subtype C was generated, in order to determine whether they were a mixture of CCR5 and CXCR4

quasispecies or dual tropic viruses at the clonal level. Sequence characteristics of these clones were also interrogated in order to better elucidate the genetic determinants of coreceptor utilization by HIV-1 subtype C viruses. CXCR4-tropic clones dominated within the dual tropic viral isolates quasispecies. A minority proportion of dual tropic clones were also identified. Unexpectedly, not a single CCR5-monotropic *env* clone from the seven primary isolates analyzed in this study was found. This is an unusual finding considering that many studies have shown that HIV-1 subtype C viruses even in late stages of disease utilize CCR5-only predominantly for cell entry. Thus a significant proportion of the remnants of these viruses among the quasispecies of the dual tropic isolates were expected to be found. Instead, all the clones detected in this study used CXCR4 as the coreceptor for cell entry, with a minority of these (14.3%) also able to mediate entry via the CCR5 receptor. Results obtained may explain why in previous studies of some of the dual tropic isolates described here (99ZACM9, 99ZASW20 and 99ZASW30); the isolates could be strongly inhibited by CXCR4 inhibitors but only modestly by CCR5 inhibitors (Cilliers et al. 2003). These earlier results can now be explained by the observation that although these isolates are dual tropic, they are dominated by X4 variant clones.

An alternative explanation of our findings is that these isolates changed their coreceptor preference during in vitro passages in PBMC cocultures as has been previously described (Voronin et al. 2007). This possible explanation is supported by the finding that isolates 99ZACM9 and 99ZASW30 displayed remarkably lower CCR5 utilization capacity (Table 2.1) than was previously described (Cilliers et al. 2003). Another explanation for our findings, and possibly a limitation in this study is that the p24 antigen ELISA method was employed and not the 50% tissue culture infective dose ($TCID_{50}$). The latter is the most accurate measure of infectious HIV-1 titers. It involves a limiting dilution-infection assay as

and a calculation of the dose required for 50% infectivity of susceptible cells in tissue culture. Previous studies have shown that there is a poor relationship between p24 content and infectious titre (Marozsan et al. 2004). This may have contributed to a selection bias. It is also worth noting that although isolates 96BW17, 99ZASW20 and 01ZADu36_5 showed a possible bias towards CCR5 utilization and were clearly dual tropic, all the *env* molecular clones generated from these isolates by bulk PCR amplification were CXCR4-only using. This finding strongly suggested that the bulk PCR could be biased towards X4 viruses. Limiting endpoint dilution PCR on one dual tropic viral isolate (01ZADu36_5) which was biased towards CCR5 utilization was therefore performed (Table 2.1). Remarkably, of 26 functional *env* clones generated by this approach, 24 exhibited dualtropism, one used CXCR4 exclusively and one used CCR5 exclusively. It can therefore be concluded that dual tropic HIV-1 subtype C isolates are dominated by X4 and R5X4 clones with negligible proportion of R5 monotropic clones.

It has been recently proposed that coreceptor switching is associated with deleterious mutations in *env* that diminish CCR5-tropism as mutations associated with CXCR4 utilization accumulate (Coetzer et al. 2008). Although coreceptor binding was not directly tested for in this study, results are consistent with the proposal by Coetzer and colleagues and with their observation that coreceptor switching is associated with a rapid decrease in the ability to use CCR5. Results obtained may suggest that in HIV-1 subtype C, the mutations required for adaptation to CXCR4 utilization significantly reduce the ability of *env* to utilize CCR5, thus resulting in reduced fitness of CCR5 utilizing viruses. This could in turn lead to the selection and amplification of clones able to utilize CXCR4. It can be speculated that given the low frequency of HIV-1 subtype C CXCR4 utilizing viruses reported in various studies, more accumulated mutations are required for switching to CXCR4 utilization for this

subtype. Alternatively, the changes required for a switch to CXCR4 utilization may result in a bigger fitness deficit for HIV-1 subtype C CCR5-tropic variants thus leading to selection against these viruses once adaptation to CXCR4 utilization has been accomplished. Further studies will be required to carefully investigate the specific localization and nature of complementary mutations required for HIV-1 subtype C *env* coreceptor switch.

The genetic characteristics associated with CXCR4-usage or dualtropism for HIV-1 subtype C viruses was also investigated. Results may be limited by founder effects since R5 sequences from the study isolates could not be generated but HIV-1 subtype C R5 sequences were available from the Los Alamos database that facilitated this comparative analysis. As described for HIV-1 subtype B, the subtype C third variable loop of gp120 (V3 region) is a major determinant of whether CXCR4 or CCR5 will be the accessory protein used by the virus for membrane fusion (Cilliers et al. 2003; Coetzer et al. 2007; Coetzer et al. 2006; Fouchier et al. 1992; Morris et al. 2007; Ndung'u et al. 2006). Typically, the V3 region consists of approximately 35 amino acids in CCR5-tropic viruses (Coetzer et al. 2006). Consistent with earlier studies, results show that V3 loop amino acid characteristics are important determinants of coreceptor tropism. In most cases, the V3 loop crown of CXCR4-utilizing clones had basic amino acid substitutions which differed from the canonical GPGQ sequence found in CCR5 HIV-1 subtype C viruses to GPGX (where X is any other amino acid), GRGH, GPGR or GPRY. X4 variants are more variable than R5 viruses in the V3 region particularly at positions 11 and 25 which tend to be mostly positively charged amino acids, often arginine (R), lysine (K) or histidine (H). The presence of a basic amino acid at both or one of these positions for the majority of clones in this study (85%) was observed. In addition, in X4 variants there may be insertions particularly between positions 13 and 14 of the V3 loop contributing to an increased length. Amino acid insertions were seen in 71% of

clones in this study. X4 variants may also be distinguished from R5 viruses as they usually have an increased net V3 charge. Consistent with these observations, 100% of X4-utilizing clones had V3 loop amino acid charges of +5 or more.

The V3 region however, is not the exclusive determinant of coreceptor usage and other regions within the *env* gene may also contribute to viral tropism. The V1/V2 and V4/V5 regions have been implicated in playing a role in determining the biological phenotype of the virus. Specifically, the number of N-linked carbohydrate moieties in these variable loops has been associated with coreceptor determination (Chohan et al. 2005; Coetzer et al. 2008; Masciotra et al. 2002; Pollakis et al. 2001). Here a strong association was found between the number of N-linked glycosylation sites and coreceptor utilization with X4 clones having a significantly higher number of these sites than R5 clones from the database overall and in the V1/V2 or V4/V5 regions (Figure 2.5A-C). In contrast in the V3 region, the number of sites was significantly higher in R5 sequences than X4/R5X4 sequences (Figure 2.5D). A previous longitudinal study of HIV-1 *env* evolution showed no significant changes in N-linked glycosylation sites of 23 viral isolates from 5 patients followed for 2-4 years (Coetzer et al. 2007). Therefore the findings in this study may suggest a rapid accumulation of N-linked glycosylation sites as coreceptor tropism switches, as opposed to a slow accumulation of these sites over time. This is consistent with recent findings of rapid decline in CCR5 utilization as alternate coreceptor utilization emerges in HIV-1 subtype B infection (Coetzer et al. 2008). In both HIV-1 subtypes A and C, shorter V1/V2 loop sequences and fewer predicted N-linked glycosylation sites have been correlated with preferential heterosexual viral transmission (Chohan et al. 2005; Derdeyn et al. 2004). No significant differences in V1/V2 length between R5 and X4 clones in this study was seen but a trend towards shorter V4/V5 for X4 clones was noted (Figure 2.5E-F). Further longitudinal studies will be

necessary in order to better understand HIV-1 subtype C transmission, coreceptor switching and the *env* genetic characteristics associated with these processes. Overall, results obtained suggest that sequence characteristics in the V3 loop, the V4/V5 loop length as well as the number of *env* predicted N-linked glycosylation sites are the primary genotypic determinants for viral tropism in HIV-1 subtype C.

It is worth noting that limiting endpoint dilution of samples in this study except for isolate 01ZADU36_5 was not performed. Therefore the presence of substantial frequencies of R5-monotropic viruses in the quasispecies of the isolates where endpoint dilution was not used cannot be completely ruled out. However, the absence of these clones in bulk amplified clones, in CCR5 only expressing cells and in endpoint diluted 01ZADu36_5 isolate that is biased towards CCR5 are all suggestive of absence of such quasispecies or presence at very low frequency. Results obtained appear to contradict the recent findings of Irlbeck and colleagues (Irlbeck et al. 2008) but it must be emphasized that in that study, samples were analyzed directly from plasma and therefore made use of viral RNA. In contrast, this study examined in vitro propagated isolates from PBMCs and hence made use of proviral DNA. Therefore this could account for the diffrernce observed. Further studies will be needed to determine whether *env* clones directly obtained from patients with dual tropic HIV-1 subtype C viruses have a bias towards CCR5 or CXCR4 tropism.

In conclusion, this study shows that dual tropic viral isolates consist of predominantly X4 and R5X4 clones. Thirty of 35 *env* clones analyzed from PBMCs utilized X4 only as the coreceptor for entry into cells, whereas 5 of 35 clones tested displayed dualtropism and no CCR5-only utilizing clones were identified. R5 monotropic clones could not be detected even

when the isolates were cultured in cells expressing CCR5 coreceptor only. A significant number of R5 monotropic clones failed to be detected when we changed our approach of viral amplification from bulk PCR to limiting endpoint dilution PCR for one dual tropic isolate showing bias towards CCR5 tropism. Viral *env* sequences from both CXCR4 and CCR5-expressing cells were indistinguishable and possessed X4/dual tropic characteristics. Furthermore, *env* sequence characteristics associated with CXCR4 utilization in HIV-1 subtype C was described. In addition to sequence changes in the *env* V3 region, the number of N-linked glycosylation sites in the V1/V2, V3 and V4/V5 regions as major determinants of coreceptor utilization in HIV-1 subtype C was identified. This study also shows that the length of the V4/V5 is a possible determinant of coreceptor utilization. It should be noted that results in this study are consistent with recent findings of the rapid loss of fitness of CCR5 envelope as coreceptor switching emerges and suggest that the sequence characteristics associated with coreceptor switch must occur rapidly *in vivo*. Further studies are needed to better characterize coreceptor switching, particularly in the context of HIV-1 subtype C, the predominant subtype in the world. Thirty-five full-length CXCR4- or dual tropic clones of HIV-1 subtype C have been generated in this study and are important elements that will facilitate further functional studies of this globally predominant subtype. Results from this study have important implications for coreceptor antagonist design and application, and further contribute to better understanding of HIV-1 pathogenesis.

CHAPTER 3

Distinct Patterns of Coreceptor Use by R5X4 HIV-1 Subtype C Clones in Primary CD4+ Lymphocytes and Macrophages versus Transformed Cell Lines

3.1. Introduction

Human immunodeficiency virus type 1 (HIV-1) entry into the cell is initiated when the viral envelope (Env) glycoprotein binds to the CD4 receptor, resulting in a conformational change in the Env surface subunit (gp120) leading to the exposure of a specific binding site for a chemokine receptor most often CCR5 or CXCR4 (Alkhatib et al. 1996a; Choe et al. 1996; Deng and Liu 1996; Dragic and Litwen 1996; Feng and Broder 1996; Kwong et al. 1998; Lusso 2006; Rizzuto et al. 1998; Wyatt et al. 1995). Thereafter, additional structural changes occur allowing the Env transmembrane protein subunit (gp41) to initiate the fusion process (Trkola et al. 1996; Wu et al. 1996).

HIV-1 infection of CD4+ lymphocytes and macrophages *in vitro* and *in vivo* is thought to be mainly mediated by the chemokine receptors CCR5 and CXCR4 (Yi et al. 2005; Zhang et al. 1998b). Studies show that the CCR5 chemokine receptor is the most commonly used coreceptor for viral transmission (Dean et al. 1996; Liu et al. 1996; Samson et al. 1996). Viruses restricted to this receptor (R5 viruses) replicate in primary lymphocytes and macrophages, are nonsyncytium-inducing (NSI) and are poorly cytopathic *in vitro* (Alkhatib et al. 1996b; Choe et al. 1996; Deng and Liu 1996; Dragic and Litwen 1996; Fenyo et al. 1989; Li et al. 1999; Valentin et al. 1994). These viruses are also classified as macrophage-

tropic (M-tropic) because of their ability to replicate in macrophages but not in transformed CD4+ lymphocyte cell lines (Asjo et al. 1986; Connor et al. 1993; Schuitmaker et al. 1992; Zhu et al. 1993). Conversely, T cell line-tropic (T-tropic) viruses replicate in primary CD4+ lymphocytes and CD4+ transformed cell lines but not in macrophages. These variants, which emerge later in infection, use CXCR4 as their principal receptor of entry (X4 viruses), are syncytium-inducing (SI) and are highly cytopathic *in vitro* (Berger 1998; Coetzer et al. 2006; Yi et al. 1999). Dual tropic viruses, which may be intermediate viruses in the evolution from R5 to X4 variants, use CCR5 and CXCR4 receptors and infect both macrophages and CD4+ T-cell lines as well as primary lymphocytes (Carrington et al. 1997; Follis et al. 1998; Li et al. 1999; Naif et al. 2002; Simmons et al. 1998; Yi et al. 1999; Zerhouni et al. 2004). The M or T-tropic paradigm was established using lab adapted X4 viruses, and it is now clear that many primary X4 variants are able to infect macrophages (Yi et al. 1999).

There have been a number of interesting studies of coreceptor use in primary cells by HIV-1. Contrary to earlier beliefs, CXCR4 is expressed on macrophages and some viral isolates use this coreceptor to infect macrophages even though T-tropic X4 strains are unable to infect these cells (Yi et al. 1999). Other studies have used R5X4 strains previously characterized in cell lines to investigate coreceptor usage in primary lymphocytes and macrophages. These studies showed that several dual tropic primary and prototype strains used CXCR4 for entry and infection in primary lymphocytes even though CCR5 was present (Bleul et al. 1997; Yi et al. 2005). However, in macrophages, these strains used both CCR5 and CXCR4. From these observations, it was concluded that although HIV-1 strains were phenotypically characterized as R5X4 viruses in transformed cell lines this does not necessarily predict dualtropism in primary cells (Yi et al. 2005).

Coreceptor usage differences between transformed cell lines and primary cells were observed for HIV-1 subtype B strains which is the most widely studied subtype although it is responsible for only approximately 12% of infections worldwide (Hemelaar et al. 2006). There are no similar studies undertaken for HIV-1 subtype C despite the higher prevalence of this subtype globally, and the apparent differences in evolution of coreceptor usage between subtypes with many studies indicating that complete switch to X4 phenotype is very rare for subtype C even in isolates obtained from late stages of chronic infection (Bjorndal and Sonnerborg 1999; Cecilia et al. 2000; Cilliers et al. 2003; Ndung'u et al. 2006; Tscherning et al. 1998). In this study, CCR5 and CXCR4 coreceptor utilization of functional HIV-1 subtype C *env* clones between transformed cell lines and primary cells (CD4+ lymphocytes and MDM) that are the main cell HIV-1 targets *in vivo* was compared. Furthermore it is suggested that there may be some important differences and heterogeneity in HIV-1 subtype C use of coreceptors in primary cells which is not always predicted or obvious by conventional experimental or genotypic analysis approaches.

3.2. Materials and Methods

3.2.1. Cells and Cell Lines

CD4+ T cells were isolated by negative selection from whole blood obtained from the University of Pennsylvania Centers for AIDS Research Immunology Core. Purified lymphocytes were maintained at a concentration of $2x10^6$ cells/ml in RPMI 1640 containing L-Glutamine with penicillin/streptomycin (50 µg/ml and 50 U/ml) (GIBCO, Grand Island, NY) and 10% heat inactivated fetal bovine serum (FBS) and stimulated with 5 µg/ml phytohaemagglutinin (PHA) (MP Biomedical, Solon, OH) at 37 C and 5% CO_2 for 72 hours in a T-75 flask. They were maintained in 300 U/ml interleukin-2 (IL-2)

(Proleukin, Novartis, Basel, Switzerland) thereafter. Monocytes were isolated from whole blood from HIV negative volunteers by density gradient centrifugation on Ficoll-Histopaque (G.E. Healthcare, Piscataway, NJ) and adherent monocytes were maintained in culture at $1x10^6$ cells/ml in RPMI 1640 containing L-Glutamine with penicillin/streptomycin (50 µg/ml and 50 U/ml), 10% heat inactivated FBS and 50 ng/ml Macrophage Colony Stimulating Factor (M-CSF) (R&D, Minneapolis, MN) in a 100 mm x 20 mm tissue culture plate at 37 C and 5% CO_2 for 7 days to allow differentiation into monocyte-derived macrophages. 293T cells were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD) and were cultured in DMEM containing L-Glutamine with penicillin/streptomycin (50 µg/ml and 50 U/ml), 10% heat inactivated FBS and kept at 37 °C in 5% CO_2. U87.CD4, U87.CD4.CXCR4 and U87.CD4.CCR5 cells were obtained from the NIH AIDS Research and Reference Reagent Program. U87.CD4 cells were maintained in selective media containing 300 µg/ml of G418 (Invitrogen, Carsbad, CA) and U87.CD4.CXCR4 and U87.CD4.CCR5 cells were maintained in selective media containing 1 µg/ml of puromycin (MP Biomedical, Solon, OH) and 300 µg/ml of G418 (Invitrogen, Carlsbad, CA).

3.2.2. Transfection

The 35 envelope molecular clones were generated as described previously (refer to 2.2.6). One day prior to cotransfection, $6x10^5$ 293T cells/well were seeded in a total volume of 2 ml per well of a 6-well flat-bottomed plate and incubated at 37 °C in 5% CO_2. Cotransfection was carried out by first combining 100 µl serum free DMEM with 12 µl Fugene reagent (Roche Applied Science, Germany) and incubating for 5 minutes at room temperature. This was then incubated at room temperature together with 100 µl

serum free DMEM and 1.3 µg pNL4-3.Luc.R-E- (Chen et al. 1994; Connor et al. 1995). pNL4-3Δ*env* is a full-length HIV plasmid with the *env* deleted. It contains the reporter firefly luciferase gene in the *nef* ORF. Thereafter 1.3 µg DNA of gp160 *env* cloned in pcDNA3.1D/V5-His-TOPO vector (Invitrogen) was added. The transfection mixture was incubated for 30 minutes, and then added to assigned wells of the plate seeded with 293T cells and incubated at 37°C and 5% CO_2 overnight. The cells were then washed twice with DMEM containing L-Glutamine with penicillin/streptomycin (50 µg/ml), 10% heat inactivated FBS and replaced with 2 ml fresh media. This was incubated at 37°C and 5% CO_2 overnight. Viral supernatants were then harvested. Supernatants were aliquotted into 15 ml tubes and centrifuged at 1,200 x g for 5 minutes. Supernatants were then removed and aliquotted into a 15 ml tube containing 100% Sucrose (final concentration of 5% sucrose) (Sigma, St. Louis, MO). Single aliquots were then stored frozen at -80°C. The titre of each of the pseudoviruses was determined by p24 antigen ELISA.

3.2.3. Infections

Infections of various cells and cell lines were performed as follows. U87.CD4, U87.CD4.CXCR4 and U87.CD4.CCR5 cells were seeded 0.15×10^5 cells/well in a 96-well plate respectively 1 day prior to infection and maintained in DMEM containing L-Glutamine with penicillin/streptomycin, 10% heat inactivated FBS. For blocking experiments, cells were pre-treated with 5 µg/ml of the CXCR4 inhibitor AMD3100 (Sigma-Aldrich, St. Louis, MO), 5 µM CCR5 inhibitor maraviroc (Pfizer Inc., New York City, NY) or a combination of both inhibitors (Loftin et al. 2010). For resistance testing on the U87 cells, an additional concentration of 15 µg/ml AMD3100 was included. Infection was done by spinnoculation at 1,200 x g for 2 hours with the HIV-1

pseudoviruses using an equivalent amount of virus (approximately 8 ng) as determined by p24 measurements. Following infection, cells were cultured at 37°C and 5% CO_2 for 3 days. Infection of monocyte derived macrophages was performed similarly, except cells were plated at $0.5x10^5$ cells/well in 96 well plates one day before infection. CD4+ T lymphocytes were plated at $2x10^5$ cells/well in 96 well plates in the presence of 10 U/ml of IL-2 and cultured at 37°C and 5% CO_2 for 4 days after infection. An R5, X4 and R5X4 control was included in all experiments. Successful infection of target cells was determined by luciferase activity measured by removing cell supernatant and lysing the cells in PBS containing 0.1% Triton X-100. 50 µl cell lysate was combined with 50 µl luciferase assay substrate (Luciferase Assay System; Promega, Madison, WI) and the luciferase relative light units (RLUs) were measured using a Dynex technologies microtiter plate luminometer (Figure 3.1). All experiments were done in duplicate. Furthermore because primary T cells and macrophages from different donors differ in their permissiveness to HIV-1, all primary cell infections were done using two different donors.

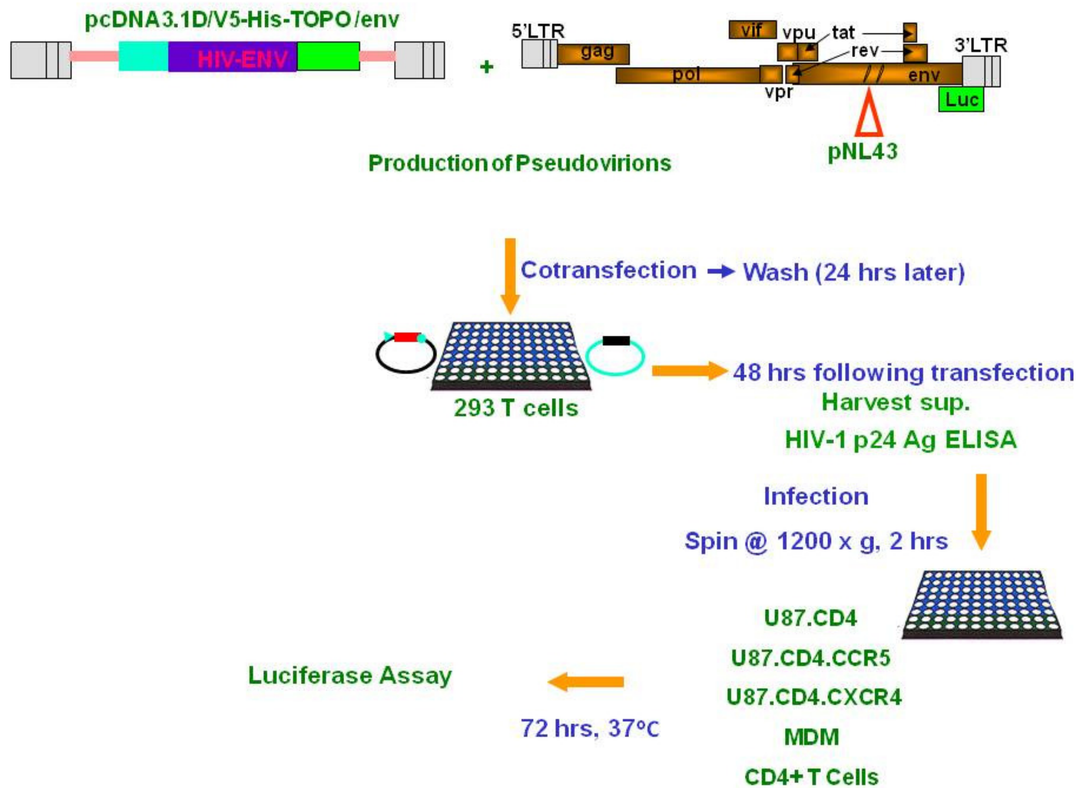


Figure 3.1: Coreceptor usage using the Spinnoculation method of infection

48 hours following tranfection infection was performed using the spinnoculation method. Transfection supernatants were centrifuged at 1200 x g gor 2 hours before the luciferase assay was carried out.

3.2.4. Genotypic Analysis

Full *env* sequences were generated and analysis was carried out as done previously (refer to 2.2.9).

3.3. Results

3.3.1. Transformed Cell Line Coreceptor Usage

Thirty five envelope clones, five each from seven dual tropic isolates were generated from each isolate, transfected into 293T cells to produce pseudoviruses and then tested for coreceptor utilization in transformed U87 cells expressing CD4 and either CCR5 or CXCR4, with coreceptor negative cells as a control. Previously coreceptor usage was performed and infection was carried out using the DEAE-dextran method (refer to 2.2.8). All 35 clones were able to infect cells expressing CXCR4. Five of 35 (14.3%) clones were also able to mediate entry via CCR5, indicating that they were dual tropic. Surprisingly, no CCR5 monotropic clones were detected from the dual tropic isolates (refer to 2.3.3). Here coreceptor usage was performed and infection was carried out using a spinnoculation method (O'Doherty et al. 2000). All 35 *env* clones-derived pseudoviruses were able to efficiently infect cells expressing CXCR4. Twenty of 35 clones (57%) tested were also able to mediate entry via the CCR5 receptor, indicating that they were R5X4 although usage of CXCR4 was higher for most. Most clones from the same isolate showed a similar coreceptor utilization profile. All clones from isolate 96BW17 were dual tropic except for 96BW17 #15 which used only CXCR4. All clones of 99ZATM1B displayed exclusive CXCR4 usage. All clones from isolate RP1 except for clone #13 used CXCR4 exclusively. RP1 #13 showed both CCR5 and CXCR4 usage. Pseudovirus clones from the RP1 isolate were generally poorly infectious as indicated by the low luciferase activity observed in target cell lines. All clones from 99ZASW20 and 99ZASW30 were R5X4 while all but one clone (#16) from 99ZACM9 also used both CCR5 and CXCR4. The 99ZACM9 #16 clone showed poor infection in cells expressing CCR5 and was therefore classified as exclusively X4. Only 1 clone (# 9) from 01ZADu36_5 used both CCR5 and CXCR4 while the remaining 4 clones used CXCR4.

Overall, although both coreceptors were used by some pseudoviruses, CXCR4 was the preferred coreceptor. These results also indicated that the spinnoculation method was better at facilitating infection of U87.CD4.CCR5 by R5X4 clones compared to the DEAE-dextran method (Table 3.1, Figure 3.2). As previously noted, there were no R5-only clones detected and the spinnoculation method confirmed this finding.

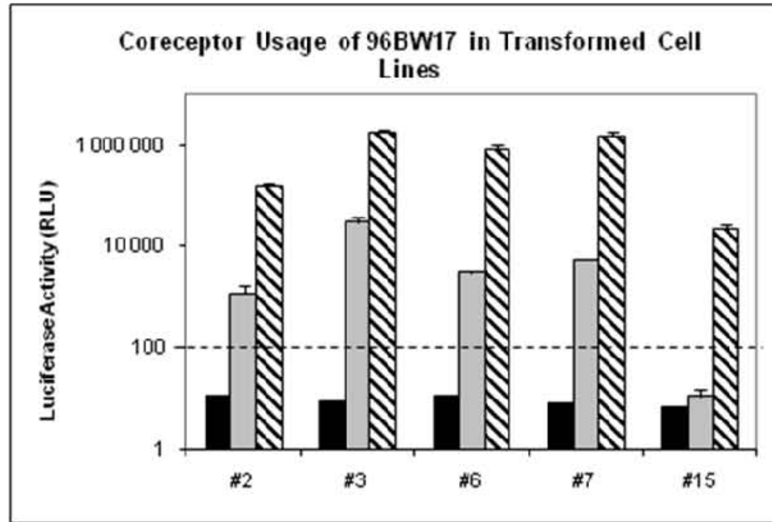
Table 3.1: Comparison of the DEAE-dextran method and Spinnoculation method of infection

	DEAE-Dextran		Spinnoculation	
Clone	**U87.CD4.CCR5**	**U87.CD4.CXCR4**	**U87.CD4.CCR5**	**U87.CD4. CXCR4**
96BW17 #2	-	+++	+	+++
96BW17 #3	-	+++	++	+++
96BW17 #6	-	+++	+	+++
96BW17 #7	-	+++	+	+++
96BW17 #15	-	+++	-	+++
99ZATM1B #3	-	+++	-	+++
99ZATM1B #5	-	+++	-	+++
99ZATM1B #6	-	++	-	+++
99ZATM1B #8	-	++	-	+++
99ZATM1B #13	-	++	-	+++
RP1 #5	-	++	-	++
RP1 #6	-	++	-	++
RP1 #8	-	++	-	++
RP1 #10	++	++	-	++
RP1 #13	++	++	++	++
99ZASW20 # 2	-	++	+	++
99ZASW20 #3	-	++	+	++
99ZASW20 #11	-	++	+++	+++
99ZASW20 #14	-	+++	+++	+++

99ZASW20 #15	-	++	+++	+++
99ZASW30 #1	-	++	++	+++
99ZASW30 #2	-	+++	++	+++
99ZASW30 #3	-	+++	++	+++
99ZASW30 #6	-	+++	++	+++
99ZASW30 #9	-	+++	++	+++
99ZACM9 #1	++	+++	++	+++
99ZACM9 #2	++	+++	++	+++
99ZACM9 #16	-	+++	-	++
99ZACM9 #18	-	+++	++	+++
99ZACM9 #21	+	+++	+	++
01ZADu36_5 #2	-	+++	-	+++
01ZADu36_5 #7	-	+++	-	+++
01ZADu36_5 #8	-	++	-	++
01ZADu36_5 #9	-	++	+	+++
01ZADu36_5 #10	-	++	-	+++

Note: +; ++ and +++ indicate the degree of coreceptor usage per clone with + being utilization and +++ representing strong interaction as measured by luminescence units. For the DEAE-dextran method, a positive result was considered to be 2 × the average relative luminescence units (RLUs) of the negative control wells+standard deviation. RLUs above this but less than 3 × this cut off value are indicated by "+", values 3 × to 10 × above cut off are shown as "++" and values above 10 × the cut off are indicated as "+++". For the spinnoculation method, + indicates a value of 100 obtained on the log scale with 1 000-10 000 being represented by ++ and >10 000 being represented by +++.

