In vitro selection of CD4-independent HIV-1 subtype C: relevance for HIV pathogenesis and therapeutic intervention

Ms Bridgette Janine Connell

A dissertation submitted to the Department of Molecular Medicine and Haematology, Faculty of Health Sciences, University of the Witwatersrand, Medical School, Johannesburg, in fulfilment of the requirements for the degree of Master of Science

Supervisor – Dr. Maria A. Papathanasopoulos

Johannesburg, June 2007

Declaration

I, Bridgette Janine Connell declare that this thesis is my own, unaided work. This thesis is being submitted for the degree of Masters of Science at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination in any other University.

Bridgette Janine Connell

Date

Dedication

This dissertation is dedicated to my unconditionally loving parents, Simon and Geraldine who have loved, supported and encouraged me throughout my life to realize the great potential I have within me.

Publications and Conference Proceedings

Publications

Bell CM, **Connell BJ**, Capovilla A, Venter WDF, Stevens WS and Papathanasopoulos MA. 2007. Molecular Characterization of the HIV-1 Subtype C Accessory Genes *vif*, *vpr* and *vpu*. AIDS Research and Human Retroviruses, 23(2), p322-330 .

Connell BJ, Michler KL, Capovilla A, Venter WDF, Stevens WS and Papathanasopoulos MA. Emergence of X4 Usage amongst HIV-1 Subtype C: Evidence for an Evolving Epidemic in South Africa. (In Preparation).

Michler KL, **Connell BJ**, Venter WDF, Stevens WS, Papathanasopoulos MA, and Capovilla A. Genetic characterization and comparison of full-length envelope glycoproteins from South African HIV-1 subtype C primary isolates that utilize CCR5 and/or CXCR4. (In preparation).

Conference Presentations

Connell BJ, Michler KL, Capovilla A, Venter WDF, Stevens WS and Papathanasopoulos MA, (2006). Emergence of X4 Usage Amongst HIV-1 Subtype C: Evidence for an Evolving Epidemic in South Africa, 13th Conference on Retroviruses and Opportunistic Infections, February 2006, Denver, CO.

Abstract

There are approximately 5.5 Million individuals in South Africa infected with HIV-1, predominantly subtype C (HIV-1C). The emergence of drug resistance to the current Antiretroviral (ARV) regimes is of great concern, thus development of novel, effective drugs/vaccines is vital. Certain conserved and thus vulnerable epitopes within the viral envelope (Env) involved in coreceptor binding are usually protected from the immune system in peripheral blood by the variable loops. However, in immune-privileged sites the Env of CD4-independent viruses may exist in a pre-triggered state where these coreceptor binding epitopes are exposed. Targeting the conserved sites could effectively neutralize HIV-1. This study aimed to adapt an HIV-1C primary isolate towards CD4-independence in the Cf2Th cell line through serial *in vitro* passage. Primary viruses from 20 drug-naïve HIV-1 AIDS patients were isolated and genotypically and phenotypically characterized. The highest percentage (30%) of CXCR4-usage amongst primary isolates from HIV-1C (and CD recombinant) infected AIDS patients worldwide was detected. These data may illustrate the increasing frequency of HIV-1C CXCR4-utilizing (X4) viruses with time and may support the theory that *env* is capable of evolving. The emergence/evolution of HIV-1C X4 viruses may have profound implications for viral pathogenesis, disease progression and future use of CCR5 antagonists as ARVs. Longitudinal follow-up studies on larger cohorts may confirm this finding. The CXCR4-utilizing isolate 05ZAFV03 was successfully adapted and serially passaged 12 times through Cf2Th cells, whilst gradually decreasing amounts of CD4 expressing cells numbers over time. Viral growth was detected with 10% CD4 expressing cells however, 100% CD4-independence was not reached. Proviral DNA from each stage of the adaptation process was sequenced and analyzed for mutations acquired within *env*. The only amino acid change noted was an E152K mutation within the V1 region at passage 4. Overall, the extent of *env* diversity appears to be a complex relationship between isolate-specific and cell-type specific factors. Future attempts to obtain and characterize an HIV-1C CD4-independent isolate will provide potential sites for therapeutic intervention by compounds such as small molecule inhibitors and/or neutralizing antibodies against the most globally prevalent HIV-1 subtype.

Acknowledgements

There are many outstanding individuals who contributed intellectually and psychologically to my MSc, and I would like to thank each person individually.

To my Supervisor, Dr. Maria Papathanasopoulos, thank you for conceptualizing this incredible MSc project and for teaching me so many invaluable skills, both in the laboratory and in life.

To my co-supervisor, Dr. Alexio Capovilla, Thank you for your ever-open door policy, for any question I had and for always being the pillar of positivety for me and for your untainted insight for the truth.

Thank you to Prof Wendy S Stevens for your intellectual input and support throughout my MSc.

To an incredible person, brilliant scientist and best friend, Koo. Words of thanks and appreciation will never do justice to amount you have helped me through everything in the last three years. I am honoured and privileged to have met you and happy to adopt you as my sister.

To Catherine Bell, thank you for willingly helping me with all my gp160 sequencing (from reaction set-up to tedious analysis), for your encouraging moral support and great chats and discussions over coffee and other beverages.

To Gill Baker and Sheena Saayman for all our coffee breaks, laughs and for making the hard times seem meaningless as if I were in a lucid dream.

To Sonja Lauterbach, Sam Barichievy and Nichole Cerutti, thank you for always encouraging me, your willingness to apply your mind to any question or problem, thanks for making me laugh regularly and for your ever-unselfish, generous natures.

To Umesh and Dael for always and willingly helping me with the frequent computer “hiccups” I encountered and for their support, time and advice.

I am greatly indebted to all the AIDS patients from the Johannesburg Hospital who volunteered to donate their blood samples to this study.

Thanks to Elevation Biotechnology (Grant Napier), Poliomyelitis Research Foundation (PRF) and the University of the Witwatersrand for their financial contributions to my project.

To Barry Mendelow for always being my mentor in this field and encouraging me to go for my dreams, no matter what stands in my way.

To Sergio Ballestrero for always providing me with Toblerone chocolate, humour and home truths about life, science and people, whenever I needed to be brought back down to earth or get myself out of depressive slumps! Thank you for going through pages and pages of “foreign HIV language” and correcting my English from an Italians’ point of view!

To my amazing Fabio Masiello for changing my life and loving me – Ti amo Tantissimo!

Thank you my Bro! You show me where I can grow as a person and you build me up from within. I look up to you for showing me how to cope with the hard times in life and thank you for supporting and loving me throughout!

To my unconditionally loving parents, Simon and Geraldine Connell. Thank you for the endless already made breakfasts and dinners, ever-understanding my freak-out-tantrums, providing internet, and copious amounts of coffee, acting as my personal psychologists and re-assuring me that “I CAN”!! The least I can do as a sign of my appreciation for your love, generosity and faith in me, is to dedicate my hard work to you both, thank you.

Table of contents

DECLARATION III
DEDICATION IV
PUBLICATIONS AND CONFERENCE PROCEEDINGS V
ABSTRACT VI
ACKNOWLEDGEMENTS I
TABLE OF CONTENTS III
LIST OF FIGURES VI
LIST OF TABLES XII
LIST OF ABBREVIATIONS XIII

Chapter 1 INTRODUCTION 1

1.1 The Global HIV/AIDS pandemic 2
1.2 Consequences of the HIV replication strategy 4
1.2.1 Subtypes 5
1.3 Structure of the HIV-1 Virion and Genome 6
1.4 The HIV Life Cycle 8
1.4.1 Entry 8
1.4.2 Reverse Transcription of Viral RNA 9
1.4.3 Integration and Transcription of Proviral DNA 10
1.4.4 Translation, Assembly and Budding 11
1.5 Disease Pathogenesis and Progression 12
1.5.1 Transmission 13
1.5.2 Acute/Primary infection 14
1.5.3 Clinical Latency/Asymptomatic Infection 15
1.5.4 Progression towards AIDS 15
1.6 Envelope Glycoprotein 17
1.6.1 Structure and Function 17
1.6.2 Glycosylation of gp120 20
1.6.3 Envelope Heterogeneity and Disease Progression 21
1.6.4 Determinants of Coreceptor Usage and Switching 23
1.6.4.1 Structure of CCR5 and CXCR4 23
1.6.4.2 V3 Loop and coreceptor Usage 25
1.6.5 The HIV-1 Env glycoprotein represents an Antiviral and Vaccine Target 29
1.6.5.1 Neutralizing Antibodies 30
1.6.5.2 Drug Targeting HIV-1 Entry 31
1.6.5.3 Development of Drug Resistance 33
1.7 CD4-independent Entry 34
1.7.1 CD4-independence 34
1.7.2 CD4-independence *in vivo* 36
1.7.3 CD4-independence *in vitro* 37

1.7.4 Relevance for Neutralizing Antibodies and Drug Targets 39
1.8 Objectives and Aims of the Study 41
Chapter 2 ISOLATION AND CHARACTERIZATION OF HIV-1 PRIMARY ISOLATES FROM ANTIRETROVIRAL DRUG-NAÏVE SOUTH AFRICAN AIDS PATIENTS 42
2.1 Abstract 43
2.2 Introduction 44
2.2.1.1 Objectives 49
2.3 Materials and Methods 50
2.3.1 Patients used in this Study 51
2.3.2 Processing of patient samples 51
2.3.3 Primary HIV-1 Viral Isolation 52
2.3.3.1 PBMC Isolation from HIV negative Blood Donor 52
2.3.3.2 Isolation of Primary HIV-1 53
2.3.4 Murex p24 HIV-1 Antigen ELISA 54
2.3.4.1 Primary antibody 54
2.3.4.2 Secondary Antibody 55
2.3.4.3 Adding the substrate 55
2.3.4.4 Absorbance Reading 55
2.3.5 MT-2 Phenotypic Assay 56
2.3.6 U87.CD4 Coreceptor Assay 57
2.3.7 Proviral DNA Extraction from PBMCs 58
2.3.8 Nested Conventional PCR 59
2.3.9 Agarose Gel Electrophoresis 60
2.3.10 PCR Product Purification 60
2.3.11 Sequencing 61
2.3.11.1 Thermal Cycle Sequencing of the gp160 region 61
2.3.11.2 Isopropanol Purification 62
2.3.11.3 Sequencing Electrophoresis 62
2.3.12 Subtyping and amino acid sequence analysis 63
2.4 Results 64
2.4.1 Clinical baseline profiles of South African ARV drug-naïve AIDS patients 65
2.4.2 HIV-1 primary virus Isolation 67
2.4.3 Phenotyping of HIV-1 primary virus isolates 67
2.4.4 Coreceptor Usage of HIV-1 primary virus isolates 68
2.4.5 Full Length gp160 Amplification 69
2.4.6 Genotypic characterization of primary virus isolates 69
2.4.7 Prediction of phenotype using C-PSSM 73
2.5 Discussion 75

Chapter 3 *IN VITRO* ADAPTATION OF AN HIV-1 SUBTYPE C CXCR4-UTILIZING ISOLATE TO CD4-INDEPENDENCE 85

3.1 Abstract 86
3.2 Introduction 87
3.2.1 Objectives 98
3.3 Materials and Methods 99
3.3.1 Primary virus Selection 100
3.3.2 Thymocyte Cell Lines and Growth Conditions 100
3.3.2.1 Basic Cell Growth and Storage 101
3.3.3 Viral Infection of Thymocyte Cells 101
3.3.4 Murex p24 HIV-1 Antigen ELISA 102
3.3.5 Adaptation to CD4-independence 102
3.3.6 Proviral DNA Isolation 103
3.3.7 Amplification and Sequencing of Full-length gp160 104
3.4 Results 106
3.4.1 Adaptation of Primary CCR5 and CXCR4 utilizing viruses to Cf2Th.CD4.CXCR4/CCR5 cell lines 107
3.4.2 Adaptation of 05ZAFV03 towards CD4-Independence 108
3.4.3 Passage 1 (P1) an P2 50% Cf2Th.CD4.CXCR4 : 50% Cf2Th.CXCR4 110
3.4.4 P1, P2 and P3 25% Cf2Th.CD4.CXCR4 : 75% Cf2Th.CXCR4 112
3.4.5 Cell-Cell Infection 115
3.4.6 P1, P2, P3 and P4 10% Cf2Th.CD4.CXC4 : 90% Cf2Th.CXCR4 115
3.4.7 P1 1% Cf2Th.CD4.CXC4 : 99% Cf2Th.CXCR4 and P1 100% Cf2Th.CXCR4 117
3.4.8 Uninfected and Infected Cf2Th cells 118
3.4.9 Amplification and Sequencing of Full-Length gp160 119
3.5 Discussion 125

Chapter 4 SUMMARIZING DISCUSSION AND CONCLUSION 137

REFERENCES 142

Appendix A: Ethics clearance ..... A

Appendix B: Amino Acid Abbreviations ..... B

Appendix C: Base Pair Abbreviations C

Appendix D: Media, Buffers and Solutions ..... D

Appendix E: GenBank Accession Numbers for gp160 sequences ..... E

List of Figures

Figure 1.1 Diagrammatic representation of the global number of HIV infected adults and children living with HIV at the end of 2006. The total number of people living with HIV/AIDS continues to grow and is currently 39.5 Million (34.1 Million- 47.1 Million). Figure adapted from [16]. 4

Figure 1.2 Schematic illustration of the HIV-1 virion structure and genome organization. The HIV-1 genome is approximately 9.7 kb in length and complex in its organization. It is comprised of 9 genes, which encode open reading frames for at least 16 structural, regulatory, accessory and enzymatic proteins. 7

Figure 1.3 Schematic representation of the HIV-1 life cycle within a $CD4^+$ T lymphocyte. The initial attachment to a primary CD4 receptor and a chemokine coreceptor is shown, thereafter penetration of the virus through the host membrane. Reverse transcription of the viral RNA into cDNA ensures compatibility of the transcripts for integration. The viral proteins are synthesized by means of hijacked host replication machinery and finally the virus assembles in the cytoplasm and proceeds to bud from the cell membrane. Maturation of the virion occurs post budding. 10

Figure 1.4 Schematic diagram showing the three classic stages of disease progression in an HIV-1 infected individual over several years. HIV-1 systematically depletes $CD4^+$ T cells until the immune system is severely compromised and AIDS results. The viral load peaks shortly after infection during the acute phase, whereupon the $CD4^+$ T cell numbers rapidly decline. After a few weeks, the immune system partially recovers in response to the nascent infection and the $CD4^+$ T cell count improves slightly. The chronic/asymptomatic phase of infection can persist for a few years, with steadily increasing viral load and declining $CD4^+$ T cell count. In the final

AIDS phase, viral loads increase significantly and the immune system is depleted to low levels. Figure adapted from [120]. 13

Figure 1.5 Graphical representation of the three-dimensional structure of the gp120 protein. The inner domain (red) variable loops V1-V5 and outer domain (yellow) and bridging sheet (blue) are shown and participate in gp120 binding to the chemokine coreceptor. Figure adapted from [158]..... 18

Figure 1.6 Schematic representation of the HIV-1 envelope protein. Epitopes that are capable of inducing neutralizing antibodies are coloured and the variable loops are designated in boxes (V1 – V5). Mannose-type or hybrid type glycosylation sites are indicated by the branched black structures, complex-type oligosaccharides are shown by the U-shaped black structures. Some epitopes that are capable of eliciting neutralizing antibodies are coloured in orange (V2 loop) and blue (V3 loop). The numbering of the amino acid residues in this figure was not used in the text and the figure was adapted from [185]. 21

Figure 1.7 (A) Schematic representation of the CCR5 chemokine coreceptor, a 7-transmembrane major coreceptor for HIV-1 gp120 with amino terminus (NH_2) on the outside and the carboxyl terminus (COOH) on the inside of the cytoplasmic domain of the cell. The cysteine residues are indicated in red and the three extracellular loops (ECL) are depicted [80]. (B) Schematic representation of the 7-transmembrane chemokine CXCR4 coreceptor. The amino terminus (NH_2) is on the outside and the carboxyl terminus (COOH) on the inside of the cytoplasmic domain of the cell. The cysteine residues are indicated in red and the three extracellular domains are depicted ECL1, ECL2, ECL3, respectively. Figure adapted from [216]..... 24

Figure 1.8 (A) Crystal structure of a CD4-triggered gp120 in complex with the V3 loop (in red) at the host cell surface, superimposed onto a structure of the four-domain CD4 receptor. The highly conserved P-G tip of the V3 loop is projected 30 Å towards the host membrane. (B) Schematic representation of the CCR5 coreceptor interaction (green) with its tyrosine-sulphated N-

terminus and extracellular loops (ECLs) and V3 (red). Figure adapted from [174]. 25