A



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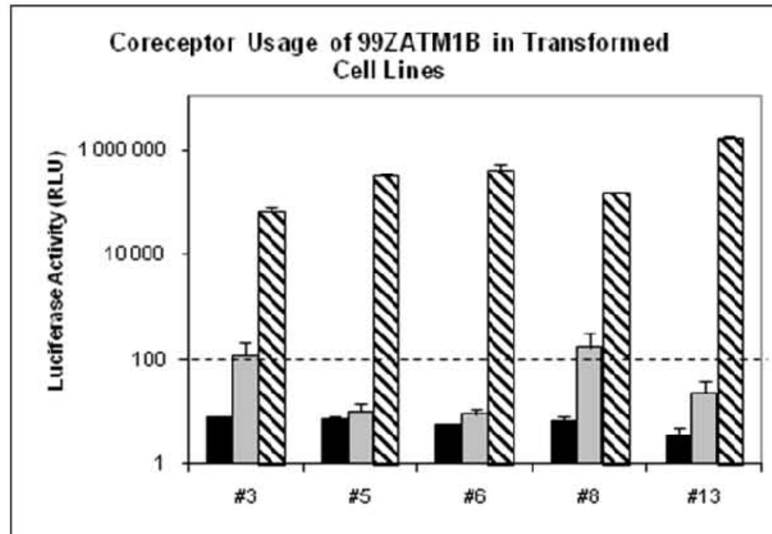

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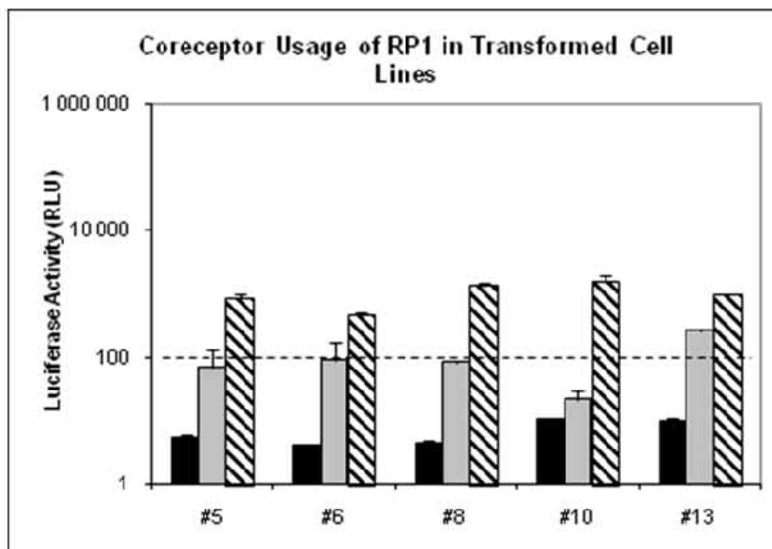

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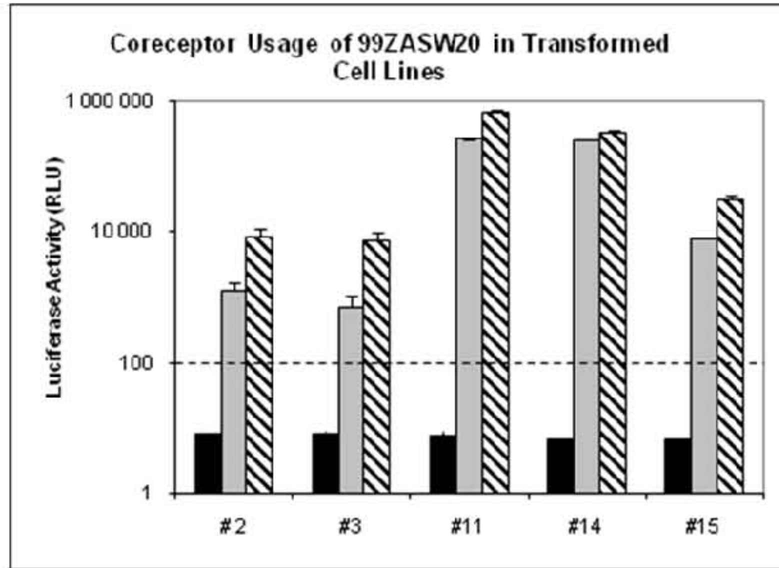

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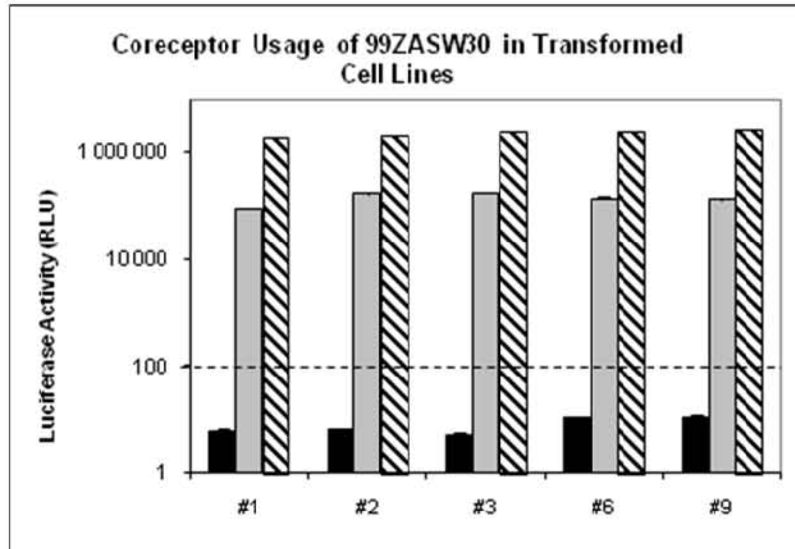


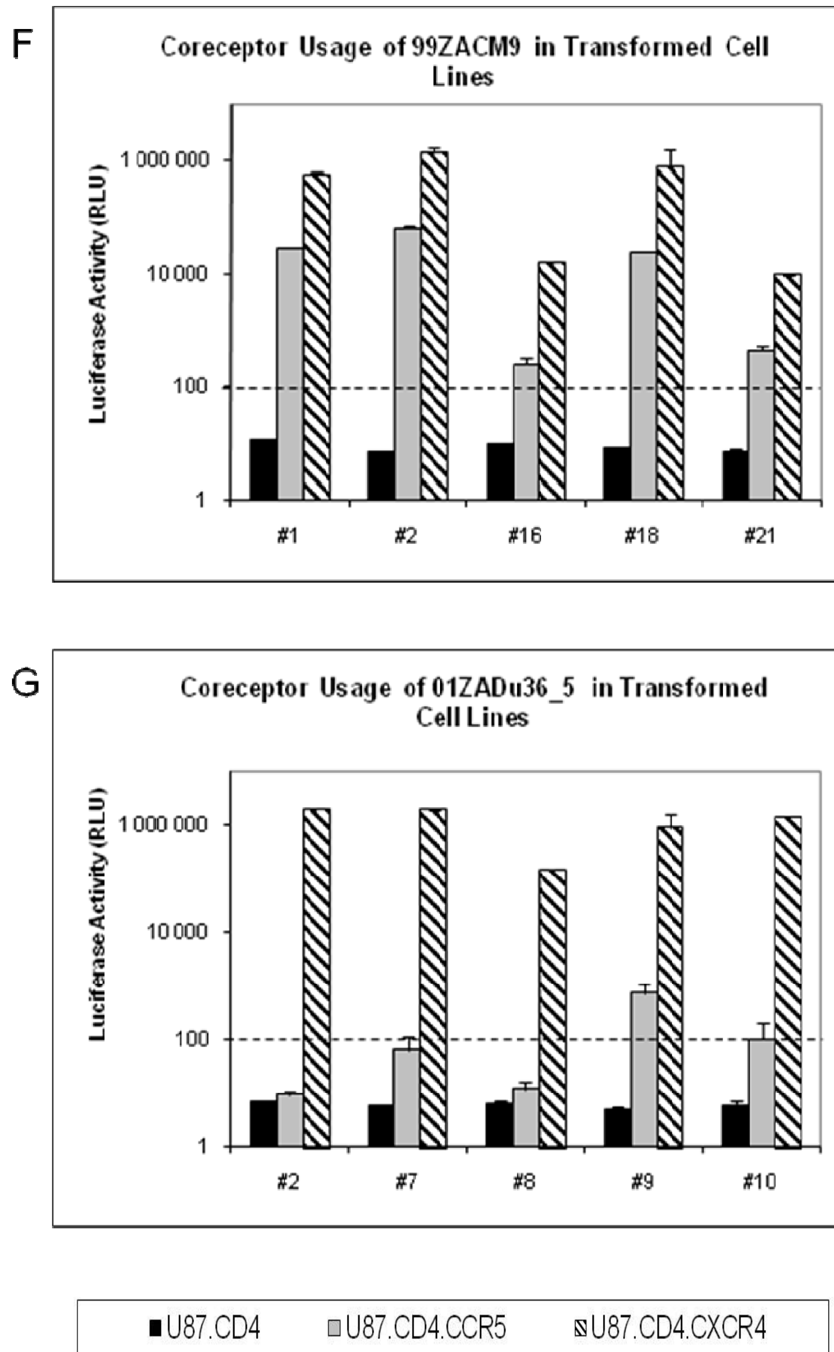
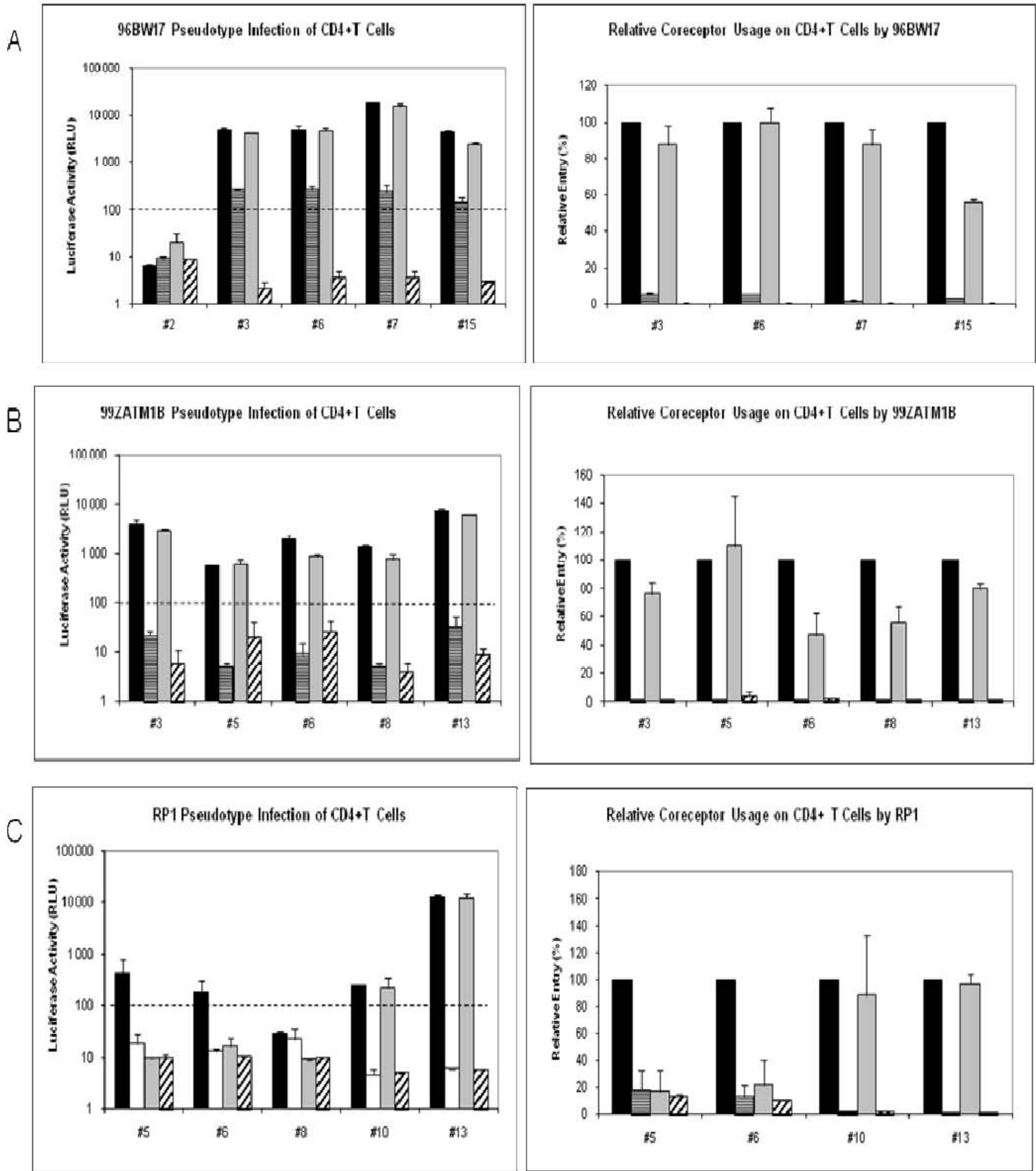


Figure 3.2: Coreceptor usage in transformed cell lines

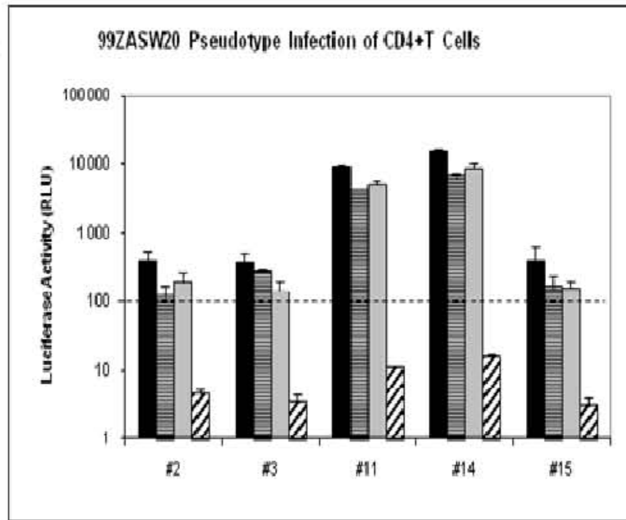
Figure 3.2 indicates the coreceptor usage characterization of each of the clones of the dual tropic viral isolates. Majority of the clones (57%) exhibit dualtropism whereas 43% show exclusive usage of CXCR4. No R5 monotropic clones were observed. The results are expressed in relative light units (RLU) and represent means +/- standard error (SEM) for experiments performed in duplicate. RLU of 100 was used as the cut-off value. Values greater than 100 RLU was regarded as infectious. A-G represent the coreceptor usage of clones of each isolate i.e the clones of each isolate are represented separately in each figure.

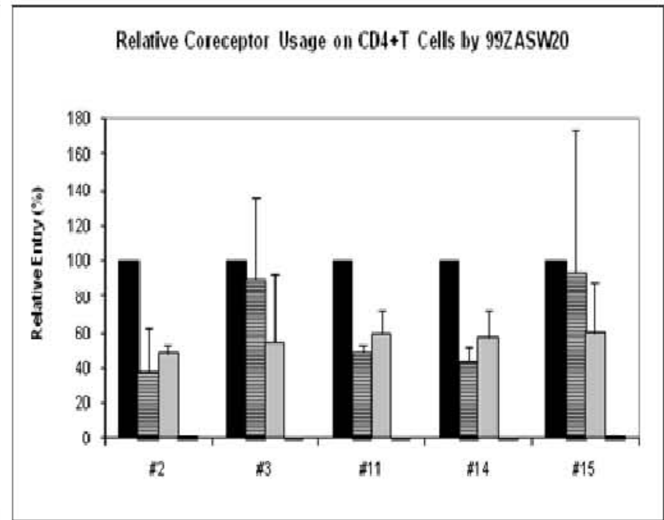
3.3.2. Coreceptor Usage in CD4+ T Lymphocytes and Monocyte Derived Macrophages

Transformed cell systems are conventionally used to establish virus coreceptor utilization profiles, however, the major targets of HIV infection *in vivo* are primary CD4+ lymphocytes and macrophages (Yi et al. 2005). Therefore, coreceptor usage of the previously characterized HIV-1 subtype C *env* clones in CD4+ T lymphocytes and monocyte derived macrophages was investigated. To determine the pathways utilized on primary cells, the CXCR4 antagonist AMD3100, the CCR5 antagonist maraviroc and a combination of these two inhibitors was used. For each inhibition assay experiment, not only the pathways of infection used but also the coreceptor utilization preference of the pseudoviruses as calculated relative to the entry of cells not blocked by antagonists was examined. The pathways of infection used by the 35 clones in CD4+ T lymphocytes and monocyte derived macrophages are shown in Figures 3.3 and 3.4 respectively.


A
96BW17 Pseudotype Infection of CD4+T Cells
Luciferase Activity (RLU)
#2
#3
#6
#7
#15
Relative Coreceptor Usage on CD4+T Cells by 96BW17
Relative Entry (%)
B
99ZATM1B Pseudotype Infection of CD4+T Cells
#5
#8
#13
Relative Coreceptor Usage on CD4+T Cells by 99ZATM1B
C
RP1 Pseudotype Infection of CD4+T Cells
#10
Relative Coreceptor Usage on CD4+ T Cells by RP1

D





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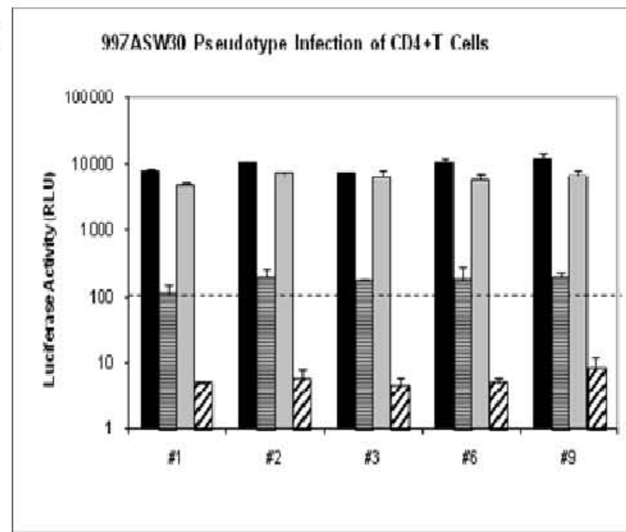

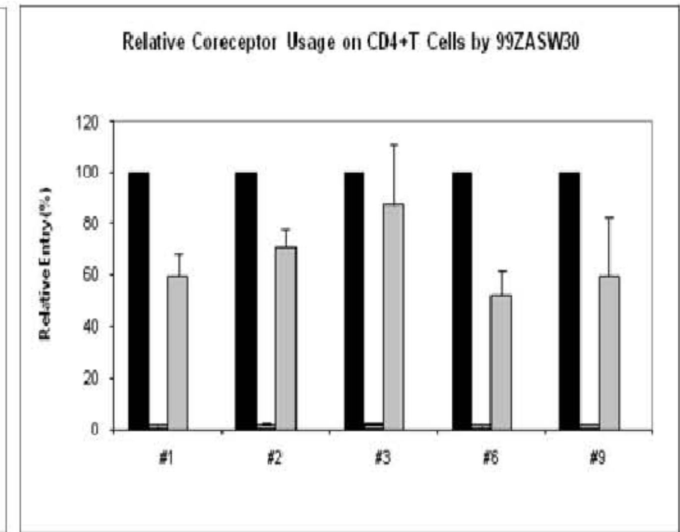

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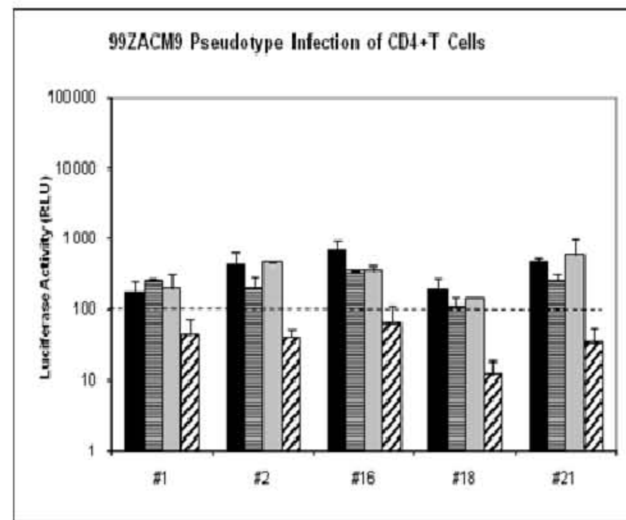
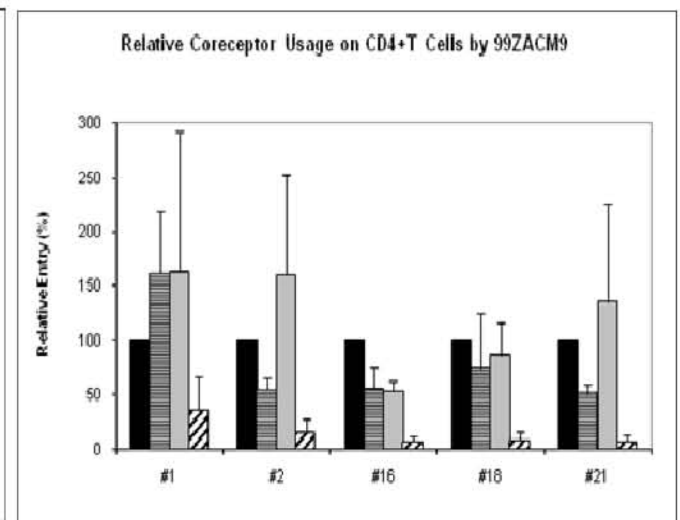




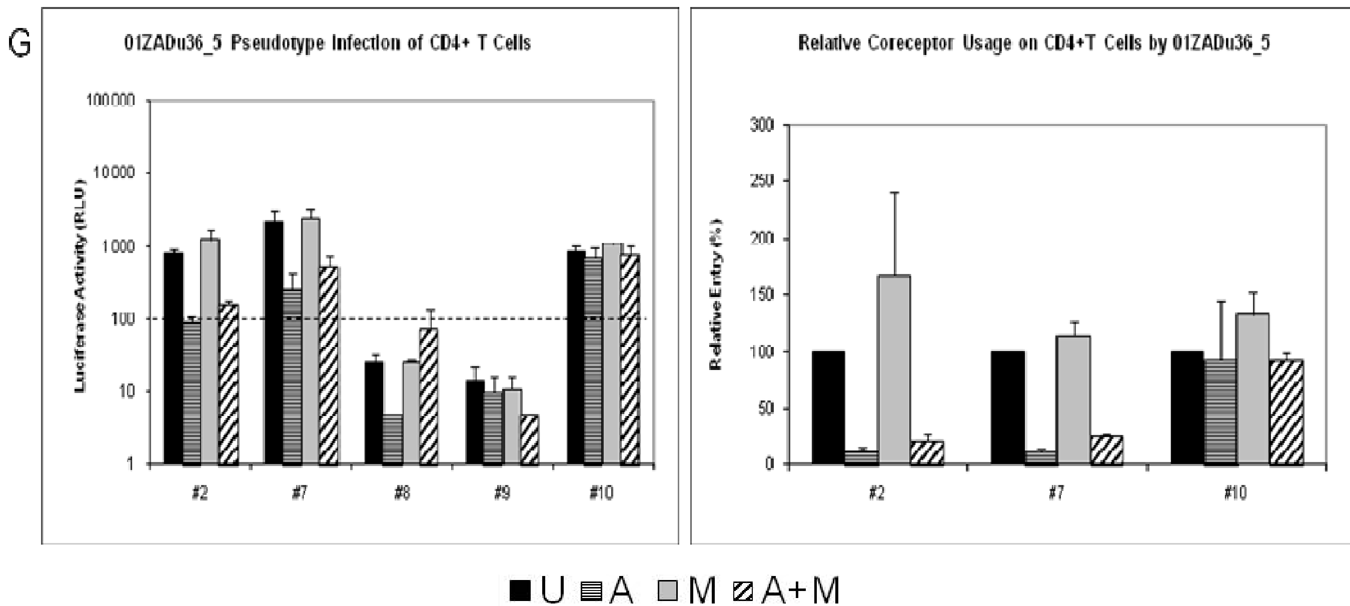
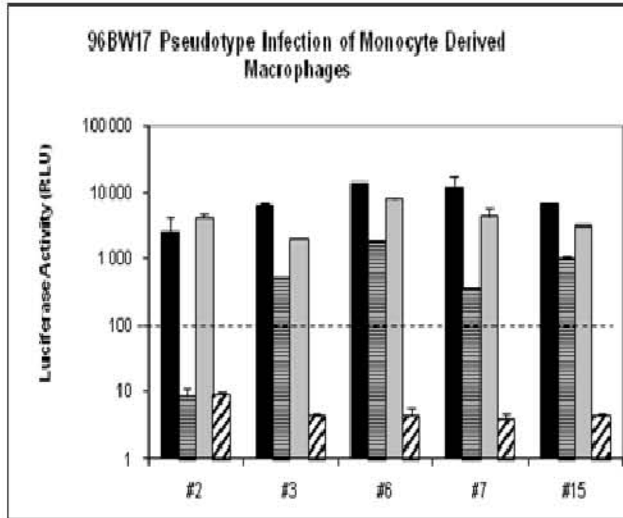
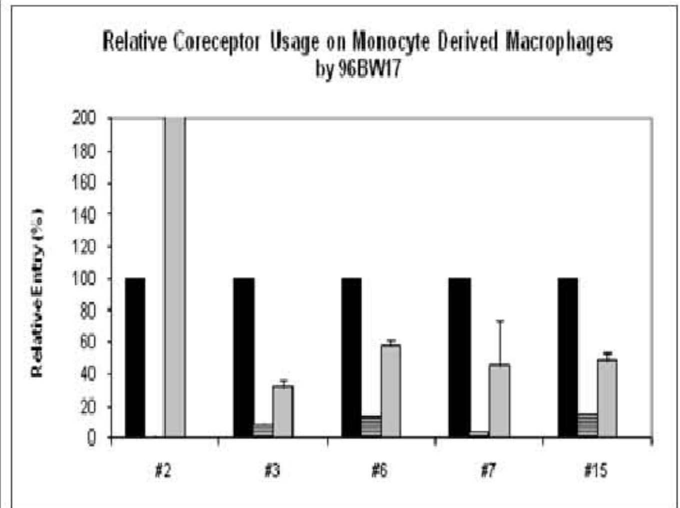


Figure 3.3: Viral infection of CD4+ T cells

The panel on the left indicates the pathways of infection used by the respective virus i.e. the coreceptor pathways used for cell entry. Data are means +/- SEM of duplicate wells and representative of 2 separate experiments using lymphocytes from different donors. Results shown for each virus are presented in RLU where a RLU of 100 was used as the cut-off value. Values greater than 100 RLU was regarded as infectious. The panel on the right shows the coreceptor pathway preference of the particular virus as calculated relative to the entry of untreated cells i.e. as a percentage of the RLU seen in the absence of coreceptor blockers. Those that were not infectious were excluded from the graphs illustrating coreceptor preference (right panel). A-G represent the infection of clones of each isolate i.e the clones of each isolate are represented separately in each figure. U – cells that are untreated, A – cells that are treated with AMD3100, M – cells that are treated with maraviroc and A+M – cells that are treated with a combination of AMD3100 and maraviroc.

A





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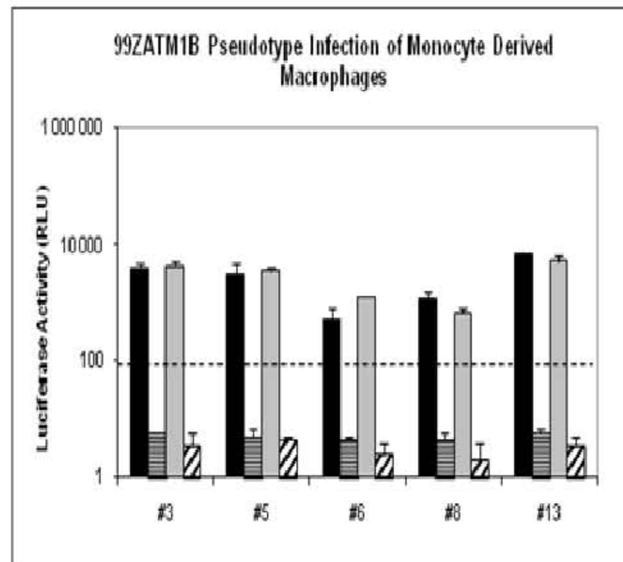

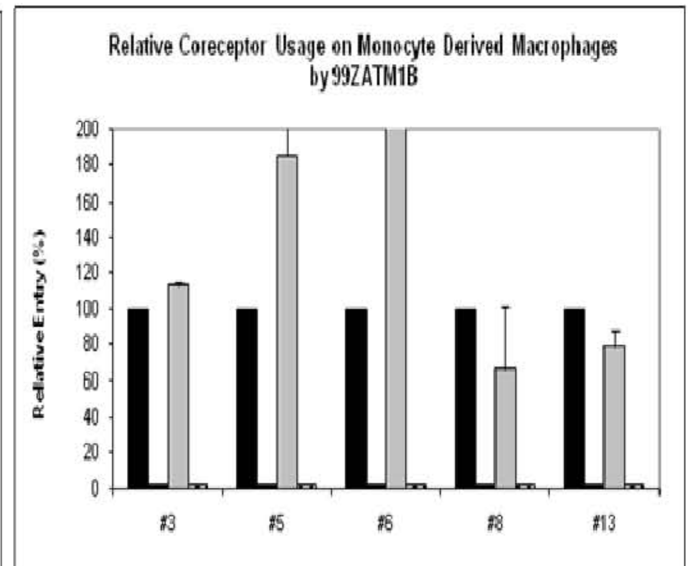

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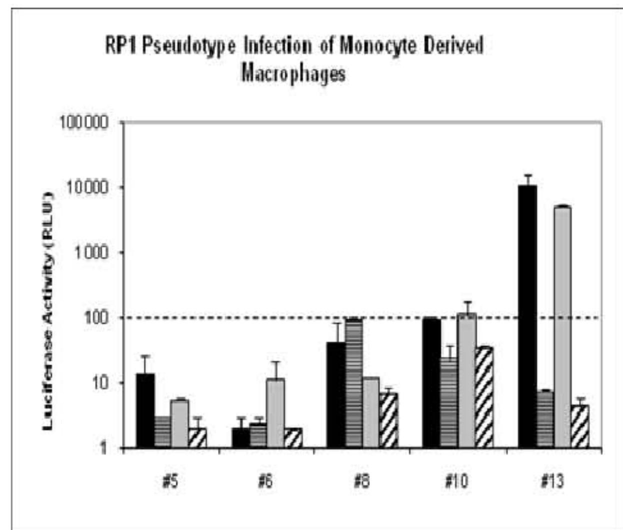

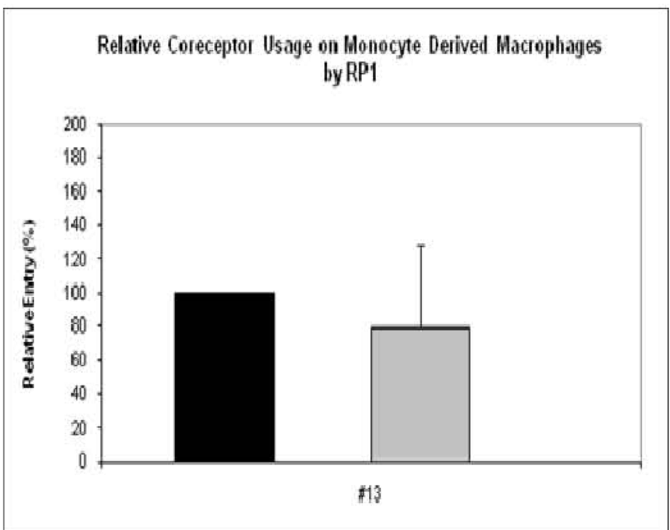

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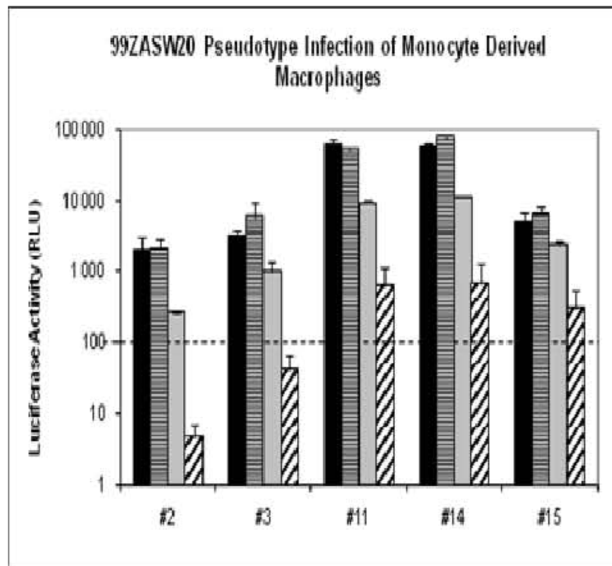

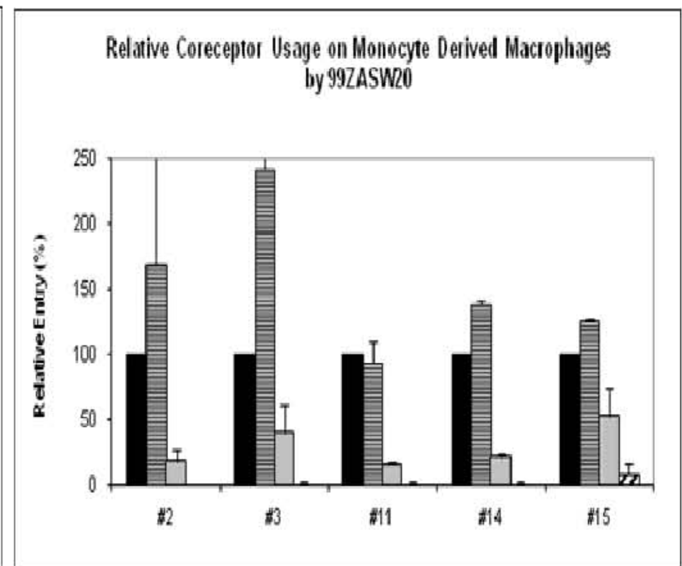

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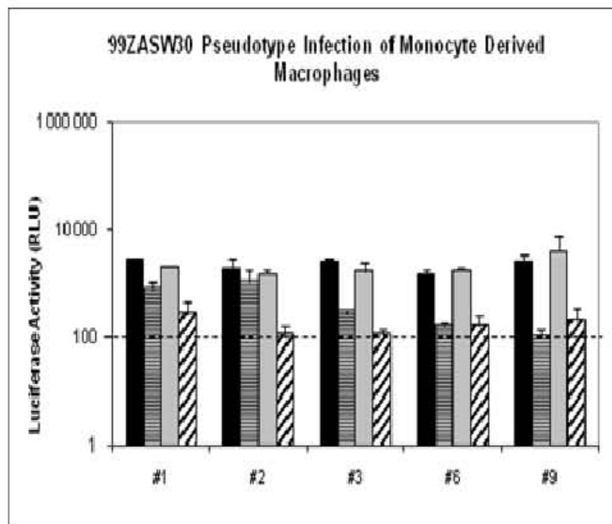

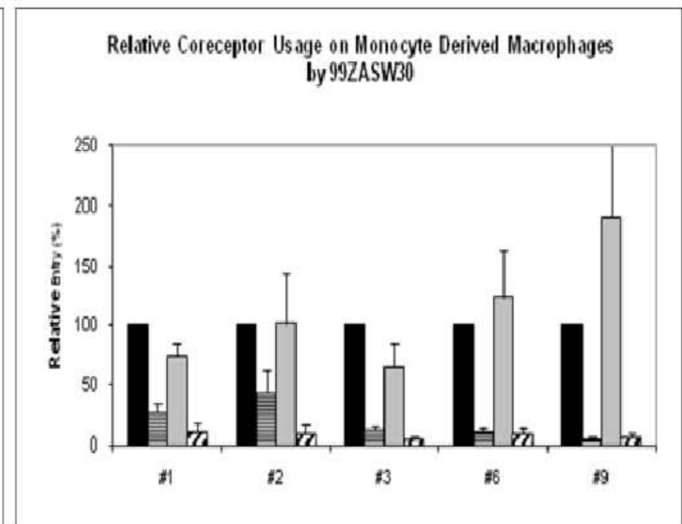

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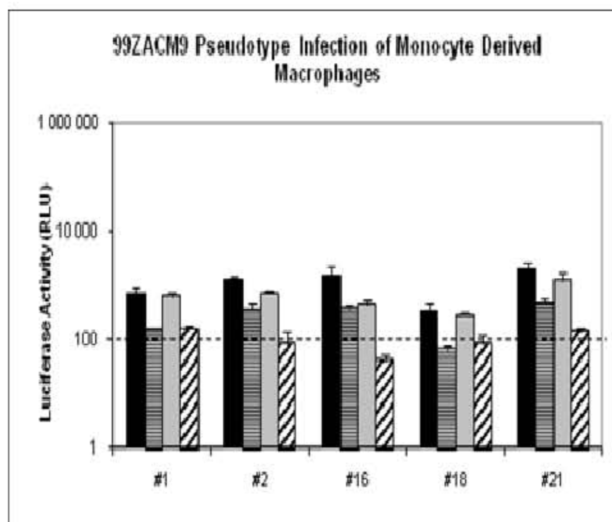
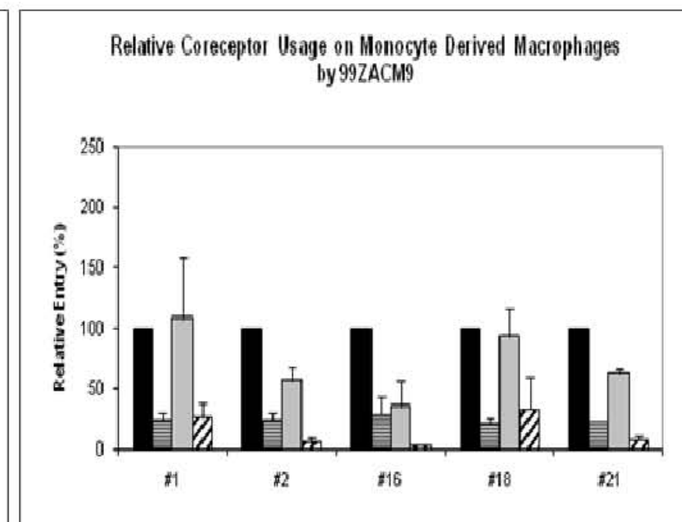




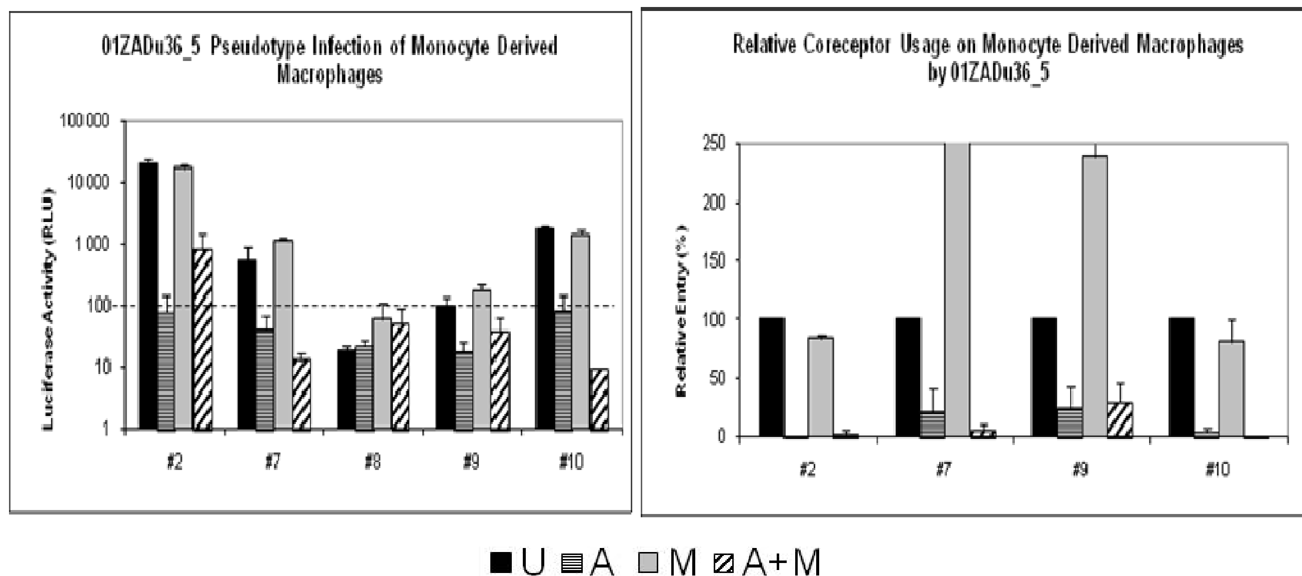


Figure 3.4: Viral infection of monocyte derived macrophages

The panel on the left indicates the pathways of infection used by the respective virus i.e. the coreceptor pathways used for cell entry. Data are means +/- SEM of duplicate wells and representative of 2 separate experiments using monocyte derived macrophages from different donors. Results shown for each virus are presented in RLU where a RLU of 100 was used as the cut-off value. Values greater than 100 RLU was regarded as infectious. The panel on the right shows the coreceptor pathway preference of the particular virus as calculated relative to the entry of untreated cells i.e. as a percentage of the RLU seen in the absence of coreceptor blockers. Those that were not infectious were excluded from the graphs illustrating coreceptor preference (right panel). A-G represent the infection of clones of each isolate i.e the clones of each isolate are represented separately in each figure. U – cells that are untreated, A – cells that are treated with AMD3100, M – cells that are treated with maraviroc and A+M – cells that are treated with a combination of AMD3100 and maraviroc.

As shown in Figure 3.3, most clones from each dual tropic viral isolate showed a similar pattern of CD4+ T lymphocyte infection. The panel on the left hand side shows the extent of CD4+ T lymphocyte infection in the absence of coreceptors antagonists, in the presence of AMD3100, maraviroc and both coreceptor inhibitors combined in order to decipher the coreceptor pathway(s) used for cell entry. AMD3100 is a CXCR4 antagonist and blocks CXCR4 entry into cells. Maraviroc is a CCR5 antagonist thereby preventing entry of CCR5 into cells. The panel on the right hand side displays coreceptor preference as calculated by determination the relative amount of luciferase in cells with

and without the coreceptor antagonist. Four of 5 clones from 96BW17 used both CCR5 and CXCR4 with a preference for CXCR4 whereas 96BW17 #2 was not infectious in CD4+ T lymphocytes. All clones from 99ZATM1B used only CXCR4 for entry into CD4+ T lymphocytes. Clones # 5, 6, 10 and 13 from RP1 were infectious in CD4+ T lymphocytes. Blockade with either of the coreceptor antagonists significantly reduced infectivity for clones 5 and 6 suggesting co-dependency on both coreceptors. Clones RP1 # 10 and 13 were X4. All clones of 99ZASW20 used both CCR5 and CXCR4 equally while clones of 99ZASW30 were R5X4 although CXCR4 preference was observed for all of them. Clones from 99ZACM9 were R5X4 with clones # 1 and 16 using both coreceptors equally with the remaining 3 displaying preferential CXCR4 utilization. Two clones of 01ZADu36_5 were noninfectious in CD4+ T lymphocytes while the remaining 3 used CXCR4 only. Figure 3.3 (right panel) shows that CXCR4 is the preferred pathway used by clones from most isolates (96BW17, 99ZATM1B, RP1, 99ZASW30 and 01ZADu36_5) in CD4+ T lymphocytes as blocking with maraviroc (CCR5 antagonist) did not significantly impact the degree of cell entry, whereas blocking with the CXCR4 antagonist AMD3100 did relative to unblocked cells. For clones from some isolates, CXCR4 use predominates with little contribution coming from CCR5 mediated infection (96BW17, 99ZASW30). Two clones from one isolate (99ZACM9 # 2 and 21) exhibit a preference for CXCR4, but CCR5 use by these clones also noticeably contributes to CD4+ T cell infection. Only two isolates (99ZASW20 and 99ZACM9 # 1 and 16) had clones with approximately equal preference for both CCR5 and CXCR4.

Overall the majority of clones infected CD4+ T cells using both the CXCR4 and CCR5 pathways, however CXCR4 was typically used more efficiently than CCR5. In contrast, clones from 2 isolates displayed nearly equal CCR5 and CXCR4 usage. Additionally,

some clones infected CD4+ lymphocytes exclusively using CXCR4, while a number of clones were very poorly infectious or did not infect CD4+ T cells at all. As expected, viruses that were X4 on the U87 cell line (99ZATM1B, RP1 and clones # 2 and 7 from 01ZADu36_5) used CXCR4 exclusively on primary CD4+ lymphocytes. Surprisingly, when a combination of inhibitors was used, cell entry was still observed in clones from isolates 99ZACM9 and 01ZADu36_5 indicating that the use of both antagonists concurrently did not efficiently inhibit viral entry. Similar to the results obtained on U87 cells, no virus displayed exclusive CCR5 mediated entry and infection in CD4+ T lymphocytes.

Most clones from each dual tropic viral isolate showed a similar pattern of infection in monocyte derived macrophages (MDM) (Figure 3.4). All but one clone (#2) from 96BW17 displayed the R5X4 phenotype with a preference for CXCR4. 96BW17 # 2 was exclusively X4. All clones from 99ZATM1B displayed exclusive CXCR4-utilization. Majority of clones from RP1 were noninfectious in macrophages except for clone #13 which showed some CXCR4 usage. All clones from 99ZASW20 were R5X4 in macrophages with a preference for CCR5. Three clones from 99ZASW30 (clones #3, 6, 9) used only CXCR4 while 2 (clones #1 and 2) used both CCR5 and CXCR4 with more efficient CXCR4 usage. Three clones from 99ZACM9 (clones #2, 16, 21) used both CCR5 and CXCR4 with a preference for CXCR4 while 2 (clones #1 and 18) clones used only CXCR4. One clone of 01ZADu36_5 (clone #8) was not infectious in macrophages while the remaining 4 were CXCR4-utilizing. Overall, the majority of clones infected macrophages using both CXCR4 and CCR5. CXCR4 usage by many of these clones was more efficient than CCR5 use (96BW17 # 3, 6, 7 and 15, 99ZASW30 # 1 and 2 and 99ZACM9 # 2, 16 and 21). Additionally, a number of clones from five isolates

(99ZATM1B and 01ZADu36_5 and clones 96BW17 # 2, 99ZASW30 # 3, 6 and 9, 99ZACM9 # 1 and 18, RP1 # 13) show no contribution of CCR5-usage to macrophage entry. In contrast to the results for CD4+ T lymphocytes where no clones showed more efficient entry through CCR5 compared to CXCR4, clones from one isolate (99ZASW20) displayed more efficient CCR5 utilization on macrophages. This was in contrast to the coreceptor phenotype for this isolate on CD4+ T lymphocytes where both pathways were used approximately equally.

In a similar divergence, clones from two isolates used CXCR4 exclusively on macrophages although these clones infect CD4+ T lymphocytes using both coreceptors (99ZASW30 # 3, 6 and 9 and 99ZACM9 # 1 and 18), while another CXCR4 using clone was noninfectious on lymphocytes (96BW17 # 2). Again, clones that were X4 using on U87 cells (99ZATM1B, RP1 and 01ZADu36_5) used CXCR4 exclusively on macrophages as well. With the exception of one clone (RP1 #13), viruses that were minimally infectious or noninfectious on CD4+ lymphocytes (RP1 # 5, 6 and 8 and 01ZADu36_5 # 8) were also not infectious in MDM. Exclusive CCR5 usage was not observed in MDM similar to the observation in CD4+ T lymphocytes.

Interesting patterns emerged when infection in the presence of both antagonists on CD4+ lymphocytes and macrophages was compared. When a combination of inhibitors was used, entry into macrophages was reduced for all clones of 99ZACM9, 99ZASW30, 01ZADu36_5 #2, 99ZASW20 #11, 14 and 15. Clones from isolate 99ZACM9 displayed a similar pattern of apparent resistance in both cell types however, for the clones from the other isolates poor inhibition seen in the presence of both antagonists was cell type

dependent. All clones of isolate 01ZADu36_5 appeared to be resistant to inhibition on CD4+ lymphocytes, but only clone # 2 had a similar phenotype on macrophages. Alternatively, clones from isolates 99ZASW20 and 99ZASW30 were inhibited in CD4+ T lymphocytes when both antagonists were used, yet complete inhibition was not observed in monocyte derived macrophages.

Recent reports have shown that some primary isolates are naturally resistant to AMD3100 (Harrison et al. 2008), and in the experiments carried out there were clones that were not completely inhibited in the presence of AMD3100 and maraviroc, which led to the hypothesis that these viruses may be resistant to these coreceptor antagonists. An alternative explanation is that these clones that appear resistant to CXCR4 and CCR5 antagonists use alternate coreceptors in primary cells. In order to determine whether these pseudovirus clones were resistant to the coreceptor antagonists as observed in primary cells, we performed inhibition assays (as done in 3.2.3) in the U87.CD4 cell line expressing either CCR5 or CXCR4. For this experiment, a virus that appeared to be resistant to AMD3100 (01ZADu36_5 # 10) as well as one clone each from the other isolates that displayed incomplete inhibition (99ZASW20 #14, 99ZASW30 #1 and 99ZACM9 #16) was selected. Also included were the remaining clones derived from 01ZADu36_5 as well as a virus that appeared to be sensitive to inhibition by the antagonists (96BW17 # 15). An R5 control JRFL and X4 control TYBE (Collman et al. 1992; Yi et al. 2003) were used in parallel. Those viruses that exhibited partial or complete resistance in the primary cell lines also exhibited a similar resistance profile in the transformed cell lines, and the virus that was inhibited was also blocked on U87.CD4.CXCR4 cells. Furthermore, the pattern of infection was the same irrespective of the concentration of the drug used (Figure 3.5A). These observations indicate that

some of these viruses truly exhibit resistance to AMD3100. Three viruses that were R5X4-using in transformed cell lines (01ZADu36_5 #9, 99ZASW30 #1 and 99ZASW20 #14) were also tested for resistance to maraviroc in the U87.CD4.CCR5 cell line. 01ZADu36_5 #9 displayed resistance to inhibition by maraviroc (Figure 3.5B).

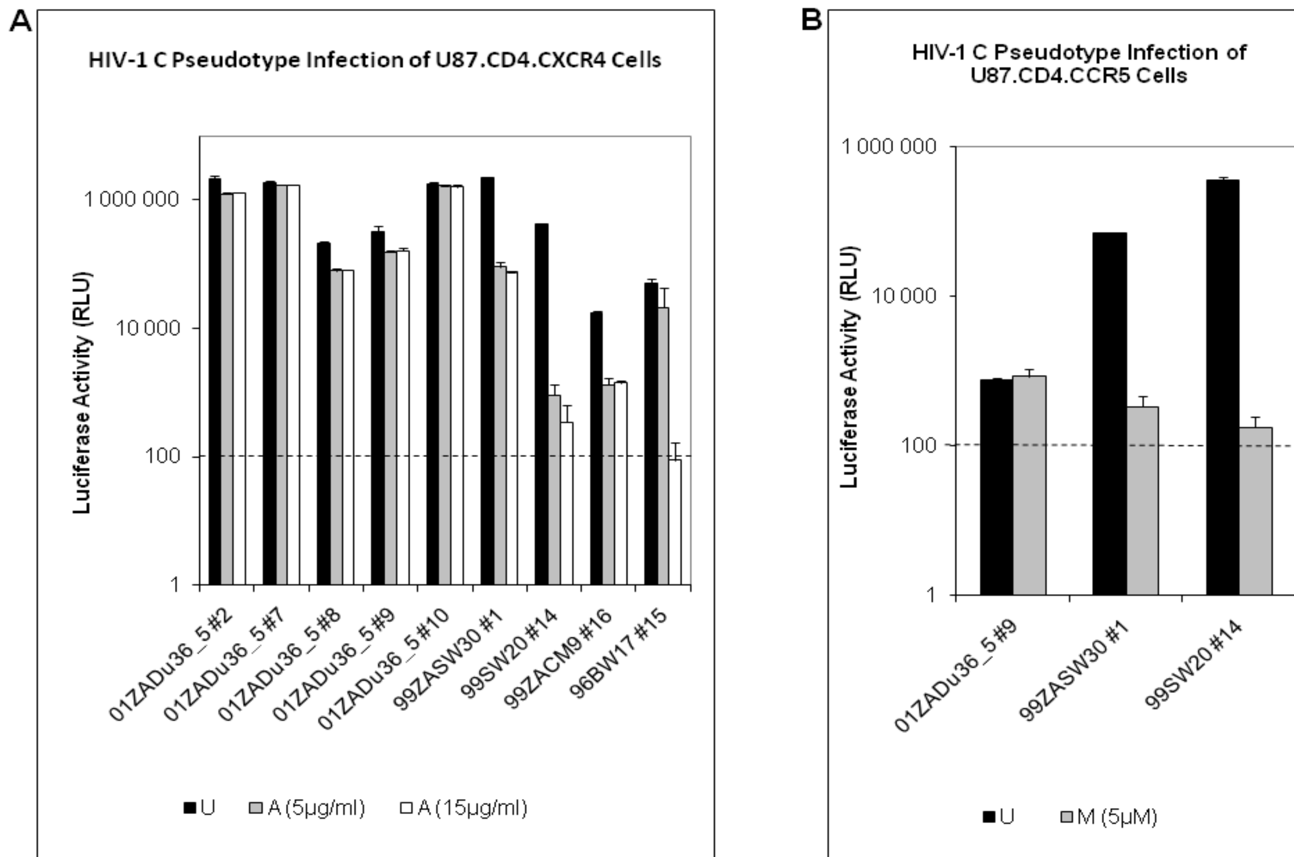


Figure 3.5: Viral infection of U87.CD4.CXCR4 cells in the presence of AMD3100 and U87.CD4.CCR5 cells in the presence of maraviroc

A) Nine virus clones were selected and tested for resistance to CXCR4 antagonist AMD3100. U87.CD4 cells expressing CXCR4 were infected in the presence of two different concentrations of AMD3100 (5 and 15 µg/ml). B) Three R5X4-using virus clones were selected and tested for resistance to CCR5 antagonist maraviroc. U87.CD4 cells expressing CCR5 were infected in the presence of maraviroc (5 µM). Results shown for each virus are presented in RLU where a RLU of 100 was used as the cut-off value. Values greater than 100 RLU was regarded as infectious. U – cells that are untreated, A (5 and 15 µg/ml) – cells that

are treated with 5 and 15 µg/ml AMD3100 respectively, M (5 µM)– cells that are treated with 5 µM maraviroc.

3.3.3. Genotypic Analysis

Full-length *env* sequences of all the clones analyzed here were previously reported (2.3.3). Here it was investigated whether coreceptor usage in primary cells was associated with specific *env* sequence characteristics. Since the V3 loop region has been shown to be an important determinant of coreceptor usage we first focused on this region. There were no obvious *env* V3 loop sequence characteristics that distinguished between clones with differential capability to mediate entry into primary cells. The full *env* sequence was also analyzed to determine if any patterns that correlated with preferential coreceptor usage in primary cells or primary cell infectivity could be observed. There were no obvious amino acid differences that correlated with preferential coreceptor utilization or infectivity in primary cells for clones from isolates 96BW17, 99ZATM1B, 99ZASW20, 99ZASW30 and 99ZACM9. However, there were numerous amino acid sequence changes throughout the *env* noted in 01ZADu36_5 #8, the only clone of isolate 01ZADu36_5 that was noninfectious in both primary cell types although the number of these amino acid differences made it difficult to pinpoint residues responsible for the unique phenotype of clone #8 in primary cells.. RP1 #13 was the only clone of isolate RP1 displaying CXCR4-usage in both CD4+ T lymphocytes and MDM. In contrast, RP1 #10 was CXCR4-using in CD4+ T lymphocytes and noninfectious in MDM whereas clones #5, 6 and 8 were noninfectious in both cell types. Differences in amino acid residues at various positions were observed for RP1 #10 which had threonine (T) at position 591, proline (P) at position 612 and asparagine (N) at position 618 whereas all other clones of this isolate had methionine (M) at position 591 and serine (S) at both positions 612 and 618 (Figure 3.6A).

A

HxB2 position	591																					612						618		
RP1 Consensus	L	M	D	Q	Q	L	L	G	M	W	G	C	S	G	K	L	I	C	T	T	A	V	P	W	N	S	S	W	N	N	R
RP1 #10	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	-	-	-	S	-	-

B

HxB2 position	31 34 44	238 248	332	443
99ZASW20 consensus	MGNLWVTVYYGVPVWT	NNKTFNGTGPCHNVST	IGDVRQ	PPIAGN
99ZASW30 consensus	VGLWVTVYYGVPVWRE	NVSTVQCTHGIKPVVS	EKDWNK	TSNITG
99ZACM9 consensus	NLWVTVYYGVPVWKEA	FNGTGPCNNVSTVQCT	AHCNIS	GNITCT
01ZADu36_5 #2,7 & 9	NLWVTVYYGVPVWKEA	NVSTVQCTHGIKPVVS	PDWNET	GGTTNG
01ZADu36_5 #8	GGNLWVTVYYGVPVWK	CTNVSTVQCTHGIKPV	SPPDWN	TRDGGN
01ZADu36_5 #10	NLWVTVYYGVPVWKEA	FNGTGPCNNVSTVQCT	RPDWNE	DGGTTN

Figure 3.6: Genotypic analysis

A and B) Differences in amino acids at various positions are represented with the HxB2 positions indicated above.

C

Strain	**V3 Sequence**	**CD4+PBL% CCR5 use***	**CD4+MDM% CCR5 use***	**PSSM Phenotype****
R-5 only virus 96BWM01_5	CTRPNNNTRKSIRI..GPGQTFYATGDIIGDIRQAHC	100	100	0
96BW17#2	-----------M--GIGRGQ----M-R----------	NI***	0.3	1
96BW17#3	-----------M--GIGRGQ----M-R----------	5	8	1
96BW17# 6	-----------M--GIGRGQ----M-R----------	6	14	1
96BW17#7	-----------M--GIGRGQ----M-R----------	1	3	1
96BW17#15	-----------M--GIGRGQ----M-R----------	3	15	1
99ZATM1B#3	----------NV--GIGRGQ----N-----N------	0.5	0.2	1
99ZATM1B#5	----------NV--GIGRGQ----N-----N------	0.8	0.2	1
99ZATM1B#6	----------NV--GIGRGQ----M-R---N------	0.4	0.8	1
99ZATM1B#8	----------NV--GIGRGQ----N-----N------	0.4	0.3	1
99ZATM1B# 13	----------NV--GIGRGQ----N-----N------	0.5	0.1	1
RP1# 5	-I--G-----RV-LGIGPGQ------RV-R-------	4	NI***	1
RP1# 6	-I--G-----RV-LGIGPGQ------RV-R-------	7	NI***	1
RP1#8	-I--G-----RV-LGIGPGQ------RV-R-------	NI***	NI***	1
RP1# 10	-I--G-----RV-LGIGPGQ------RV-R-------	2	NI***	1
RP1# 13	-I--G-----RV-LGIGPGQ------RV-R-------	0.05	0.1	1
99ZASW20#2	-------------TGIGRGQ------Q---V-----	30	42	1
99ZASW20#3	-------------TGIGRGQ------Q---V-----	72	60	1
99ZASW20# 11	-------------TGIGRGQ------Q---V-----	48	75	1
99ZASW20# 14	-------------TGIGRGQ------Q---V-----	43	75	1
99ZASW20# 15	-----------V-IGIGRGHA--T—KV--N-------	42	80	1
99ZASW30#1	-----------V--GIGRGHA--T-GKV--N------	1	28	1
99ZASW30#2	-----------V--GIGRGHA--T-GKV--N------	2	53	1
99ZASW30#3	-----------V--GIGRGHA--T-GKV--N------	2	12	1
99ZASW30#6	-----------M--GIGRGHA--T-GKV--N------	2	10	1

99ZASW30#9	-----------V--GIGRGHA--T-GKV--N------	2	3	1
99ZACM9 #1	-A--G---I-R---..GPRYA---KET----------	49	21	1
99ZACM9 #2	-A--G---I-R---..GPRYA---KET----------	50	25	1
99ZACM9#16	-A--G---I-R---..GPRYA---KET----------	49	23	1
99ZACM9#18	-A--G---I-R---..GPRYA---KET----------	56	19	1
99ZACM9#21	-A--G---I-R---..GPRYA---KET----------	52	23	1
01ZADu36_5#2	----D-KINMKRIKI.GPGRA-V--KG-R---R--Y-	12	0.3	1
01ZADu36_5#7	----D-KINMKRIKI.GPGRA-V--KG-R---R--Y-	12	7	1
01ZADu36_5#8	----D-KINMKRIKI.GPGRA-V--KG-R---R--Y-	NI***	NI***	1
01ZADu36_5#9	----D-KINMKRIKI.GPGRA-V--KG-R---R--Y-	NI***	17	1
01ZADu36_5#10	----D-KINMKRIKI.GPGRA-V--KG-R---R--Y-	41	4	1

Figure 3.6: Genotypic analysis

C) V3 sequence alignment and viral phenotype prediction. * Percentage of total entry in the presence of CXCR4 blocking. ** Predicted coreceptor phenotype based on R5X4 PSSM algorithm. 0=R5, 1=X4 or R5X4. ***NI=Non-infectious.

Full *env* sequence analysis revealed that no major amino acid changes were observed in clones 99ZASW20 #3, 11, 12 and 15 and clones from 99ZASW30 where drug resistance in macrophages was seen. These clones all had amino acid valine (V) at position 248 and aspartic acid (D) at position 332. Valine was also present at position 248 in 01ZADu36_5 #2 and 9 which also showed resistance to inhibition in macrophages. The same amino acid substitution was present in 01ZADu36_5 #7 which showed resistance in CD4+ T lymphocytes. Aspartic acid was also located at position 332 in 01ZADu36_5 # 10, where resistance to inhibition was seen in CD4+ T lymphocytes. However, clones from 99ZACM9 displayed resistance to inhibition in macrophages but did not possess the above mentioned amino acids at the respective positions, suggesting a complex and subtle basis for resistance.

It was also investigated whether any similarities could be found between clones from 99ZACM9, 99ZASW20 and 99ZASW30 in which resistance to inhibition by a combination of antagonists was observed in macrophages. Amino acid threonine (T) at position 238 was a common feature of all these clones. This was also observed in 01ZADu36_5 # 2 and 9 which showed resistance in macrophages and #7 where resistance was seen in CD4+ T lymphocytes. Clones from 99ZACM9 and clones 01ZADu36_5 #2, 7 and 10 all displayed resistance to inhibition in CD4+ T lymphocytes. All of these clones have shared unique amino acids of leucine (L) at position 31, threonine (T) at position 34, glutamic acid (E) at position 44 and threonine (T) at position 443. Clone #8 of 01ZADu36_5 which was noninfectious in both cell types and all other clones did not have these amino acids located at these respective positions (Figure 3.6B).

The relationship between V3 sequences and CD4+ lymphocyte and monocyte derived macrophage coreceptor preference was further investigated (Figure 3.6C). A substantial percentage of CCR5-usage was seen in both primary cell types despite C-PSSM prediction being X4/R5X4 for clones of 99ZASW20 and 99ZACM9. Significant CCR5 usage was also noted for 99ZASW30 #1 and 2 and 01ZADu36_5 #10 in monocyte derived macrophages, despite a C-PSSM score predictive of X4/R5X4 phenotype. For most clones a C-PSSM score of 1 indicating X4/R5X4-usage correlated with the calculated percentage of CCR5-use in both CD4+ T lymphocytes and monocyte derived macrophages.

3.4. Discussion

Chemokine receptors play an important role in the initiation of HIV-1 infection at the cellular level. Chemokine receptor utilization is also an important determinant of the rate of disease progression. Due to the emerging availability of entry inhibitors such as the CCR5 antagonists for the clinical management of HIV-1 infection, it has become increasingly important to better characterize coreceptor utilization and cellular tropism by HIV-1 isolates as well as to understand the mechanisms underlying coreceptor switch and evolution in the course of infection particularly in regions heavily affected by HIV/AIDS. Previously, 35 full-length *env* clones from seven dual tropic isolates of HIV-1 subtype C were generated. It was found that in transformed cell lines that express either CXCR4 or CCR5, most of the pseudovirus clones from these isolates were CXCR4 utilizing (85.7%), with a minority of R5X4 clones (14.3%) and virtually no R5 clones detected (refer to 2.3.3). Here, the coreceptor utilization profile of these clones using the spinnoculation method of cell infection was re-evaluated and based on this approach, 57% of the clones were R5X4 and 43% were X4. However, even dual tropic clones generally preferred CXCR4 usage. Nevertheless, both approaches confirmed the absence of R5-only pseudovirus clones from the 35 clones generated from the 7 dual tropic isolates. For a significant majority of clones, results were exactly the same in spite of the methodology used for infection. The finding that the spinnoculation method resulted in higher infectivity of U87.CD4.CCR5 cells is consistent with the observation by O'Doherty and colleagues (O'Doherty et al. 2000) where it was noted that the spinnoculation method which involves allowing cell/virus contact by centrifugation at 1,200 x g for 2 hours is better at facilitating productive cell infection. This study shows that differences in coreceptor usage classification can be obtained from using different methodologies to infect target cells and highlight the need for caution in interpreting and comparing data across studies that use varied infection protocols.

It is generally assumed that virus coreceptor usage determination in transformed cells predicts coreceptor preference *in vivo* where the main target cells of HIV-1 are primary lymphocytes and macrophages although significant differences have previously been reported (Yi et al. 2005). In addition, genotypic methods do not always predict coreceptor usage especially in distinguishing X4 and R5X4 viruses (Garrido et al. 2008; Low et al. 2007). Data on coreceptor usage in primary cells and genotypic prediction algorithms is particularly lacking for HIV-1 subtype C which is the predominant subtype globally and which appears to be unique in coreceptor evolution.

In the current study therefore, the coreceptor utilization profile of HIV-1 subtype C clones in transformed cell lines was compared to the coreceptor utilization profile in primary CD4+ T lymphocytes and monocyte derived macrophages. HIV-1 subtype C Env clones determined to be R5X4 or X4 in transformed cell lines used either both the CCR5 and CXCR4 or exclusively the CXCR4 pathway for entry. The coreceptor utilization profiles were the same in transformed cell lines and in both primary cell types, CD4+ T lymphocytes and MDM for 20 of 35 (57%) clones. One clone (2.9%) was dual tropic (R5X4) in transformed cell lines but CXCR4-only using in both primary cell types. One clone (2.9%) was X4 in U87 cells and CD4+ T lymphocytes but was not infectious in MDM. Five clones (14.3%) were R5X4 in U87 cells and CD4+ T lymphocytes but used only CXCR4 in MDM. Two clones (5.7%) were CXCR4-using in transformed cell lines but were R5X4 in both primary cell types. Two clones (5.7%) that were R5X4 in U87 cells used CXCR4 only in MDM but were not infectious in CD4+ T lymphocytes. The remaining four (11.4%) of 35 clones showed CXCR4 usage in transformed cell lines but were non-infectious in both primary cell types. Thus while results showed that in a majority of cases (57%) there is a concurrence in coreceptor utilization profile between transformed and primary cells, there was discordance between

results obtained in transformed versus primary cells in 43% of cases. These results underline the need to further study the determinants of coreceptor usage in primary cells since coreceptor antagonists will need to prevent infection of target cells *in vivo*.

When both the CCR5 and CXCR4 pathways were utilized for entry there was a striking bias towards CXCR4 preference in both CD4+ T lymphocytes and MDM for the majority of the pseudoviruses as was the case in transformed cell lines. This observation, combined with the observation that X4 and dual tropic but nor R5 clones were predominant among dual tropic isolates are consistent with the suggestion that CCR5 coreceptor utilization is rapidly lost as dual tropic isolates emerge during HIV-1 infection (Coetzer et al. 2008). However, it is worth noting that clones from isolate 99ZASW20 used both CCR5 and CXCR4 almost equally in CD4+ T lymphocytes and were biased towards CCR5 in MDM. Two clones derived from isolate 99ZACM9 (# 1 and 16) used both CCR5 and CXCR4 with similar levels of efficiency in CD4+ T lymphocytes but clone #1 was exclusively CXCR4-using in macrophages and clone #16 preferred using CXCR4 in macrophages. Further studies will be needed to address whether such dual tropic clones with preferential CCR5 utilization represent earlier phases in virus coreceptor evolution, suggesting a sequential shift and adaptation where CCR5 usage is progressively lost as CXCR4 usage emerges as opposed to a sudden shift to X4 phenotype.