Figure 1.9 Schematic illustration of the 35 amino acid V3 loop (in blue). The GPGQ tetrapeptide crown/tip motif is indicated in pink. The 11th and 25th amino acid residues are highlighted in red, showing their importance in coreceptor binding. The N-linked glycosylation site at NNT and the disulphide linkage between the two cysteine residues are also shown. The V3 loop sequence depicted here is based on the Consensus subtype C sequence used in Section 2.4.6. 27

Figure 2.1 Cytopathic effects of the 05ZAFV03 isolate in MT-2 cells lines; (A) uninfected and (B) infected showing extensive formation of syncytia (see arrow), with marked ballooning at the edge of the clumps (200 X). 67

Figure 2.2 Representative electrophoresis gel of the PCR amplification of the envelope region (~2.7kb) from the HIV-1 subtype C isolates. Ten µl of second round PCR product was loaded onto a 0.8% Agarose gel. The sizes of the molecular weight marker (MW; 1kb Ladder, New England Biolabs) are indicated on the left. 69

Figure 2.3 Phylogenetic relationships of the V3 loop regions (~100 bp) of Env from the 20 newly characterized HIV-1 primary viral isolates. Subtype reference sequences from the Los Alamos database are shown. Nineteen out of 20 of the isolates clustered distinctly with the subtype C reference sequences and one isolate, 05ZAFV10, was confirmed subtype D within the V3 loop. The phylogenetic tree was constructed from nucleotide sequences, using the neighbour-joining method and bootstrap values were used to estimate the stability of the nodes. Only bootstrap values of 70% or higher are shown. The phylogenetic analysis does not show all significant associations due to the relatively small fragment size..... 70

Figure 2.4 Alignment of the predicted V3 amino acid sequences of 20 newly characterized South African HIV-1 subtype C and D (05ZAFV10) isolates (14

R5 and 6 R5X4/X4). The sequences are compared to a HIV-1 subtype C consensus sequence. The overall positive charge, number of amino acids and phenotype of each isolate is shown on the right. Dashes (-) indicate deletions/insertions, and positions 11 and 25 and the V3 crown/tip are highlighted. 71

Figure 2.5 Comparison of the (A) V3 loop net amino acid length and (B) net amino acid charge of the 20 HIV-1 subtype C CCR5 and CXCR4 utilizing isolates from this study. 72

Figure 2.6 Alignment of the predicted amino acid sequences of the V3 loop region of the two dual tropic primary isolates from this study and dual tropic reference sequences obtained from the Los Alamos database [47]. 73

Figure 3.1 Unliganded SIV gp120 core structure. All carbohydrates are depicted as stick models and polypeptides as ribbons. The outer domain (in blue) and inner domain is coloured according to substructure (N terminus is orange; α1 is yellow; three-strand sheet is cyan; outer/inner domain transition is purple; α5 is green). The four strands that form the bridging sheet in the CD4-bound conformation are depicted (in red). Disordered residues are shown as dashed lines and the stumps of truncated variable loops V1, V2 and V3 are indicated and the intact variable loops V4 and V5 are also labelled. Figure adapted from [82]. 89

Figure 3.2 Schematic illustration of the molecular arrangement of the gp120 proteins on the surface of the HIV-1 virion during CD dependent virus infection (A) and CD4-independent Viral infection (B). Figure adapted from **[423]**. 91

Figure 3.3 Graphical representation of successful infection of the P1 Cf2Th.CD4.CXCR4 cell line with 05ZAFV03 by day 9 post infection (red graph) and unsuccessful infection of the Cf2Th.CD4.CCR5 cell line with 05ZAFV22 (blue graph) (A). Graph representing successful infection of the

second generation (P2) of Cf2Th.CD4.CXCR4 cells with 05ZAFV03 by day 3. Infection had reached a plateau by day 4-5 (B). 108

Figure 3.4 Schematic overview of the serial Cf2Th cell line passages of 05ZAFV03 grown in decreasing amounts of CD4 cell surface expression. As described in the Section **3.3**, there were sequential passages were performed with a volume of infectious cell-free viral supernatant from the preceding passage. 109

Figure 3.5 Graphical representation of successful P1 infection within the cell population comprising 50% Cf2Th.CD4.CXCR4 expressing cells and 50% Cf2Th.CXCR4 expressing cells (A). Graphical representation of the successful P2 50% Cf2Th.CD4.CXCR4 : 50% Cf2Th.CXCR4 infection (B). 112

Figure 3.6 Successful infection by day 7 of the first passage of 25% Cf2Th.CD4.CXCR4 : 75% Cf2Th.CXCR4 (A). The viral supernatant was then used to infect the second passage, in parallel (B) and to further strengthen the viral infectivity of this percentage cell line, the viral supernatant was taken through a passage 3, again in parallel (C)..... 114

Figure 3.7 Co-culture of infected cells from P3 25% Cf2Th.CD4.CXCR4 : 75% Cf2Th.CXCR4 with uninfected CXCR4 expressing cell in a ratio of 1:1400. 115

Figure 3.8 Graphical representations of the P1 – P4 of 10% Cf2Th.CD4.CXCR4 : 90% Cf2Th.CXCR4 viral growth over 7 – 8 days represented by graphs A – D respectively..... 116

Figure 3.9 (A) Graphical representation of the P1 1% Cf2Th.CD4.CXCR4 : 99% Cf2Th.CXCR4 viral growth curve over almost 2 weeks. The viral culture was expanded on day 6 and the days were continually number from one, one day post expansion. (B) Graphical representation of the minimal 05ZAFV03 viral growth in the 100% Cf2Th.CXCR4 expressing cell line by day 5. 118

Figure 3.10 Uninfected Cf2Th cells (A) Cf2Th.CD4.CXCR4 expressing and (B) Cf2Th.CXCR4 expressing, respectively, and infected Cf2Th cells with 05ZAFV03 (C) Cf2Th.CD4.CXCR4 expressing and (D) Cf2Th.CXCR4 expressing, respectively. Cytopathic effects and cell death can be seen in Cf2Th.CD4.CXCR4 expressing cells, panel C. However, there are negligible, if any, cytopathic effects in infected Cf2Th.CXCR4 expressing cells, panel D. All images were acquired at 10 X magnification on the Zeiss Axiovert 100 M microscope (Zeiss, Göttingen, Germany). 119

Figure 3.11 Cleaned gp160 PCR Products (~ 2.7 kb) of all the isolates chosen for sequence analysis for the adaptation towards CD4-independence. The DNA Molecular Weight Marker (MW) X (Roche, Mannheim, Germany) was used and 10 µl of each PCR product was electrophoresed in a 0.8% Agarose gel (Sigma, St Louis, MO). 120

Figure 3.12 Alignment of the predicted amino acid sequences of the parental (primary) sequence with the sequences obtained from the Cf2Th cell line serial passage variants with decreasing amounts of CD4 expression. Variable (V), constant (C) and other regions of the gp160 region are indicated. Synonymous mutations are indicated at amino acids 295 (in green) and at 454 (in pink). The non-synonymous mutation within the V1 loop (in yellow) is indicated by the E to K mutation (in red) shown at position 152. Primary refers to the primary 05ZAFV03 gp160 sequence (section 2.4.5); P2 100% refers to gp160 sequence from the P2 100% Cf2Th.CD4.CXCR4; P2 rep refers to the repeated P2 100% sequence; P2 50%50% refers to gp160 sequence from the P2 50% Cf2Th.CD4.CXCR4 : 50% Cf2Th.CXCR4; P3 25%:75% refers to gp160 sequence P3 25% Cf2Th.CD4.CXCR4 : 75% Cf2Th.CXCR4; P4 25%:75% refers to gp160 sequence from the co-culture; P1 10:90% refers to gp160 sequence from the P1 10% Cf2Th.CD4.CXCR4 : 90% Cf2Th.CXCR4; P4 refers to gp160 sequence from the P4 10% Cf2Th.CD4.CXCR4 : 90% Cf2Th.CXCR4 and P4 rep refers to the repeated P4 10%:90% sequence. The dots represent identical amino acids..... 124

List of Tables

Table 2.1 Epidemiological data of 31 AIDS patients attending a clinic in Johannesburg, South Africa. 66

Table 2.2 Phenotype and biotype of the 20 HIV-1 ARV drug-naïve primary isolates. 68

Table 2.3 Raw data generated from the 20 HIV-1 subtype C isolates in this study from the C-PSSM phenotype prediction algorithm web site. 74

Table 3.1 Primers used for gp160 cycle sequencing. 105

Table 3.2 Cell culture data for 05ZAFV03 growth as the CD4 cell surface concentration decreased towards CD4-independence. 110

List of Abbreviations

Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral therapy
BMVEC's	Brain Microvascular Endothelial Cells
bp	base pair
°C	Degrees Celsius
CA	Capsid
C2	Second Constant region
CC	Linker between first and second heptad repeat regions
CCR5	CC Chemokine Receptor 5
cDNA	Complementary Deoxyribonucleic acid
CXCR4	CXC Chemokine Receptor 4
CD4	Cluster of Differentiation number 4
CD8	Cluster of Differentiation number 8
CDR2	Second complementarity-determining region
Cf2Th.CD4. CCR5	Canine thymocyte expressing CD4 and CCR5
Cf2Th.CD4. CXCR4	Canine thymocyte expressing CD4 and CXCR4
CM	Cryptococcal Meningitis
CMV	Cytomegalovirus
CNS	Central Nervous System

COOH	Carboxyl terminus
C-PSSM	Subtype C Position Specific Scoring Matrices
CRF	Circulating Recombinant Forms
CSF	Cerebrospinal Fluid
CTL	Cytotoxic T-lymphocyte
DC	Dendritic cells
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	deoxy Nucleotide Triphosphate
ECL	Extracellular loops
EDTA	Ethylenediaminetetraacetic acid
EI	Entry Inhibitor
ELISA	Enzyme linked immunosorbent assay
Env	Envelope glycoprotein
env	Envelope gene
EthBr	Ethidium Bromide
FCS	Fetal calf serum
FDC	Follicular Dendritic Cells
FMDV	Foot and Mouth Disease Virus
FP	Fusion protein
Gp	Glycoprotein
GPCR	G-protein coupled receptor

GRIDS	Gay-Related Immune Deficiency Syndrome
GS	Glutamine synthetase
H_2SO_4	Sulphuric Acid
HAART	Highly active antiretroviral therapy
HADC	HIV-1 Associated Dementia Complex
HIV-1	Human Immunodeficiency virus type 1
HIV-2	Human Immunodeficiency virus type 2
HIVD	HIV Dementia
HR-1	First heptad repeat region
HR-2	Second heptad repeat region
IL-2	Interleukin-2
IN	Integrase
kb	kilo base
kDa	Kilo Dalton
LC	Langerhans cells
LTNP	Long Term Non Progressor
LTR	Long Terminal Repeat
MA	Matrix
mAbs	Monoclonal antibodies
mg	Milligram
$MgCl_2$	Magnesium Chloride

ml	millilitre
MTCT	Mother to Child Transmission
MVB	multivesicular body
MW	Molecular weight
ng	Nanogram
nM	Nanomolar
NH	Amino terminus
NLS	Nuclear Localization Signal
NSI	Non-Syncytium Inducing
OI	Opportunistic Infections
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PCP	Pneumocystis carinii pneumonia
PCR	Polymerase chain reaction
ρg/ml	Picogram per millilitre
PHA	Phytohemagglutinin
PI	Protease Inhibitor
PIC	Pre-integration complex
PML	Progressive Multifocal Leukoencephalopathy
PR	Protease
PSSM	Position Specific Scoring Matrices
R5	CCR5 using HIV
RANTES	Regulated upon activation, normal T-cell expressed and

	secreted
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
RT	Reverse transcriptase
RTI	Reverse Transcriptase Inhibitor
RT-PCR	Reverse transcription polymerase chain reaction
SANBS	South African National Blood Service
sCD4	Soluble CD4
SDF-1α	Stromal cell-Derived Factor – 1 alpha
SI	Syncytia inducing
SIV	Simian Immunodeficiency virus
$TCID_{50}$	50% tissue culture infectious doses
TB	Tuberculosis
TM	Transmembrane
V3	Third variable loop
VL	Viral load
V5	Fifth variable region
wt	Wild type
X4	CXCR4 using HIV
µg	Microgram
µM	Micromolar
UNAIDS	The Joint United Nations Programme on HIV and AIDS
V	Variable Region
Vif	Viral Infectivity Factor

Vpr	Viral protein R
Vpu	Viral protein U
VRC	Vaccine Research Centre
VSV	Vesicular Stomatitis Virus
WHO	World Health Organization
WITS	University of the Witwatersrand

Chapter 1 INTRODUCTION

1.1 The Global HIV/AIDS pandemic

Acquired Immunodeficiency Syndrome (AIDS) was first detected in May 1981 among four homosexual men in Los Angeles, United States of America who presented with infections such as *Pneumocystis carinii* pneumonia (PCP), Kaposi's sarcoma, prolonged fever and Candida infections [1, 2]. The apparent sexually transmitted immune deficiency in these patients was thought to be due to cytomegalovirus (CMV) infections in homosexual men, and called Gay-Related Immune Deficiency Syndrome (GRIDS) [1-5]. However, this disease was not only seen in homosexual men, by 1983 groups of intravenous drug abusers, individuals receiving blood and blood products and heterosexual Haitians in America, presented with AIDS [6, 7].

The causative agent of AIDS is a retrovirus that was first isolated from patients and demonstrated cytopathic effects on $CD4^+$ T cells [8]. This virus was initially referred to as Human T-cell leukaemia virus (HTLV-III) [8-10] and is now called Human Immunodeficiency Virus (HIV), the etiologic agent of AIDS. HIV-1 crossed the species barrier from chimpanzees to humans during the early twentieth century and has since infected millions of humans. Origins of HIV have thus been linked to the simian immunodeficiency virus (SIV) from the genus *Lentiviruses* of the family *Retroviridae* [11-14]. Currently, one percent of the world's population is infected with the worlds' fastest evolving pathogen, HIV [15].

AIDS is characterized by the progressive depletion of $CD4^+$ T lymphocytes which play an important role in establishing and enhancing the cell-mediated and the humoral immune response [2, 5]. When an individual suffers severe damage to their immune system, their vulnerability to opportunistic infections (OIs) and malignancies are heightened due to the loss of the individuals' ability to mount an effective immune response. Ultimately death results after many years of untreated infection [9].

There was an estimated 39.5 million people worldwide living with HIV at the end of 2006. An estimated 4.3 million became newly infected with HIV and an estimated 2.9 million lost their lives to AIDS that year. These figures are almost equivalent to 11,780 new infections and 7,945 deaths per day due to AIDS [16]. The emergence of this pandemic has arguably been the most catastrophic event in medicine in the last 25 years.

Africa remains the global epicentre of the AIDS pandemic and the major burden of HIV/AIDS is currently borne by sub-Saharan Africa. Almost two-thirds (24.7 million) of all individuals infected with HIV globally, live in sub-Saharan Africa (Figure 1.1). By the end of 2006, 34% of all AIDS deaths globally were occurring in Southern Africa [16]. About fourteen percent (5,5 million) of the current global HIV-1 infected population is living in South Africa [16]. Antenatal surveillance suggests that the prevalence of HIV in South Africa among pregnant women (15-24 years) is 22.5%. Furthermore women bear a disproportionately large part of South Africa's HIV/AIDS burden. It is one of the worst in the world and shows neither evidence of a decline, nor a decrease/plateau in HIV-1 prevalence [16].

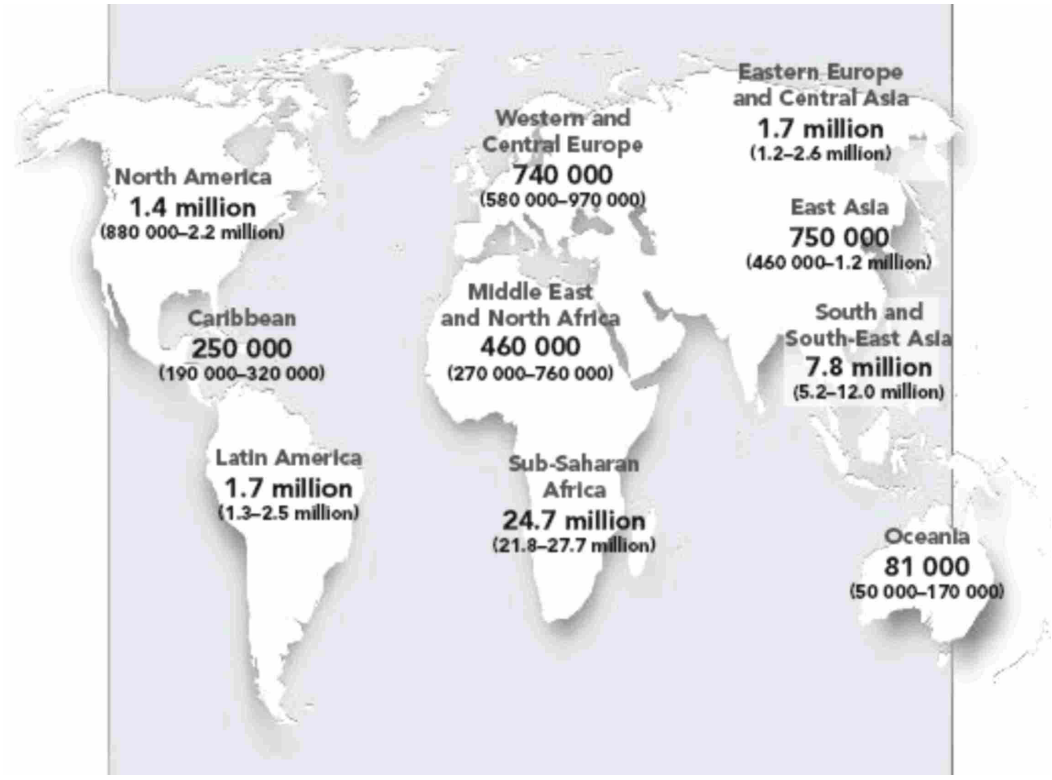


Figure 1.1 Diagrammatic representation of the global number of HIV infected adults and children living with HIV at the end of 2006. The total number of people living with HIV/AIDS continues to grow and is currently 39.5 Million (34.1 Million- 47.1 Million). Figure adapted from [16].

1.2 Consequences of the HIV replication strategy

Genetic diversity of HIV-1 exists along the entire length of the genome between viral isolates from different individuals and between viral quasispecies within the same individual. The most unique and precarious characteristics of HIV-1 is its inherent variability and capability of generating quasispecies as a direct result of two features; lack of a proof-reading mechanism by the viral reverse transcriptase (RT) enzyme during replication [17] and its rapid replication rate [18, 19]. The error prone RT has an estimated misincorporation (insertions/deletions) rate of 1 x 10^{-4} - 3.4 x 10^{-5} per base pair per replication cycle [17, 20-24]. This equates to about one nucleotide being miss-incorporated per replication cycle of 9.7 kb. This

process is exacerbated by the high production of approximately 1 x 10^{10} viral particles daily and in the absence of proof-reading mechanisms, this results in extensive viral heterogeneity [18, 19, 22, 25-27].

Recombination between two RNA genomes also results in major gene-rearrangements and generation of diversity within the subpopulations within the host [28-30]. Together, these features allow HIV to rapidly mutate its genome enabling the virus to constantly evolve and increase genetic variability. This impacts on factors such as the genotypic viral diversity amongst different isolates, immune escape and emergence of Antiretroviral (ARV) drug resistance [20].

1.2.1 Subtypes

To date, two main types of HIV have been identified with origins as zoonotic lentiviruses; HIV-1 is believed to have originated from a SIV_{CPZ} from the chimpanzee (*Pan troglodytes*) population [13, 31] and HIV-2 is believed to have originated from the SIV_{SM} sooty mangabey (*Cercocebus atys*); SIV originated from macaques (SIV_{MAC}) [32, 33].

HIV-1 and 2 are transmitted in the same fashion (section 1.5.1), yet HIV-2 has a lower rate of transmission, longer asymptomatic period, lower viral load and hence it is less pathogenic [34, 35]. HIV-2 is endemic in West Africa and to a lesser extent elsewhere in the world such as Europe and the West coast of India [36, 37]. However, HIV-1 predominates worldwide and has a three times higher mortality rate compared to HIV-2 [38].

In addition to the two main types of HIV, further classification systems have been constructed from the copious phylogenetic data analyses of the many strains of HIV-1 and HIV-2 isolated and analyzed worldwide. There are four sub-classifications for HIV-1; groups, subtypes, sub-subtypes and circulating recombinant forms (CRFs). Of the groups; the Major group (Group M) is responsible for the current global pandemic and the Outlier Group (Group O) and

New group (Group N; consisting of non-O and non-M viruses) are less globally distributed. Group O and N are both highly genetically divergent from group M and sparsely distributed in Cameroon and West Central Africa [39-42].

Group M is further subdivided up into 9 distinct subtypes, namely A, B, C, D, F, G, H, J, K wherein there are two sets of sub-subtypes A1, A2 and F1, F2 respectively [43, 44]. The emergence of CRFs has resulted from many recombination events between different HIV-1 viruses and already 34 CRFs have been described [45-47]. These viruses share an identical mosaic structure in their genomes as they have descended from the same recombination events [48]. The following criteria should be fulfilled in order for an isolate to be considered a CRF; firstly, there must be full-length genome sequences for at least two isolates, secondly they should only resemble each other and no other existing CRF in their subtype structure and finally they should have been found in at least two epidemiologically unlinked individuals [48-51].

HIV-1 subtype C is the most prevalent subtype as well as the most rapidly spreading HIV-1 subtype worldwide, according to the latest WHO/UNAIDS statistics [52, 53]. Subtype C infections mainly occur in developing countries where viral transmission routes are mostly heterosexual and perinatal, however, this subtype has only recently been studied in depth. HIV-1 subtype C now causes the vast majority of new HIV-1 infections worldwide (55%) [52] and has thus become a major focus of drug development and vaccine efforts [16].

1.3 Structure of the HIV-1 Virion and Genome

An intact, mature T-lymphotropic HIV-1 retrovirus is spherical in shape, with a diameter of approximately 80 to 120 nm [8]. As in all retroviruses, HIV-1 has two copies (diploid) of identical plus-strand genomic RNA. The viral regulatory, structural and accessory proteins and enzymes are encoded by 9 genes spanning approximately 9.7 kb of genetic material. These 9 open reading frames code for at

least 16 distinct proteins. Three of these genes encode structural proteins (Gag, Pol and Env), 2 genes encode regulatory proteins (Tat and Rev) and 4 genes encode accessory proteins (Vif, Vpr, Vpu and Nef) (Figure 1.2) [54, 55]. The viral envelope (Env), encoded by *env*, encases a matrix protein membrane (p17/MA), which provides further structure to the virion as well as encompasses the single layer of structural capsid/core proteins (p24/CA), encoded by *gag*. The capsid contains the viral RNA, the protease (PR), RT heterodimer (comprised of two subunits; the RNAse H (p66) subunit and the reverse transcriptase (p51) subunit), and integrase (IN), encoded by *pol*.

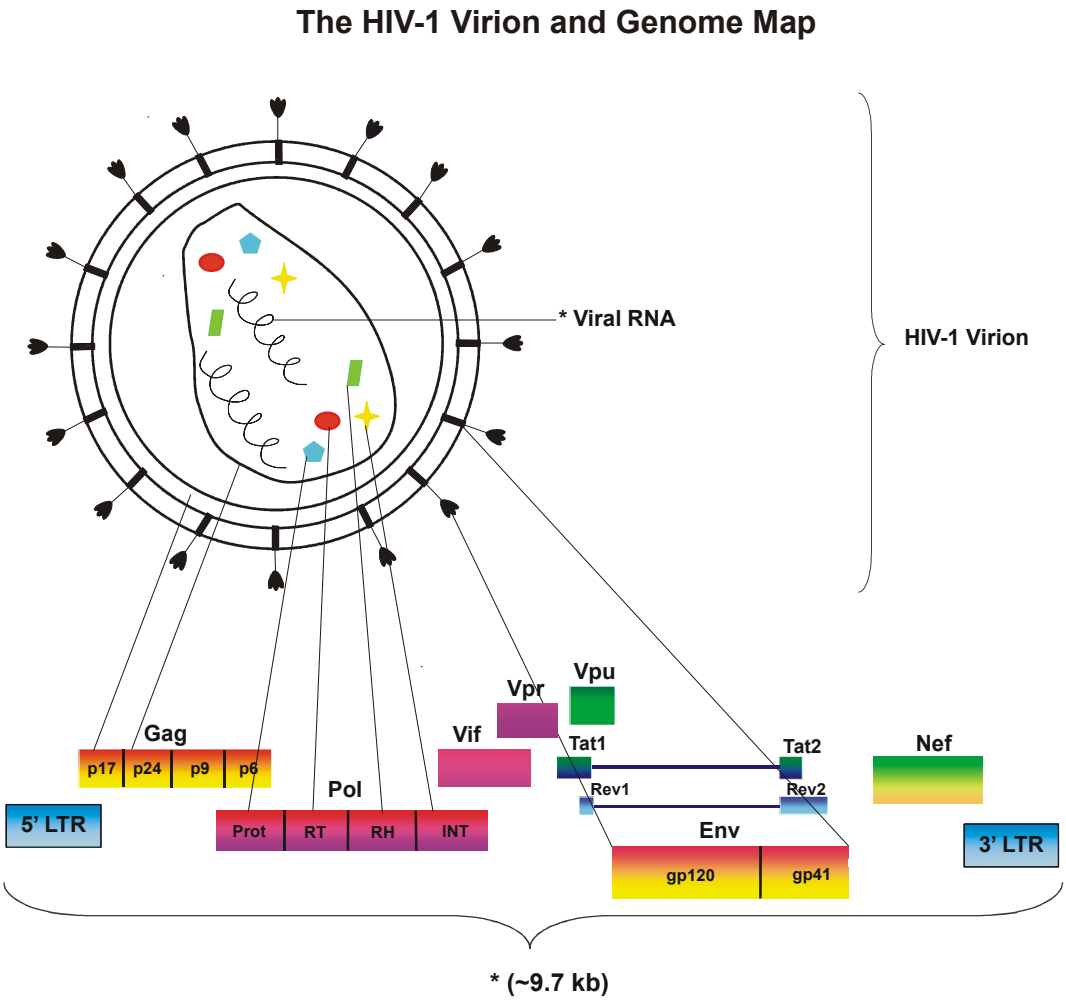


Figure 1.2 Schematic illustration of the HIV-1 virion structure and genome organization. The HIV-1 genome is approximately 9.7 kb in length and complex in its organization. It is comprised of

9 genes, which encode open reading frames for at least 16 structural, regulatory, accessory and enzymatic proteins.

1.4 The HIV Life Cycle

HIV-1 predominantly infects T Lymphocytes and/or primary monocytes/macrophages that express CD4 glycoproteins on their surfaces [56, 57]. This commonly results in progressive loss of $CD4^+$ T lymphocytes and a decline in their function within the host. An in-depth understanding of the HIV-1 life cycle is paramount to designing potential therapeutic drugs for treatment of this disease as well as developing preventative approaches such as microbicides and vaccines.

1.4.1 Entry

The *env* is initially translated into a 160 kDa envelope glycoprotein (gp160/Env) precursor. This polypeptide precursor becomes N-linked glycosylated in the rough endoplasmic reticulum and is then cleaved by host cellular proteases in the Golgi apparatus to yield two functional glycoproteins [58]. The cleavage site is an arginine-rich hydrophobic region between the surface glycoprotein subunit (gp120) and the transmembrane glycoprotein subunit (gp41) [59-61]. Clusters of three protruding gp120 proteins associate non-covalently with three gp41 subunits on the viral surface and assemble as trimers [62-64]. The HIV-1 Env/gp160 plays a pivotal role in the viral life cycle, and is responsible for viral transmission, host cell entry, tropism and influences replication kinetics.

HIV and SIV entry into a host cell is a dynamic and complex, multi-step, cascade process. Viral entry is initiated with the binding of the viral gp120 to the primary $CD4^+$ receptor on a $CD4^+$ T cell which is followed by the sequential binding of a chemokine coreceptor [56, 57, 62, 65, 66]. Once the glycoprotein has bound CD4, conformational molecular rearrangements within the viral gp120 are triggered, exposing specific CD4 induced (CD4i) conserved coreceptor binding epitopes [66-

68]. This structural intermediate is responsible for the exposure and/or formation of a chemokine coreceptor binding site. Among the variety of coreceptors, either the β-chemokine receptor 5 (CCR5) and/or α-chemokine receptor 4 (CXCR4) are most commonly used [69-71]. Generally, HIV-1 viral isolates that are transmitted and those that predominate during the early stages of infection/asymptomatic phase, utilize the CCR5 coreceptor [72]. However, CXCR4 utilizing viruses appear to emerge in the later stages of the disease progression in about 60% of HIV-1 subtype B infected patients [73-76]. Viral tropism of HIV-1 can largely be linked to these two coreceptors [66, 69-71, 77-81].

Once the coreceptor is bound, the gp120 protein may dissociate from the gp41 protein which is stably anchored/inserted within the viral membrane [82]. This induces further conformational rearrangements within the gp41, involving the antiparallel association of the two coiled heptad repeats (HR-1 and HR-2), forming a fusion active six-stranded helix bundle/hairpin structure. This fusion peptide brings the viral and host cell membranes into close proximity. Finally the hydrophobic gp41 amino terminus is harpooned into the host cell membrane, leading to the fusion of viral and host outer lipid membranes [63, 83-87].

1.4.2 Reverse Transcription of Viral RNA

Once the two membranes of the virus and target cell have fused, the viral capsid/core is injected into the cytoplasm of the host cell, rapidly uncoats and releases the viral RNA and other viral enzymes required for viral replication [88-90]. The viral reverse transcriptase enzyme then transcribes the single stranded RNA molecules into double stranded complementary DNA molecules (cDNA) (Figure 1.3) [91-93].

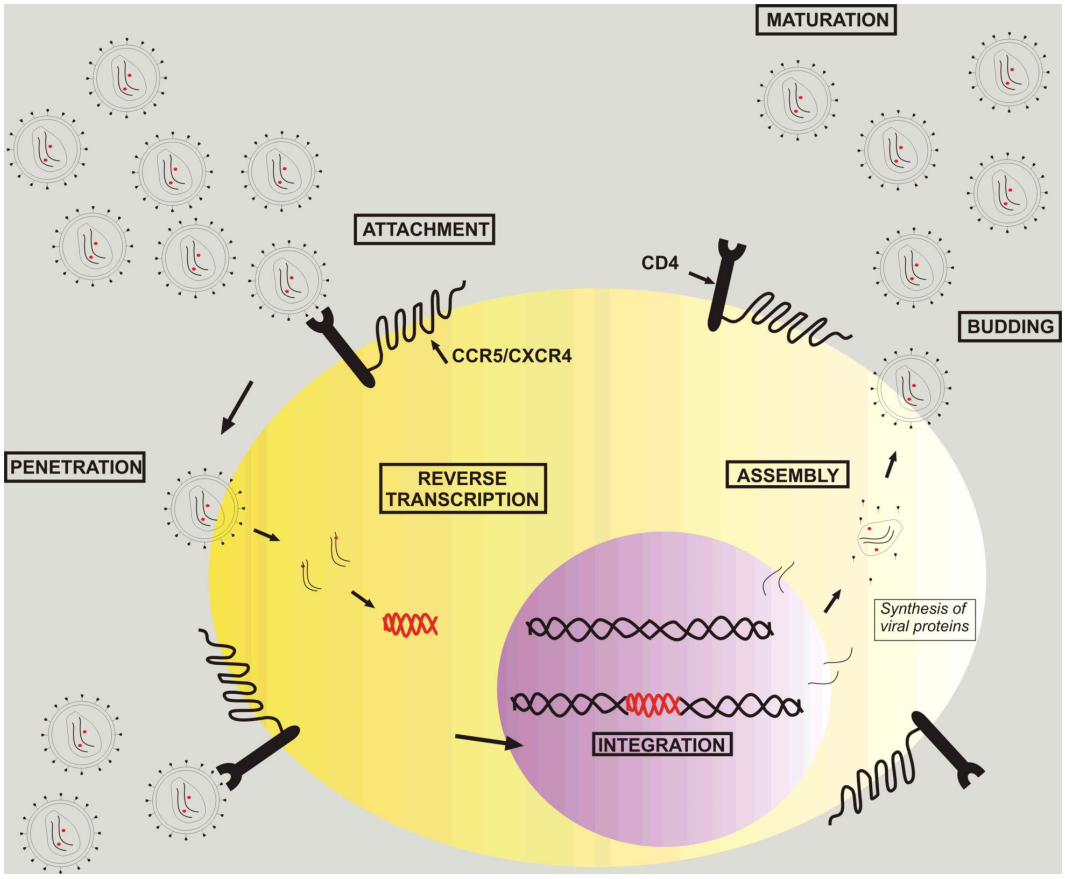


Figure 1.3 Schematic representation of the HIV-1 life cycle within a $CD4^+$ T lymphocyte. The initial attachment to a primary CD4 receptor and a chemokine coreceptor is shown, thereafter penetration of the virus through the host membrane. Reverse transcription of the viral RNA into cDNA ensures compatibility of the transcripts for integration. The viral proteins are synthesized by means of hijacked host replication machinery and finally the virus assembles in the cytoplasm and proceeds to bud from the cell membrane. Maturation of the virion occurs post budding.