The results on the ability of dual tropic strains to use both CCR5 and CXCR4 on primary cells, albeit with a high degree of heterogeneity in efficiency of utilization are consistent with the findings of Ghezzi and colleagues who reported similar diverse dual tropic HIV-1 subtype B Env phenotypes in primary cells (Ghezzi et al. 2001). However, results on HIV-1 subtype C viruses are somewhat in contrast to the findings of Yi *et al.* who showed that while several

subtype B R5X4 primary and prototype strains used both CCR5 and CXCR4 for infection of MDM, they were restricted to usage of CXCR4 only for entry and infection in peripheral blood lymphocytes even though CCR5 was present and could be utilized by R5-using strains (Yi et. al., 2005). A possible explanation for these differences could be the particular virus strains studied. In turn, these virus strain differences could be associated with the specific timing or phase of coreceptor evolution. Longitudinal studies in patients undergoing coreceptor usage switch will be needed to further understand these differences.

Most clones in this study were sensitive to coreceptor inhibitors but some appeared to be resistant as they did not exhibit complete inhibition in both primary cell types even when a combination of antagonists was used. This was observed for some clones of isolates 99ZASW20, 99ZASW30, 99ZACM9 and 01ZADu36_5. It is noteworthy that although clones from isolates 99ZASW20 and 99ZASW30 were inhibited in CD4+ T lymphocytes, complete inhibition was not observed in MDM. Clones from isolates 99ZACM9 and 01ZADu36_5 displayed a similar pattern of inhibition in both cell types. This led to the investigation of whether these viruses were truly resistant to the inhibitors. Results from transformed cell line infections confirmed that some of the *env* clones had a natural resistance to the CXCR4 inhibitor AMD3100. Results obtained suggest that it is unlikely that alternate coreceptors played a role since experiments in U87.CD4 cells confirmed the pattern observed in primary cells. It can therefore be concluded that some HIV-1 subtype C envelope proteins display natural resistance to chemokine receptor antagonists. Further studies will be needed to investigate the extent of this phenomenon and to define associated genetic determinants.

Genotypic analysis of *env* clones in this study suggested that subtle or complex amino acid patterns likely contribute to the phenotype differences observed between transformed cell lines and primary cells since no obvious amino acid changes or patterns were noted. A limitation of this study is that primary cells from diverse donors may differ in expression levels and cell surface configuration of coreceptors, making it difficult to assess the extent to which the results obtained in this study can be generalized. However, it is noteworthy that for PBMCs from 2 different donors used in this study, results were comparable suggesting that the Env properties described here are unlikely to have been influenced by the donor phenotype.

This is the first study to examine correlation of coreceptor usage between transformed cell lines and primary T cells for HIV-1 subtype C envelope clones. It can be concluded that while coreceptor usage assessment in reporter cell lines accurately predicts coreceptor usage in a majority of cases, important differences exist in a minor but significant proportion of cases. In addition, as shown in this study most HIV-1 subtype C dual tropic env clones are capable of using both CCR5 and CXCR4 in both CD4+ T lymphocytes and MDM. However, a diverse repertoire of phenotypes existed, with most clones displaying a bias for the CXCR4 coreceptor over CCR5, although there were also notable exceptions. Finally, it is also noted that there are several HIV-1 subtype C Env clones with apparent natural resistance to coreceptor antagonists. Further studies to address genetic basis for biased coreceptor utilization in primary cells and for resistance to chemokine receptor antagonists are warranted.

CHAPTER 4

Drug Resistance and Viral Tropism in HIV-1 Subtype C-Infected Patients in KwaZulu-Natal, South Africa: Implications for Future Treatment Options

4.1. Introduction

South Africa has the highest number of HIV infections worldwide and the province of KwaZulu-Natal accounts for the highest estimated percentage (39%) of infections among women attending antenatal clinics in the country (UNAIDS 2007). The most frequently encountered HIV-1 genetic strain in South Africa and in KwaZulu-Natal is HIV-1 subtype C (HIV-1C) and this subtype accounts for more than half of the global infections (Esparza 2005; Hemelaar et al. 2006; Visawapoka et al. 2006). Access to HAART in South Africa has increased dramatically since the launch of the Operational Plan for Comprehensive HIV and AIDS Care and Treatment for South Africa in 2003 (http://www.info.gov.za/otherdocs/2003/aidsplan/report.pdf.). As a consequence of this uptake, South Africa now has the largest antiretroviral therapy programme in the world although the proportion of people on HAART to those needing treatment remains low with only an estimated 28% of infected people receiving HAART by the end of 2007 (www.doh.gov.za).

It is important to note that even in the presence of HAART and undetectable viremia in chronic HIV-1 infection, viral transcriptional activity persists, suggesting that there is ongoing low-level replication or transcriptionally active latent infection (Furtado et al. 1999;

Tobin et al. 2005). This ongoing viral replication can lead to emergence and persistence of drug resistance, which poses a significant public health threat. Drug resistance is of particular concern in resource-poor countries, because intense Virologyogical monitoring is not always affordable or feasible resulting in the use of clinical or immunological algorithms to guide the use of HAART (WHO guidelines, 2006). The use of these algorithms may inadvertently lead to a delay in the switching of failing HAART regimens. The delay in the switch of failing HAART regimens in the developing world, where WHO guidelines recommend the use of 2 nucleoside reverse-transcriptase inhibitors (NRTIs) and one non-nucleoside reverse-transcriptase inhibitor (NNRTI) as first line therapy may in turn result in the unintended consequences of the accumulation of thymidine analog mutations (TAMs) which are associated with broad cross resistance and may therefore severely limit the options available for second line therapy. A recent study from Malawi has highlighted this emerging problem although it remains unclear how widespread the problem is and there exists data to suggest that the accumulation of TAMs in resource-poor settings is lower than would have been anticipated under current guidelines and practices (Cozzi-Lepri et al. 2009; Hosseinipour et al. 2009).

In addition to concerns regarding the emergence of TAMs in resource-poor settings, there is inconclusive evidence that individuals failing HAART may have a higher proportion of chemokine receptor CXCR4-utilizing viruses compared to antiretroviral naïve patients (Hunt et al. 2006; Johnston et al. 2003; Marconi et al. 2008). During the early stages of HIV-1 infection, CCR5-utilizing viruses (R5 viruses) predominate whereas X4 and R5X4 variants, which are associated with rapid disease progression, emerge in the late chronic phase of disease in a significant proportion of patients (Connor et al. 1997; Scarlatti et al. 1997). Several studies have shown that although all subtypes can undergo the switch from the

utilization of CCR5 to CXCR4, this is less frequently observed in HIV-1C infections even in late stages of disease (Bjorndal and Sonnerborg 1999; Cecilia et al. 2000; Cilliers et al. 2003; Ndung'u et al. 2006; Tscherning et al. 1998). The predominant utilization of CCR5 by HIV-1C could be interpreted to suggest that CCR5 antagonists would be more efficacious in this setting as salvage therapy in patients failing the current classes of drugs. However, in a study in Zimbabwe, where HIV-1 subtype C predominates, Johnston and coworkers found a high frequency (50%) of CXCR4-tropic viruses in patients failing partially suppressive ARV therapy while no such viruses were found in ARV-naïve patients although the CD4 cell counts were comparable between the two groups (Johnston et al. 2003). This study suggested that ARV therapy may be selecting for the more virulent X4 strains in subtype C infection. A higher proportion (41%) of dual/mixed or X4-tropic viruses was also encountered in HIV-1 subtype B treated individuals as compared to 18% of treatment-naïve individuals (Hunt et al. 2006). Due to the emergence of drug resistance to NRTIs, NNRTIs and PIs, CCR5 entry inhibitors may represent an important salvage option but only if individuals failing treatment harbor exclusively R5 viruses (Heera et al. 2009; Vandekerckhove et al. 2009). Alternatively, if duration of infection or HAART facilitates the emergence of X4 viruses, CCR5 inhibitors may have to be applied as part of first-line/early regimens for maximum benefit. Maraviroc, a CCR5 inhibitor has been approved by the Food and Drug Administration (FDA) as long as the tropism assay has been performed prior to its use (http://www.fda.gov/ForConsumers/ByAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm124198.htm). Hoever, since the tropism assay is an expensive procedure it is not feasible to perform this assay on all patients in South Africa.

Here the prevalence and patterns of drug resistance mutations in individuals failing antiretroviral therapy in a resource-poor setting where HIV-1 subtype C predominates was

studied. The drug resistance mutational pathways and factors associated with failure of first-line HAART in an area where monitoring relies mainly on clinical and immunological algorithms was sought to be understood. Furthermore, coreceptor utilization profiles of HIV-1C viruses from individuals initiating or failing HAART was determined to assess the usefulness of CCR5 antagonists either as first-line or salvage therapy in this setting. The accuracy of *env* sequence based genotypic predictive algorithms in assessing the prevalence of R5 and X4 viruses in patients initiating or failing HAART was also explored.

4.2. Materials and methods

4.2.1. Study Participants:

Study participants were recruited from the Sinikithemba outpatient HIV/AIDS clinic at McCord hospital in Durban, KwaZulu-Natal, South Africa. Patients were included in the ARV-naïve cohort if they were at least 18 years of age, were HIV positive and had no prior history of ARV therapy (the use of single dose nevirapine for the prevention of mother to child HIV transmission was not an exclusion criteria). Patients who met these criteria as well as had a CD4+ T-cell count of $\leq$ 200 cell/µl or displayed AIDS defining clinical features according to WHO staging irrespective of CD4 counts or viral loads were recruited into this study. Patients were included in the ARV-failing arm if they were at least 18 years of age, had a known HIV infection, an HIV-1 RNA of $\geq$5,000 copies/ml, a CD4 cell count of <200/µl and had at least 6 months of uninterrupted ARV therapy. HAART-failing patients were also recruited into the study if they clinically assessed to be failing therapy irrespective of CD4+ T-cell counts. A total of 45 HAART-failing and 45 HAART-naive patients were recruited. All study participants gave written informed consent and the study was approved by the Biomedical Research Ethics

Committee (BREC), UKZN. Following consent to participate in the study, further clinical and demographic data were collected and these are summarized in Table 4.1.

Table 4.1: Patient information

Patient Characteristic	ARV-Experienced Patients failing Treatment (n=45)	ARV-Naïve Patients (n=45)	p-value
Age, median years (Q1-Q3)	36 (24-51)	36 (20-78)	0.65
Gender: Female	28 (65%)	27 (60%)	
Black race	45 (100%)	45 (100%)	
CD4 cell count, median cells/mm^3 (Q1-Q3)			
Current	174 (9-718)	123 (8-660)	0.036
Nadir	57 (3-197)		0.0004
Vial load, median copies/ml	6, 653 (225-220,010)	44,042 (1,702-1,167,759)	0.0010
WHO stage at visit			
I	29 (64 %)	2 (4%)	
II	3 (7 %)	3 (7%)	
III	0 (0 %)	4 (9%)	
IV	13 (29 %)	36 (80%)	
Current treatment regimen:			
Regimen 1A			
(d4T, 3TC, EFV)	23 (51.1%)		
Regimen 1B			
(d4T, 3TC, NVP)	2 (4.4%)		
ZDV/AZT, d4T, ddI, NVP	1 (2.2%)		
ZDV/AZT, d4T, 3TC, EFV	2 (4.4%)		
ZDV/AZT, 3TC, EFV	13 (29.0%)		
ZDV/AZT, 3TC,NVP	4 (8.9%)		

Change in ARVs from initial regimen	27 (60%)		
Duration of ARV therapy, median months	29 (7-100)		

4.2.2. Sample collection, Viral Load and CD4 Measurement

Thirty-five milliliters of blood was obtained in an ethylene-diaminetetraacetic acid (EDTA) vacutainer or acid citrate dextrose (ACD) tubes for viral load quantification, CD4 cell counts, genotypic resistance testing and viral tropism studies. CD4 + T cell counts were determined from fresh blood from all HIV-infected participants using the FACS MultiSET System and analyzed on the four-colour FACS Calibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. Plasma viral load measurements were done using the quantitative COBAS AmpliPrep/COBAS Amplicor HIV-1 Monitor Test, version 1.5 (Roche Diagnostics, Rotkruez, Switzerland).

4.2.3. Genotypic resistance testing

Vacutainer tubes containing approximately 10 ml blood were gently inverted to ensure that the blood was properly mixed. The tubes were then centrifuged at 2,200 rpm for 10 minutes (Jouan C412) and the plasma aliquotted and stored for genotypic resistance testing using the Viroseq HIV-1 Genotyping System (Celera Diagnostics, CA, USA) as directed by the manufacturer.

4.2.4. Phenotypic coreceptor analysis

Coreceptor usage of viruses from patient plasma samples was determined using the Trofile Coreceptor Tropism Assay (Monogram Biosciences, San Francisco, California, USA). The Trofile assay is a commercial, standardized cell-based approach which assists in determining coreceptor tropism by testing all determinants of tropism in the gp160 coding region of the envelope protein (Coakley et al. 2005; Westby et al. 2006).

4.2.5. cDNA synthesis, envelope amplification and cloning

PBMCs were separated from whole blood by density-gradient centrifugation on Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden). The plasma layer was removed and used for RNA extraction using the QIA-amp Viral RNA Kit (Qiagen, Germany) and cDNA was synthesised using the Superscript III Reverse Transcriptase Kit (Invitrogen Carisbad, CA). The following components were pipetted into a 0.5 ml RNase-free tube: 8.75 µl water, 1.25 µl 20 µM primer (OMF19 - 5'GCACTCAAGGCAAGCTTTATTGAGGCTTA3'), 5µl dNTP mix (10 mM) and 50 µl RNA template. This mixture was placed at 65ºC for 3-5 minutes and then cooled on ice for 1 minute. The following components were then combined in a second tube: 20 µl 5 x buffer, 5 µl DTT (100 mM), 5 µl RNaseOUT (40 u/µl) and 5 µl SSIII RT (200 u/µl). These ingredients were then added to the original tube, mixed and incubated at 50ºC for one hour and the temperature then increased to 55ºC for one hour. SSIII RT was then inactivated by heating at 70ºC for 15 minutes. Thereafter, 1 µl RNase H was added to the tube and incubated at 37ºC for 20 minutes.

The 3 kb *env* gene was amplified by a nested polymerase chain reaction (PCR) using the Platinum Taq High Fidelity System (Invitrogen, Carisbad, CA) and the following primers in the first round: OMF19 -5'GCACTCAAGGCAAGCTTTATTGAGGCTTA3' and VIF1 – 5'GGGTTTATTACAGGGACAGCAGAG3'. Cycling conditions were as follows: a 4 minute denaturation at 94°C, followed by 35 cycles of 15 seconds at 94°C, 30 seconds at 55°C, and 4 minutes at 68°C. The final extension was at 68°C for 20 minutes. For the second round PCR, 5 µl of first round template was then amplified using the Phusion Phusion Hot Start High-Fidelity DNA Polymerase kit (Finnzymes, Finland) together with the following primers: Env1A 5'-CACCGGCTTAGGCATCTCCTATGGCAGGAAGAA-3' and EnvM 5'-TAGCCCTTCCAGTCCCCCCTTTTCTTTTA-3'. The forward primer Env1A was designed to include the 4 base pair sequences (CACC) necessary for directional cloning on the 5' end. Cycling conditions were as follows: a 5 minute denaturation at 94°C, followed by 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 4 minutes at 72°C. The final extension was at 72°C for 10 minutes (as in 2.2.5). The amplified product was then run on a 1% agarose gel and gel purified using the Qiaquick gel extraction kit (Qiagen, Germany).

Once the DNA was purified, one *env* from 20 ARV-failing patients and 20 ARV-naïve patients was cloned into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen, Carisbad, CA) as previously described (refer to 2.2.6).

4.2.6. Envelope sequencing and sequence analysis

After cloning, the *env* gene was sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit version 3.1 (Applied Biosystems, CA, USA). Sequences were assembled and edited using Sequencher 4.8 and aligned with Mega 4. To evaluate the clustering of these sequences with each other and with subtype references, phylogenetic trees were constructed in Paup 4.0 and visualized using Treeview 1.6.6. Coreceptor utilization was predicted using the web-based subtype C-specific position specific scoring matrix (C-PSSM) programme (http://indra.mullins.microbiol.washington.edu/pssm/), a bioinformatics tool that reliably predicts coreceptor phenotype using V3 loop sequences (Jensen et al. 2006). Predicted N-linked glycosylation sites were examined using the web-based programme N-GLYCOSITE (www.hiv.lanl.gov).

4.2.7. Statistical Analysis

All statistical analysis was done using Graph Pad Prism 5. Factors associated with tropism were assessed using unpaired t tests and Fisher's exact tests.

4.3. Results

4.3.1. Patient Demographics

Forty five HAART-naïve and 45 HAART-failing patients were recruited for this study. Table 4.1 shows patient information as well as drug regimens, viral loads and CD4+ T-cell counts at the time of recruitment. For patients failing therapy, nadir CD4 counts are also represented. All patients were Black African. The median age for both groups was

36 years. Majority of patients in both groups were female - 60% of HAART-naïve patients and 65% HAART-failing patients. The median CD4+ T-cell count at the time of recruitment was 123 cells/mm^3 for HAART-naïve patients and 174 cells/mm^3 for HAART-failing patients, a significant difference between the two groups (p=0.036). CD4+ T cell counts at recruitment were also significantly different between the two groups, with HAART-naïve individuals having a median of 123 cells/μl compared to HAART-failing patients with a median of 174 cells/μl (p=0.0004). The median nadir CD4+ T cell count for HAART-failing study subjects was 57 cells/μl. HAART-naïve patients had significantly higher median plasma viral load of 44,042 copies/ml compared to 6,653 copies/ml for HAART-experienced participants (p=0.001). For patients failing treatment, the median duration on therapy was 29 months. Drug regimens at the time of sampling are listed in Table 4.1.

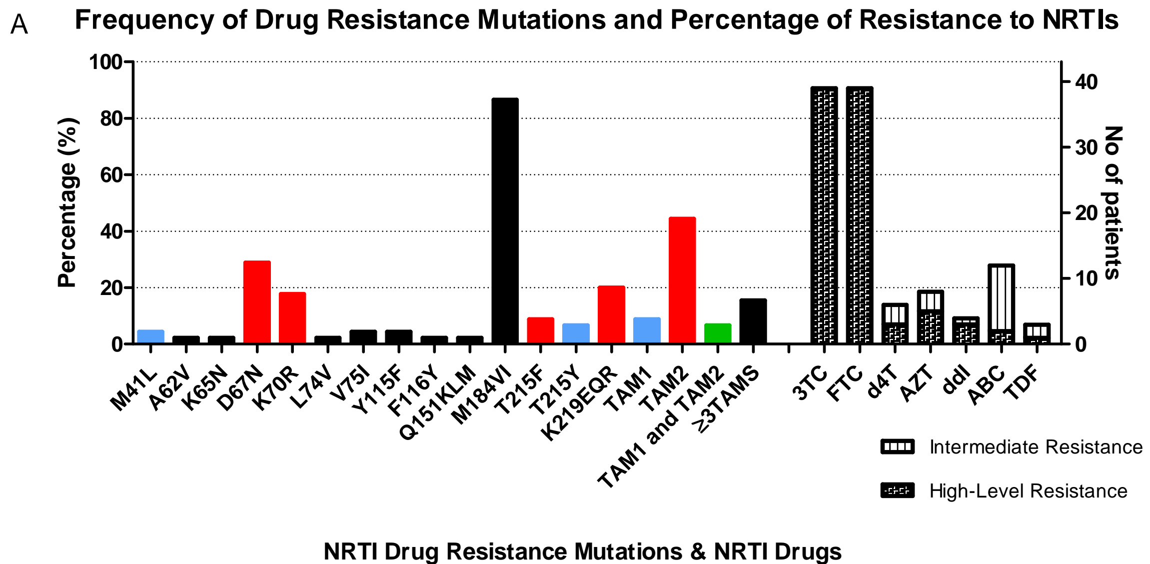
4.3.2. Genotypic Drug Resistance Typing

Drug resistance results were obtained for 43 of the 45 HAART-failing patients. Resistance testing was also performed for ten HAART-naïve patients. The only major drug resistance mutation observed in the HAART-naïve individuals was in one patient with the NNRTI-associated E138A mutation. Three HAART-naive patients had minor protease inhibitor (PI) resistance mutations, one patient with mutations L10L/V and T74S/T, another had mutation A71T and the third patient had the T74S/T mutation. Of the 45 HAART-failing patients, 51.1% were on South African national treatment guidelines Regimen 1A (d4T, 3TC and EFV); 4.4% were on Regimen 1B (d4T, 3TC and NVP), 29% were on ZDV/AZT/ZDV 3TC and EFV; 8.9% were on ZDV/AZT/ZDV, 3TC and NVP; 2.2% were on ZDV/AZT/ZDV, d4T, ddI and NVP and 4.4% were on

ZDV/AZT/ZDV, d4T, 3TC and EFV. Twenty seven (60%) of the patients were on previous ARV therapy. Of this, 11/27 (40.8%) had previous history of Regimen 1A. 2/27 (7.4%) had a previous history of d4T, 3TC, NVP and EFV; 2/27 (7.4%) had a previous history of d4T and NVP and 1/27 (3.7%) each had previous history of the following: ZDV/AZT, d4T, 3TC, ddI, EFV; d4T, NVP, EFV; ZDV/AZT, 3TC, NVP, Lopinivir/Ritonivir; ZDV/AZT, d4T, EFV; ZDV/AZT, 3TC, Lopinivir/Ritonivir; ZDV/AZT, d4T, 3TC, NVP; d4T, 3TC; ZDV/AZT, d4T, NVP; 3TC, ddI, EFV; ZDV/AZT, 3TC, EFV; d4T, 3TC, ddI, EFV; and ZDV/AZT, 3TC,ddI (Table 4.1).

The specific drug resistance amino acid substitutions detected, mutation frequency and pathways in HAART-failing patients are shown in Figure 4.1. Mutations to all three major classes of drugs were noted. The prevalence of mutations and mutational pathways are summarized in Figure 4.1. Forty one of the 43 (95%) ARV-failing patients possessed at least one drug resistance mutation. Ninety one percent of patients had at least one drug resistance mutation against 2 classes of drugs (nucleoside reverse transcriptase inhibitors (NRTI) and non-nucleoside reverse transcriptase inhibitors (NNRTI). Nineteen percent had at least one resistance mutation against all 3 classes of drugs (NRTI, NNRTI and PI). For PI, only one minor mutation (T74S) was present in 9 of the HAART-failing patients (20%). M184V/I, present in 87% of HAART-failing patients was the most common NRTI mutation detected. Thymidine analog resistance mutations (TAMs) were detected in 55% of patients. The TAM1 pathway NRTI mutations M41L and T215Y, associated with intermediate to high level resistance to ZDV/AZT and d4T and low level resistance to ddI, ABC and TDF (Whitcomb et al. 2003) were present in approximately 9% of patients. Neither an insertion at codon 69 nor the L210W mutation, both also indicative of the TAM1 pathway was noted. The TAM2 pathway mutations present were D67N, K70R, T215F and K219EQR. Forty four percent of patients had TAM2 pathway

mutations. Seven percent of patients possessed both TAM1 and TAM2 mutations and 16% had three or more TAMs. Approximately 91% of patients had high level resistance to 3TC and FTC, 19% had high or intermediate level resistance to ZDV/AZT. Fourteen percent had high or intermediate level resistance to d4T while 9% had high level resistance or intermediate resistance to ddI. High or intermediate level resistance to ABC was noted in 28% of patients, while only 7% displayed high or intermediate level resistance to TDF (Figure 4.1A).



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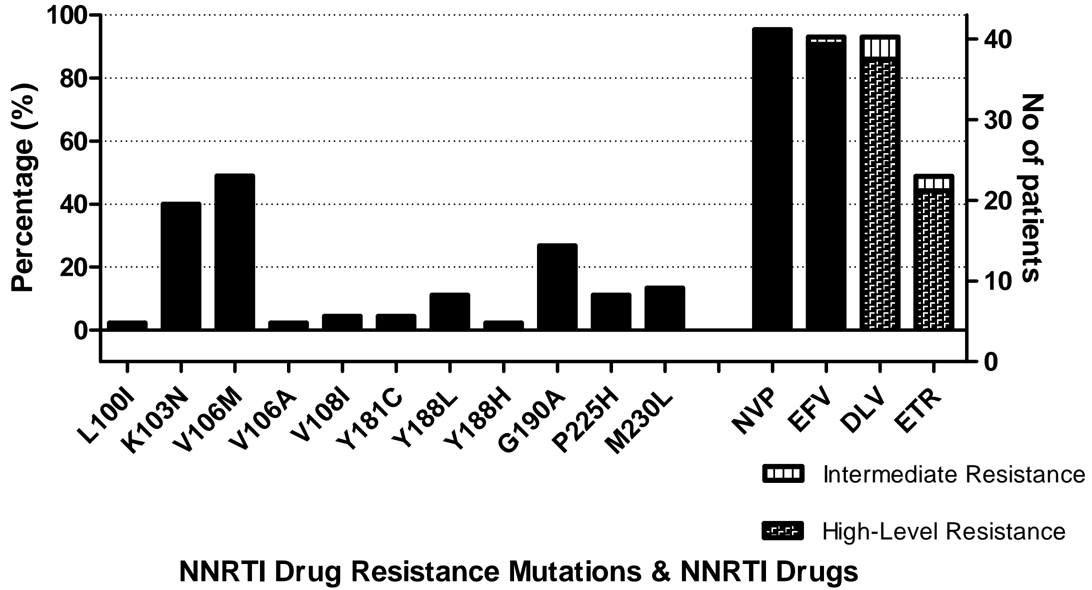

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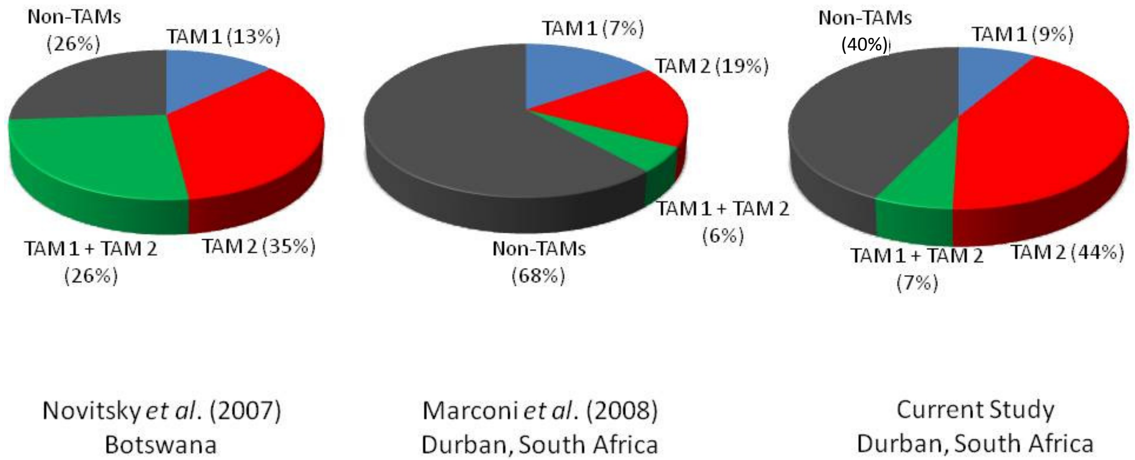


Figure 4.1: Frequency of drug resistance mutations and thymidine analog resistance mutations (TAMs)

A) shows the frequency of NRTI resistance mutations, thymidine analogue mutation frequencies and number of patients displaying high and intermediate level resistance to specific NRTIs and B) shows the frequency of NNRTI resistance mutations and the number of patients showing resistance to specific NNRTIs. C) shows the frequency of TAMs comparing the current study to 2 recently published HIV-1C studies. The current study had a

higher percentage of TAM2 than the other two studies, and there is a sharp increase in proportion of patients with TAMs in patients failing therapy in this study compared to the and earlier study (Marconi et al, 2008) from the same health setting.

NNRTI mutations noted are summarized in Figure 4.1B. The most common NNRTI resistance mutation was V106M, found in 49% of HAART-failing participants. The K103N (40%) and G190A (27%) mutations were also relatively common but no G190S mutations were present in any of the patients. V106M and K103N both cause high-level resistance to 3 of the 4 NNRTI: nevirapine (NVP), delavirdine (DLV) and efavirenz (EFV) but has no effect on etravirine (ETR). G190A causes high level resistance to NVP, intermediate resistance to EFV and low level resistance to ETR. This mutation also increases susceptibility to DLV (http://hivdb.stanford.edu). Ninety-five percent of patients had mutations associated with high level resistance to NVP, 93% had high/intermediate level EFV resistance mutations. Ninety three percent displayed high or intermediate level resistance to DLV, with 49% displaying high/intermediate level resistance to ETR (Figure 4.1B).

Recent studies in southern Africa have highlighted the growing problem of thymidine analog mutations in patients receiving the World Health Organization (WHO) or national antiretroviral programmes recommended first-line therapy (Hosseinipour et al. 2009; Marconi et al. 2008; Novitsky et al. 2007). The prevalence of these mutations may be increasing as antiretroviral roll-out accelerates, accompanied by mainly clinical and immunological based monitoring of treatment. The proportion and patterns of TAM mutations observed in this study was therefore compared to data reported from earlier studies from similar geographic and social-economic background. Fifty-five percent of

individuals failing therapy had TAM mutations compared to 32% and 74% reported from the South Africa and Botswana respectively (Marconi et al. 2008; Novitsky et al. 2007) (Figure 4.1C). Patients in the current study were recruited from the same healthcare facility as those analyzed in the study by Marconi and colleagues and generally followed the same treatment guidelines and overall clinical care. In the Botswana study, patients were predominantly on a regimen containing ZDV/AZT and ddI. In all three studies, there were higher proportions of TAM2 compared to TAM1 mutations, with substantially higher percentage of mixed TAM1 and TAM2 noted in the Botswana study. It was further investigated whether the duration of treatment was associated with the development of TAMs. A non-significant trend between the presence of TAMs and the duration of treatment (p=0.08) was obtained.

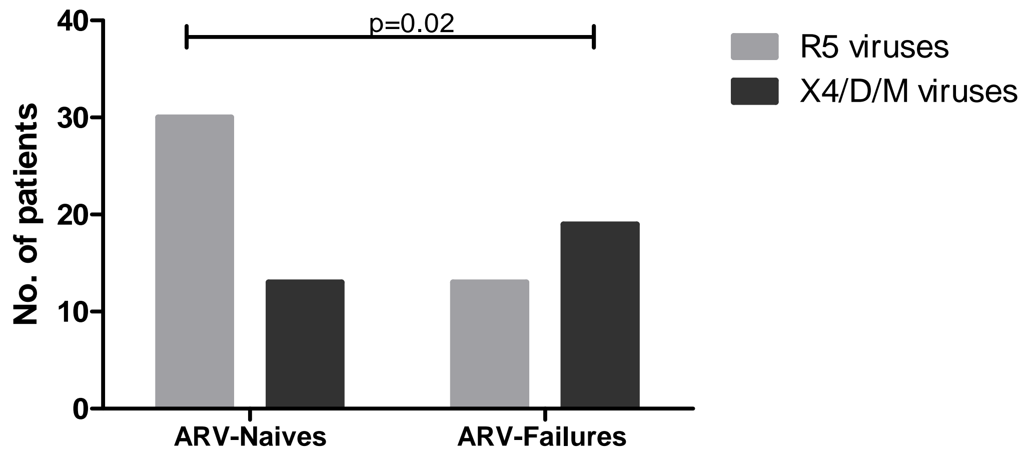
4.3.3. Coreceptor utilization

4.3.3.1. Phenotypic Coreceptor Analysis

The Trofile coreceptor tropism assay was used to determine viral tropism of plasma derived viruses. Only 75 samples (32 from ARV-experienced patients failing treatment and 43 from ARV-naive patients) yielded reportable data. Overall, 31/75 (41%) were dual/mixed viruses, 43/75 (57%) were CCR5-using, and only 1 (1%) was CXCR4-utilizing. Of the 43 ARV-naive patients, 30 (70%) possessed R5 viruses compared to 13 (30%) with dual/mixed viruses. No ARV-naive patients exhibited exclusive X4 viruses in this assay. Of the 32 ARV-experienced patients failing treatment, 13 (41%) possessed R5 viruses, 18 (56%) had dual/mixed infections while one patient (3%) had X4-only viruses (Figure 4.2A, Table 4.2). Thus patients failing treatment had a higher percentage (59%) of X4/dual/mixed viruses compared to ARV-naive patients with 30% and

conversely, ARV-naive patients had higher proportion of R5 viruses (70%) compared to patients failing therapy with 41% (p=0.02).