1.4.3 Integration and Transcription of Proviral DNA

The reverse transcription complex or pre-integration complex (PIC) forms, consisting of an aggregateion of the nascent cDNA, integrase, and other viral proteins. Unique nuclear localization signals (NLS) on karyophiles associated with the cell's microtubule network direct the PIC through the host cell nuclear pores to its destination within the host nucleus [94-96]. The newly synthesized viral cDNA is then irreversibly integrated into an actively transcribed region within the cleaved

host genomic DNA, via integrase [97, 98]. The proviral DNA remains integrated within the host genetic material indefinitely.

Host machinery is subsequently hijacked by viral proteins to transcribe and translate the newly inserted HIV genome [99, 100]. Transcriptional control is exerted by the Long Terminal Repeat (LTRs) regions, which contain enhancer and promoter sequences to up-regulate viral protein production. Viral transcription may occur in two separate stages [101, 102]; firstly, transcription is mediated by RNA-Polymerase II directly interacting with host transcription factors and *cis*-acting elements, found on the LTRs of the HIV genome. The second phase of transcription is also activated from the LTR regions, only once the viral Tat protein has been synthesized [103, 104]. Some infected cells remain dormant and contribute to the organization of immune privileged latent viral reservoirs within lymphoid tissues, e.g. spleen, lymph nodes [105, 106].

1.4.4 Translation, Assembly and Budding

Viral proteins are subsequently translated, from the 5' to 3' direction of mRNA, into polypeptide sequences that are then intracellularly cleaved into functional shorter proteins by the viral and/or host proteases within the host's cytoplasm. Once all the viral proteins have been translated and cleaved into their correct functional sizes, certain viral proteins undergo specialized post-translational modifications whereby glycosylation, phosphorylation and myristoylation occurs [107-109].

Structural and enzymatic proteins collect and assemble into immature HIV virions at the inner surface of the host cell membrane in cholesterol rich lipid rafts [110, 111]. During the process of assembly and budding from a host cell, HIV incorporates host lipids and glycoproteins in the viral Env [112, 113]. Generally, HIV assembles at, and buds from the plasma membrane of T cells and epithelial cells [114]. However, in macrophages HIV assembles at, and buds into internal

late endosomal and multivesicular body (MVB) membranes which are then transported to the cell surface and exocytosed [115-117]. The outer Env of each virion consists of a lipid layer that was derived from the previous infected host $CD4^+$ T lymphocyte or macrophage, post virion budding. This allows HIV to remain similar in phenotype to the host cell, contributing to the viral strategy of avoiding recognition by the hosts' immune system.

As the virion matures via the Gag polypeptide processing cascade [118], an infectious, post-budding particle forms that can then infect further host cells. Many $CD4^+$ T lymphocytes become infected and can produce between 10.3×10^9 and 10×10^{10} new virions per day [18, 19, 27]. Mathematical models have shown that the life span for productively infected lymphocytes is between one and two days until ultimate latent viral reservoir stabilization within the host cell or death [18, 19, 25-27, 119].

1.5 Disease Pathogenesis and Progression

The clinical course of disease progression is well characterized and can be separated into three distinct phases; the acute phase (primary infection), the asymptomatic phase (clinical latency) and finally AIDS. Figure 1.4 shows the typical disease progression depicting primary infection, clinical latency and final stage AIDS showing the relative viral phenotype associated with each stage.

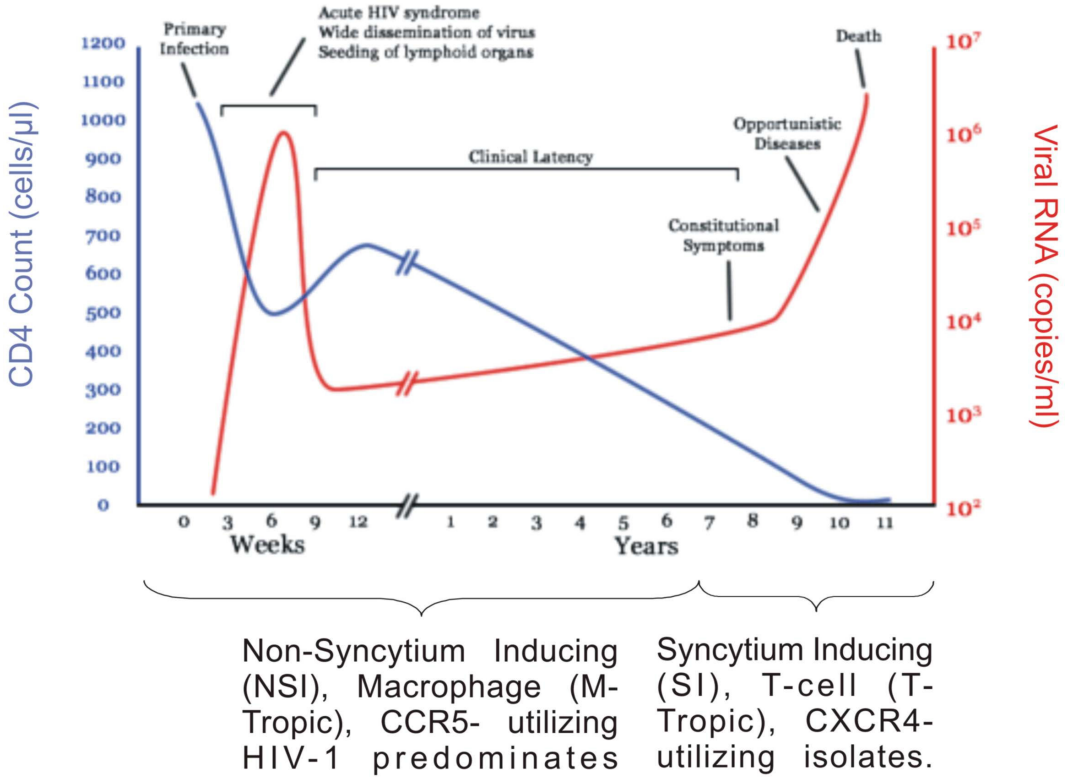


Figure 1.4 Schematic diagram showing the three classic stages of disease progression in an HIV-1 infected individual over several years. HIV-1 systematically depletes $CD4^+$ T cells until the immune system is severely compromised and AIDS results. The viral load peaks shortly after infection during the acute phase, whereupon the $CD4^+$ T cell numbers rapidly decline. After a few weeks, the immune system partially recovers in response to the nascent infection and the $CD4^+$ T cell count improves slightly. The chronic/asymptomatic phase of infection can persist for a few years, with steadily increasing viral load and declining $CD4^+$ T cell count. In the final AIDS phase, viral loads increase significantly and the immune system is depleted to low levels. Figure adapted from [120].

1.5.1 Transmission

HIV-1 is most commonly transmitted through infected bodily fluids during homosexual or heterosexual intercourse. There are however other means of transmitting the virus through non-sexual channels, such as transfusion of infected blood samples and/or products, sharing of infected needles among infected

intravenous drug users and vertical transmission; mother to child transmission (MTCT). Transmission from mother to child can occur *in-utero*, through the natural birth process, puerperal and/or through delivery of breast milk. During sexual transmission of HIV-1, the virus crosses the mucosal barrier and attaches to host cells expressing the necessary primary receptor and coreceptors for infection. The virus attaches to Langerhans cells (LC), which are defined as dendritic cells (DC), present at the vaginal/anal mucosa (stratified squamous epithelia) where virions are passed onto $CD4^+$ T cells in systemic circulation [56, 57, 121, 122].

Having attached and gained entry into the target cells, the virus is rapidly transported via the blood stream to the lymph node reservoirs where it replicates uncontrollably and disseminates throughout the body, rapidly depleting the $CD4^+$ T cell pool [123, 124]. HIV-1 transmission is generally associated with the transfer of relatively homogenous viral populations to the host [125-127], upon where viral tropism may influence the selection of specific sub-population variants.

1.5.2 Acute/Primary infection

This phase lasts between 2 and 12 weeks and is associated with relatively high levels of viral titer and a decrease in total $CD4^+$ T cell count [128-130]. Unfortunately, many HIV-1 positive patients are not diagnosed correctly within this phase. The acute stage of infection is referred to as the "window period"; a period when the patient is HIV-1 positive, but no patient antibodies have yet been made against the viral antigens.

Within days after primary infection, cytotoxic T-lymphocyte (CTL) production is the first host immune response against HIV-1 [131]. Binding antibodies appear within weeks after infection and their presence is detected by p24 Enzyme Linked Immunosorbent Assays (ELISA). The CTL response is followed by neutralizing antibodies that partially block HIV-1 entry into host cells [132, 133], however

these antibodies may take months to develop. Due to the host cellular immune response, the viral load is partially curtailed [131, 132].

This acute clinical phase/primary infection (Figure 1.4) usually presents in patients with a flu-like illness, referred to as seroconversion illness, which may also affect the central nervous system (CNS) [134] and is the most infectious stage of disease [135]. Most individuals may develop headache, myalgia, fever, malaise, lymphadenopathy and pharyngitis during primary infection. During this phase, large amounts of virus collect in the follicular dendritic cells (FDC) within the lymphoid organs and the Cerebrospinal Fluid (CSF), these regions are referred to as viral reservoirs [136-140]. Generally, CCR5 utilizing, Non-Syncytium Inducing (NSI) isolates are transmitted and predominate during the acute stages of infection (Figure 1.4) [141, 142].

1.5.3 Clinical Latency/Asymptomatic Infection

During this stage, HIV-1 is actively replicating within the body without causing noticeable symptoms (Figure 1.4). Patients are infectious during this period for up to 10 years, however, the duration of clinical latency may vary between individuals and the relative pathogenicity of the particular strain of HIV-1. Previous studies have shown that HIV-1 replication in lymph nodes is particularly high during this stage [143]. However, the immune system gradually deteriorates as $CD4^+$ T cells are progressively depleted during later stages and this correlates with an exponential increase in viral load in the peripheral blood (Figure 1.4).

1.5.4 Progression towards AIDS

AIDS is the most severe manifestation of HIV infection within the host. AIDS is the end stage of the disease progression and is associated with CD4 counts below 200 cells/mm^3, and the onset of OIs [144]. The rate at which the initial infection

within an individual is controlled by the immune system, is a good indicator of how quickly the individual progresses to AIDS [145]. The higher the viral load set point of the individual, the higher the probability they will develop AIDS at a faster rate [145]. Prognosis in treatment-naïve patients in South Africa is rarely over two years from presentation of AIDS and the life expectancy (at birth) for men and women in Zimbabwe is now 37 and 34 years of age, respectively [146].

Symptomatic HIV-1 infection is primarily caused by the acquisition of OIs and diseases that a healthy immune system could usually overcome. Some of the more common OIs that are seen in AIDS patients are, Tuberculosis, Mycobacterium avium complex, Cryptococcus meningitis, *Pneumocystis carinii* pneumonia (PCP), diarrhoea, candidiasis, CMV retinitis, histoplasmosis, progressive multifocal leukoencephalopathy (PML), Kaposi's sarcoma and Non-Hodgkin's lymphoma [147-151]. Generally, CXCR4 utilizing, Syncytium Inducing (SI) isolates are detected during the late stages of disease as an individual progresses towards AIDS (Figure 1.4) [141, 142].

The current global definition of AIDS includes a positive test result for HIV as well as one or more of the 26 recorded OIs or a $CD4^+$ lymphocyte count less than 200 cells/mm^3 or less than 14% of the total lymphocyte count [152]. In South Africa, AIDS is identified by the clinical diagnosis of a $CD4^+$ T cell count of less than 200 cell/mm^3 blood and the presentation of one or more OIs [153].

The clinical criteria for patients to receive treatment, subsidized by the South African government, is to present with a $CD4^+$ T cell count less than 200 cells/mm^3 irrespective of WHO stage disease or present with WHO stage IV disease irrespective of CD4 count [154, 155]. The National Government Antiretroviral Rollout in South Africa was initiated in April 2004 and at the end of September 2006, 235 000 HIV-1 infected individuals were on the roll-out of Highly Active Anti-Retroviral Therapy (HAART); this is a 2-fold increase since 2004 [154]. However, less than 5% of 5.5 million HIV-1 infected people in South Africa in need of treatment are receiving it [16].

The HIV/AIDS epidemic has devastating effects on all aspects of the population from national development, social and economic consequences, to health, transport and education. Despite cases where HAART has been successful, there is an overwhelming rate of emergence of drug resistant strains of HIV-1 and incomplete suppression of HIV-1 replication in infected patients [156]. Patients endure adverse side effects from the therapy, toxicities from its long-term use [157] and adherence and affordability are part of the multitude of the urgent reasons for alternative treatment options. This is why the search for a novel effective therapy and/or a vaccine is so essential.

1.6 Envelope Glycoprotein

1.6.1 Structure and Function

The gp120 protein structure consists of three distinct domains, the inner domain, outer domain and bridging sheet (Figure 1.5) [66]. The bridging sheet is responsible for the connectivity of the inner and outer domains and all three domains are conformationally rearranged in such a way as to form a docking pocket within gp120 where the CD4 molecule binds. Post-CD4 binding, the four antiparallel β-pleated strands of the bridging sheet electrostatically interact/bind with either the CCR5 and/or CXCR4 chemokine receptor [66].

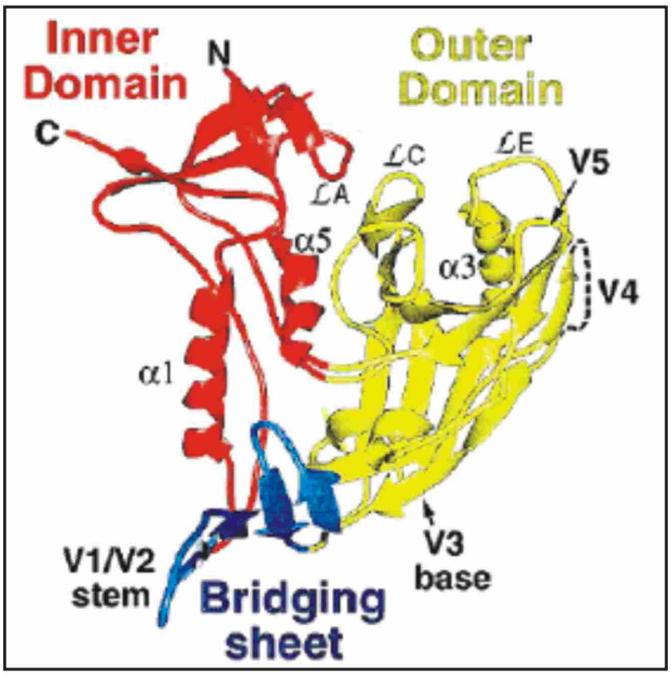


Figure 1.5 Graphical representation of the three-dimensional structure of the gp120 protein. The inner domain (red) variable loops V1-V5 and outer domain (yellow) and bridging sheet (blue) are shown and participate in gp120 binding to the chemokine coreceptor. Figure adapted from [158].

The crystal structure of the unliganded SIV gp120 core [82, 159] shows that there are significant structural re-arrangements compared to the CD4-bound gp120 [66, 160], confirming the conformational flexibility of gp120. Previous studies on trimer models based on gp120, suggest that the V3 region is conformationally flexible and adjusts its position relative to the V1-V2 domains post CD4 binding [82] (discussed in further detail later). The V1 and V2 loops partially mask the conserved CD4 and chemokine coreceptor binding sites and upon CD4-gp120 binding, then the V2 loop is displaced, exposing the previously concealed V3 loop, CD4i epitopes and stabilizing the bridging sheet [68, 161]. These CD4-induced conformational changes have been shown to contribute significantly to the specificity and binding of the coreceptors [162-167].

The inner domain of gp120 contains the N and C termini and is believed to interact with the transmembrane gp41 [168]. The outer domain includes the variable regions 3, 4 and 5 (V3 - V5) which are anchored at their bases by disulphide bonds. All the cystiene residues within gp120 are completely conserved [169, 170]. Many insertions, deletions and substitutions in these regions contribute to the high degree of sequence variability and amino acid diversity, allowing flexibility within these loops [168].

The five conserved regions (C1 - C5) are interspersed between the five variable regions and fold into the gp120 core [66, 171]. These five constant regions within *env* have far less sequence variability and therefore harbour the more conserved function of chemokine coreceptor binding [171, 172]. The first and fifth conserved regions (C1 and C5) are implicated in the interaction between gp120 and the fusion peptide transmembrane glycoprotein gp41 [66, 173]. The conserved regions of gp120 are masked by the variable regions and are thus protected from attack by the hosts' immune response. The host immune response may be evaded by the variable outer domain of gp120 which is exposed to the hosts' immune system concealing the conserved regions. The outer domain thus avoids the humoral immune response and is referred to as the "silent face" [64].

Kwong *et al.,* [66] elucidated the specific contact residues involved in the CD4-gp120 interaction. Structure analysis elucidated 26 conserved gp120 amino acid residues that interact with 22 CD4 amino acid residues [66]. In culmination, this interaction buries 802 $Å^2$ surface area of gp120 and 742 $Å^2$ from CD4 [66, 67, 82, 174]. However, the surface areas that are actually in contact are much smaller, leading to the exposure of the gp120 core which is then readily able to bind to the chemokine coreceptor [62, 77, 164]. The chemokine binding residues are conformationally masked and not fully elucidated, however these sites are believed to be highly sensitive to antibody neutralization [175, 176].

Genetic, immunological and structural studies of the HIV-1 Env have revealed its incredibly diversity in the form of immunodominant loops, and its cunning

mechanisms of humoral and cellular immune system evasion. Immune evasion is achieved by the conformational masking of conserved coreceptor binding regions by the highly variable loops and "self-masquerading glycan shield" [61, 64, 176-178].

1.6.2 Glycosylation of gp120

The gp120 envelope protein undergoes post-translational modifications by enzymatic attachment of asparagine (N)-linked polysaccharide chains. The N-linked glycosylation targets any peptide sequence motif that is defined by $NX_1(S/T)X_2$, where X represents any amino acid, except Proline [179]. The gp120 is highly glycosylated harbouring approximately 24 potential complex glycans within the variable regions and high mannose/hybrid glycans within the conserved regions of gp120 (Figure 1.6) [169, 180]. Over half of the molecular mass of gp120 is due to the significant N-linked glycosylation addition onto the protein [181]. Glycosylation of the gp120 molecule is essential for the correct folding and processing of the three dimensional envelope protein [182] and thus the correct structural conformation is assumed for CD4 binding.

The heavy carbohydrate moieties form a "glycan shield" that sterically masks/protects the vulnerable surfaces on the viral envelope from host neutralizing antibody attack [171, 177, 183]. The position and amount of glycosylation sites and variation thereof, differ over time and thus provide an indication of the degree of escape of an isolate from the hosts' immune system [184].

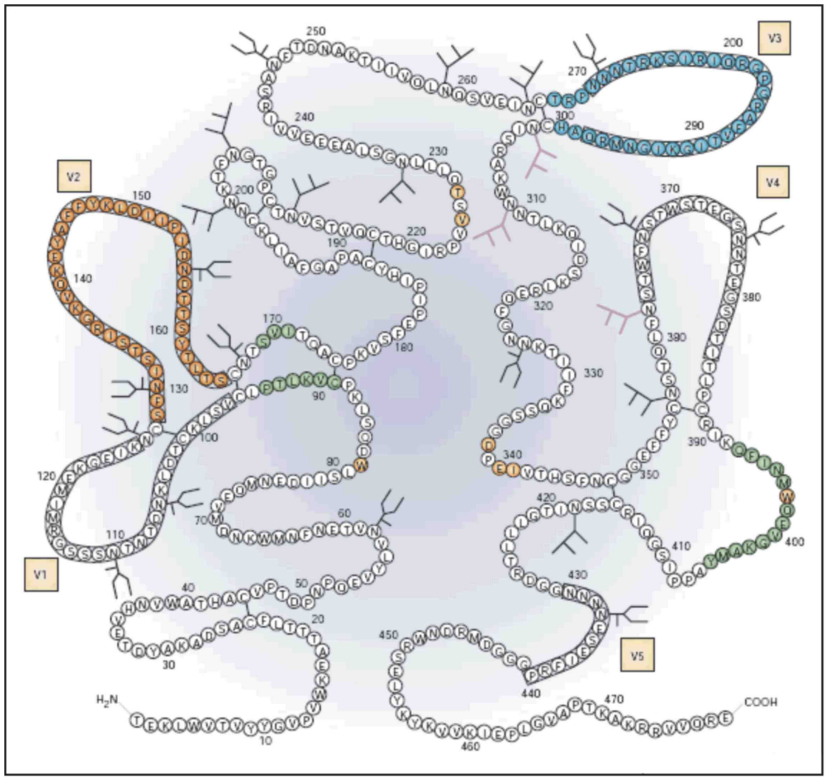


Figure 1.6 Schematic representation of the HIV-1 envelope protein. Epitopes that are capable of inducing neutralizing antibodies are coloured and the variable loops are designated in boxes (V1 – V5). Mannose-type or hybrid type glycosylation sites are indicated by the branched black structures, complex-type oligosaccharides are shown by the U-shaped black structures. Some epitopes that are capable of eliciting neutralizing antibodies are coloured in orange (V2 loop) and blue (V3 loop). The numbering of the amino acid residues in this figure was not used in the text and the figure was adapted from [185].

1.6.3 Envelope Heterogeneity and Disease Progression

Many studies have investigated the correlation between disease progression and viral heterogeneity. The variable regions of the envelope glycoprotein (particularly focusing on the V3 region) are major determinants in host cell tropism and elicit humoral and cellular immune responses against specific recognition sites [186, 187].

When the host is initially infected with HIV-1, the virus is transmitted as a homogeneous population of quasispecies with relatively low sequence diversity [125-127]. After primary infection in a treatment naïve individuals, the viral sequence diversity rapidly increases with disease progression and then decreases in viral diversity in the late stages of disease when the immune system ultimately deteriorates [188]. The HIV-1 populations within an individual are constantly evolving and sequences may differ as much as 10% from one-another at the end stages of disease [76].

Viral escape may be a function of host immune pressure or it may be purely a chance event due to the highly error-prone, poor proof-reading capabilities of the HIV-1 reverse transcriptase coupled with the high viral replication rate (see section 1.2). These changes may result in changes in length and overall charge of the variable loops (and other regions within gp120) as well as changes in number and position of glycosylation sites within the envelope [177]. Whatever the cause of immune evasion, the virus undergoes extensive viral heterogeneity and this is a determining factor for disease progression [189, 190].

The classical early stages of HIV-1 infection are characterized by predominating NSI, macrophage (M)-tropic, slow/low replicating, CCR5 utilizing (R5) variants (Figure 1.4) [191-195]. The R5 variants predominate during the acute and asymptomatic phases of infection [72] and play an important role in transmission and the infection of macrophages at the site of infection [126, 195-198]. R5 variants have been found to dominate even after vertical and sexual transmission [198] even when the dominant cell type during early and late infection is $CD4^+$ T cells.

However, T-cell (T)-tropic, SI, rapid/high replicating CXCR4 utilizing (X4) variants may emerge during the later phases of disease pathogenesis leading to accelerated loss of naïve and resting $CD4^+$ T cells, broadening of the coreceptor usage profile of HIV-1 isolates and rapid progression to AIDS (Figure 1.4). This is

observed in at least 50%-60% of HIV-1 subtype B patients [73, 193, 194, 196, 199-207].

Dual tropic viruses, capable of gaining entry into a host cells via the CXCR4 and/or the CCR5 coreceptor are termed R5X4 strains [141, 208]. CCR5 is expressed on activated lymphocytes, macrophages, dendritic cells and microglial and neuronal cells, however CXCR4 is ubiquitously expressed on all somatic cells [209]. Dual tropic variants may be an important structural intermediate during the switching process between R5 utilizing and X4 utilizing HIV-1 isolates [210, 211]. CXCR4 utilizing viruses have shown increased cytopathicity *in vitro*, which may be a fundamental link between coreceptor switching and the increased pathogenicity in late stage AIDS patients [212, 213].

1.6.4 Determinants of Coreceptor Usage and Switching

1.6.4.1 Structure of CCR5 and CXCR4

Both chemokine receptors CCR5 (previously named LESTR/fusin) and CXCR4 contain 352 amino acids, and are G-Protein Coupled receptors (GPCRs) that transverse the cellular membrane seven times [192]. These heptahelical large protein receptors transduce extracellular signals into intracellular signals via the activation of a G protein, bound to Guanosine triphosphate (GTP) [192]. The three extracellular domains of the receptor are glycosylated and contain four highly conserved cysteine moieties, which form disulphide bonds that stabilize the receptor within the membrane (Figure 1.7 A and B). Moore and Stevenson have elucidated the subtle differences in charge between the surfaces of the two coreceptors, CCR5 has a neutral charge, as opposed to the negatively charged CXCR4 [214]. Despite this low homogeneity between the two major coreceptors, dual tropic viruses can use both CXCR4 and CCR5 relatively efficiently [80, 215].

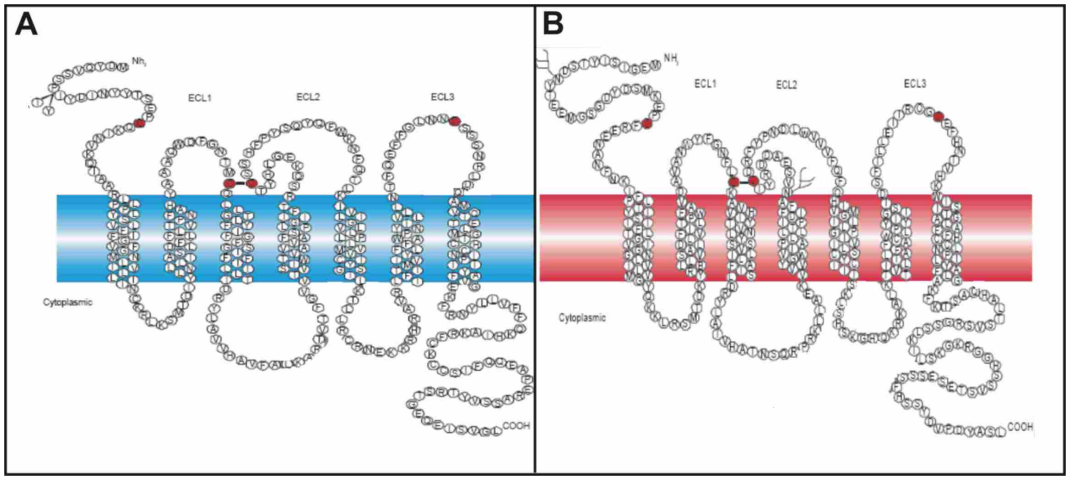


Figure 1.7 (A) Schematic representation of the CCR5 chemokine coreceptor, a 7-transmembrane major coreceptor for HIV-1 gp120 with amino terminus (NH_2) on the outside and the carboxyl terminus (COOH) on the inside of the cytoplasmic domain of the cell. The cysteine residues are indicated in red and the three extracellular loops (ECL) are depicted [80]. (B) Schematic representation of the 7-transmembrane chemokine CXCR4 coreceptor. The amino terminus (NH_2) is on the outside and the carboxyl terminus (COOH) on the inside of the cytoplasmic domain of the cell. The cysteine residues are indicated in red and the three extracellular domains are depicted ECL1, ECL2, ECL3, respectively. Figure adapted from [216].

Further analysis of CCR5 and CXCR4 has shown that negatively charged amino acids, including tyrosine, in the amino termini of the coreceptors are critical for binding of the viral envelope [217]. The second extracellular loop of CXCR4 is implicated in expanded host cell tropism and yields a more negative charge, which may lead to the greater attraction to the more positive CXCR4 tropic envelope [217-219]. The association between gp120 and the coreceptor is likely one based on charge, indicating that the higher the positive charge of the envelope, the higher the likelihood of the isolate using CXCR4 to gain entry.

The Env of a R5 utilizing HIV-1 may evolve to use CXCR4 over time and this may be intimately associated with the common structural features of the two coreceptors and the ability of gp120 to adapt/mutate to influence this interaction as disease progresses.

1.6.4.2 V3 Loop and coreceptor Usage

The V3 loop plays a critical role in coreceptor binding of HIV-1 and host cell tropism [220-222]. Coreceptor usage has been extensively studied in HIV-1 subtype B and certain characteristics that influence coreceptor binding have been elucidated. The intact crystal structure of HIV-1 V3-containing gp120 core in complex with CD4 and an antigen-binding fragment of the R5 antibody, has been elucidated [174]. This crystal structure elegantly depicts how the V3 loop extends outwards and downwards away from the gp120 glycoprotein towards the host cell membrane (Figure 1.8).

This V3 loop was described as a "molecular hook" that engages the N-terminus of the CCR5 coreceptor [174]. The conserved Proline (P) - Glycine (G) motif on the tip of the V3 loop projects 30 Å towards to host cell membrane from the gp120 core (Figure 1.8). This allows interaction of the V3 loop with the N-terminus of the CCR5 coreceptor; as the tip binds to the ECL2 of CCR5 and the conserved base interacts with the sulphated CCR5 N-terminus (Figure 1.8) [174].

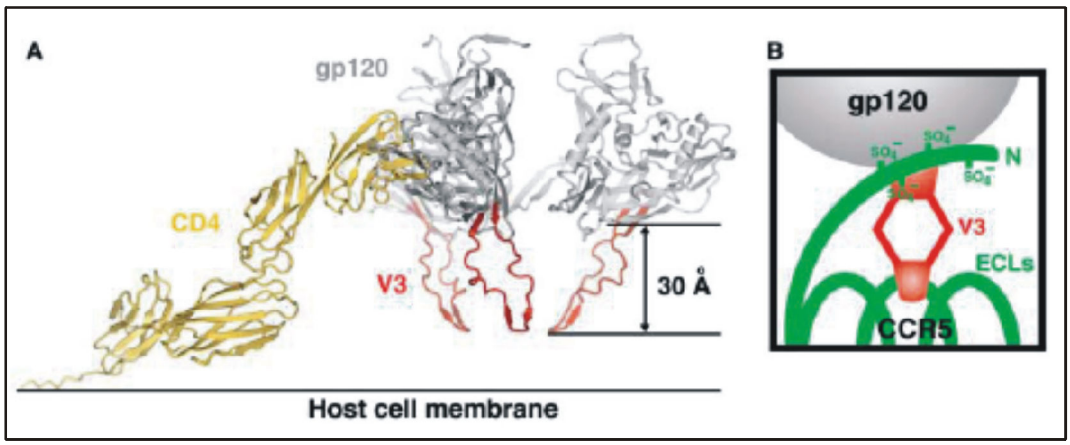


Figure 1.8 (A) Crystal structure of a CD4-triggered gp120 in complex with the V3 loop (in red) at the host cell surface, superimposed onto a structure of the four-domain CD4 receptor. The highly conserved P-G tip of the V3 loop is projected 30 Å towards the host membrane. (B) Schematic representation of the CCR5 coreceptor interaction (green) with its tyrosine-sulphated N-terminus and extracellular loops (ECLs) and V3 (red). Figure adapted from [174].

The V3 loop amino acid charge, amino acid length variation, glycosylation patterns, tetrapeptide motif and its secondary structure have all been found to influence viral coreceptor usage [223, 224].

The V3 region is hypervariable and induces both highly specific as well as broadly cross reactive antibodies (e.g. 447-52D and 2219) due to its high degree of antigenicity [225, 226]. Previous studies have shown more specifically, that it is the overall amino acid charge of the V3 loop which determines coreceptor phenotype [167, 175]. A higher positive charge will correlate with CXCR4 usage (X4 using virus) and SI phenotype, similarly a lower positive charge associates with the NSI CCR5 coreceptor usage (R5 using virus) [69-71, 77, 80, 164, 201, 227-229].