A



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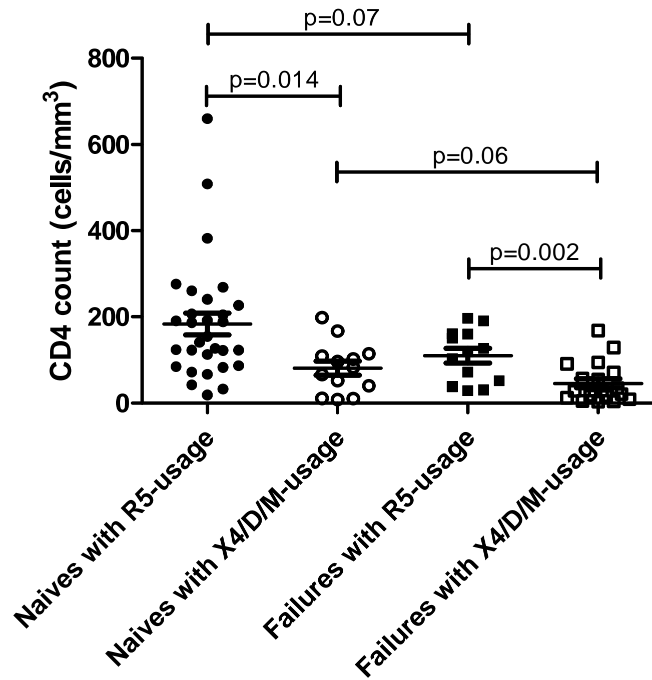


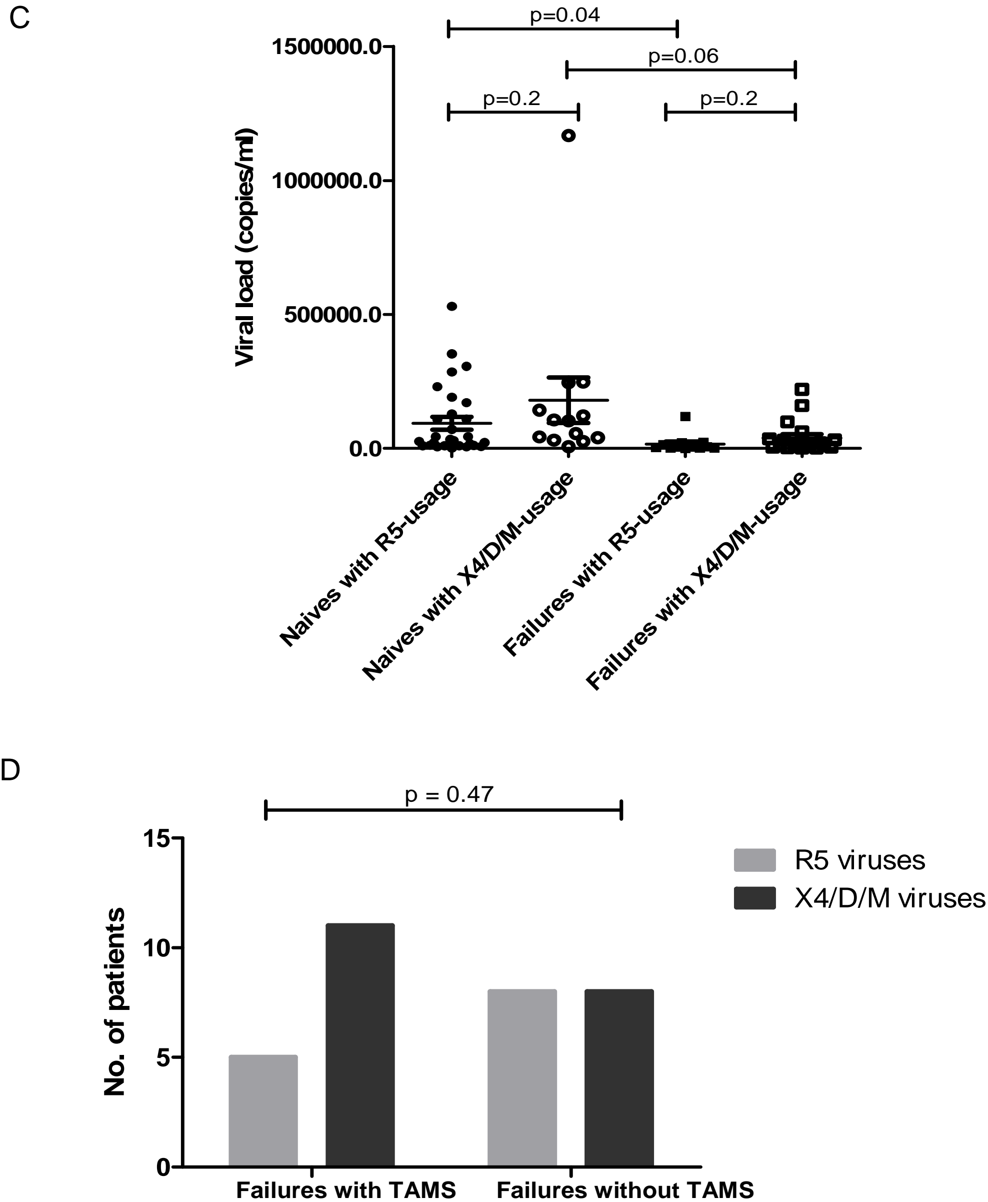


Figure 4.2: Relationship of viral tropism of ARV-naive and ARV-failing patients

A) Frequency of X4/dual/mixed- and R5-utilizing viruses in patients failing treatment and treatment-naive individuals. Bar graph indicating results from the trofile assay. A significant p value of $p<0.02$ was obtained. B) CD4 counts and coreceptor usage in HAART-naive patients and HAART-failing patients. Dot graph indicating results from trofile assay. ARV-naive patients with X4/D/M viruses had a lower CD4 count than ARV-naive patients with R5 viruses (p=0.014). ARV-failing patients with X4/D/M viruses had a lower CD4 count than ARV-failing patients with R5 viruses (p=0.02). For ARV-failing patients nadir CD4 counts are respresented C) Viral loads and coreceptor usage in HAART-naive patients and HAART-failing patients. Dot graph indicating results from trofile assay. ARV-naive patients with R5 viruses had a higher viral load than ARV-failing patients with R5 viruses (p=0.04). D) Association of TAMs in X4/D/M viruses. Bar graph indicating trofile assay results. D/M indicates dual/mixed viruses.

Table 4.2: Functional Coreceptor Utilization

ARV-Failing Patient Sample	Trofile Coreceptor Tropism Assay	ARV Naive Patient Sample	Trofile Coreceptor Tropism Assay
704MC001F	R5X4-using	704MC001N	CCR5-using
704MC002F	R5X4-using	704MC002N	CCR5-using
704MC003F	CCR5-using	704MC003N	CCR5-using
704MC004F	CCR5-using	704MC004N	CCR5-using
704MC005F	R5X4-using	704MC005N	R5X4-using
704MC006F	Non-reportable	704MC006N	R5X4-using
704MC007F	Non-reportable	704MC007N	R5X4-using
704MC008F	CCR5-using	704MC008N	CCR5-using
704MC009F	R5X4-using	704MC009N	CCR5-using
704MC010F	Non-reportable	704MC010N	R5X4-using
704MC011F	CCR5-using	704MC011N	R5X4-using
704MC012F	Non-reportable	704MC012N	CCR5-using
704MC013F	Non-reportable	704MC013N	CCR5-using
704MC014F	R5X4-using	704MC014N	CCR5-using
704MC015F	CCR5-using	704MC015N	CCR5-using
704MC016F	R5X4-using	704MC016N	R5X4-using
704MC017F	R5X4-using	704MC017N	CCR5-using
704MC018F	CCR5-using	704MC018N	R5X4-using
704MC019F	Non-reportable	704MC019N	CCR5-using
704MC020F	CXCR4-using	704MC020N	CCR5-using
704MC021F	CCR5-using	704MC021N	CCR5-using
704MC022F	R5X4-using	704MC022N	Non-reportable
704MC023F	Non-reportable	704MC023N	R5X4-using

704MC024F	Non-reportable	704MC024N	CCR5-using
704MC025F	R5X4-using	704MC025N	CCR5-using
704MC026F	CCR5-using	704MC026N	CCR5-using
704MC027F	R5X4-using	704MC027N	R5X4-using
704MC028F	Non-reportable	704MC028N	R5X4-using
704MC029F	CCR5-using	704MC029N	CCR5-using
704MC030F	CCR5-using	704MC030N	CCR5-using
704MC031F	CCR5-using	704MC031N	CCR5-using
704MC032F	R5X4-using	704MC032N	CCR5-using
704MC033F	CCR5-using	704MC033N	CCR5-using
704MC034F	Non-reportable	704MC034N	CCR5-using
704MC035F	R5X4-using	704MC035N	CCR5-using
704MC036F	R5X4-using	704MC036N	CCR5-using
704MC037F	R5X4-using	704MC037N	R5X4-using
704MC038F	R5X4-using	704MC038N	CCR5-using
704MC039F	R5X4-using	704MC039N	Non-reportable
704MC040F	Non-reportable	704MC040N	CCR5-using
704MC041F	Non-reportable	704MC041N	CCR5-using
704MC042F	R5X4-using	704MC042N	CCR5-using
704MC043F	Non-reportable	704MC043N	R5X4-using
704MC044F	R5X4-using	704MC044N	CCR5-using
704MC045F	CCR5-using	704MC045N	R5X4-using

It was then sought to determine if there was a relationship between CD4 counts, viral loads and viral tropism. Using the nadir CD4 count for patients failing treatment, it was found that patients with X4/dual/mixed viruses had significantly lower CD4+ T cell

counts compared to those with R5 viruses in both the ART-naive and ART-failing groups with significant p values of 0.014 and 0.002 respectively, whereas no significant differences were found between failures and naives with R5 viruses (p=0.07) and X4/dual/mixed viruses (p=0.06). ART-naive patients harbouring R5 viruses had a significantly higher viral load than patients failing treatment (p=0.04) (Figure 4.2C). Studies have suggested that the emergence of CXCR4-using viruses is a consequence of duration of infection. Thus it was next investigated whether the presence of dual/mixed/X4 viruses was associated with age in this cohort of patients. No significant correlation (p=0.29) was observed. It was then sought to determine if the length of treatment was associated with the presence of dual/mixed/X4 viruses, but no such relationship (p=0.95) was observed. It was also investigated whether patients with TAMs were more likely to harbor dual/mixed/X4 viruses. Eleven of 16 (69%) HAART-failing patients with TAMs had X4/dual/mixed viruses and 5 (31%) had CCR5-using viruses, compared to the respective proportions of 50% dual/mixed/X4 versus 50% R5 among patients without TAMs (p=0.47) (Figure 4.2D).

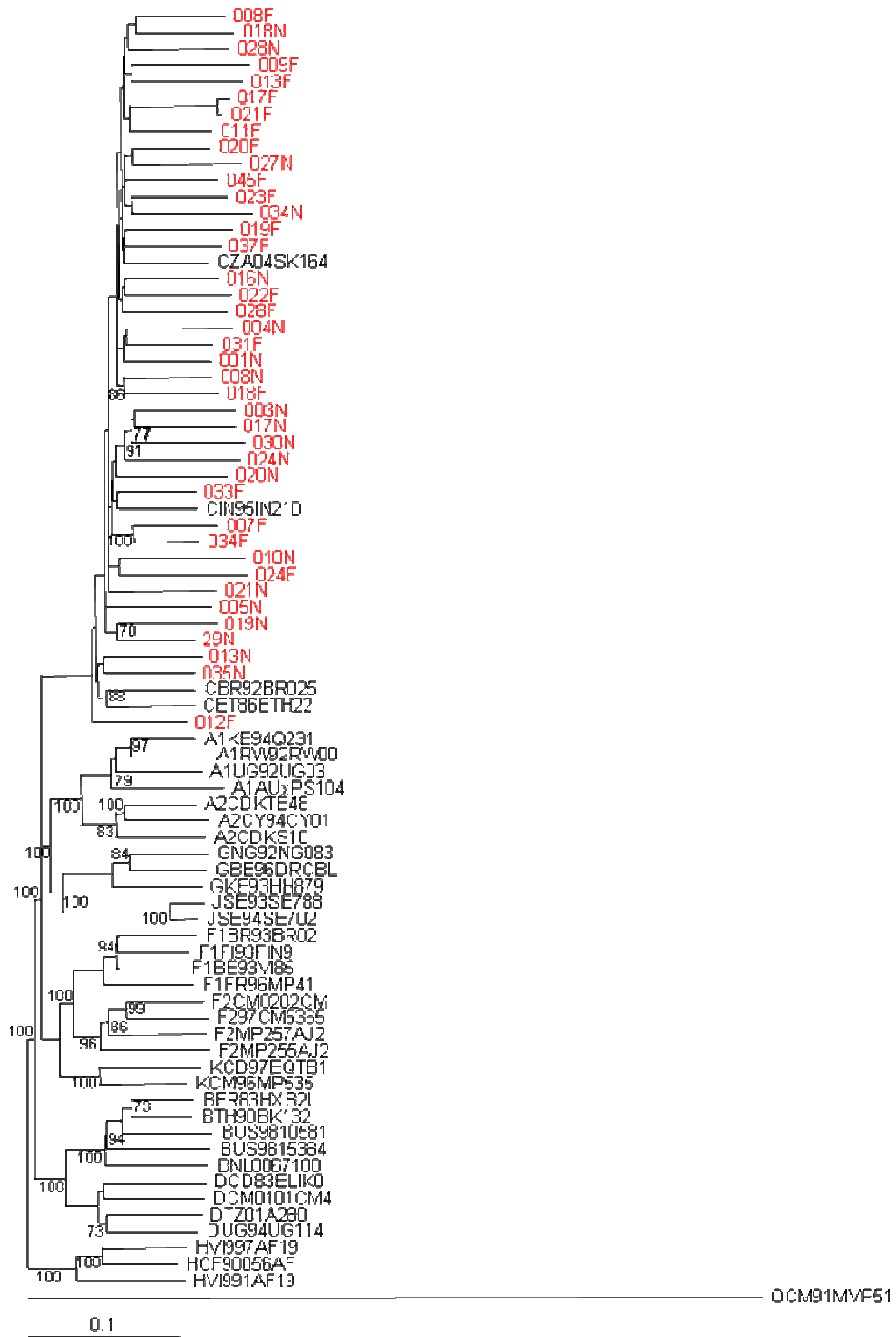
4.3.3.2. Genotypic Analysis of the *env* gene

HIV-1 envelope sequence determines coreceptor utilization (Briggs et al. 2000; Cann et al. 1992; Fouchier et al. 1992; Rizzuto et al. 1998; Wu et al. 2006). Envelope sequence based genotypic coreceptor prediction algorithms offer a simpler and less expensive means of analyzing viral tropism in patients and could facilitate easier determination of whether a patient can be treated with CCR5 antagonists or not, particularly in resource-limited settings where phenotypic assays are too expensive and not readily available. The extent to which viral tropism could be predicted by *env* sequence characteristics was next assessed. Randomly 20 Virologyogically failing and 20 ARV-naïve patients were

selected and analyzed full-length *env* sequences for predictive coreceptor utilization profiles.

All 40 full-length *env* clones generated in this study phylogenetically clustered with HIV-1 subtype C references with a high degree of confidence (Figure 4.3A). Phylogenetic analysis of the *pol* region also showed that all patients with the exception of one participant (704MC012F) were infected with HIV-1 subtype C. Patient 704MC012F was infected with subtype G as identified by a web-based Rega HIV Subtyping tool (www.bioafrica.net) (Figure 4.3B).

A



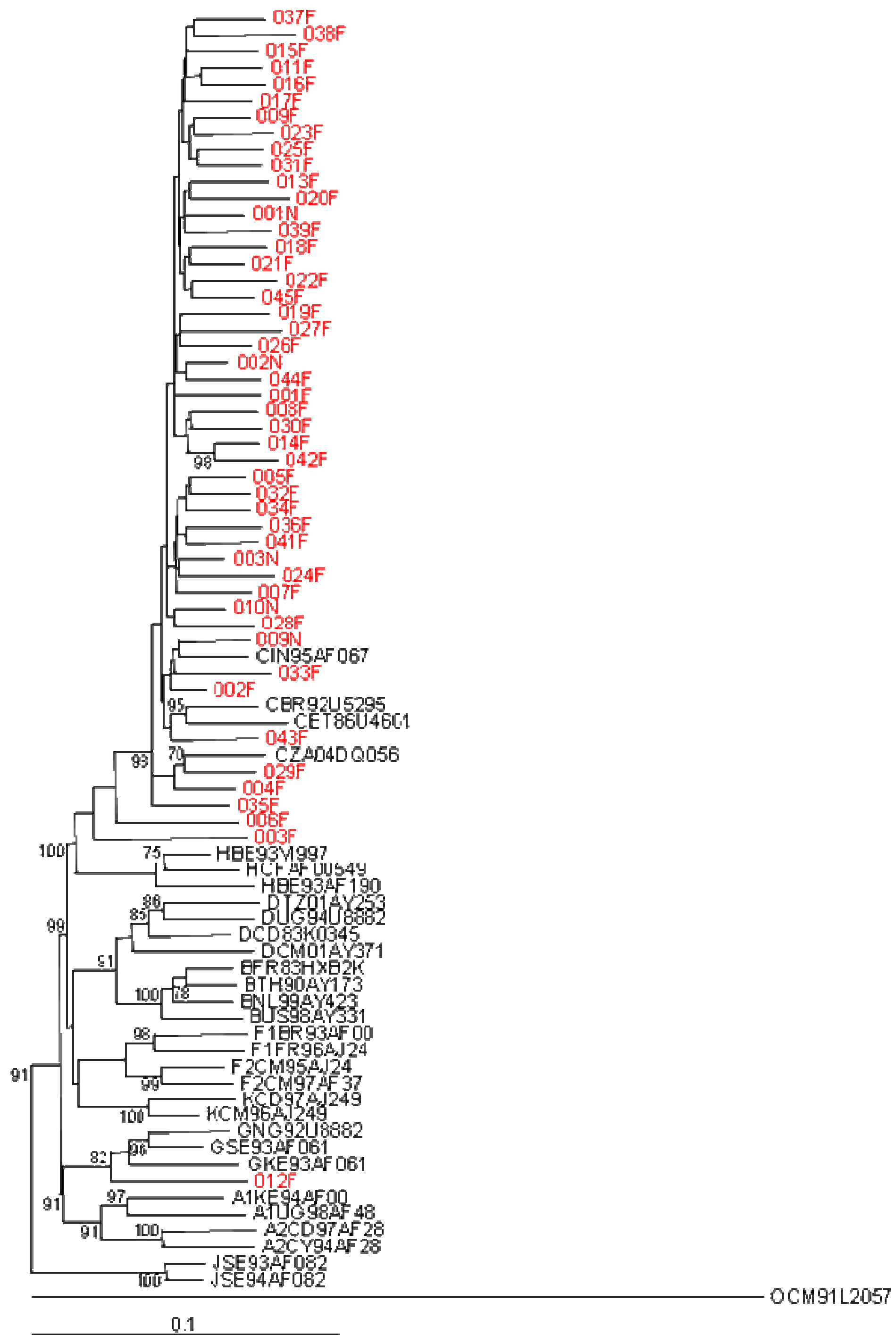


Figure 4.3: Neighbour-Joining phylogenetic trees

A) Neighbour-Joining phylogenetic tree constructed from the *env* gene sequences. All clones highlighted in red cluster closely with subtype C. B) Neighbour-Joining phylogenetic tree constructed from the *pol* gene sequences. All patient samples highlighted in red cluster together with the subtype C reference with the exception of 704MC012F which clusters with subtype G. Bootstrap values of more than 70% are shown.

The *env* V3 loop sequences were first analyzed because this region is an important determinant of coreceptor usage (Figure 4.4 and Table 4.3). The overall V3 consensus sequence generated for ARV-naive patients had 2 more amino acids than the consensus sequence generated for ARV-failing patients. Amino acid substitutions in the crown motif, a conserved tetrapeptide located at the tip of the V3 loop may influence coreceptor utilization. The conventional V3 loop crown motif sequence in R5 HIV-1 subtype C sequences is GPGQ whereas in CXCR4-tropic sequences the crown motif may change from the conventional GPGQ to GPGX where X is any other amino acid (Coetzer et al. 2006). Thus CXCR4 utilization in HIV-1 subtype C is commonly associated with a basic amino acid substitution in the V3 loop. The crown motifs for most clones generated were GPGQ. Thirty-three (83%) clones produced this crown motif, 16 of which were from patients failing therapy and 17 were from ARV naïve individuals. Of the 16 clones from ARV-failing patients displaying the GPGQ crown motifs, 8 were predicted to be CCR5-usage only. Of the 17 ARV-naïve clones displaying the GPGQ crown motifs, all except 1 (704MC020N) showed CCR5-usage only. Crown motifs for the clones from the remaining failures were GPGR (704MC009F; 704MC020F; 704MC028F and 704MC034F) all of which showed CXCR4-usage or dual tropism according to C-PSSM. Clone from patient 704MC012F and possessed crown motifs that read GRGQ and showed CXCR4-usage or dual tropism. From the clones produced from the ARV-naïve individuals, 2 had crown motifs that read GPGR and both of these showed CXCR4-usage or dual tropism according to C-PSSM. One clone (704MC027N) had a crown motif that read GGPG and showed X4-usage or dual tropism. In the study by Johnston and colleagues where 50% of the patients were X4-using, crown motifs of GPGQ predominated followed by GPGR as in our study (Johnston et al. 2003). One clone (704MC035N) had a V3 sequence identical to the subtype C consensus.

Consensus C	CTRPNNNTRKSIRI..GPGQTFYATGDIIGDIRQAHC
Consensus (Failures)	---------.------GPGQ.----..------.-.-
007F	-V-------T----..GPGQ-----NG------E---
008F	----G----R-V--..GPGQ-----------------
009F	-S-GQ–KRR.STRI..GPGRQI—GRS-RT---GK-Y-
011F	-I—G---------...GPGQ---.-NK----------
012F	---------R-M—.GIGRGQ----M-R----------
013F	----GDHRKRI---..GPGQA-H-RDN------K-Y-
017F	----G----R---V..GPGQSI---NR------K-Y-
018F	-I—G------V--...GPGQ------EV---------
019F	-L—G---------...GPGQ------A----------
020F	-------ITTR-ISI.GPGRP--TKNIGRDIK---Y-
021F	----G----R---V..GPGQSI---NR------K-Y-
022F	-------R-R-M--..GPGQV-----A---N----Y-
23F	----G----R----..GPGQ------------K---
024F	----G----RG---..GPGQ--F--RT--N-------
028F	-------K-RR-K-..GPGRA-VTNN---N-----Y-
031F	----G----Q----..GPGQ------------K---
033F	-----------V--..GPGQ-----N---------Y-
034F	---------R----..GPGR--FT----....-----
037F	----G--I--R-G-..GPGQA-R--SG---N----Q-
045F	---------Q----..GPGQ----R------------

```
Consensus C     CTRPNNNTRKSIRI..GPGQTFYATGDIIGDIRQAHC

Consensus       ----.---------..GPGQ.----------------
(Naives)

001N            ----G---------..GPGQ----NN-----------
003N            -I--G------V--..GPGQ----N.-------K---
004N            ---------Q--GF..GPGQA----------------
005N            -I--G---------..GPGQ-----------------
008N            ----G------V--..GPGQ----------NT-----
010N            -----I-K-QR---..GPGRA-V-I-K------K---
013N            ----S------VG-..GPGQ-I---------------
016N            -----------V--..GPGQA-----G--------Y-
017N            ---VG------V--..GPGQA----N-----------
018N            -I--G---------..GPGQA---H-E---N------
019N            ----G------V--..GPGQ----------N------
020N            ----G--I------..GPGQA-FT-----------Y-
021N            --------------..GPGQA----E-----------
024N            -----------V-L..GPGQ-------------E---
027N            ------TR-TR---GPGGPGHAFY-NTV-----K-Y-
028N            -I---SH--QEGVRI.GPGRA--VR-K------K-Y-
029N            ----G----T----..GPGQ------AVT--------
030N            ----G---------..GPGQ----------N------
034N            ----G----------.GPGQ-L-TN.-------K-Y-
035N            --------------..GPGQ-----------------
```

Figure 4.4: Alignment of V3 sequences of clones of ARV-failing and ARV-naïve patients
The crown motif for each sequence is indicated in red. All sequences from viruses determined to be X4/dual/mixed by the Trofile assay are highlighted in green.

Table 4.3: Summary table of V3 characteristics of clones ARV-failing and ARV-naïve patients

Clone	A.A Length	Calc V3 net charge	PSSM V3 net charge	Amino Acid (11/25)	Crown Motif	PSSM Coreceptor usage*	Prediction based on the 3 rules**
R-5 only virus 96BWM01_5	35	4	4	Ser(S)/Asp(D)	GPGQ	CCR5-using only	CCR5-using
704MC007F	35	3	3	Ser(S)/Gly(G)	GPGQ	CCR5-using only	CCR5-using
704MC008F	35	4	4	Ser(S)/Asp(D)	GPGQ	CCR5-using only	CCR5-using
704MC009F	36	7	7	Ser(S)/Asp(D)	GPGR	CXCR4-using	CXCR4-using
704MC011F	34	6	6	Ser(S)/Lys(K)	GPGQ	CXCR4-using	CXCR4-using
704MC012F	37	7	7	Ser(S)/Arg(R)	GRGQ	CXCR4-using	CXCR4-using
704MC013F	35	7	7	Iso(I)/Asn(N)	GPGQ	CXCR4-using	CXCR4-using
704MC017F	35	6	6	Ser(S)/Arg(R)	GPGQ	CXCR4-using	CXCR4-using
704MC018F	35	4	4	Ser(S)/Glu(E)	GPGQ	CCR5-using only	CCR5-using
704MC019F	35	5	5	Ser(S)/Ala(A)	GPGQ	CCR5-using only	CCR5-using
704MC020F	35	5	5	Iso(I)/Asn(N)	GPGR	CXCR4-using	CXCR4-using
704MC021F	35	6	6	Ser(S)/Arg(R)	GRGQ	CXCR4-using	CXCR4-using
704MC022F	35	5	5	Ser(S)/Ala(A)	GPGQ	CXCR4-using	CXCR4-using
704MC023F	35	5	5	Ser(S)/Asp(D)	GPGQ	CCR5-using only	CCR5-using
704MC024F	34	7	7	Gly(G)/-	GPGQ	CXCR4-using	CXCR4-using
704MC028F	34	8	8	Arg(R)/Asn(N)	GPGR	CXCR4-using	CXCR4-using
704MC031F	35	4	4	Ser(S)/Asp(D)	GPGQ	CCR5-using only	CCR5-using
704MC033F	34	4	4	Ser(S)/Asn(N)	GPGQ	CCR5-using only	CCR5-using
704MC034F	31	6	6	Ser(S)/-	GPGR	CXCR4-using	CXCR4-using
704MC037F	35	6	6	Arg (R)/Gly(G)	GPGQ	CXCR4-using	CXCR4-using
704MC045F	35	4	4	Ser(S)/Asp(D)	GPGQ	CCR5-using only	CCR5-using

704MC001N	35	4	4	Ser(S)/Asp(D)	GPGQ	CCR5-using only	CCR5-using
704MC003N	35	5	5	Ser(S)/Asp(D)	GPGQ	CCR5-using only	CCR5-using
704MC004N	35	2	2	Ser(S)/Asp(D)	GPGQ	CCR5-using only	CCR5-using
704MC005N	35	4	4	Ser(S)/Asp(D)	GPGQ	CCR5-using only	CCR5-using
704MC008N	35	5	5	Ser(S)/Asp(D)	GPGQ	CCR5-using only	CCR5-using
704MC010N	35	9	9	Arg (R)/Lys(K)	GPGR	CXCR4-using	CXCR4-using
704MC013N	35	3	3	Ser(S)/Asp(D)	GPGQ	CCR5-using only	CCR5-using
704MC016N	35	4	4	Ser(S)/Gly(G)	GPGQ	CCR5-using only	CCR5-using
704MC017N	35	4	4	Ser(S)/Asp(D)	GPGQ	CCR5-using only	CCR5-using
704MC018N	35	6	6	Ser (S)/Glu(E)	GPGQ	CCR5-using only	CCR5-using
704MC019N	35	5	5	Ser(S)/Asp(D)	GPGQ	CCR5-using only	CCR5-using
704MC020N	35	3	3	Ser(S)/Asp(D)	GPGQ	CXCR4-using	CCR5-using
704MC021N	35	3	3	Ser(S)/Asp(D)	GPGQ	CCR5-using only	CCR5-using
704MC024N	35	3	3	Ser(S)/Asp(D)	GPGQ	CCR5-using only	CCR5-using
704MC027N	37	7	7	Arg(R)/The(T)	GGPG	CXCR4-using	CXCR4-using
704MC028N	36	7	7	Gly(G)/Lys(K)	GPGR	CXCR4-using	CXCR4-using
704MC029N	35	4	4	Ser (S)/Ala(A)	GPGQ	CCR5-using only	CCR5-using
704MC030N	35	5	5	Ser(S)/Asp(D)	GPGQ	CCR5-using only	CCR5-using
704MC034N	34	4	4	Ser(S)/Asp(D	GPGQ	CCR5-using only	CCR5-using
704MC035N	35	4	4	Ser(S)/Asp(D	GPGQ	CCR5-using only	CCR5-using

*C-PSSM cannot distinguish between CXCR4-using and dual tropic and classifies these as CXCR4-using. **The 3 rules include the overall net V3 charge, the amino acids at positions 11 and/or 25 and C-PSSM prediction.

Another feature of the *env* V3 loop associated with coreceptor usage and tropism determination is the property of amino acids at positions 11 and/or 25 (Fouchier et al. 1992). Of the 20 failures 6 clones had a negative amino acid at either one of these

positions with a neutral amino acid at the other. Five of these clones showed CCR5-usage according to C-PSSM. Six clones had a positive amino acid at either one of the positions with a neutral amino acid at the other. All 6 clones showed CXCR4-usage or dual tropism according to C-PSSM. The remaining 8 clones all had neutral amino acids at both positions. Five of these showed X4-usage or dual tropism and 3 showed R5-usage according to C-PSSM. Majority (15) of the naïve clones had a negative amino acid at either position 11 or 25 and a neutral charge at the other. All but one (704MC020N) showed CCR5-usage according to C-PSSM. Two (704MC027N and 704MC028N) had a positive amino acid at either one of these positions with a neutral amino acid at the other. Both exhibited CXCR4-usage or dual tropism. Clone from 704MC010N had positive amino acids at both positions 11 and 25 and both showed X4-usage or dual tropism. 704MC016N and 704MC029N had neutral amino acids at both positions and showed R5-usage according to C-PSSM (Table 4.3).

The number of amino acids in the V3 loop can also be indicative of tropism. The typical V3 loop from CCR5 tropic viruses has 35 amino acids. Thirty of the 40 V3 clonal sequences had an amino acid of 35. Thirteen (43%) of these belonged to the group failing therapy. Majority of the clones in the ARV-naïve group (17; 57%) had a V3 length of 35 amino acids. Five clones had an amino acid length of 34. Four of the 5 belonged to the ARV-failing group. Two clones had 36 amino acids, one from each group of patients and two clones had 37 amino acids – again, one from each group of patients. One clone (704MC034F) had only 31 amino acids within the V3 region (Table 4.3).

Amino acid insertions in the V3 loop, particularly at positions 13 and 14 are features consistent with CXCR4 usage as previously described (Coetzer et al. 2006). This was

found this in three of the four clones that had a V3 length of greater than 35 (704MC012F, 704MC027N, 704MC028N). All of these show CXCR4-using or dual tropic according to C-PSSM. The fourth clone (704MC009F) shows an insertion at position 19 and was also indicated as CXCR4-using or dual tropic by C-PSSM.

The V3 region was also analyzed by manually calculating the overall net amino acid charge, another indicator of *env* coreceptor utilization (Table 4.3). C-PSSM was also used. Both manual and C-PSSM calculations were comparable for all clones. A higher overall net V3 charge is associated with X4-usage. As a general rule a charge less than +4.5 is regarded as R5-using and charges greater than +4.5 are regarded as X4-using (Coetzer et al. 2006; Fouchier et al. 1995; Fouchier et al. 1992; Kuiken et al. 1992). When we looked at the charge obtained to the C-PSSM coreceptor prediction, we saw this in 32 cases (80%). The remaining 9 showed discrepancies to this rule. However, 6 of these (704MC019F, 704MC023F, 704MC003N, 704MC008N, 704MC019N and 704MC030N) had charges of (+5) and showed CCR5-usage, only slightly above +4.5. Clone 704MC018N had a charge of +6 and displayed X4-usage or dual tropism and 704MC020N had a charge of 3 even though C-PSSM predication was CXCR4-using or dual tropic.