An accurate prediction of CCR5 coreceptor usage/NSI phenotype, may also be determined by the presence of a neutral amino acid (i.e. serine (S)) at position 11 in the V3 loop as well as a negatively charged amino acid (either aspartic acid (D) or glutamic acid (E)) at position 25 [201, 202, 229-232]. Conversely, if a basic/positive amino acid is found at position 11 and/or 25 (i.e. arginine (R), lysine (K) and Histidine (H)), this overall positive charge will strongly correlate with the SI phenotype (Figure 1.9) [201, 202, 229, 233-235]. The higher the net charge of the V3 loop, the higher the correlation is of the positive V3 loop interacting with the negatively charged CXCR4 coreceptor [203].

The V3 region is usually 35 amino acids in length and located between amino acids 296 and 331 of gp120, forming a distinctive “finger-like” loop (in subsequent discussions, amino acid positions 1 through 35 correspond to 296 through 332 in the standard reference HXBc2) (Figure 1.9). Generally CXCR4 utilizing V3 loop sequences are slightly longer in length, due to the increased amino acid insertions [236, 237].

There are two potential N-linked glycosylation sites within the HIV-1 subtype B V3 loop reference sequence, one appears at the N-terminal cysteine residue at the base of the loop (NCT) and the other is found from position 6-8 (NNT) (Figure

1.9). However, the N-linked glycosylation site (NCT) is rarely present in HIV-1 subtype C V3 loop sequences [238-240]. The glycan at position 6-8 of the V3 loop, has been implicated in CCR5 usage and decreasing sensitivity to antibody neutralization [241, 242]. The loss of this N-linked glycan is associated with less frequent use of the CCR5 coreceptor and in some cases, lack of the glycan is associated with exclusive CXCR4 usage [243, 244].

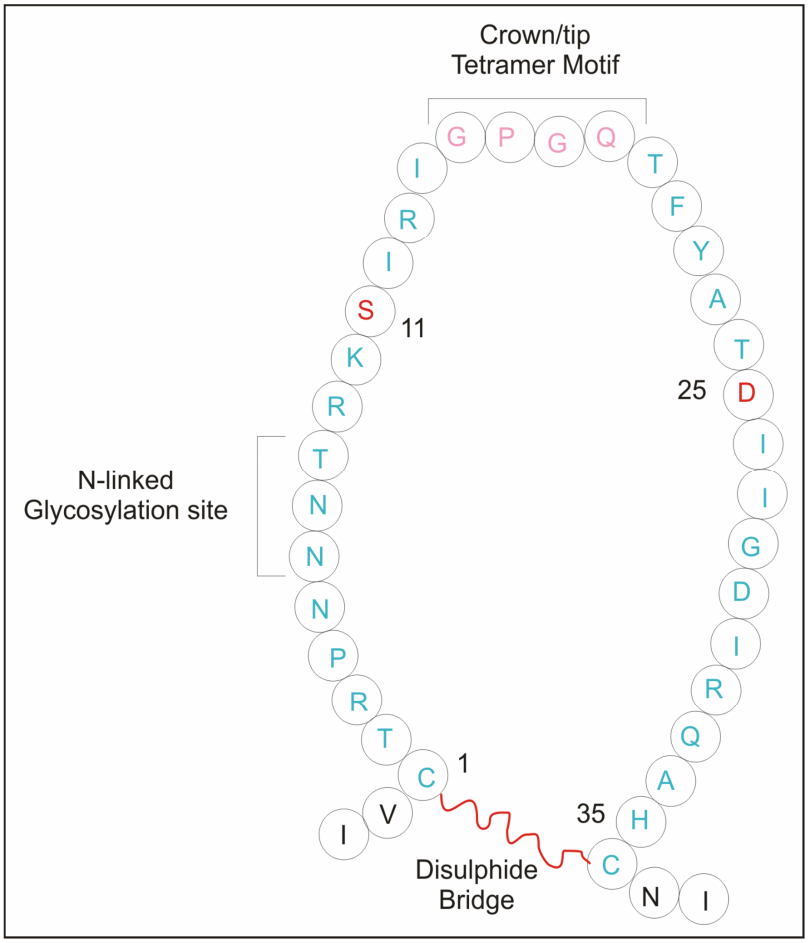


Figure 1.9 Schematic illustration of the 35 amino acid V3 loop (in blue). The GPGQ tetrapeptide crown/tip motif is indicated in pink. The 11th and 25th amino acid residues are highlighted in red, showing their importance in coreceptor binding. The N-linked glycosylation site at NNT and the disulphide linkage between the two cysteine residues are also shown. The V3 loop sequence depicted here is based on the Consensus subtype C sequence used in Section 2.4.6.

The highly conserved crown/tip of the V3 loop (residues 15 – 18) is also a strong predictive region of HIV-1 subtype C cellular tropism. The consensus subtype B

tetrapeptide crown motif is generally GPGR/K, irrespective of coreceptor usage [234]. However, in HIV-1 subtype C isolates, the tetramer motif GPGQ has shown great conservation in CCR5 utilizing HIV-1 isolates, whereas a more variable motif is displayed in CXCR4 utilizing isolates such as GPGR or GRGQ (Figure 1.9) [245].

The secondary structure conformation of the V3 loop also plays a crucial role in viral biological phenotype/cellular tropism [202, 246]. According to neural network modelling, the V3 loop has a predicted secondary structure composed of a short carboxyl-terminal α–helix and two antiparallel β–sheets which is formed by the disulphide linkage between two conserved cysteine residues at its base [247], [201, 248].

There is still debate as to whether the V3 loop is directly or indirectly involved in the initial gp120-CD4 binding interaction [222]. Whether the V3 loop sequence, or its overall conformation, define chemokine coreceptor usage is still uncertain and thus further investigations into the specific requirements of the V3 loop that influence coreceptor usage are necessary. Studies on the correlation between chemokine coreceptor usage/cellular tropism and V3 loop characteristics of HIV-1 subtype C isolates, remains to be extensively evaluated. To date, only a few CXCR4 utilizing HIV-1 subtype C viruses have been identified, and the majority of HIV-1 subtype C viruses have shown almost exclusive CCR5 usage [164, 200, 232, 239, 249-263].

Some studies have shown that there is a marked increase in the frequency of CXCR4-using HIV-1 viral isolates in HIV-1 infected patients receiving/following antiretroviral treatment [264, 265]. A study has shown that host cell availability, expressing the various coreceptors used by HIV-1 is unlikely to be the sole driving force of this described R5 tropic to X4 tropic switch. This may be due to a delayed X4 tropism development associated with higher levels of CXCR4 expression on total and naïve $CD4^+$ T cells [266].

Coreceptor usage of HIV-1 isolates is established with 100% accuracy in an MT-2 phenotypic cell assay [267-269]. However, phenotypic assays do not elucidate genotypic changes that are responsible for the change in viral tropism. The advent of bioinformatic algorithms as molecular software packages have enabled the identification of empirically predetermined sequence motifs in the variable regions of HIV-1 coreceptor binding sites. A score is mathematically produced which is interpreted by indicating the likelihood of the sequence of interest rendering CXCR4 or CCR5 usage [270, 271]. However, the evolutionary pathways, sequences of mutations and biological and genotypic selective pressures that are required for the switch in viral phenotype from R5 to X4 usage are not fully understood, despite being extensively studied.

Coreceptor switching has become arguably relevant as it may be the reason for drug resistance to novel CCR5 antagonists that are currently in advanced stages of clinical development [272-275].

1.6.5 The HIV-1 Env glycoprotein represents an Antiviral and Vaccine Target

The sequence variability and glycosylation of the gp120 glycoprotein surface may vary the immunogenicity and antigenicity of the envelope [276]. The gp120 glycoprotein is the primary target for neutralizing antibodies elicited by the hosts' immune system during the course of natural infection [177, 276]. Since certain conserved residues of gp120 are concealed from the host's immune system and have demonstrated increased sensitivity to neutralizing antibodies, these epitopes present as ideal candidates for vaccine studies and or targets of novel antiretroviral drugs. Neutralizing antibody determinants can thus be elucidated and used as vaccine immunogens to elicit strong memory immune responses within an uninfected individual.

1.6.5.1 Neutralizing Antibodies

Broadly neutralizing monoclonal antibodies to HIV-1 infection recognize specific epitopes found on the envelope glycoprotein namely, 2G12, 2F5, b12, 4E10 and Z13 [178, 277-284]. Both 2G12 and b12 bind the conformationally invariant surface of the outer domain of gp120 and have shown efficient neutralization against diverse HIV-1 primary isolates [280, 283, 285, 286]. 2G12 contains mannose rich glycosylation sites and recognizes glycan residues on the outer region on gp120 in positions N295, N332, N339 and N392 (numbering based on the HXBc2 viral genome) [277, 281, 287]. 2F5 recognizes a conserved linear sequence (ELDKWA) on the C-terminal region of gp41 [278], and 4E10 recognizes the conserved sequence (NWF(D/N)IT) on gp41 close to the 2F5 epitope [282]. Recently, a high-affinity variant of Z13, named Z13e1, was isolated and found to have 100 times stronger neutralization potency against sensitive HIV-1 and stronger binding affinity for the membrane-proximal external region of gp41, compared to its parental Z13 [280].

Recently, Zhou *et al.,* [178] elucidated the structure of the gp120 glycoprotein in complex with the broadly neutralizing antibody b12 at 2.3Å resolution [178]. The vulnerable site on gp120 that is functionally required for CD4 binding was targeted. This recessed site is located between the glycan-shielded silent face and the conformationally flexible inner domain/bridging sheet and is accessible by CD4 for functional entry [178]. This crystal structure is of profound importance, as it details the accessibility and ability of b12 to bind and disable the highly protected HIV-1 Env and this has implications for an HIV-1 vaccine [178].

The coreceptor switch from NSI (R5) to SI (X4) results in both biological and physical changes within Env. Interestingly, the neutralizing antibodies target conserved epitopes in the V3 loop and R5 viruses are considerably more resistant to neutralizing antibodies as compared to their X4 counterparts [183, 276, 288-292]. The increased sensitivity of X4/SI Env to some broadly neutralizing antibodies is due to the enhanced binding capacity and accessibility of the antibody

for the V3 loop. Therefore, the V3 loop conformational state associated with the X4 Env is more accessible and susceptible to neutralizing antibodies [293]. This information may have profound implications for vaccine design and drug development protecting against SI/X4 variant evolution and/or emergence.

1.6.5.2 Drug Targeting HIV-1 Entry

The three main classes of Food and Drug Administration (FDA) approved current Antiretroviral Therapies (ARTs) for use in HIV infection are; Reverse Transcriptase Inhibitors (RTIs), Protease Inhibitors (PIs) and Entry Inhibitors (EIs) [294]. However, viral resistance to current therapies has prompted the emergence of four new classes of drug targets for ART; Inhibitors targeting (i) NCp7 Zn Finger inhibitors, (ii) integrase, (iii) rev/tat and (iv) viral maturation inhibitors [295-299].

Several novel compounds acting as entry inhibitors have been designed to target the three distinct stages of the entry process; namely attachment of gp120 to the primary CD4 receptor, interaction of the gp120-CD4 complex with either CCR5 and/or CXCR4 chemokine coreceptors and finally gp41 mediated membrane fusion.

T-20 (Enfuvirtide/Fuzeon) is a novel entry inhibitor peptide that interferes with the formation of the six-helix bundle in gp41 thereby preventing fusion between viral and host membranes [300, 301]. T-20 was initially used as a salvage therapy, however it is now used in combination with other antiretroviral drugs due to the detection of drug resistant mutations and incomplete viral suppression in HIV-1 positive patients taking T-20 [302].

Several drugs have been designed to target the gp120-CD4 interaction. PRO 542 (CD4-IgG2), is a recombinant tetravalent CD4-immunoglobulin fusion peptide which comprises four gp120 binding sites [303]. BMS-378806 is the first small indole-based molecular compound that inhibits the gp120-CD4 interaction through

binding to the conserved CD4-binding pocket on gp120 [304, 305]. Cyanovirin (CV-N) is a protein compound (lectin) that targets virion attachment to host cells via blocking the gp120-CD4 interaction by competitively binding to high-mannose carbohydrates on the viral glycoproteins [306]. This protein was extracted from the cyanobacterium *Nostoc ellisporum* and may be useful as a therapeutic agent or as a topical microbicide [306, 307].

Many entry-inhibiting compounds that inhibit gp120 from interacting with chemokine coreceptors are in various stages of development. There are two main classes within the coreceptor inhibitors, namely those targeting CCR5 and those targeting CXCR4. Most HIV-1 strains are sensitive to inhibition by CCR5 proinflammatory chemokines, CCL5 (RANTES), CCL3 (MIP-1α) and CCL4 (MIP1β) [77]. RANTES is a β-chemokine that is naturally secreted by cytotoxic T cells and binds to CCR5 and inhibits cellular entry of R5 variants [308]. TAK779 prevents gp120 interactions with the CCR5 coreceptor via binding one of the grooves formed by the seven transmembrane helices of CCR5 [309, 310] and TAK 220 is a small molecule CCR5 antagonist that is orally bioavailable [311].

There are also CXCR4 inhibitors that are highly cationic and specifically target CXCR4. However, CXCR4 plays important biological roles (embryogenesis) and immunological roles and thus targeting CXCR4 as a therapy is somewhat controversial [312]. Stromal cell-Derived Factor - 1 alpha (SDF-1α) is a natural ligand for CXCR4 and highly specific for CXCR4 [313]. Therefore, this is cause for concern relating to the undesirable effects of blocking SDF-1α *in vivo*. AMD3100 is a bicyclam cationic compound that binds to the extracellular loops of CXCR4 and can prevent both X4 and R5X4 isolates from binding to CXCR4 [314, 315]. Both T140 (precursor peptide T-22) and ALX40-4C are specific for targeting X4 viruses [316, 317]. Other CXCR4 antagonists include AMD 070 and KRH 2731 [318, 319].

Currently, many entry and fusion inhibitors are being investigated in controlled clinical trials and some are bioavailable as oral compounds in human clinical trials.

Fusion and attachment inhibitors include; AK602 [320], a CCR5 antagonist in early human trials, Maraviroc [272], a CCR5 antagonist in Phase III trials, AMD070 [321], a CXCR4 antagonist in Phase II trials, BMS-378806 [304, 305], is in Phase II trials, HGS004 [322], a monoclonal antibody CCR5 antagonist, successfully completed a Phase I trial , PRO 140 [323] and PRO 542, Schering C, SP01A, an entry inhibitor in Phase III trials, TAK-652, another CCR5 antagonist in Phase I trials, TNX-355 is a monoclonal antibody in Phase II trials and finally Vicriviroc (SCH 417690) [47, 324, 325]. However, Phase III trials of vicriviroc, a non-protein molecule that binds to the extracellular cavity of CCR5, has been halted due to its inferior antiviral efficacy [326]. The study will however be repeated with higher doses of vicriviroc [47].

1.6.5.3 Development of Drug Resistance

In an attempt to prevent the inevitable progression to AIDS, HAART has been developed. However, despite individuals who respond successfully to HAART, treatment failure is common and due to the failure of almost 100% viral suppression by the current treatment regimes and the emergence of drug resistant HIV-1 strains is inevitable [327]. Thus, the development of effective antiretroviral drugs and/or vaccines is vital. Treatment failure on HAART results from a combination of different factors.

Firstly, drug-resistant mutations within the HIV-1 genomic material my pre-exist and under the pressure of partially-effective antiretroviral regimens, may be selected for [328]. Many factors result in the development of drug-resistant strains of HIV-1. The high replication capacity of HIV and its error-prone reverse transcriptase are major factors that contribute to viral escape from the immune system and the emergence of drug resistant strains [46]. Another significant source of genetic variation in HIV-1 is recombination between different HIV-1 variants within the host. Simultaneous infection of an individual by two or more distinct

HIV-1 strains may occur *in vivo* and recombination between these viruses may lead to highly resistant strains [329, 330].

Secondly, sub-inhibitory drug levels allow resistant strains to populate within the host. This can arise from a range of events, such as poor patient adherence to the drug regime, poor drug absorption, incorrect drug dose or inhibitory interactions between drugs within the host [327].

Lastly, the exposed surfaces of the gp120 molecule contain hypervariable domains that are highly glycosylated with carbohydrate moieties that serve as immunologic decoys for the humoral immune response [331, 332]. This hypervariability of the HIV-1 surface may also lead to drug resistance.

The phenomenon of HIV-1 drug resistance may either significantly impair viral fitness or lead to the emergence of more fit and more pathogenic viral variants. The observed genetic diversity of HIV-1 may also be influenced by selective pressure such as the host's immune response, cell tropism of the virus and the genetic heterogeneity of the host. Resistance to all of the currently availably ARVs has been documented [333-343] and thus new classes of drugs directed against more conserved target sites is imperative if the resistance to therapy is to be curtailed.

1.7 CD4-independent Entry

1.7.1 CD4-independence

HIV entry into a host cell is a dynamic and complex multi-step cascade process which is initiated with binding of the viral envelope glycoprotein to the CD4 receptor [56, 57]. However, some primary HIV-1, HIV-2 and SIV isolates can, to some extent, enter cells *in vivo* without the requirement for CD4 and bind directly

to coreceptors to initiate infection [344-347]. These viruses are termed CD4-independent and this naturally occurring phenomenon *in vivo* is relatively rare.

The viral envelopes are 'partially triggered', allowing direct binding of the coreceptor to initiate host cell infection. Overall, CD4-independent viruses express gp120 in a 'pre-triggered' conformation in which the coreceptor binding site is exposed and can bypass the CD4 receptor and interact directly with the coreceptor [345, 346, 348-350].

The CD4-independent Envs generally possess multiple amino acid substitutions and/or deletions when compared to the CD4-dependent wild type (wt) virus. These mutations ultimately lead to the exposure of conserved epitopes that mediate direct binding to the CCR5 and/or CXCR4 coreceptor [167, 175, 351-353]. Consequently, CD4-independent viruses infect target cells with a much higher efficiency compared to wt viruses. However, these CD4-independent viruses exhibit heightened sensitivity to neutralizing antibodies that recognize conserved epitopes, which are essential for coreceptor engagement [351, 354-357].

The CD4-independent phenotype can be a natural consequence of infection of some cell types. Some cells express low levels of CD4, or do not possess CD4 in their cellular membrane. For example, macaque macrophages have been shown to express low levels of CD4 [358, 359]. Additionally, cells such as brain macrophages, oligodendrites, brain microvascular endothelial cells, astrocytes and microglia do not express CD4 [360]. HIV-1 is capable of infecting human brain cells which results in severe cognitive behavioural and motor destruction, termed HIV-Associated Dementia Complex (HADC) [361]. However, the mechanism of viral entry into astrocytes and neurons is currently unclear and elucidation of this entry pathway will aid in HIV-1 disease pathogenesis control.

Studying the structure and function of CD4-independent HIV-1 viruses, may provide insight into the cascade of events that occur during Env-mediated membrane fusion with host cells. The specific residues involved in CD4 binding and subsequent conformational changes that allow for coreceptor exposure may be

genotypically and phenotypically identified. Further information regarding the interaction of the CD4-independent structural intermediate envelope and coreceptor, will provide a unique opportunity to determine which mutational changes in a CD4-independent envelope govern tropism for the primary receptor, CD4. Once the conserved coreceptor binding residues are elucidated, there will be potential for exploitation of these regions or analogues thereof for potential use as novel entry inhibitors or therapeutic vaccines.

1.7.2 CD4-independence *in vivo*

Several CD4-independent SIV, HIV-2 and HIV-1 isolates have been characterized *in vivo*. CD4-independent CCR5 utilization *in vivo* is relatively common in SIV and HIV-2 strains [345, 348, 362, 363] and CD4-independent, CCR5 tropic SIVs have a reduced affinity for CD4 and have been previously isolated from rapid progressor rhesus macaques [356, 357, 364].

To date, only the HIV-1 primary isolate, (R2) has been shown to infect CCR5 positive CD4 negative cells, but not efficiently. This isolate harbours a rare mutation in the V3 loop of gp120 [365].

The CD4-independent phenotype in HIV and SIV can thus be attributed to one of two changes within gp120. Firstly, insertions and/or deletions can cause repositioning of the variable V1/V2 loop so as to unmask the coreceptor binding site. Secondly, an accumulation of successive mutations in V3 and the base of V4 might increase envelope affinity for coreceptor binding (or pre-folding of the gp120 bridging sheet) [344, 366, 367]. Mutational changes in gp41 may also trigger conformational rearrangements and fusion to occur after a weaker gp120-coreceptor interaction [348]. However, the extent to which CD4-independent viruses enter cells via a CD4-independent mechanism and have an expanded tropism for CD4-negative cells *in vivo* is currently unclear.

1.7.3 CD4-independence *in vitro*

There have also been several reports of *in vitro* studies showing that strains of SIV, HIV-2 and laboratory adapted HIV-1 strains can bind either CXCR4 or CCR5 coreceptors in the absence of CD4 [344-346, 349, 350, 367-371]. Zerhouni *et al.,* [367] hypothesized that these CD4-independent quasispecies would exist at low concentrations (in primary culture) compared to the more abundant CD4-dependent strains, and that they may be enriched through serial propagation. However, infection of T cell lines lacking CD4 expression is much less efficient; hence, the significance and relevance of such viruses *in vivo* is relatively unclear.

CD4-independent HIV-1 and HIV-2 variants that utilize CXCR4 for cell entry have been isolated *in vitro* by passage through cells lacking CD4. Hoffman *et al.,* [351] derived an HIV-1 CD4-independent virus, IIIBx that interacted directly with the CXCR4 coreceptor and this phenotype was correlated with mutations throughout *env*. The IIIBx exhibited a heightened sensitivity to neutralization by HIV-1 positive human sera, as opposed to its CD4-dependent counterpart, IIIB [346, 372].

The cloned *env* from HIV-1 IIIBx (8x) and HIV-1/IIIB (HXB2R) variants were generated by serially passaging cloned *envs* through CD4-negative, CXCR4 positive cells [350, 369]. The 8x carried a gp120 with a stable, constitutively exposed coreceptor binding site enabling it to bind directly to CXCR4 and was significantly more sensitive to neutralization by human sera from HIV-1 positive individuals [346, 351]. High affinity CXCR4 coreceptor usage was shown in CD4-independent HIV-2 variants ROD/B and VCP [346, 369, 370]. The critical amino acid substitutions that were responsible for the CD4-independent phenotype and exposure of the coreceptor binding site, were changes in the V3 loop sequence, around the base of the V4, and in gp41 [350, 373].

The CD4-independence in the HIV-1 m7NDK variant correlated with the acquisition of two glycosylation sites in V1/V2 and 7 amino acid changes within

the C2, V3 and C3 regions of *env* [344]. Another study was performed on a CXCR4 tropic HIV-1 iNDK recombinant clone, derived from the same parental virus as the m7NDK variant. The clone showed the acquisition of two N-linked glycosylation sites in the V1/V2 region, three amino acid changes in the V3 loop and the loss of one N-linked glycan from the C2 region [374].

Kolchinsky *et al.,* [349] and colleagues have shown that CD4 binding in the entry cascade of HIV-1 isolates can be made redundant by alteration and acquisition of glycosylation sites in the V2 loop and V1-V2 stem elements [349]. These changes allow the envelope glycoproteins to assume a pre-triggered state whereby the coreceptor binding site is exposed and CCR5 binding can occur [349]. The HIV-1 ADA variant infected cells via CCR5 in the absence of CD4, and removal of a single glycosylation site at the base of the V2 loop was found to confer the CD4-independent phenotype [355]. Structural models of gp120 suggest that removal of this glycan presumably results in the repositioning of the V1/V2 loops, so as to expose the highly conserved coreceptor binding site. The CD4-independent HIV-1 NDK variant correlated with the acquisition of two glycosylation sites in the V1/V2 loops [374].

CD4-independent usage of the CCR5 coreceptor by an HIV-2/vcpm variant mapped to a positively charged amino acid in the C4 region of *env* [375]. Other CCR5 tropic, CD4-independent infection of host cells, has been shown in SIV [345, 357, 376-378]. Puffer *et al.,* [379] determined that two amino acid changes in the V1/V2 loop and gp41 are responsible for the CD4-independent phenotypic effects on the SIVmac239 Env [379].

The genotypic determinants for the CD4-independent phenotype *in vitro* are mapped to *env*, however the underlying mechanisms of this phenomenon are largely unknown. The changes acquired by different HIV-1 recombinant clones post CD4-independent adaptation vary considerably between isolates. There does not seem to be a common, standard motif associated with *env* that is required for CD4-independent *env* clones. In general, mutations in the coreceptor binding

domains and mutations within the trans-membrane region of *env* as well as an alteration of the N-linked glycosylation profile will render an isolate CD4-independent [344-346, 349, 350, 367-371]. The changes observed *in vitro* may not be a direct reflection of the exact changes that are taking place *in vivo,* due to the lack of immune pressure *in vitro*.

1.7.4 Relevance for Neutralizing Antibodies and Drug Targets

CD4-independent viruses are more susceptible to antibody neutralization, possibly explaining the rarity of the occurrence of the CD4-independent phenotype *in vivo* [355]. The relationships between CD4-independence, antibody neutralization sensitivity, and exposure of CD4-induced epitopes that are associated with the coreceptor binding site of gp120 have been investigated [351, 354]. The conserved determinants of the neutralization sensitivity of the CD4-independent phenotype have largely been mapped to the gp160 region.

There is thus broad potential for the exploitation of these highly neutralization sensitive CD4-independent envelopes that are partially triggered, exposing their coreceptor binding domains. Neutralization may take the form of antibodies and/or small molecular inhibitors of the Env-coreceptor interactions. Monoclonal antibodies directed at the conserved coreceptor binding regions of the envelope may be used as immunological surrogates for inhibition viral entry.

As mentioned previously, 50% of worldwide HIV-1 infections are caused by subtype C and one-third (32%) of the worlds' HIV-1 infected population lives in Southern Africa [16]. These data taken together with the vast and rapid genetic variability rate of HIV-1, highlight the urgency and importance for studying subtype C in order to elucidate common drug targets and prevention strategies between subtypes that may aid in producing broadly cross-reactive antiviral treatments and/or vaccines.

It is expected that CD4-independent viruses will have an exposed coreceptor binding site, providing a number of potential sites for therapeutic intervention by compounds such as small molecule inhibitors and/or neutralizing antibodies.

To date, there are no reports in the literature on HIV-1 subtype C, CD4-independent viruses. A CD4-independent HIV-1 subtype C viral isolate would be extremely valuable for use as a potential vaccine immunogen, and a target to identify novel entry inhibitors. In addition, it would provide useful information on the viral entry process. Thus, this study focused on the adaptation of primary HIV-1 subtype C isolates towards CD4-independence.

1.8 Objectives and Aims of the Study

The overall aim of this study was to isolate a CD4-independent HIV-1 subtype C isolate. We attempted to generate this isolate using the following two procedures:

1. Isolation and characterization (genotypic and phenotypic) of primary HIV-1 subtype C isolates from antiretroviral drug-naïve AIDS patients.

2. Adaptation of selected primary isolates to CD4-independence through serial *in vitro* passage in Cf2Th cell lines.

The significance of this study is that CD4-independent viruses are expected to possess exposed coreceptor binding sites, providing potential conserved sites for therapeutic intervention by compounds such as small molecule inhibitors, neutralizing antibodies and/or therapeutic vaccines. This is a novel study performed on HIV-1 subtype C primary isolates.

Chapter 2 ISOLATION AND CHARACTERIZATION OF HIV-1 PRIMARY ISOLATES FROM ANTIRETROVIRAL DRUG-NAÏVE SOUTH AFRICAN AIDS PATIENTS

2.1 Abstract

HIV-1 gains entry into host cells through the envelope glycoprotein binding to CD4 and a coreceptor, either CCR5 and/or CXCR4. HIV-1 subtype C viruses predominantly use CCR5, unlike other group M subtypes that often switch to using CXCR4 during disease progression to AIDS. This study investigated the genetic and phenotypic properties of 20 antiretroviral drug-naïve advanced AIDS patients attending a clinic in Johannesburg, South Africa. Blood was collected over 6 months between January and June 2005 and primary viral isolates were established and propagated using standard co-culture techniques. Coreceptor usage for each isolate was determined, elucidating biotype and phenotype, using the U87.CD4 and MT-2 cell assays respectively. Viral phenotype was shown to be either Non-syncytium inducing (NSI) or syncytium inducing (SI). Proviral DNA was extracted, the full-length *env* genes were PCR amplified and the V3 loop region was sequenced and extensively analyzed. Fourteen isolates utilized CCR5 and exhibited the NSI phenotype and the remaining six of the 20 (30%) primary isolates utilized CXCR4 for cell entry and exhibited the SI phenotype. Phylogenetic analysis of V3 loop sequences from 19/20 isolates confirmed they were HIV-1 subtype C and isolate 05ZAFV10 was found to be subtype D in the V3 loop region. Predicted amino acid analysis of the V3 loop sequences indicated specific residues and motifs relating to coreceptor usage. The SI viruses had significant genetic changes in the V3 loop, characteristic of CXCR4 usage, when compared to NSI viruses from the same cohort. We report the highest percentage of CXCR4 usage amongst primary isolates from HIV-1 subtype C infected AIDS patients in South Africa. These results imply that the frequency of HIV-1 subtype C CXCR4 utilizing viruses may be increasing with time. This detected emergence/evolution of CXCR4 usage amongst HIV-1 subtype C may have profound implications for viral pathogenesis, disease progression and future use of CCR5 antagonists as antiretroviral agents.

2.2 Introduction

HIV-1 entry is initiated by the envelope glycoprotein (Env/gp160), located on the surface of the virion, binding to host cellular receptors. Env also plays a pivotal role in the viral life cycle, and is responsible for transmission, viral tropism and viral replication kinetics. HIV-1 gains entry into host cells through the Env binding to its primary receptor, CD4 and a coreceptor, either CCR5 and/or CXCR4. Viral tropism of HIV-1 can largely be linked to these two coreceptors.

Several studies have shown that viruses isolated from HIV-1 positive individuals early in the time course of disease progression, are non-syncytium inducing (NSI), macrophage (M)-tropic, CCR5-utilizing HIV-1 (R5) strains. While T-cell (T)-tropic, syncytium inducing (SI), CXCR4-utilizing isolates (X4) may emerge during the later phases of infection [141, 142]. Some HIV-1 viruses are capable of gaining entry into the host cell by binding to CD4 and CCR5 and/or CXCR4, such viruses are termed dual-tropic (R5X4). These phenotypic characteristics of HIV-1 have been closely associated with the growth characteristics of viral isolates in co-culture experiments with donor peripheral blood mononuclear cells (PBMCs) [267, 380, 381] and in cell lines expressing the relevant coreceptors [197, 268, 269, 382].

During late stage disease, approximately 50% - 60% of the isolates from AIDS patients infected with HIV-1 subtype B showed multiple coreceptor usage [73-76, 193, 194, 196, 200-207]. After the appearance of CXCR4 utilizing viruses, both R5 and X4 tropic variants may co-exist within the individual. Changes in cellular tropism and the emergence of X4 coreceptor usage by HIV-1 strains *in vivo* seem to be a key event in disease pathogenesis. This promiscuous nature of the HIV-1 virus may be associated with accelerated $CD4^+$ T cell decline, broader target cell range, accelerated disease progression to AIDS and decreased survival time in antiretroviral (ARV) treatment-naive patients [73, 194, 196, 206, 207]. Despite extensive research regarding the cellular and molecular mechanisms of coreceptor switching observed in conjunction with disease progression towards AIDS, the mechanisms remain unknown.

Several hypotheses have been described that attempt to elucidate the phenomenon of coreceptor switching and the late appearance of X4 variants: Firstly, the 'transmission-mutation hypothesis' suggests that CCR5 utilizing viruses are preferentially transmitted and mutate to CXCR4 utilizing variants over time [219, 383]. It has been shown that dual tropism may evolve in CCR5 utilizing strains of HIV-1 through the acquisition of the ability to use the first and second extracellular loops of the CXCR4 coreceptor. The HIV-1 *env* may undergo several mutations to acquire dual tropism and maintains its use of the CCR5 coreceptor despite these changes. A second 'target-cell-based hypothesis' suggests that the availability of CCR5 and CXCR4 expressing cells shift in favour of X4 utilizing virus infection, enabling a pre-existing pool of CXCR4 utilizing viruses to expand [383]. Ribeiro *et al.,* [384] suggested that naïve and memory cell turnover rates throughout disease progression are responsible for the observed tropism switch in HIV-1 [384]. Finally, the 'immune-system-based hypothesis' suggests that during the early stages of infection, CXCR4 utilizing viruses are easily neutralized by the host immune system and thus suppressed [383]. The X4 populations will emerge later during infection, because of the deterioration in function of the immune system.