Therefore, based on the multiple V3 loop sequence-based algorithms available for phenotype prediction, the clones generated suggest that there are discrepancies among the various methods in terms of predictability when using C-PSSM as not all results indicated the same coreceptor prediction. Such observations have also been recorded previously (Johnston et al. 2003), where in one case a R5 virus showed X4 characteristics. For the purpose of comparing the predictive coreceptor usage obtained

for the cloned samples in this study as well as the drug resistance mutations, a series of predictors of coreceptor usage was employed. These included the 11/25 rule, the overall net V3 charge and C-PSSM. A combined algorithm which made use of all 3 predictive rules was used and was calculated taking into account 2 correlating predictions from the 3 rules.

Using the combined algorithm, the V1/V2, V3 and V4/V5 regions of the *env* gene was further analyzed as these regions have been implicated in playing a role in coreceptor utilization. Sequence features in these regions that may influence viral tropism are the amino acid length and the number of predicted N-linked glycosylation sites (Chohan et al. 2005; Coetzer et al. 2007; Coetzer et al. 2008; Masciotra et al. 2002; Pollakis et al. 2001). The total number of predicted N-linked glycosylation sites in clones from this study varied from 24 to 37. Various groups were compared and the predicted R5-using failures had a significantly greater amount of predicted N-linked glycosylation sites within the *env* (p=0.0123). No other significant findings with regard to predicted N-linked glycosylation sites within the specific regions i.e. the V1/V2, V3 and V4/V5 regions were observed (Figure 4.5).

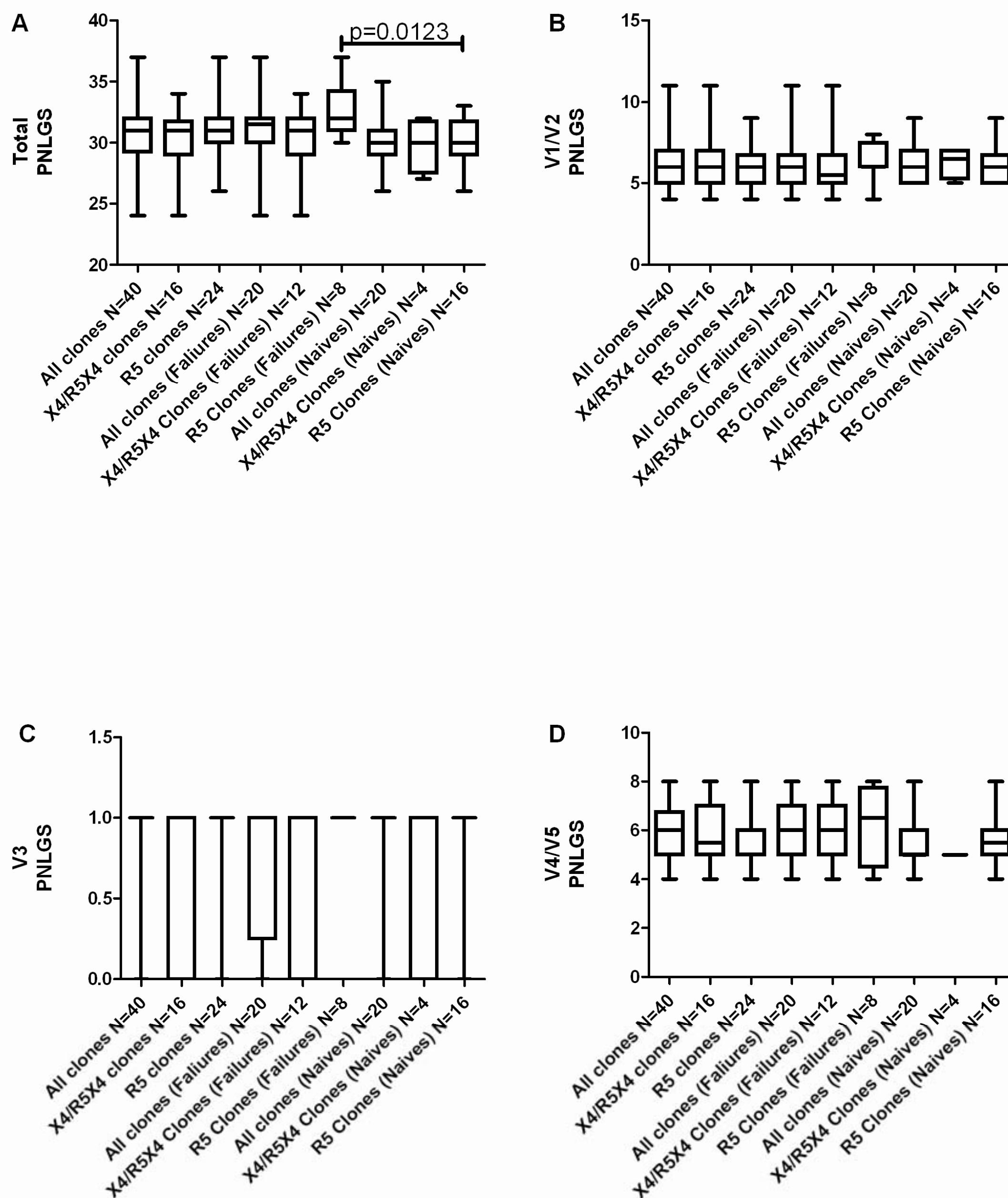


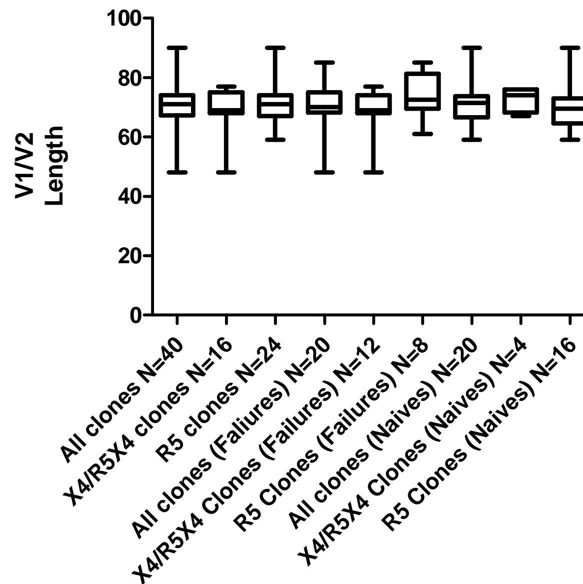
Figure 4.5: Box plots of predicted N-linked glycosylation sites (PNLGS)

A) shows the total number of PNLGS within the *env*. B) the number of PNGLS within the V1/V2 region C) the number of PNGLS within the V3 region D) the number of PNLGS within the V4/V5 region. The line within each box represents the median value for each group.

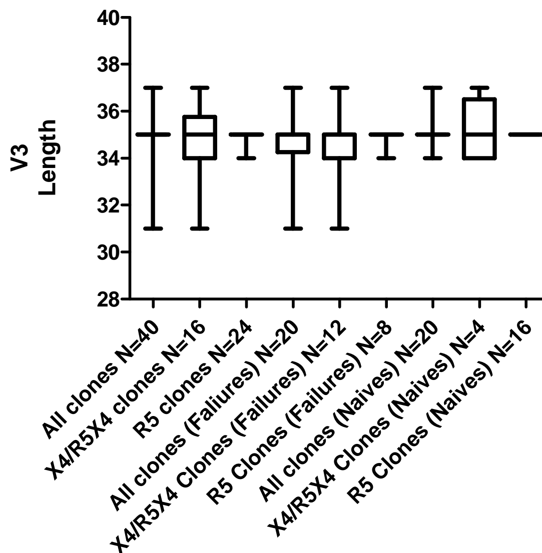
Previous reports have suggested that a lack of predicted N-linked glycosylation sites at positions 6-8 of the V3 loop may be indicative of CXCR4-utilization (Coetzer et al. 2006). Seven generated clones had a lack of this site in our study (704MC009F, 704MC018F, 704MC022F, 704MC028F, 704MC010N, 704MC020N and 704MC028N). Of these majority (5) were predicted to be CXCR4-using.

Differences in the loop lengths between R5, X4 and R5X4 sequences were next analyzed. The overall V1/V2 length ranged from 48-90 amino acids and the combined V4/V5 loop length ranged from 34-47. The V3 loop length ranged from 31-37 amino acids. No significant observations were obtained regarding loop length (Figure 4.6).

A



B



C

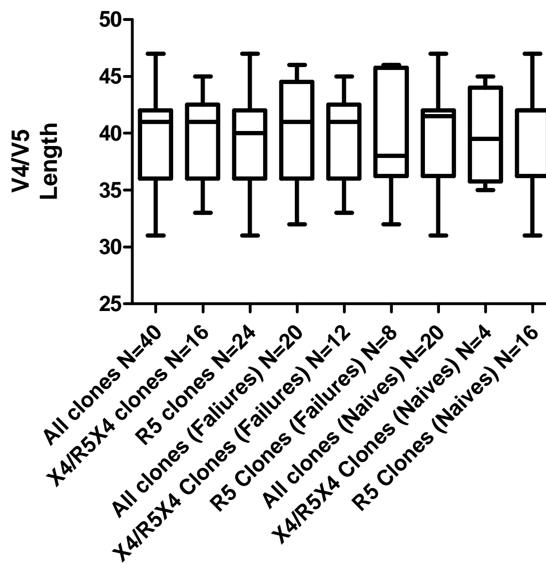


Figure 4.6: Box plots of *env* variable loop lengths.
A) the V1/V2 loop length. B) the V3loop length C) the V4/V5 loop length. The line within each box represents the median value for each group.

Using the combined algorithm method for coreceptor prediction, it was then investigated whether those patients failing treatment had a higher percentage of predicted CXCR4 usage. With the entire group of patients from which sequence data has been generated it was found that 60% of failures were predicted to be CXCR4-using and of the ARV-naïve patients 85% were predicted to be CCR5-using (p=0.0079). This also produced a significant result in our functional assay. No significant association between CD4 counts and X4-usage was observed (p=0.65). However, when X4/dual/mixed-usage in failures verses naive patients were examined, patients failing treatment harboured a significantly higher amount of X4/dual/mixed viruses (p=0.0028) as compared to R5 viruses where no significance was obtained (p=0.49). It was also investigated whether predicted CXCR4-usage was associated with older patients. No significant result (p=0.41) was obtained. It was then further investigated if the length of treatment could possibly be a driving force towards predicted CXCR4-usage but once again no significant result was obtained for this (p=0.88). It was next investigated whether those patients with TAMs were mostly predicted to have CXCR4-using viruses. Five (56%) of failures with TAMs were predicted to use CXCR4 and 4 (44%) were predicted to use CCR5. Although TAMs were found in more patients that harboured X4/dual/mixed viruses no significance was reported (p=0.31). It is noteworthy that for the predicted results a much smaller group of patients were analyzed.

The various predictive algorithms were also assessed in their ability to correctly predict sequences. Functional results for 32 of the 40 patients were obtained and on which predictive analysis was done using the Trofile Coreceptor Tropism Assay. Twenty-seven samples (84%) produced comparable results when the predictive data using the combined algorithm and the functional data were analyzed. Only 5 samples (16%)

produced results that did not correlate between the two methods. Overall the combined algorithm predicted 84% of sequences correctly followed by C-PSSM (81%), 11/25 rule (78%) and overall net V3 charge (75%) (Table 4.4). Both the combined algorithm and the 11/25 rule produced the highest percentage (90%) of R5 sequences correctly predicted with C-PSSM being 85% and overall net V3 charge of 71%. This was in contrast for X4/dual/mixed sequences where the overall net V3 charge displayed the highest percentage (81%) of correct prediction followed by the combined algorithm and C-PSSM (72%) and then the 11/25 rule (55%). In summary these predictive methods have proved to be reliable as analysis of results from both the functional assay as well as the predicted coreceptor utilization method were comparable.

Table 4.4: V3 loop-based methods for coreceptor usage prediction

Method	% of sequences correctly predicted	% of R5 sequences correctly predicted	% of X4/D/M sequences correctly predicted
11/25	78	90	55
Overall net V3 charge	75	71	81
C-PSSM	81	85	72
Combined algorithm*	84	90	72

*The combined algorithm makes use of all 3 predictive rules i.e. the amino acids at positions 11 and/or 25, the overall net V3 charge and C-PSSM prediction. For the combined algorithm, results were calculated taking into account 2 correlating predictions from the 3 rules.

4.4. Discussion

Highly active antiretroviral therapy has saved lives and improved the quality of lives for millions of people living with HIV/AIDS worldwide. However, the emergence and spread of drug resistance could limit and reverse some of the gains experienced so far in the clinical management of HIV/AIDS, particularly in the developing world where the options of affordable and easily accessible antiretroviral drugs are limited. Monitoring the patterns of antiretroviral drug use and emerging drug resistance is therefore crucial for the success and sustainability of treatment programmes. Moreover, as new drugs become available, there is a growing need to better characterize viruses from both drug naïve and Virologyogically failing patients in order to better understand the suitability of these new drugs either as part of the current regimens or as salvage therapy options. This study investigated the prevalence and pattern of drug resistance mutations in a cohort of HIV-1 subtype C-infected individuals failing therapy. In addition, since it has been previously reported that suboptimal antiretroviral treatment or certain classes of drugs may select for the more virulent X4 virus variants (Johnston et al. 2003), and in an attempt to better understand coreceptor usage evolution for HIV-1 subtype C viruses, the determinants of viral tropism in antiretroviral therapy-naïve and therapy experienced Virologyogically failing patients were analyzed.

Results show that in a South African setting where patients are receiving antiretroviral therapy according to national and WHO guidelines, 95% of patients failing therapy had at least one drug resistance mutation. More than half of the patients had a previous history of ARV therapy and majority of the patients were on Regimen 1A (d4T, 3TC, EFV). The most common NRTI mutation noted was M184V/I in 87% of patients, consistent with other recent subtype C studies (Marconi et al. 2008), although the percentage of patients with this

mutation was higher in this cohort. V106M was the commonest NNRTI resistance mutation, occurring in 49% of Virologyogically patients and at a much higher frequency compared to the V106A (2%) mutation. This pattern is also consistent with reports from previous HIV-1C studies (Brenner et al. 2003; Kantor et al. 2005; Loemba et al. 2002; Marconi et al. 2008). The K103N mutation was also relatively common at (40%), and unsurprisingly, there were hardly any protease inhibitor mutations since none of the patients were on protease inhibitors. Data from this study also revealed that 55% of patients failing therapy had thymidine analog mutations (TAMs) compared to 32% of patients studied from the same city in 2005-2006 (Marconi et al. 2008). A recent study in Malawi also reported a similar high percentage (56%) of TAMs in persons failing therapy under the public sector antiretroviral programme (Hosseinipour et al. 2009), and these data are in contrast to earlier studies where lower levels of TAMs were reported (Marconi et al, 2008 and Novitsky et al, 2007). There was a higher percentage of TAM2 pathway mutations (44%) in this cohort compared to the Marconi cohort from the same population (19%) whereas in the studies by Novitsky *et al*. and Cozzi-Lepri *et al*., 35% and 52% of the TAM2 pathway mutations were identified respectively (Cozzi-Lepri et al. 2009; Marconi et al. 2008; Novitsky et al. 2007). A higher TAM2 versus TAM1 percentage similar to previous studies was observed (Marconi et al. 2008; Novitsky et al. 2007) but very low when compared to Cozzi-Lepri *et al*. where 65% of TAM1 pathway mutations were seen but this is explained by the longer treatment period in this study (Cozzi-Lepri et al. 2009) as compared to the other studies. From these findings, we see that TAM mutations appear to be occurring at high frequencies particularly in patients following government/national/WHO guidelines in resource-poor settings, which may be severely limiting in terms of switch regimes. In addition, the same mutations are seen in other subtype C studies, and TAM2 is more common despite different regimens. This is in contrast to subtype B. This may require further studies. Overall, it can be concluded that there is a high

level of mutations in ART-experienced patients failing therapy suggesting the development of drug resistance rather than non-adherence whereas no significant mutations in ART-naïve individuals were identified (although sample size is small).

Numerous studies have previously reported that X4 viruses are rare in HIV-1C infections. The data obtained here appear to be consistent with these previous studies as only one sample contained X4 only variants. However, it has recently been shown that X4 clones dominate in dual tropic primary HIV-1C isolates propagated in peripheral blood mononuclear cells (Singh et al. 2009) , while in contrast others have described that R5 viruses predominate in plasma from X4/dual/mixed subtype B (Irlbeck et al. 2008) . Therefore, further studies will be needed to address which envelope clones dominate in plasma from the X4/dual/mixed phenotypes identified in this study. However, it can be concluded that in most HIV-1C infections, there is residual CCR5 utilization by HIV-1 despite evolution to CXCR4 usage, and this is in contrast to HIV-1 subtype B where complete switch to CXCR4 usage is commonly observed (Bjorndal and Sonnerborg 1999; Cecilia et al. 2000; Cilliers et al. 2003; Ndung'u et al. 2006; Tscherning et al. 1998). It was also investigated whether HAART-failing HIV-1C-infected patients had higher proportion of X4/dual/mixed viruses compared to HAART-naïve patients. Although this was found to be the case, the patients failing HAART had lower median (nadir) CD4+ counts compared to HAART-naïve patients, and as shown in Figure 4.2B, individuals possessing X4/dual/mixed viruses had significantly lower CD4+ T cell counts compared to those with R5-only viruses, in both the HAART-naïve and HAART-failing arms of this study. These data therefore suggest that CD4+ T cell counts (and perhaps length of infection), rather than HAART is the possible main cause of X4/dual/mixed viruses, consistent with data from HIV-1 subtype B studies (Briz et al. 2008; Hunt et al. 2006). However, only a longitudinal study can decisively determine whether there is higher

proportion of emergence of X4/dual/mixed viruses in treated versus HAART-naïve patients with similar CD4 T cell counts. This study underlines the importance of introducing CCR5 inhibitors relatively early in the course of HIV-1 subtype C infection for possible maximum benefit and to preserve other drugs for later use. Clinical trials are needed to determine the equivalence or superiority of CCR5 inhibitors as part of first line or early regimens, rather than as salvage therapy in HIV-1 subtype C settings.

Finally, the availability of virus phenotype and genotype data allowed us to assess the utility of V3 loop sequenced based methods for predicting viral tropism. Data shows that while genotypic methods are reliable for a majority of cases, they failed to correctly predict tropism in 10 to 45% of cases. It was also noteworthy that sequence based algorithms were better at predicting R5 compared to X4/dual/mixed phenotypes. There remains an urgent need to further investigate and develop better predictive algorithms, perhaps taking into account sequences outside of the V3 and more detailed analysis of V3 loop sequences using newer technologies able to better characterize V3 loop quasispecies diversity.

In summary, this study examines coreceptor tropism directly in patients failing currently recommended regimens, and compares this with ARV-naïve patients in a HIV-1 subtype C setting. The presence of high proportions of patients with TAMs suggests that these mutations may be accumulating over time in this population as a result of inadequate viral suppression, most likely as a consequence of immunological and clinically driven monitoring. These results may suggests that in situations where Virologic monitoring is not possible, measures need to be put in place to improve adherence and to develop new monitoring tools. Comparison of the prevalence of CXCR4-utilizing viruses between ARV-

naive prior to initiating ART with the prevalence among treated patients revealed that there was a high prevalence of X4/dual/mixed utilizing viruses in patients failing treatment, possibly due to lower nadir CD4 counts in these patients, underlining the need for investigating the possible earlier use of CCR5 inhibitors before the development of X4/dual/mixed viruses. Data also highlights the usefulness and limitations of genotypic coreceptor prediction methods in assessing whether HIV-1C infected patients can be put on regimens that include CCR5 inhibitors. Longitudinal studies on viral coreceptor evolution in HIV-1C infections are warranted.

CHAPTER 5

Discussion and Conclusion

Infection with human immunodeficiency virus type 1 (HIV-1) is characterized by high genomic diversity of the virus. Because of this diversity several distinct groups of the virus each with its own unique DNA sequence characteristics have emerged. HIV-1 is divided into three groups – M (major) which accounts for 90% of infections, O (outlyier) and N (new or non-O, non-M) (Lihana et al. 2009; Penn et al. 2008; Simon et al. 1998; Wainberg 2004). The major group is further subdivided into 9 subtypes (Peeters and Sharp 2000; Thomson et al. 2002) and 43 circulating recombinant forms (CRFs) (http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html). Particular viral subtypes predominate in specific geographical regions with subtype C, the focus of this study, accounting for more than half of all new infections worldwide and being the most predominant circulating subtype in southern Africa (Hemelaar et al. 2006; Penn et al. 2008). Viral entry into cells is mediated by binding of the viral envelope (Env) gp120 with a CD4 receptor and a chemokine receptor most often CCR5 and CXCR4 (Alkhatib et al. 1996a; Choe et al. 1996; Deng and Liu 1996; Dragic and Litwen 1996; Feng and Broder 1996; Kwong et al. 1998; Lusso 2006; Rizzuto et al. 1998; Wyatt et al. 1995). HIV-1 viral isolates are differentiated based on their ability to use these coreceptors – R5 viruses use CCR5, X4 viruses use CXCR4 and R5X4 (dual tropic) viruses use both CCR5 and CXCR4. According to various published studies HIV-1 subtype C has a unique viral coreceptor evolution pattern in that a switch from the predominant CCR5 (R5) to CXCR4 (X4) phenotype is less common for this subtype compared to other subtypes. However, although rare, dual tropic HIV-1C

isolates have been described previously. Furthermore, reports have suggested that certain highly active antiretroviral drugs (HAART) may select for X4 viral variants. Therefore, this study was undertaken to better understand the functional and genotypic characteristics of dual tropic HIV-1C isolates, and to characterize drug resistance and coreceptor usage patterns in HAART-naïve versus HAART-failing HIV-1C infected patients.

Coreceptor utilization has been characterized extensively in previous studies. However, most previous studies have focused on viral isolates, although HIV-1 exists as distinct quasispecies within isolates (Coetzer et al. 2006; Ping et al. 1999; Yi et al. 1999; Yi et al. 2005). Since most previous studies have shown that HIV-1 subtype C maintains preferential CCR5 utilization even in late stages of clinical AIDS, it is hypothesized that R5 clones would dominate in dual tropic HIV-1 subtype C isolates. In order to test this hypothesis and further determine whether dual tropic HIV-1 subtype C isolates exist as a mixture of CCR5- and CXCR4- tropic quasispecies, truly dual tropic viruses or both at the clonal level, 35 functional HIV-1 full-length *env* clones derived from seven dual tropic HIV-1C strains were first generated. The coreceptor usage of the clones in transformed cell lines was characterized using two different phenotypic methods of transfection and infection. The sequence characteristics of these clones were also examined in order to better elucidate the genetic determinants of coreceptor utilization by HIV-1 subtype C viruses. Using the DEAE-dextran method of infection, 30 of 35 (85.7%) clones were CXCR4-tropic clones, thus demonstrating that X4 clones dominated within the dual tropic viral isolates quasispecies. A minority proportion of dual tropic clones (14.3%) were also identified. However, when the approach of coreceptor utilization was changed to a spinnoculation method of infection, 20 of 35 (57%) of the clones were dual tropic with 43% exhibiting exclusive X4 phenotype. Therefore the method of infection used may result in significant differences in phenotypic determination of

viral coreceptor usage, and this finding has important implications for the comparison of data across different studies that use different methods. However, surprisingly and importantly, using both methods of infection, there was not a single CCR5-monotropic *env* clone identified from the seven primary isolates analyzed. This result was unusual considering that previous studies have shown that HIV-1 subtype C viruses utilize CCR5-only predominantly for cell entry, even in late stages of disease. Predominance or at least a significant proportion of the CCR5 clones among the quasispecies of the dual tropic isolates was therefore expected to be found. Instead, majority of the clones were CXCR4-utilizing with a substantial percentage being able to mediate cell entry via the CXCR4 receptor exclusively.

The finding that X4 clones dominated in these dual tropic strains was further confirmed by analysis of sequences from both transformed cell lines (U87.CD4 cells expressing both CXCR4 and CCR5) and peripheral blood mononuclear cells were examined. All of these sequences were classified as X4/dual tropic, including clones generated from the U87.CD4 cell line expressing CCR5 that were classified as dual tropic. Single genome amplification of clones from one dual tropic isolate also produced sequences that were predominantly X4/dual tropic.

Phenotypic analysis is expensive and not practical to carry out in heavily burdened populations, particularly in a poor resource setting. Genotypic sequence analysis would mitigate some of the cost and feasibility concerns. However, it has been shown that genotypic methods may not always be reliable as they do not always predict coreceptor usage especially in distinguishing X4 and R5X4 viruses (Garrido et al. 2008; Low et al. 2007), so there is a continuing need to develop better sequence based genotypic algorithms. The next aim

therefore was to establish how consistent these currently available genotypic methods for HIV-1 subtype C are by comparing the phenotypic data obtained to genotypic data analysis. Coreceptor utilization is genetically determined by sequence characteristics within the *env* gene. These include specific amino acid changes particularly within the V3 variable loop as well as the number and pattern of predicted N-linked glycosylation sites. The V3 region is critical for coreceptor binding and is the major determinant of which chemokine receptor (CCR5 or CXCR4) will be the accessory protein used by the virus for membrane fusion (Cilliers et al. 2003; Coetzer et al. 2007; Coetzer et al. 2006; Fouchier et al. 1992; Huang et al. 2005; Morris et al. 2007; Ndung'u et al. 2006). This region typically consists of approximately 35 amino acids in CCR5-tropic viruses (Coetzer et al. 2006; Mefford et al. 2008) and variations such as variations in the length, the amino acids at positions 11 and 25, changes within the crown motif and the overall net charge may all contribute to coreceptor usage (Coetzer et al. 2007; Coetzer et al. 2006; Jensen et al. 2006; Jensen et al. 2003; Renjifo et al. 1999). A web based programme C-PSSM (http://indra.mullins.microbiol.washington.edu/pssm/) which also assists in determining viral phenotype was used. PSSM is a bioinformatic tool used for predicting HIV-1 coreceptor usage from the amino acid sequence of the V3 loop. PSSM is in most part highly accurate but there have been studies where not all results were completely accurate particularly when clonal samples were studied (Jensen et al. 2003; Low et al. 2007).

Other sequence characteristics outside of the V3 loop may also assist in coreceptor utilization. These include specific amino acid changes within the remaining variable regions V1/V2, V3, V4/V5 as well as the pattern and number of predicted N-linked glycosylation sites. All of these genotypic methods of analysis proved to be reliable in this study and there was a strong correlation between the phenotypic and genotypic methods used, although

limitations were noted. Hence it is suggested from these observations that the genotypic methods investigated in this study were consistent.

The findings from this study have many implications on HIV-1 subtype C pathogenesis particularly on coreceptor evolution. It was found that at the clonal level, dual tropic strains appear to be dominated by X4 virus variants. Further, a substantial number (57%) of subtype C-infected patients investigated in this study harbored dual tropic viruses. CCR5 inhibitors would not be suitable for managing such individuals with dual tropic HIV-1 subtype C infection. The initial hypothesis was that in a subtype C setting R5 viruses would dominate in these strains but observations from this study overwhelmingly refutes that hypothesis and clearly suggests that CCR5 antagonists are unsuitable even as salvage therapy in dual tropic HIV-1 subtype C. All of these observations impact on coreceptor evolution and may imply that a rapid loss of CCR5 tropism is occurring as CXCR4 usage emerges. This is consistent with recent findings of rapid decline in CCR5 utilization as alternate coreceptor utilization emerges in HIV-1 subtype B infection (Coetzer et al. 2008). However one would question that if viruses had been cloned directly from plasma rather than using primary isolates cultured in peripheral blood mononuclear cells, would X4 viruses still dominate? This may be a question for further study especially since a previous study has shown that R5 viruses predominated in dual tropic HIV derived from plasma samples (Irlbeck et al. 2008).

Most studies on viral coreceptor usage have been done using stably transfected reporter cell lines. However, transfected cell systems may differ from coreceptor utilization *in vivo* where the main target cells of infection are primary lymphocytes and macrophages (Yi et al. 2005). The ability of the HIV-1C *env* clones to facilitate infection of lymphocytes and macrophages

was next investigated and their coreceptor utilization profiles in primary CD4+ T lymphocytes and monocyte-derived macrophages characterized. Clones generated from dual tropic isolates irrespective of whether they were CXCR4- or R5X4-using in transformed cell lines used either both the CCR5 and CXCR4 pathways of entry or exclusively the CXCR4 pathway of entry. When both pathways were utilized there was a striking bias towards CXCR4-usage in both CD4+ T lymphocytes and monocyte derived macrophages for majority of the viruses. A few viruses displayed comparable CCR5 and CXCR4 usage and clones from one virus isolate preferred CCR5 usage in macrophages. It is noteworthy that some viruses particularly those that show lower levels of infection in transformed cell lines were not infectious in primary cells. Thus although in most instances (57%) coreceptor usage in primary cells was consistent with coreceptor utilization in transformed cell lines, a few cases (43%) showed that coreceptor phenotyping in transformed cell lines does not always predict usage in primary cells. It should also be noted that pseudoviruses were used for transfection and then infection. Perhaps infection with primary viruses may produce a different outcome.

Finally, the patterns of drug resistance mutations and coreceptor usage among HAART-naïve and HAART-failing HIV-1C infected patients were studied and analyzed. At least one drug resistance mutation was observed in 95% of HAART-failing patients with thymidine analog resistance mutations (TAMs) being present in 55% of patients. A high percentage of patients had mutations from the TAM2 pathway (44%) whereas 9% of patients had mutations from the TAM1 pathway. Overall the level of TAMs in patients from this study was high particularly when this study is compared to the study by Marconi *et al.*, (32%) which used patients from the same population. However the duration of treatment in each study may provide an explanation for this as patients from the Marconi study had a mean duration of treatment of 11 months (Marconi et al. 2008) versus this study of 29 months. Hence, shorter

exposure to failing regimens may reduce the opportunity for the accumulation of TAMs. Also, because more than half of the patients were receiving thymidine analogs (stavudine and zidovudine) and majority of patients were previously on a regimen containing either one or both of these drugs, it is an indication that these mutations were highly favoured. The fact that most patients failing HAART had drug resistance mutations shows that these patients are truly failing therapy and the problem is not that patients are being non-compliant.

In addition a high percentage of patients had high level resistance to 3TC and FTC followed by ABC. This is consistent with the fact that the M184VI mutation occurred most frequently and this is associated with high level resistance to 3TC and FTC (http://hivdb.stanford.edu).

NNRTI mutations V106M and K103N also occurred at high frequency. These are associated with high level resistance to NVP, DLV, EFV and ETR (http://hivdb.stanford.edu) all of which showed a substantial percentage of high level resistance.

HAART-failing patients had significantly higher prevalence (59%) of X4/dual/mixed-utilizing viruses compared to HAART-naïve patients (30%) using the Trofile coreceptor tropism assay while 41% of HAART-failing patients harbored viruses that used CCR5 and 70% of HAART-naïve patients used CCR5. This is in keeping with previous studies where X4/dual tropic viruses dominated in patients failing treatment (Hunt et al. 2006; Johnston et al. 2003). This suggests that ARVs may be creating an environment for the emergence of X4/dual/mixed-utilizing viruses. However, patients failing treatment had lower nadir CD4 counts so it may be suggested that X4/dual/mixed viruses occurred at high frequency because these patients had lower CD4 counts. Only a longitudinal study can decisively determine

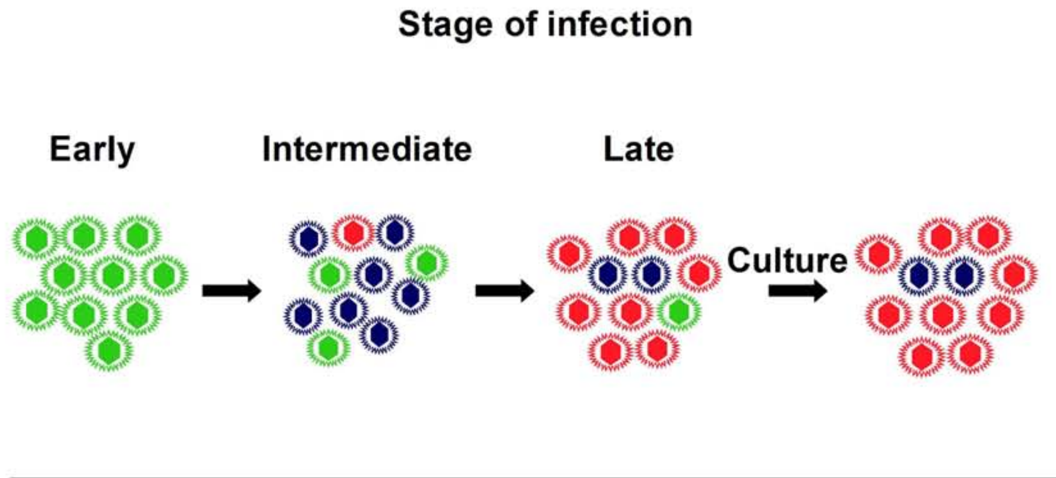
whether there is higher proportion of emergence of X4/dual/mixed viruses in treated versus HAART-naïve patients with similar CD4 T cell counts. As most of these virus variants were dual/mixed variants and only 1 exclusive CXCR4-utilizing virus was found, it can be suggested that coreceptor evolution clearly occurs but also that X4-only viruses are rare. However, as the presence of exclusive CCR5-using viruses has not been observed in this study, it is suggested that CCR5 inhibitors may not be appropriate for a significant proportion of HIV-1 subtype C infected people with low CD4 counts. There is therefore a need for clinical trials to test coreceptor antagonists in early phases of infection before X4 viruses emerge. This study suggests that maximum benefit is more likely to be achieved when drugs such as maraviroc are used early in infection rather than waiting to use these drugs as salvage therapy. This study also shows that coreceptor testing will be necessary for HIV-1 subtype C infections before coreceptor antagonists can be used. This however, is currently expensive and may not be feasible.

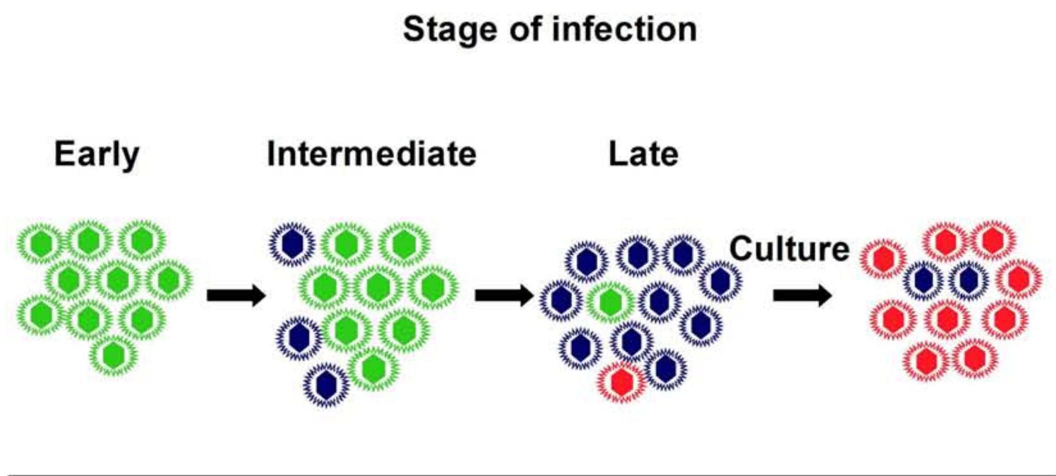
The correlation of phenotypic and genotypic methods was further investigated for patient plasma derived viruses that had not been cultured. There was an 84% correlation between the functional assay and the combined predictive algorithm method. This method correctly predicted 90% of R5 sequences and 72% of X4 sequences. C-PSSM predicted 81% of sequences correctly with 85% of R5 sequences and 72% of X4 sequences being correctly predicted. The 11/25 rule predicted 78% of sequences correctly with 90% of R5 sequences and 55% of X4 sequences being correctly predicted. The overall net V3 charge predicted 75% of sequences correctly with 71% of R5 sequences and 81% of X4 sequences being correctly predicted. It should be noted that there is a clear pattern that R5 sequences were better predicted than X4/dual/mixed sequences. However from these observations it is

suggested that although there were discrepancies, the genotypic methods investigated in this study were in most part consistent.

In summary, while X4 variants arose, R5 and dual/mixed viruses still dominated in both the naïve and treated patients. Further studies will be needed to address whether X4 clones dominated in the plasma of the patients studied here as the results do appear to suggest there are some differences between viruses derived from plasma and primary isolates cultured in peripheral blood mononuclear cells. Perhaps the culturing of primary isolates in peripheral blood mononuclear cells creates an environment that favors/selects for X4-virus variants thereby accounting for the high prevalence of CXCR4-utilizing viruses derived from peripheral blood mononuclear cells. From these observations, the following model had been hypothesized. For subtype B infection, an evolutionary shift suggests that these dual/mixed-utilizing viruses are in the intermediate stage of viral switching before CXCR4-usage associated with advanced disease progression and AIDS emerges and dominates (Figure 5.1A). Subtype C behaves differently where dual/mixed-utilizing viruses begin to emerge during the intermediate stage of infection although R5 viruses dominate during this stage. In the late stage of infection these dual/mixed viruses dominate possibly with the emergence of some X4-using viruses (Figure 5.1B). However, culturing of primary viral isolates in peripheral blood mononuclear cells may introduce some bias in somehow selecting for CXCR4-utilizing viruses (Figure 5.1A and B). This proposed model contributes to the existing knowledge of the HIV-1 epidemic but further investigation is required.

This study enhances our understanding of HIV-1 subtype C pathogenesis and the results have important implications for the use of coreceptor antagonists for the clinical management of HIV-1C infection.





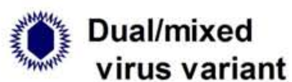


Figure 5.1: Viral evolution of HIV-1 subtype B and C

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Functional and genetic analysis of coreceptor usage by dualtropic HIV-1 subtype C isolates

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ABSTRACT

It is widely documented that a complete switch from the predominant CCR5 (R5) to CXCR4 (X4) phenotype is less common for HIV-1 subtype C (HIV-1C) compared to other major subtypes. We investigated whether dualtropic HIV-1C isolates represented dualtropic, mixed R5 and X4 clones or both. Thirty of 35 functional HIV-1 *env* clones generated by bulk PCR amplification from peripheral blood mononuclear cells (PBMCs) infected with seven dualtropic HIV-1C isolates utilized CXCR4 exclusively. Five of 35 clones displayed dualtropism. Endpoint dilution of one isolate did not yield a substantial proportion of R5-monotropic *env* clones. Sequence-based predictive algorithms showed that *env* sequences from PBMCs, CXCR4 or CCR5-expressing cell lines were indistinguishable and all possessed X4/dualtropic characteristics. We describe HIV-1C CXCR4-tropic *env* sequence features. Our results suggest a dramatic loss of CCR5 monotropism as dualtropism emerges in HIV-1C which has important implications for the use of coreceptor antagonists in therapeutic strategies for this subtype.

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Introduction

HIV/AIDS is the most serious infectious disease challenging the public health sector in sub-Saharan Africa. The disease remains the leading cause of death in the region and in 2007 alone, 76% of deaths were as a result of HIV/AIDS, with 68% of all new HIV infections occurring in this region. Southern Africa is the most seriously affected sub-region and in 2007 accounted for 32% of all new infections and AIDS-related deaths worldwide. South Africa constitutes the highest number of HIV infections globally (UNAIDS, 2007). The main circulating HIV-1 subtype in South Africa is HIV-1 subtype C (HIV-1C), which accounts for approximately 56% of infections worldwide (Esparza, 2005; Hemelaar et al., 2006; Visawapoka et al., 2006). Subtype groupings are based on sequence variations that occur within all gene and non-protein coding regulatory regions, but the most dramatic differences are found in the envelope (*env*) gene (Gao et al., 1998). The envelope is an important target of humoral immune responses and is a crucial determinant of overall viral fitness (Ball et al., 2003; Marozsan et al., 2005). The *env* gene plays an important role in viral transmission by determining which coreceptor the virus uses to mediate entry. During transmission and subsequent to infection, viral fitness and target cell tropism properties are thought to be important determinants of infectivity and the rate of disease progression (Troyer et al., 2005). The importance of the envelope as a major target of humoral immunity, its contribution to overall fitness during transmission, and its role in the rate of disease progression make it a particularly attractive target for vaccine and drug development. However, progress towards these goals has been greatly hindered by the extreme genetic variability of the *env* gene.

HIV-1 utilize members of the seven transmembrane chemokine receptor family as coreceptors for entry into target cells (de Roda Husman et al., 1999; Oppermann, 2004; Ross and Cullen, 1998; Vila-Coro et al., 2000; Xiao et al., 1999). The virus gp120 envelope glycoprotein first binds to the primary CD4 receptor on target cells, which induces conformational changes on the envelope exposing the coreceptor binding site (Rizzuto et al., 1998; Wyatt et al., 1995). The two main coreceptors that the HIV-1 envelope binds to subsequent to the conformational change are CCR5 or CXCR4 (Alkhatib et al., 1996; Choe et al., 1996; Deng and Liu, 1996; Doranz et al., 1996; Dragic and Litwen, 1996; Feng and Broder, 1996). HIV-1 strains can be classified based on their coreceptor utilization, with CCR5 tropic viruses termed R5, CXCR4 tropic viruses termed X4 and viruses that use both coreceptors (dualtropic viruses) termed R5X4 (Berger, 1998; Berger, Murphy, and Farber, 1999). R5 viruses predominate in the early stages

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of HIV-1 infection, whereas dualtropic and X4 variants, which are associated with rapid disease progression, emerge in the late chronic phase of disease in a significant proportion of patients (Connor et al., 1997; Scarlatti et al., 1997). It is well established that while all subtypes are capable of undergoing coreceptor utilization switch from CCR5 to CXCR4, this is less frequently encountered in HIV-1 subtype C infections, even in late stages of disease (Bjorndal and Sonnerborg, 1999; Cecilia et al., 2000; Cilliers et al., 2003; Ndung'u et al., 2006; Tscherning et al., 1998). Furthermore, expanded coreceptor usage beyond CCR5 and CXCR4 has also been occasionally reported but its significance for HIV-1 replication in vivo and disease progression is unclear (Aasa-Chapman et al., 2006; Cilliers et al., 2005; Dash et al., 2008).

Coreceptor utilization is genetically determined by sequence characteristics within the *env* gene, primarily specific amino acid changes within three of the five hypervariable regions namely the V1/V2 and V3 loops, as well as the number and pattern of predicted N-linked glycosylation sites (Fouchier et al., 1992; Pastore and Nedellec, 2006; Pollakis et al., 2001). Additional sequence changes within the *env* gene have also been implicated in coreceptor determination or the coreceptor switching process (Aasa-Chapman et al., 2006; Coetzer et al., 2008).

In several instances where HIV-1 subtype C isolates able to mediate cell entry via CXCR4 have been described, dualtropic (R5X4) strains that utilize both CCR5 and CXCR4 have been more frequently encountered compared to X4 monotropic viruses (Cilliers et al., 2003; Coetzer et al., 2006; Dash et al., 2008; Johnston et al., 2003; Ndung'u et al., 2006; van Rensburg et al., 2002). However, despite the occasional isolation of dualtropic HIV-1C viruses, such viruses have rarely been extensively characterized at both the functional and genetic clonal level. It is therefore largely unknown whether dualtropic HIV-1C strains represent a mixture of R5 and X4 viruses or truly dualtropic strains (or both) at the clonal level. Furthermore, genetic determinants associated with change in coreceptor usage have rarely been described for HIV-1 subtype C, particularly those that may reside outside of the V3 loop region. In this study we investigated whether dualtropic HIV-1C primary isolates represented truly dualtropic viruses at the clonal level, or mixed R5 and X4 clones. We describe the generation of functional envelope clones from dualtropic HIV-1C isolates and the sequence characteristics in the HIV-1C *env* gene, both within and outside of the V3 region that are associated with coreceptor utilization phenotype.

Results

Viral infection of stimulated PBMCs

Seven HIV-1 subtype C dualtropic isolates from individuals in South Africa (Cilliers et al., 2003) and Botswana (Ndung'u et al., 2006) were selected for this study. In addition, a well-characterized CCR5-only utilizing HIV-1 subtype C isolate, BWM01_5 was used as a positive control (Ndung'u et al., 2006). Infection of the stimulated PBMCs was assessed by HIV-1 p24 antigen ELISA over a 14-day culture period. As shown in Fig. 1, p24 antigen concentration increased in culture supernatant for all the isolates. The isolates replicated to different levels and with different replication kinetics. On day 14, culture supernatants were removed and genomic DNA was extracted from the cells for *env* gene amplification.

Confirmation of dualtropism of primary viral isolates

We first analyzed the seven primary viral isolates propagated in PBMCs for their ability to use multiple coreceptors on cell lines. Specifically, we analyzed for ability to mediate cell entry via CCR5 or CXCR4 because these are the main coreceptors previously described for a significant proportion of HIV-1 primary isolates. In order to assess the ability of the isolates to utilize these coreceptors, virus equivalent to 2 ng of p24 antigen each was used to infect U87.CD4 glioma cell lines with or without the co-expression of the coreceptors. Table 1 shows the highest amounts of p24 antigen reached by the primary isolates over a 10-day period in culture. All 7 primary viral isolates replicated in cells expressing CXCR4 and CCR5. It was noteworthy that while all the isolates replicated efficiently in CXCR4 expressing cells, 3 isolates (RP1, SW30 and CM9) replicated to relatively low titers in cells expressing CCR5. Isolates CM9 and SW30 were previously shown to replicate efficiently in both CCR5 and CXCR4 expressing cell lines (Cilliers et al., 2003) and therefore our results could indicate that in vitro passages of the isolates is selecting against CCR5 utilization.

Determination of coreceptor usage by env clones

The *env* gene (approximately 3 kb) was then amplified from PBMC genomic DNA by PCR, gel purified and cloned into a mammalian cell expression plasmid vector (pcDNA3.1D/V5-His-

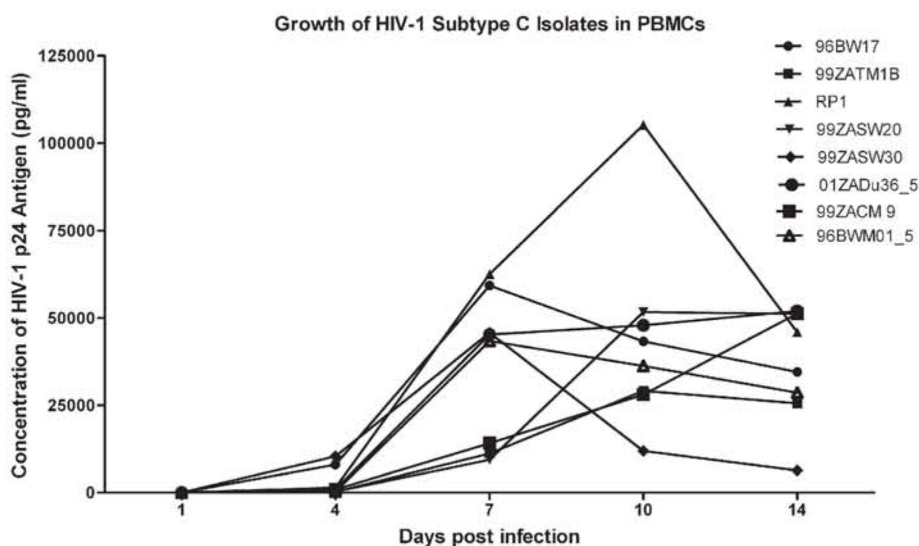

Fig. 1. Viral infection of PBMCs. 5000 pg p24 antigen equivalent of virus was used to infect stimulated PBMCs. Virus growth was monitored by p24 antigen concentration increased over a 14-day period. BWM01_5 is an R5 monotropic viral isolate and was used as a positive control. All isolates and clones are referred to by their shorter names within the text (BW17, TM1B, RP1, SW20, SW30, Du36_5, CM9 and BWMO1_5).

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Table 1
Coreceptor usage characterization of primary viral isolates.

Isolate	CD4 count (cells/μl)	Viral load (copies/ml)	Highest p24 antigen (pg/ml) reached by primary isolates over 10-day period in culture		
			U87.CD4	U87.CD4.CCR5	U87.CD4.CXCR4
96BW17	NA	NA	*(19)* 630	*(25)* 707,297	*(10)* 209,119
99ZATM1B	NA	190,000	*(13)* 610	*(151)* 32,012	*(126)* 98,654
RP1	7	178,830	*(0.9)* 610	*(1.2)* 2120	*(646)* 961,835
99ZASW20	2	43,595	*(0.2)* 0	*(0.2)* 160,683	*(1.5)* 47,787
99ZASW30	2	73,860	*(0.8)* 1	*(0.8)* 2141	*(1.0)* 158,623
99ZACM9	24	NA	*(1.1)* 2	*(1.2)* 2201	*(1.1)* 11,979
01ZADu36_5	25	54,944	*(1.3)* 1	*(2.1)* 1,658,728	*(1.0)* 46,525
96BWM01_5	NA	NA	–	–	–
Du179	279	2640	–	–	–

NA—not available. All patients were ART naive.
2000 pg of p24 viral supernatants from PBMC co-cultures was used to infect U87.CD4 cells expressing different coreceptors i.e. CCR5 or CXCR4 coreceptors. The U87.CD4 cell line was used as a control cell line. We used 2000 pg of HIV-1 p24 equivalent of virus to infect and cells were washed 3 times after overnight incubation with infecting stock. Virus growth above 2000 pg (amount used to infect) was considered productive infection of target cells. All italicized figures in brackets indicate background level i.e. the p24 antigen (pg/ml) on day 0.

TOPO). Five clones for each patient isolate were generated. Pseudoviruses were prepared by cotransfecting 293T cells with each of the 35 *env* plasmid clones with the pNL4-3.Luc.R-E-construct (Connor et al., 1995; He et al., 1995). This construct contains the infectious NL4-3 provirus backbone but is *env* and *vpr* deleted and *nef* has been replaced by the firefly luciferase gene. Virus supernatant from the transfected 293T cells equivalent to 2 ng p24 antigen was then used to infect U87 cells expressing the chemokine receptors CCR5 or CXCR4. A previously characterized dualtropic *env* clone Du179 (Coetzer et al., 2006) was used in parallel as a positive control. Productive entry of target cells was determined by measurement of firefly luciferase activity in cell lysates of infected U87 cells. All 35 *env* clones were able to efficiently infect cells expressing CXCR4 (Table 2). Five of 35 (14.3%) clones tested were also able to mediate entry via the CCR5 receptor, indicating that they were dualtropic. Two of the clones able to utilize CCR5 were from isolate RP1 while the other three were from isolate CM9. Surprisingly, there were no CCR5 monotropic clones detected from the bulk PCR envelope analysis of dualtropic isolates in this study.

We then reasoned that there were two possibilities to explain these results; one is that CCR5 monotropic envelopes are present at very low frequencies and therefore are virtually undetectable as clones from amongst the primary isolate viral quasispecies or that the CXCR4 viruses may be more fit and outcompeting CCR5 clones when propagated in PBMCs. In order to differentiate between these two possibilities, we infected U87.CD4.CCR5 cells with each of the primary viral isolates, with the objective of amplifying or favoring the CCR5-tropic clones in CCR5-only expressing cells. RNA was then extracted from the viral supernatant from U87.CD4.CCR5 cells, functional *env* clones generated and coreceptor usage determined as described for the PBMC-derived clones. All clones generated from U87.CD4.CCR5 cell supernatants showed dualtropism (data not shown). This result suggests that *env* clones using CCR5 only were virtually absent or present at very low frequencies within the quasispecies of the 7 primary isolates analyzed here.

Limiting endpoint dilution PCR

We also considered the possibility that bulk PCR *env* amplification and cloning could result in resampling bias and explain the absence of CCR5-only *env* clones. We thus used a single genome amplification approach to generate diverse clones from isolate Du36_5. This clone was selected for this analysis because it showed a bias towards CCR5 utilization and yet we had failed to identify CCR5-only tropic clones from this isolate. Thirty clones of DU36_5 were amplified and cloned by this limiting endpoint dilution PCR approach. These clones were then tested for coreceptor usage in the U87.CD4.CCR5 and U87.CD4.CXCR4 cell lines. Of the 26 functional clones, 24 were dualtropic, one clone used CXCR4 exclusively and one clone showed exclusive R5-usage.

Genotypic analysis of the env gene

All 35 *env* full-length clones generated in this study by bulk PCR were sequenced to investigate phylogenetic relationships and to correlate coreceptor usage phenotype to genotype data. Phylogenetic analysis showed that all clones clustered with subtype C references with a high degree of confidence (Fig. 2). Furthermore, the clones from each primary viral isolate clustered together. As described above, sequences were also generated from U87.CD4.CCR5 and U87.CD4.CXCR4 cells infected with each of the primary isolates. These clones utilized both CCR5 and CXCR4, and their sequences were virtually phylogenetically indistinguishable from those obtained from PBMC cultures. Results obtained by using position-specific scoring matrix for HIV-1 subtype C (C-PSSM), a phenotype predictive tool based on HIV-1 subtype C sequences (Jensen et al., 2006) indicated CXCR4 or dualtropic phenotype and high net V3 charges (data not shown).

Table 2
Coreceptor usage characterization of the HIV-1C *env* clones.

Clone	U87.CD4.CCR5	U87.CD4.CXCR4	Accession number
96BW17 #2	–	+++	FJ846633
96BW17 #3	–	+++	FJ846634
96BW17 #6	–	+++	FJ846635
96BW17 #7	–	+++	FJ846636
96BW17 #15	–	+++	FJ846637
99ZATM1B #3	–	+++	FJ846653
99ZATM1B #5	–	+++	FJ846654
99ZATM1B #6	–	++	FJ846655
99ZATM1B #8	–	++	FJ846656
99ZATM1B #13	–	++	FJ846657
RP1 #5	–	++	FJ846658
RP1 #6	–	++	FJ846659
RP1 #8	–	++	FJ846660
RP1 #10	++	++	FJ846661
RP1 #13	++	++	FJ846662
99ZASW20 #2	–	++	FJ846643
99ZASW20 #3	–	++	FJ846644
99ZASW20 #11	–	++	FJ846645
99ZASW20 #14	–	+++	FJ846646
99ZASW20 #15	–	++	FJ846647
99ZASW30 #1	–	++	FJ846648
99ZASW30 #2	–	+++	FJ846649
99ZASW30 #3	–	+++	FJ846650
99ZASW30 #6	–	+++	FJ846651
99ZASW30 #9	–	+++	FJ846652
01ZADu36_5 #2	–	+++	FJ846628
01ZADu36_5 #7	–	+++	FJ846629
01ZADu36_5 #8	–	++	FJ846630
01ZADu36_5 #9	–	+++	FJ846631
01ZADu36_5 #10	–	++	FJ846632
99ZACM9 #1	++	+++	FJ846638
99ZACM9 #2	++	+++	FJ846639
99ZACM9 #16	–	+++	FJ846640
99ZACM9 #18	–	+++	FJ846641
99ZACM9 #21	+	+++	FJ846642
96BWM01_5[a]	+++	–	
96BW17#10[a]	–	+++	
Du179[a]	+++	+++	
pNL4-3.Luc.R-E-[a]	–	–	

For each experiment, a positive result was considered to be 2 × the average relative luminescence units (RLUs) of the negative control wells + standard deviation. RLUs above this but less than 3 × this cut off value are indicated by "+", values 3 × to 10 × above cut off are shown as "++" and values above 10 × the cut off are indicated as "+++".
[a] 96BWM01_5 is an R5-only control, 96BW17#10 is an X4-only control and Du179 is an R5X4 contol. pNL4-3.Luc.R-E- is the negative control.

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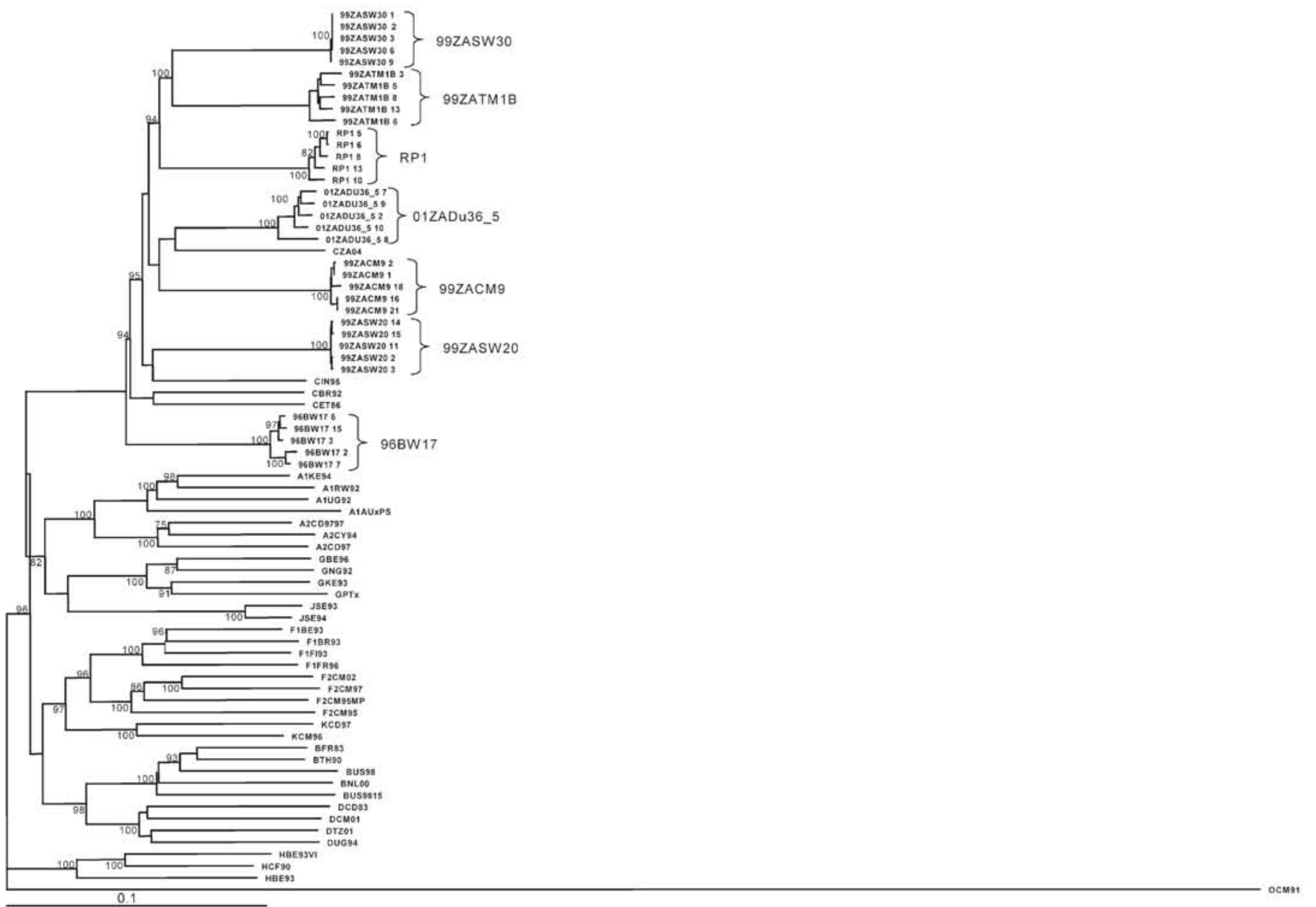

Fig. 2. Neighbour-Joining phylogenetic tree constructed from the *env* gene sequences. All clones of a particular viral isolate cluster closely together. Furthermore, all clones cluster with the subtype C reference with a high degree of confidence.

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Consensus C	C T R P N N N T R K S I R I . . G P G Q T F Y A T G D I I G D I R Q A H C
96BW17 #2	- - - - - - - - - - - M - - G I G R G Q - - - - M - R - - - - - - - - - -
96BW17 #3	- - - - - - - - - - - M - - G I G R G Q - - - - M - R - - - - - - - - - -
96BW17 #6	- - - - - - - - - - - M - - G I G R G Q - - - - M - R - - - - - - - - - -
96BW17 #7	- - - - - - - - - - - M - - G I G R G Q - - - - M - R - - - - - - - - - -
96BW17 #15	- - - - - - - - - - - M - - G I G R G Q - - - - M - R - - - - - - - - - -
Consensus	- - - - - - - - - - - M - - G I G R G Q - - - - M - R - - - - - - - - - -
99ZATM1B #3	- - - - - - - - - - N V - - G I G R G Q - - - - N - - - - - N - - - - - -
99ZATM1B #5	- - - - - - - - - - N V - - G I G R G Q - - - - N - - - - - N - - - - - -
99ZATM1B #6	- - - - - - - - - - N V - - G I G R G Q - - - - M - R - - - N - - - - - -
99ZATM1B #8	- - - - - - - - - - N V - - G I G R G Q - - - - N - - - - - N - - - - - -
99ZATM1B #13	- - - - - - - - - - N V - - G I G R G Q - - - - N - - - - - N - - - - - -
Consensus	- - - - - - - - - - N V - - G I G R G Q - - - - N - - - - - N - - - - - -
RP1 #5	- I - - G - - - - - R V - L G I G P G Q - - - - - - R V - R - - - - - - -
RP1 #6	- I - - G - - - - - R V - L G I G P G Q - - - - - - R V - R - - - - - - -
RP1 #8	- I - - G - - - - - R V - L G I G P G Q - - - - - - R V - R - - - - - - -
RP1 #10	- I - - G - - - - - R V - L G I G P G Q - - - - - - R V - R - - - - - - -
RP1 #13	- I - - G - - - - - R V - L G I G P G Q - - - - - - R V - R - - - - - - -
Consensus	- I - - G - - - - - R V - L G I G P G Q - - - - - - R V - R - - - - - - -
99ZASW20 #2	- - - - - - - - - - - - - T G I G R G Q - - - - - - Q - - - V - - - - -
99ZASW20 #3	- - - - - - - - - - - - - T G I G R G Q - - - - - - Q - - - V - - - - -
99ZASW20 #11	- - - - - - - - - - - - - T G I G R G Q - - - - - - Q - - - V - - - - -
99ZASW20 #14	- - - - - - - - - - - - - T G I G R G Q - - - - - - Q - - - V - - - - -
99ZASW20 #15	- - - - - - - - - - - V - I G I G R G H A - - T - K V - - N - - - - - - -
Consensus	- - - - - - - - - - - - - T G I G R G Q - - - - - - Q - - - V - - - - -
99ZASW30 #1	- - - - - - - - - - - V - - G I G R G H A - - T - G K V - - N - - - - - -
99ZASW30 #2	- - - - - - - - - - - V - - G I G R G H A - - T - G K V - - N - - - - - -
99ZASW30 #3	- - - - - - - - - - - V - - G I G R G H A - - T - G K V - - N - - - - - -
99ZASW30 #6	- - - - - - - - - - - M - - G I G R G H A - - T - G K V - - N - - - - - -
99ZASW30 #9	- - - - - - - - - - - V - - G I G R G H A - - T - G K V - - N - - - - - -
Consensus	- - - - - - - - - - - V - - G I G R G H A - - T - G K V - - N - - - - - -
01ZADu36_5 #2	- - - - D - K I N M K R I K I . G P G R A - V - - K G - R - - - R - - Y -
01ZADu36_5 #7	- - - - D - K I S M K R I K I . G P G R A - V - - K G - K - - - R - - Y -
01ZADu36_5 #8	- - - - D - K I N M K R I K I . G P G R A - V - - K G - K - - - R - -Y -
01ZADu36_5 #9	- - - - D - K I N M K R I K I . G P G R A - V - - K G - R - - - R - - Y -
01ZADu36_5 #10	- - - - D - K I S M K R I K I . G P G R A - V - - K G - K - - - R - - Y -
Consensus	- - - - D - K I N M K R I K I . G P G R A - V - - K G - K - - - R - - Y -
99ZACM9 #1	- A - - G - - - I - R - - - . . G P R Y A - - - K E T - - - - - - - - - -
99ZACM9 #2	- A - - G - - - I - R - - - . . G P R Y A - - - K E T - - - - - - - - - -
99ZACM9 #16	- A - - G - - - I - R - - - . . G P R Y A - - - K E T - - - - - - - - - -
99ZACM9 #18	- A - - G - - - I - R - - - . . G P R Y A - - - K E T - - - - - - - - - -
99ZACM9 #21	- A - - G - - - I - R - - - . . G P R Y A - - - K E T - - - - - - - - - -
Consensus	- A - - G - - - I - R - - - . . G P R Y A - - - K E T - - - - - - - - - -

Fig. 3. Alignment of V3 sequences of clones of primary viral isolates. The crown motif for each sequence is indicated in blue and dualtropic clone sequences are indicated in green.

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Table 3
Summary table of V3 characteristics of clones of primary viral isolates.

Clone	A.A length	Calc V3 net charge	PSSM V3 net charge	Amino acid (11/25)	Crown motif	PSSM coreceptor usage	Phenotype prediction
R-5 only virus 96BWM01_5	35	4	4	Ser (S)/Asp (D)	GPGQ	CCR5-using only	CCR5-using
96BW17 #2; 3; 6; 7; 15	37	7	7	Ser (S)/Arg(R)	GRGQ	CXCR4-using	CXCR4-using or dt
99ZATM1B #3; 5; 6; 8; 13	37	8	8	Asp(N)/Arg(R)	GRGQ	CXCR4-using	CXCR4-using or dt
P1 #5; 6; 8; 10; 13	37	8	8	Arg(R)/Arg(R)	GPGQ	CXCR4-using	CXCR4-using or dt
99ZASW20; #2; 3; 11; 14; 15	37	6	6	Ser(S)/Glut(Q)	GRGQ	CXCR4-using	CXCR4-using or dt
99ZASW30 #1; 2; 3; 6; 9	37	9	8	Ser(S)/Lys(K)	GRGH	CXCR4-using	CXCR4-using or dt
01ZADu36_5 #2; 9	36	8	8	Arg(R)/Gly(G)	GPGR	CXCR4-using	CXCR4-using or dt
01ZADu36_5 #7; 8; 10	36	7	7	Arg(R)/Gly(G)	GPGR	CXCR4-using	CXCR4-using or dt
99ZACM9 #1; 2; 16; 18; 21	35	6	6	Thr(T)/Arg(R)	GPRY	CXCR4-using	CXCR4-using or dt

The envelope V3 loop is an important determinant of coreceptor utilization (Briggs et al., 2000; Cann et al., 1992; Fouchier et al., 1992; Rizzuto et al., 1998; Wu et al., 2006). Therefore, we further analyzed the V3 loop of the functionally characterized *env* clones in order to identify and describe sequences associated with dualtropism and CXCR4 utilization (Fig. 3 and Table 3). Of particular interest was the crown motif, a conserved tetrapeptide located at the tip of the V3 loop. Changes within this region may influence coreceptor usage. The consensus crown motif for clones from isolate RP1 was GPGQ, which is the conventional V3 loop crown sequence observed in CCR5-tropic subtype C sequences. The crown motifs for clones generated from Du36_5 and CM9 were GPGR and GPRY respectively, sequence substitutions that are indicative of CXCR4 tropism (Coetzer et al., 2006). Clones from BW17, TM1B and SW20 each displayed consensus crown motif sequences that read GRGQ. The consensus crown motifs of SW30, Du36_5 and CM9 read GRGH, GPGR and GPRY respectively. Thus CXCR4 utilization in HIV-1 subtype C is commonly associated with a basic amino acid substitution in the V3 tetrapeptide although this is not an absolute requirement.

Another feature of the *env* V3 loop associated with tropism determination is the property of amino acids at positions 11 and/or 25 (Fouchier et al., 1992). The consensus sequences for all isolates with the exception of SW20 showed a positively charged amino acid substitution at one or both of these positions. BW17 has serine (S) (neutral charge) and arginine (R) (positively charged); TM1B has asparagine (N) (neutral) and arginine, SW20 has serine and glutamine (Q) both of which carry neutral charges, SW30 has serine and lysine (K), Du36_5 has arginine and glycine (G) and CM9 has arginine and threonine (T) at positions 11 and 25 respectively. RP1 has arginine at both positions. The number of amino acids in the V3 loop can also be indicative of coreceptor usage. The typical V3 loop from CCR5 tropic viruses has 35 amino acids. Clones from CM9 were 35 amino acids long in the V3 loop, whereas clones from Du36_5 were 36 amino acids long. Clones from isolates TM1B, SW30 and BW17 had 2-amino acid insertions, increasing the length of the V3 loop to 37 amino acids. The insertions occurred at positions 13 and 14 of the V3 loop for clones from isolates RP1 and SW20 and at positions 6 and 7 for clones from Du36_5. Clones from TM1B, SW30 and BW17 had insertions between positions 15 and 16. Amino acid insertions in the V3 loop, particularly at positions 13 and 14 are features consistent with CXCR4 utilization as previously described (Coetzer et al., 2006). None of the insertions observed in the V3 loop of the clones from this study was noted in HIV-1 subtype C R5 sequences downloaded from the Los Alamos database (www.hiv.lanl.gov) (data not shown). The V3 region was also analyzed by manually calculating the overall net amino acid charge, another indicator of *env* coreceptor tropism (Table 3). C-PSSM, a web-based bioinformatic tool used for predicting HIV-1C coreceptor usage from the amino acid sequences of the V3 loop (Jensen et al., 2006) was also used. Both manual and C-PSSM calculations were comparable except for the clones from SW30 where calculated scores were slightly higher than C-PSSM generated scores. Higher overall net V3 charges are associated with X4-usage. A charge less than +4.5 is regarded as R5-using and charges above +4.5 are regarded as X4-using (Coetzer et al., 2006; Fouchier et al., 1995; Fouchier et al., 1992; Kuiken et al., 1992). Therefore, based on the multiple V3 loop sequence based algorithms available for phenotype prediction, all clones generated in this study were either only CXCR4-using or dualtropic, consistent with the functional data.

We next analyzed the V1/V2 and V4/V5 regions of the *env* gene as these regions have also been implicated in playing a role in viral tropism. Sequence features in these regions that may influence coreceptor utilization are the amino acid length and the number of predicted N-linked glycosylation sites (Chohan et al., 2005; Coetzer et al., 2007; Coetzer et al., 2008; Masciotra et al., 2002; Pollakis et al., 2001). The number of predicted N-linked glycosylation sites in clones from this study varied from 23 to 33. Clones for RP1, SW20 and SW30 all had 30 predicted N-linked glycosylation sites. Within the V1/V2 and V3 regions, the N-linked glycosylation sites varied between isolates but occurred at the same positions for all clones of the same isolate irrespective of whether they were X4-using or dualtropic except for one clone from TM1B which had 2 predicted N-linked glycosylation sites in the V2 region whereas the other 4 clones of this isolate had 3. The sites within the V4/V5 regions for all clones of all isolates showed slight variations in position. However, all clones from Du36_5 exhibited CXCR4-usage and showed variation in the positions of the sites in all five hypervariable regions. The positions of N-linked glycosylation sites varied from clone to clone and based on these positions no pattern emerged that could distinguish CXCR4-using clones from those that used both CCR5 and CXCR4.

When the total number of predicted N-linked glycosylation sites within the *env* as well as within the V1/V2 and V4/V5 regions was analyzed, no significant difference was observed between the CXCR4-using clones and dualtropic clones. However, the median number of N-linked glycosylation sites for X4/X4R5 clones from this study was significantly higher at (30) compared to (21) for R5 clones (30 sequences downloaded from the Los Alamos HIV-1 database) ($p<0.0001$) (Figs. 4A–C). R5 sequences showed a lower number of predicted N-linked glycosylation sites within the entire *env* as well as within the V1/V2 and V4/V5 regions when compared to R5X4/X4 clonal sequences.

Previous reports have suggested that a lack of predicted N-linked glycosylation sites at positions 6–8 of the V3 loop may be indicative of CXCR4-usage (Coetzer et al., 2006). We found this site to be conserved in the clones analyzed in this study, despite the utilization of CXCR4 by all the clones. All clones (except those from isolate Du36_5) contained a predicted N-linked glycosylation site at position 6 although they were CXCR4-using. This was also observed in a previous study by Johnston et al. (2003) where all but one X4 sequence maintained this site. We found a significant reduction in the number of predicted N-linked glycosylation sites within the V3 region of clones generated in this study as compared to the R5 sequences from the database (Fig. 4D).

The entire *env* sequence i.e. gp160 of all CXCR4- and CCR5/CXCR4-using clones were compared to determine if any distinguishing features could be identified. Specifically, we analyzed for unique

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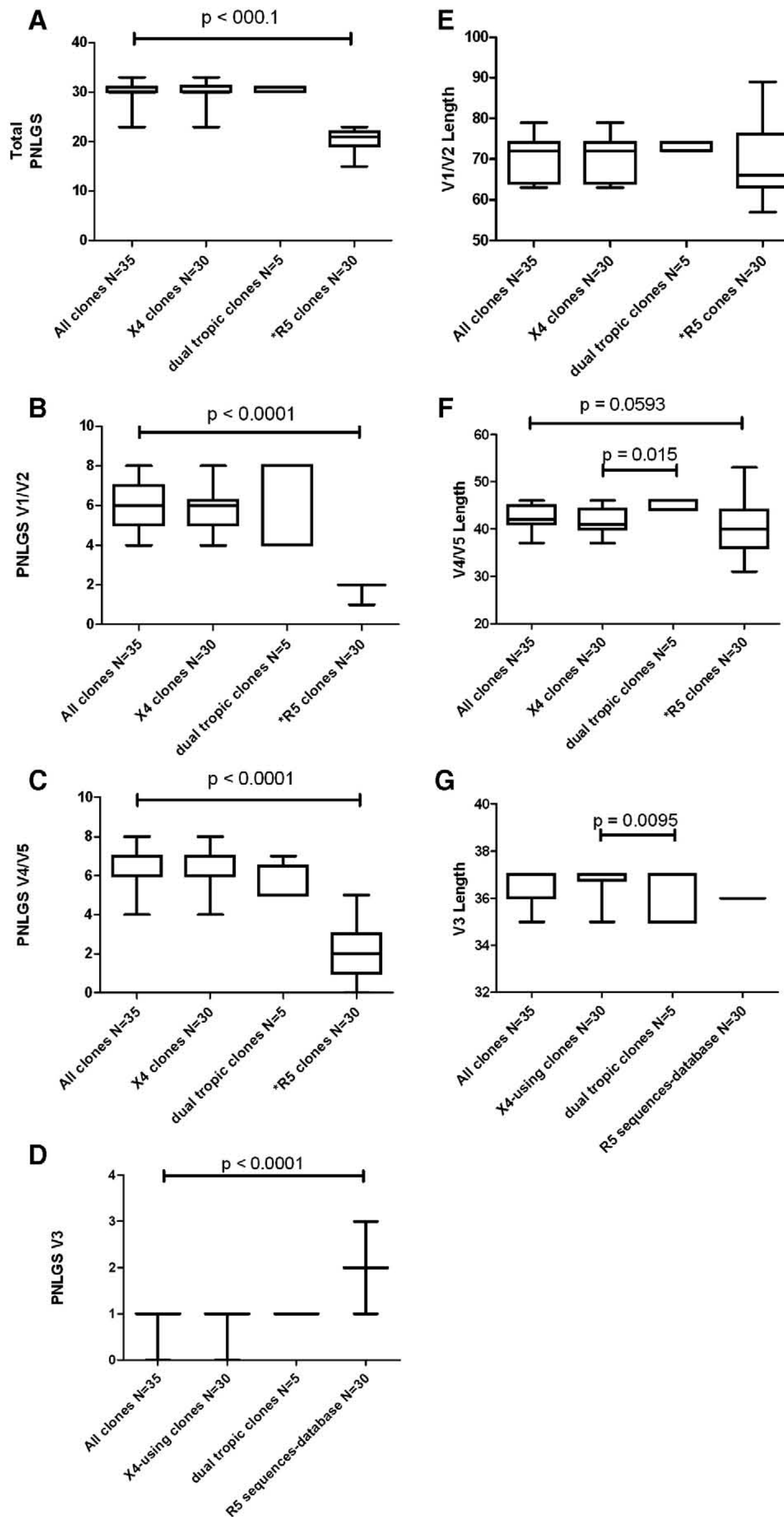

Fig. 4. Box plots of putative N-linked glycosylation sites (PNLGS) and *env* variable loop lengths. (A) Shows the total number of PNLGS within the *env*. (B) The number of PNGLS within the V1/V2 region. (C) The number of PNGLS within the V4/V5 region. (D) The number of PNLGS within the V3 region. (E) The V1/V2 loop length. (F) The V4/V5 loop length. (G) The V3 loop length. The line within each box represents the median value for each group. *Indicates R5 sequences downloaded from the Los Alamos database (www.hiv.lanl.gov).

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signature patterns such as conservation of amino acids with a specific charge or physical property at a particular position, putative N-linked glycosylation sites, deletions, insertions or number of amino acids. The 2 clones of RP1 displaying dualtropism (i.e. clones #10 and 13) had leucine (L) at position 373 whereas the CXCR4-using clones of this isolate (clones #5; 6; 8) had proline (P) at this position (data not shown). The other isolate that produced clones exhibiting dualtropism was CM9. No distinguishing signature sequences were noted that could differentiate between X4 and X4R5 sequences.

We next analyzed for differences in the loop lengths between X4, X4R5 and R5 sequences. Most variation was seen in V1 which ranged from 16 to 27 amino acids. V2 had a relatively constant loop length (40–45). The combined V4/V5 loop length ranged from 37 to 46. The V1/V2 and V4/V5 loop lengths of the clones produced in this study were plotted against R5 sequences from the Los Alamos database. No significant differences were observed between the V1/V2 sequences of clones generated in this study and the R5 sequences from the Los Alamos database (Fig. 4E). However, for the V4/V5 region, there was a significant difference between the generated clones using the CXCR4 coreceptor for viral entry and the dualtropic clones ($p = 0.015$) (Fig. 4F), with the dualtropic clones having an increased V4/V5 loop length. For the V3 loop, all analyzed R5 sequences had a loop length of 36 amino acids whereas X4 and R5X4 clones from this study showed variability with a range from 35 to 37 amino acids (Fig. 4G).

Discussion

The requirement by HIV-1 for specific cellular interacting factors during the entry step offers an opportunity for the development of vaccines and drugs that target this crucial step in the virus replication cycle (Dhami et al., 2009; Hunt and Romanelli, 2009; Pantophlet and Burton, 2006; Phogat et al., 2007). Coreceptors play an important role in initiating infection at the cellular level. Additionally, coreceptor utilization is an important determinant of the rate of disease progression. The emerging availability of entry inhibitors such as the CCR5 antagonists underlines the importance of better characterization of coreceptor utilization and cellular tropism by HIV-1 isolates particularly in heavily burdened countries where the drugs are likely to be required on a large scale for the clinical management of HIV/AIDS. In this study, we generated 35 full-length *env* clones from seven dualtropic isolates of HIV-1 subtype C, in order to determine whether they were a mixture of CCR5 and CXCR4 quasispecies or dualtropic viruses at the clonal level. We also interrogated the sequence characteristics of these clones in order to better elucidate the genetic determinants of coreceptor utilization by HIV-1 subtype C viruses. We found that CXCR4-tropic clones dominated within the dualtropic viral isolates quasispecies. A minority proportion of dualtropic clones were also identified. Unexpectedly, we found that there was not a single CCR5-monotropic *env* clone from the seven primary isolates analyzed in this study. This is an unusual finding considering that many studies have shown that HIV-1 subtype C viruses even in late stages of disease utilize CCR5-only predominantly for cell entry. We thus expected to find a significant proportion of the remnants of these viruses among the quasispecies of the dualtropic isolates. Instead, all the clones detected in this study used CXCR4 as the coreceptor for cell entry, with a minority of these (14.3%) also able to mediate entry via the CCR5 receptor. Our results may explain why in previous studies of some of the dualtropic isolates described here (CM9, SW20 and SW30); the isolates could be strongly inhibited by CXCR4 inhibitors but only modestly by CCR5 inhibitors (Cilliers et al., 2003). These earlier results can now be explained by the observation that although these isolates are dualtropic, they are dominated by X4 variant clones.

An alternative explanation of our findings is that these isolates changed their coreceptor preference during in vitro passages in PBMC co-cultures as has been previously described (Voronin et al., 2007). This possible explanation is supported by the finding that isolates CM9 and SW30 displayed remarkably lower CCR5 utilization capacity (Table 1) than was previously described (Cilliers et al., 2003). It is also worth noting that although isolates BW17, SW20 and Du36_5 showed a possible bias towards CCR5 utilization and were clearly dualtropic, all the *env* molecular clones generated from these isolates by bulk PCR amplification were CXCR4-only using. This finding strongly suggested that the bulk PCR could be biased towards X4 viruses. We therefore performed limiting endpoint dilution PCR on one dualtropic viral isolate (Du36_5) which was biased towards CCR5 utilization (Table 1). Remarkably, of 26 functional *env* clones generated by this approach, 24 exhibited dualtropism, one used CXCR4 exclusively and one used CCR5 exclusively. We therefore conclude that dualtropic HIV-1 subtype C isolates are dominated by X4 and X4R5 clones with negligible proportion of R5 monotropic clones.

It has been recently proposed that coreceptor switching is associated with deleterious mutations in *env* that diminish CCR5-tropism as mutations associated with CXCR4 utilization accumulate (Coetzer et al., 2008). Although we did not directly test for coreceptor binding in this study, our results are consistent with the proposal by Coetzer et al. and with their observation that coreceptor switching is associated with a rapid decrease in the ability to use CCR5. Our results may suggest that in HIV-1 subtype C, the mutations required for adaptation to CXCR4 utilization significantly reduce the ability of *env* to utilize CCR5, thus resulting in reduced fitness of CCR5 utilizing viruses. This could in turn lead to the selection and amplification of clones able to utilize CXCR4. We can speculate that given the low frequency of HIV-1 subtype C CXCR4 utilizing viruses reported in various studies, more accumulated mutations are required for switching to CXCR4 utilization for this subtype. Alternatively, the changes required for a switch to CXCR4 utilization may result in a bigger fitness deficit for HIV-1 subtype C CCR5-tropic variants thus leading to selection against these viruses once adaptation to CXCR4 utilization has been accomplished. Further studies will be required to carefully investigate the specific localization and nature of complementary mutations required for HIV-1 subtype C *env* coreceptor switch.

We also investigated the genetic characteristics associated with CXCR4-usage or dualtropism for HIV-1 subtype C viruses. Our results may be limited by founder effects since we could not generate R5 sequences from the study isolates but we nevertheless had HIV-1 subtype C R5 sequences available from the Los Alamos database that facilitated this comparative analysis. As described for HIV-1 subtype B, the subtype C third variable loop of gp120 (V3 region) is a major determinant of whether CXCR4 or CCR5 will be the accessory protein used by the virus for membrane fusion (Cilliers et al., 2003; Coetzer et al., 2007; Coetzer et al., 2006; Fouchier et al., 1992; Morris et al., 2007; Ndung'u et al., 2006). Typically, the V3 region consists of approximately 35 amino acids in CCR5-tropic viruses (Coetzer et al., 2006). Consistent with earlier studies, we found that V3 loop amino acid characteristics are important determinants of coreceptor tropism. In most cases, we found that the V3 loop crown of CXCR4-utilizing clones had basic amino acid substitutions which differed from the canonical GPGQ sequence found in CCR5 HIV-1 subtype C viruses to GPGX (where X is any other amino acid), GRGH, GPGR or GPRY. X4 variants are more variable than R5 viruses in the V3 region particularly at positions 11 and 25 which tend to be mostly positively charged amino acids, often arginine (R), lysine (K) or histidine (H). We found the presence of a basic amino acid at both or one of these positions for the majority of clones in this study (85%). In addition, in X4 variants there may be insertions particularly between positions 13 and 14 of the V3 loop contributing to an increased length. We found amino acid insertions in 71% of clones in this study. X4 variants may also be distinguished from R5 viruses as they usually have an increased net V3 charge. Consistent with these observations, we

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found that 100% of X4-utilizing clones had V3 loop amino acid charges of +5 or more.

The V3 region however, is not the exclusive determinant of coreceptor usage and other regions within the *env* gene may also contribute to viral tropism. The V1/V2 and V4/V5 regions have been implicated in playing a role in determining the biological phenotype of the virus. Specifically, the number of N-linked carbohydrate moieties in these variable loops has been associated with coreceptor determination (Chohan et al., 2005; Coetzer et al., 2008; Masciotra et al., 2002; Pollakis et al., 2001). Here we found a strong association between the number of N-linked glycosylation sites and coreceptor utilization with X4 clones having a significantly higher number of these sites than R5 clones from the database overall and in the V1/V2 or V4/V5 regions (Figs. 4A–C). In contrast in the V3 region, the number of sites was significantly higher in R5 sequences than X4/X4R5 sequences (Fig. 4D). A previous longitudinal study of HIV-1 *env* evolution showed no significant changes in N-linked glycosylation sites of 23 viral isolates from 5 patients followed for 2–4 years (Coetzer et al., 2007). Therefore our findings may suggest a rapid accumulation of N-linked glycosylation sites as coreceptor tropism switches, as opposed to a slow accumulation of these sites over time. This is consistent with recent findings of rapid decline in CCR5 utilization as alternate coreceptor utilization emerges in HIV-1 subtype B infection (Coetzer et al., 2008). In both HIV-1 subtypes A and C, shorter V1/V2 loop sequences and fewer predicted N-linked glycosylation sites have been correlated with preferential heterosexual viral transmission (Chohan et al., 2005; Derdeyn et al., 2004). We did not find significant differences in V1/V2 length between R5 and X4 clones in this study but a trend towards shorter V4/V5 for X4 clones was noted (Figs. 4E–F). Further longitudinal studies will be necessary in order to better understand HIV-1 subtype C transmission, coreceptor switching and the *env* genetic characteristics associated with these processes. Overall, our results suggest that sequence characteristics in the V3 loop, the V4/V5 loop length as well as the number of *env* predicted N-linked glycosylation sites are the primary genotypic determinants for viral tropism in HIV-1 subtype C.

It is worth noting that we did not perform limiting endpoint dilution of samples in this study except for isolate Du36_5. Therefore we cannot completely rule out the presence of substantial frequencies of R5-monotropic viruses in the quasispecies of the isolates where endpoint dilution was not used. However, the absence of these clones in bulk amplified clones, in CCR5 only expressing cells and in endpoint diluted Du36_5 isolate that is biased towards CCR5 is all suggestive of absence of such quasispecies or presence at very low frequency. Our results appear to contradict the recent findings of Irlbeck et al. (2008) but it must be emphasized that in that study, samples were analyzed directly from plasma in contrast to our study in which we examined in vitro propagated isolates. Further studies will be needed to determine whether *env* clones directly obtained from patients with dualtropic HIV-1 subtype C viruses have a bias towards CCR5 or CXCR4 tropism.

In conclusion, we show in this study that dualtropic viral isolates consist of predominantly X4 and X4R5 clones. Thirty of 35 *env* clones analyzed from PBMCs utilized X4 only as the coreceptor for entry into cells, whereas 5 of 35 clones tested displayed dualtropism and no CCR5-only utilizing clones were identified. R5 monotropic clones could not be detected even when the isolates were cultured in cells expressing CCR5 coreceptor only. We also failed to detect a significant number of R5 monotropic clones when we changed our approach of viral amplification from bulk PCR to limiting endpoint dilution PCR for one dualtropic isolate showing bias towards CCR5 tropism. Viral *env* sequences from both CXCR4 and CCR5-expressing cells were indistinguishable and possessed X4/dualtropic characteristics. Furthermore, we describe *env* sequence characteristics associated with CXCR4 utilization in HIV-1 subtype C. In addition to sequence changes in the *env* V3 region, we identify the number of N-linked glycosylation sites in the V1/V2, V3 and V4/V5 regions as major determinants of coreceptor utilization in HIV-1 subtype C. We also show that the length of the V4/V5 is a possible determinant of coreceptor utilization. We note that our results are consistent with recent findings of the rapid loss of fitness of CCR5 envelope as coreceptor switching emerges and suggest that the sequence characteristics associated with coreceptor switch must occur rapidly in vivo. Further studies are needed to better characterize coreceptor switching, particularly in the context of HIV-1 subtype C, the predominant subtype in the world. We have generated 35 full-length CXCR4- or dualtropic clones of HIV-1 subtype C, important reagents that will facilitate further functional studies of this globally predominant subtype. Our results have important implications for coreceptor antagonist design and application, and further contribute to better understanding of HIV-1 pathogenesis.

Materials and methods

Viral isolates

Seven primary viral isolates were analyzed in this study. BW17 is a dualtropic HIV-1C virus isolated in 1996 from an infected person with acquired immunodeficiency syndrome (AIDS) in Botswana (Ndung'u et al., 2006). TM1B; RP1; SW20; SW30; CM9 and Du36_5 were obtained from the AIDS Virus Research Unit, National Institute for Communicable Diseases, Johannesburg, South Africa and were from patients at various disease stages: acute infection (Du36_5), slow progressor (TM1B), rapid progressor (RP1) and AIDS (SW20; SW30; CM9) (Choge et al., 2006; Cilliers et al., 2003; Coetzer et al., 2006).

Cells and cell lines

U87.CD4 cells with or without the expression of the chemokine receptors CCR5 or CXCR4 were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD). U87.CD4 cells without chemokine receptors were cultured in Dulbecco's modified eagles medium (DMEM) containing L-glutamine (Gibco, NY, USA) supplemented with 15% heat inactivated fetal bovine serum (FBS) (Gibco, NY, USA), 300 μg/ml G418 (Sigma, Germany) and 50 μg/ml penicillin–streptomycin (Gibco, NY, USA). U87.CD4 cells expressing CCR5 or CXCR4 were propagated in the same medium but additionally supplemented with 1 μg/ml puromycin (Sigma, Germany). 0.5×10^6 cells were cultured in 6-well flat-bottomed plates in a total of 2 ml culture medium at 37 °C and 5% CO_2.

293T cells were obtained from the NIH AIDS Research and Reference Reagent Program (Rockville, MD) and were cultured in DMEM containing L-glutamine supplemented with 10% heat inactivated FBS and 50 μg/ml gentamicin (Sigma, Germany). 50,000 293T cells per well were seeded in a total volume of 0.3 ml per well of a 48-well flat-bottomed plate. These cells were incubated at 37 °C in 5% CO_2 overnight before transfection.

Viral isolates propagation and DNA extraction

PBMCs from anonymous low risk HIV-negative volunteers were separated by density-gradient centrifugation on Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden). We confirmed that the samples were HIV-negative by performing HIV RNA testing on the plasma (Ampliscreen v1.5, Roche Diagnostics, Rotkreuz, Switzerland). 5×10^6 PBMCs from 2 donors were combined and stimulated by culturing in RPMI 1640 with penicillin–streptomycin (50 μg/ml and 50 U/ml), 10% heat inactivated FBS, 5 μg/ml phytohaemagglutinin (PHA) (Sigma, Germany) and 20 U/ml interleukin-2 (IL-2) (Roche Applied Science, Germany) at 37 °C and 5% CO_2 for 72 h in a T-25 flask. For infection of the stimulated PBMCs, 5 ng p24 antigen equivalent of virus was used. On days 1, 4, 7, and 10, 50% of the media was removed and replaced with fresh medium. Aliquoted supernatant was retained for

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quantification of p24 antigen as previously described. On day 14, supernatant was removed and preserved for p24 antigen quantification. Cells were harvested and resuspended in 200 μl PBS. DNA was then extracted using the QiaAmp DNA Blood Mini kit (Qiagen, Germany).

Confirmation of dualtropism of primary viral isolates

PBMC-grown virus corresponding to 2 ng of p24 HIV-1 antigen was used for infection of U87.CD4 cells expressing either CCR5 or CXCR4. On days 0, 4, 7 and 10 half of the media was removed and replaced with fresh medium. The removed supernatant was retained for quantification of p24 antigen using the Vironostika HIV-1 Antigen Microelisa system (Biomerieux, Boxtel, Netherlands). Previously well-characterized dualtropic (Du179) and CCR5-tropic (BWM01_5) primary isolates were used as positive controls.

Amplification of envelope (env) gene

The 3 kb *env* gene was amplified by polymerase chain reaction (PCR) using Phusion Hot Start High-Fidelity DNA Polymerase (Finnzymes, Finland) with the following primers: Env1Adir 5′-CACCGGCTTAGGCATCTCCTATGGCAGGAAGAA-3′ and EnvM 5′-TAGCCCTTCCAGTCCCCCCTTTTCTTTTA-3′. The forward primer Env1Adir was designed to include the 4 base pair sequence (CACC) necessary for directional cloning on the 5′ end. Cycling conditions were as follows: a 5 minute denaturation at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 4 min at 72 °C. The final extension was at 72 °C for 10 min . The amplified product was then run on a 1% agarose gel and gel purified using the Qiaquick gel extraction kit (Qiagen, Germany).

Cloning

Once the DNA was purified, the *env* of each primary viral isolate was cloned into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen, Carlsbad, CA). For the transformation procedure, Stratagene XL-10 Gold Ultracompetent cells (Stratagene, USA) were used. Molecular clones were screened as follows. A colony PCR was performed after the colony was incubated for 1.5 h at 37 °C with continuous shaking (225 rpm) in a 96-well plate containing 100 μl Luria Bertani (LB) media (Sigma, Germany) and ampicillin (100 μg/ml) (Calibiochem, Darmstadt, Germany) to determine positivity of the cloned insert. This is a directional *env* insert-specific PCR as it uses the forward primer T7 (5′ TAATACGACTCACTATAGGG 3′) found on the vector and reverse primer Env M which is *env* specific. SuperTherm Taq Polymerase (Southern Cross Biotechnology, Cape Town, South Africa) was used and cycling conditions were as follows: denaturation for 5 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 4 min at 68 °C. The final extension was for 10 min at 68 °C. The amplified products were then run on a 1% agarose gel. Clones were considered positive if they yielded a 3 kb band on an agarose gel. These clones were then grown up at 37 °C overnight with shaking in 3 ml LB broth containing 100 μg/ml ampicillin. Plasmid DNA was then isolated using Qiaprep Spin Miniprep kit by following the manufacturer's instructions (Qiagen, Germany). The first five functional *env* clones identified from each isolate were selected for further analysis.

Limiting endpoint dilution PCR

A limiting endpoint dilution PCR was performed on one primary viral isolate, Du36_5 in order to determine whether the bulk PCR resampling bias resulted in clones biased towards either CCR5 or CXCR4 coreceptor usage. Single genome amplification was undertaken as previously described (Salazar-Gonzalez et al., 2008). Primers and cycle conditions were the same as used in the bulk PCR reactions. Once confirmed by agarose gel electrophoresis, 30 PCR products were purified and cloned and coreceptor usage was tested for the 26 clones that were functional.

Coreceptor usage assays

Cotransfection was carried out by first combining 50 μl serum free DMEM and 2.5 μl Fugene reagent (Roche Applied Science, Germany) and incubating for 5 min at room temperature. This was then incubated at room temperature together with 0.6 μg gp160 *env* DNA (i.e. cloned product) and 0.3 μg pNL4-3.Luc.R-E- (Connor et al., 1995; He et al., 1995). pNL4-3.Luc.R-E- is a full-length HIV plasmid with two frameshifts that render the clone *env* and *vpr* deleted. The reporter firefly luciferase gene has been inserted into the *nef* gene. The transfection mixture was incubated for 30 min , and then added to assigned wells of the plate seeded with 293T and incubated at 37 °C and 5% CO_2 for 48 h . The supernatant together with 7.5 μg/ml DEAE-Dextran (Sigma, Germany) was added to U87. CD4 cells as well as U87.CD4 cells expressing the coreceptors CXCR4 or CCR5. This was incubated at 37 °C and 5% CO_2 for 48 h . The cells were lysed using Glo Lysis buffer (Promega, Madison, WI, USA) and incubated with Bright-Glo Assay reagent (Promega, Madison, WI, USA). The luciferase activity was then determined using the Turner-Biosystems Modulus Microplate instrument (Promega, Madison, WI, USA). A negative control consisting of the plasmid pNL4-3.Luc.R-E- and various positive controls were used in each coreceptor expressing cell line. The positive controls were previously characterized *env* clones Du179 (dualtropic), 96BWM01_5 (R5), and 96BW17#10 (X4). In addition, for each assay plate, 100 nM of CCR5 inhibitor RANTES and 500 nM of CXCR4 inhibitor AMD3100 were used with the respective controls to confirm specificity of entry into target cells. Experiments were done in duplicate and the average relative luminescence units (RLUs) for each clone were calculated. A positive result was considered to be twice the average of the negative control plus standard deviation.

Sequencing and sequence analysis

The *env* gene was sequenced after cloning using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit version 3.1 (Applied Biosystems, CA, USA). Sequences were assembled and edited using Sequencher 4.8. They were then aligned with Mega 4. Phylogenetic trees were constructed in Paup 4.0 to evaluate the clustering of these sequences with each other and with subtype references. Phylogenetic trees were then visualized using Treeview 1.6.6. The consensus sequence for clones from each isolate was generated using BioEdit Sequence Alignment Editor Software (Tom Hall, North Carolina State University). Coreceptor utilization was predicted using the web-based subtype C-specific position-specific scoring matrix (C-PSSM) programme (http://indra.mullins.microbiol.washington.edu/pssm/), a bioinformatics tool that reliably predicts coreceptor phenotype using V3 loop sequences (Jensen et al., 2006). Predicted N-linked glycosylation sites were examined using the web-based programme N-GLYCOSITE (www.hiv.lanl.gov). All reference sequences were obtained from the Los Alamos database (www.hiv.lanl.gov).

Nucleotide sequence accession numbers

The sequence data obtained from this study have been submitted to Genbank under the following accession numbers: FJ846629–FJ846662 and selected clones will be deposited into the NIH AIDS Research and Reference Reagent Program repository.

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