Recently, Mild *et al.,* [385] suggested that the detected emergence of CXCR4 utilizing HIV-1 variants in the later stages of disease pathogenesis, may be due to intrapatient recombination between different viruses with distinct tropisms [385]. This data suggests that the pre-existing circulating R5 and X4 viruses recombine, and result in CXCR4 utilizing chimeric viruses with the potential to evade the host immune system [385]. This is the first time that intrapatient recombination has been demonstrated at such high frequencies.

Thus, there is still debate as to how these CXCR4 utilizing viruses arise. X4 emergence may be a primary or secondary pathogenic event i.e. X4 isolates may be directly responsible for accelerated disease progression or there may be an independent event that promotes pathogenesis and thus X4 isolates emerge later in disease progression. These questions relating to coreceptor change and viral

phenotypic evolution need further investigation using both phenotypic and genotypic molecular tools.

There are various culture-based and bioinformatic approaches which are used to determine HIV-1 viral coreceptor usage and biological phenotype. The use of the MT-2 assay and other indicator cell lines (U87.CD4.CCR5 and U87.CD4.CXCR4) establish viral coreceptor usage providing qualitative data represented as either NSI/CCR5 utilizing or SI/CXCR4 utilizing [69, 70, 77, 80, 164, 227, 228, 268, 269].

The MT-2 cell line has been traditionally used as a experimental surrogate phenotypic assay for the prediction of viral tropism *in vivo* [267-269]. This cell line expresses high levels of CXCR4 exclusively and does not express CCR5. Therefore, growth of CCR5 utilizing viruses is prohibited and only the CXCR4 variants will cause a phenotypic cytopathic effect (CPE) in the form of syncytia in the MT-2 cell line.

The third hypervariable loop (V3 loop) within Env is generally 35 amino acids in length, and has been implicated in coreceptor usage, and variations within V3 have been associated with changes in cell tropism, syncytium formation in MT-2 cell lines and progression to AIDS stage of disease [204, 234, 268, 386]. The specific progression of mutation acquisition by an HIV-1 viral isolate enabling a switch from CCR5 utilization to CXCR4 utilization is largely unknown.

Bioinformatic approaches suggest that there are many genotypic changes in the V3 loop that influence changes in subtype B coreceptor usage. Position Specific Scoring Matrices (PSSM's) are used to detect non-random predetermined distributions of amino acids and/or motifs in a particular sequence [271]. If a sequence has a high PSSM score, the sequence will closely resemble an X4 sequence and vice versa for R5 variants. The score will also provide an evaluation of an isolate's transition from R5 to X4 tropism. Recently, sensitive modifications have been made to the existing PSSM software, to reliably predict viral phenotype for HIV-1 subtype C sequences, C-PSSM [270].

The Briggs method is another predictive tool for HIV coreceptor usage based on subtype B sequences. This method evaluates the net charge and positions of specific amino acids: an R5/NSI variant typically possesses a net V3 loop positive charge of < 4 and that of an X4/SI variant is typically > 4 [387]. The prediction of CCR5 coreceptor usage from HIV-1 *env* sequences, is relatively accurately determined by the presence of a neutral and negatively charged amino acid at positions 11 and 25 respectively, in the V3 loop [201, 202, 229-232]. Conversely, if a basic/positive amino acid is found at position 11 and/or 25 the sequence will probably represent a SI phenotype [201, 202, 229, 233-235]. Recent data suggests that the presence or absence of a predicted N-linked glycosylation site found at position 6-8 within the V3 loop is associated with efficient CCR5 usage and inefficient CCR5 usage, respectively [243, 244]. Whether or not mutations in *env* gradually accumulate over time, or if the presence of basic amino acids at positions 11 and 25 are sufficient for R5 to X4 transition, is also still uncertain.

HIV-1 subtype C viruses now are the most prevalent subtype and cause >50% of new HIV-1 group M infections worldwide, and have therefore become a major focus of novel drug development and vaccine design [52]. Published research up to 2007 has shown that the ability of HIV-1 subtype C to switch or expand its coreceptor usage during the natural course of infection is relatively low. HIV-1 subtype C isolates from all disease stages, in different regions of the world including South Africa, isolated from a variety of cohorts, utilize the CCR5 coreceptor almost exclusively and show minimal use of CXCR4 [164, 232, 239, 249-261, 388-390]. Coreceptor usage and switching has been extensively studied in HIV-1 subtype B isolates and to a lesser extent in subtype C isolates. The therapeutic implications and monitoring of coreceptor usage of HIV-1 isolates from AIDS patients, prior to commencement of ARV treatments that incorporate coreceptor antagonists, is thus very important. This study focuses on the isolation and characterization of primary HIV-1 viruses isolates during 2005.

2.2.1.1 Objectives

The overall objective of this study was to isolate and investigate the genetic and phenotypic properties of South African HIV-1 primary isolates collected over 6 months in 2005 from randomly selected ARV drug-naïve AIDS patients. This was achieved by the following aims:

1. To isolate PBMCs and plasma from selected ARV drug-naïve AIDS patients attending an AIDS clinic at the Johannesburg General Hospital.

2. To isolate and propagate primary HIV-1 isolates by standard co-culture techniques.

3. To phenotypically and biologically characterize each primary viral isolate with respect to coreceptor usage by growth in MT-2 and U87.CD4.CCR5/CXCR4 cell lines, respectively.

4. To determine whether genotypic characterization of the V3 amino acid sequence of Env can be correlated to phenotype.

2.3 Materials and Methods

2.3.1 Patients used in this Study

Thirty-one antiretroviral drug-naïve advanced AIDS patients presenting at the HIV/AIDS clinic in the Johannesburg General Hospital, South Africa participated in this study between January and July 2005 (Table 2.1). All the patients were admitted to the clinic for purposes of initiating HAART treatment and samples for the purposes of this study were obtained prior to onset of therapy. To qualify for enlistment onto the government rollout, a patient must be diagnosed with AIDS and/or present with a $CD4^+$ T cell count of 200 cells/ml or less. Ethical approval was obtained for this study from the University of the Witwatersrand Committee for Research on Human Subjects (Protocol No. M060734). All patients within the cohort completed informed consent documentation, prior to drawing 5 ml of whole blood in ethylenediaminetetraacetic acid (EDTA) tubes from each patient and viral samples were designated 05ZAFV01 through 05ZAFV31. The plasma viral load, the $CD4^+$ T cell counts and individual clinical status were obtained from the hospital patient file (Table 2.1).

2.3.2 Processing of patient samples

Patient whole blood was centrifuged at 1 400 rpm for 10 minutes. The patient plasma was removed in 1 ml aliquots and stored in liquid nitrogen until used. The patient PBMCs were then isolated from the remainder blood as described in section 2.3.3.1. Patient HIV-1 PBMCs were stored in 1 ml aliquots in a freezing mix (50% FCS, 40% RPMI. 10% DMSO (dimethylsulphoxide)) (Sigma, Steinheim, Germany). Initially, the infected PBMCs were stored in a slow-freezing stratacooler and after 48 hours, they were relocated into a liquid nitrogen freezer (-130°C), until further use. A 1 ml aliquot of patient PBMCs was used in subsequent viral infections (primary viral isolation).

2.3.3 Primary HIV-1 Viral Isolation

2.3.3.1 PBMC Isolation from HIV negative Blood Donor

Leukocyte enriched whole HIV-1 negative blood (buffy coats) were received weekly from the South African National Blood Service (SANBS). These buffy coats were depleted of 80-90% of the red blood cells (RBC) and most of the plasma had been removed prior to receiving the packs. Random blood donor's PBMCs from two HIV-1 negative donors were isolated using Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden). Two donors were used to culture primary virus so as to exclude for host effects. The buffy coats were initially decanted into sterile 50 ml NUNC tubes (AEC Amersham, Freiburg, Germany) and centrifuged for 10 minutes at 1 200 rpm in an Eppendorf Centrifuge 5810 R (Eppendorf, Hamburg, Germany). Remaining plasma was removed from each donor and the buffy coat layer was gently layered onto 15 ml Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) and centrifuged for 30 minutes at 1 500 rpm without a brake. The PBMCs were transferred into a new sterile 50 ml NUNC tube and washed twice, or until clear, in 45 ml of 1 x Phosphate Buffered Saline (PBS) (Sigma, Steinheim, Germany) at 1 200 rpm for 10 minutes. The washed PBMC cell pellet was resuspended in appropriate growth medium (described below) and counted manually in 0.4% trypan blue (Sigma, Steinheim, Germany) using a haemocytometer.

Donor PBMCs were cultured in supplemented RPMI growth medium [Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma, Steinheim, Germany) supplemented with 10% fetal calf serum (FCS) (Gibco, Grand Island, USA), L-Glutamine (2 mM) (Gibco, Grand Island, USA) and penicillin (0.5 U/ml)/streptomycin (0.5 U/ml) antibiotics (Gibco, Grand Island, USA)]. Both donor cell isolations were adjusted to a final cell concentration of 2 x 10^6 cells/ml and each donor PBMC suspension was maintained separately in 75 cm^2 sterile NUNC flasks (AEC Amersham, Freiburg, Germany). A final concentration of 1

µg/ml of phytohaemagglutinin (PHA) was added to the isolated PBMCs to induce T Cell proliferation by upregulating the host Interleukin-2, human (IL-2) membrane receptor [391], and incubated at 37°C with 5% CO_2. Three days post stimulation the PHA containing medium was removed, and five percent IL-2 (Roche Diagnostics, Mannheim, Germany) was added to the supplemented RPMI growth medium for stimulation of cell growth during isolation. The two isolated donor PBMCs were mixed together and used in subsequent co-culture experiments.

2.3.3.2 Isolation of Primary HIV-1

The PBMCs from each patient within the cohort were isolated with the same technique as described for the HIV-1 negative donor PBMCs (section 2.3.3.1), however, starting volumes of whole blood were only 5ml and the subsequent steps in the isolation process were adjusted for this decrease in starting volume. Primary virus was generated by coculturing 2 x 10^6 cells/ml isolated patient PBMCs with 2 x 10^6 cells/ml PHA-activated donor PBMCs in 5ml RPMI growth medium, supplemented with 5% IL-2 (described in section 2.3.3.1) in 25 cm^2 sterile tissue culture flasks (AEC Amersham, Freiburg, Germany). All virus-containing supernatants were centrifuged to remove any contaminating cellular debris, aliquoted into 1 ml volumes in 2 ml NUNC storage vials (AEC Amersham, Freiburg, Germany). Vials were initially stored at -70°C for 24 hours and then stored in liquid nitrogen until further use in subsequent studies.

The T25 flasks containing the first passage (P1) of primary virus co-culture with patient and donor PBMCs, were incubated at 37°C with 5% CO_2 for up to 28 days. The viral isolates were maintained by replacing 50% of the total volume of culture medium with fresh medium containing IL-2 (Roche Diagnostics, Mannheim, Germany) on days 4, 10, 17 and 24 and with PHA-stimulated donor PBMCs on days 7, 14, 21 and 28. The primary viral cultures were monitored weekly (days 7, 14, 21 and 28) for viral growth indicated by the rise in p24 antigen. The Murex p24

enzyme-linked immunosorbent assay (ELISA) kit (Abbot Murex, Dartford, UK) was used to monitor the p24 Antigen (Ag) level of the infections (see section 2.3.4). When cultures yielded negative p24 Ag readings by day 28, they were discarded. However, when cultures reached high levels of p24 Ag, they were expanded into 10 ml of RPMI growth medium. Once the expanded cultures yielded high viral titer, they were centrifuged at 1 400 rpm, and the infected primary PBMCs and infected supernatants were stored as above for patient PBMCs and viral supernatant.

A working virus stock (second passage; P2) of the primary virus was performed by adding 500 µl of high viral titer P1 supernatant to 20 ml of 2 x10^6 cell/ml mixed donor PHA-stimulated PBMCs. The expanded P2 cultures were also incubated at 37°C with 5% CO_2 for a maximum of 28 days, and monitored for viral growth as described for the P1 culture propagation. The infected PBMCs and culture supernatant were harvested in the manner mentioned above and stored at -130°C and -70°C, respectively.

2.3.4 Murex p24 HIV-1 Antigen ELISA

The Murex p24 ELISA kit (Abbot Murex, Dartford, UK) was used to monitor the p24 level as an indicator of viral growth in all PBMCs co-culture experiments and in cell line supernatants as per manufacturers' instructions.

2.3.4.1 Primary antibody

The 20 x concentrated biotinylated murine anti-p24 monoclonal antibody (conjugate 1 – primary antibody) was diluted twenty times by adding aggregated human IgG in phosphate buffer (conjugate diluent 1). The diluted conjugate one was mixed in a 1:1 ratio with NP40, containing a bovine aprotinin protease inhibitor and casein stabilizer, to form the conjugate working solution. One

hundred µl of the conjugate working solution was added to each of the test wells used and 100 µl of each viral supernatant and the controls were added to each well. The working solution was mixed with the viral supernatant/control by gently pipetting up and down 5 times with the multi-channel pipette. The strips were covered with an adhesive plastic plate sealer for prevention of sample evaporation and cross-over contamination between wells. The plate was placed in the 5% $C0_2$ incubator for 1 hour at 37°C.

2.3.4.2 Secondary Antibody

After the first 1 hour incubation period, the adhesive plastic seal was removed and the strips were washed 5 times with the 1 x phosphate buffer wash fluid using the plate washer (ELX 50 Autostrip washer, Bio-tek Instruments, South Africa). The secondary antibody (conjugate 2 – peroxidase conjugated streptavidin) was diluted 100 x with phosphate buffer (conjugate diluent 2). Two hundred microlitres of 1 x peroxidase conjugated to streptavidin was added to each well and a new adhesive plastic plate sealer was applied to the strips. The plate was again placed in the 37°C 5% $C0_2$ incubator for 30 minutes.

2.3.4.3 Adding the substrate

After the second incubation period the plate was washed 5 times in the phosphate wash buffer. Two hundred µl of 1 x tetramethylbenzidine (TMB) dissolved in dimethyl sulphoxide (DMSO) was then added to each well and allowed to incubate for 30 minutes at room temperature (15 – 30°C).

2.3.4.4 Absorbance Reading

The ELISA was stopped by adding 50 µl of 1M Sulphuric acid (H_2SO_4) to each well after the last room temperature incubation period. H_2SO_4 (1M) was added to

each well in the same order and the same time intervals as was used when the substrate was added. The reaction was allowed between 5 and 10 minutes to stop and the strip holder was gently tapped to ensure thorough mixing of the solutions. The absorbance of the solutions is each well was read at 450 nm referenced against 690 nm using the plate reader (ELX 808, Bio-tek Instruments, South Africa). The instrument was blanked against air. A positive and negative control was included into every p24 ELISA that was performed. The positive and negative control solutions were standard solutions provided within the kit, containing recombinant p24 Ag in solution and human serum negative for HIV-1 antigens, respectively.

2.3.5 MT-2 Phenotypic Assay

The NSI/SI phenotypes of all patient primary isolates (P2), were determined by growth in MT-2 cell lines (obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Douglas Richman [392, 393]). The cells were maintained in supplemented RPMI growth medium, and infections were performed with 5 x 10^4 cells/ml that had been pre-seeded overnight in 24 well NUNC culture plates (AEC Amersham, Freiburg, Germany). Infections were performed by adding 50 µl P2 cell-free supernatant of each HIV-1 isolate to the cells to a final volume of 2 ml supplemented RPMI growth medium. The cultures were monitored daily for cytopathic effects (CPEs) (e.g. syncytia) for up to 2 weeks in a 37°C, 5% $C0_2$ incubator. Syncytia are defined and presented as "balloon-like" large cells that form when T-tropic viruses infect host cells. Viruses were termed syncytium inducing (SI) if they successfully infected the cells and formed syncytia (T-Tropic) and termed non-syncytium inducing (NSI) (Macrophage tropic) if no cellular infection/cytopathic effects were observed. Both R5 (HIV-1 96ZM651, negative control) and R5X4 (HIV-1 96USNG31, positive control) viruses were included in the MT-2 assay. Viruses were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH HIV-1 96ZM651 (Subtype C) from Dr. Feng Gao and Dr. Beatrice Hahn and

a dual tropic HIV-1 96USNG31 (Subtype C) from Drs. D. Ellenberger, P. Sullivan, and R.B. Lal [394];.

2.3.6 U87.CD4 Coreceptor Assay

Viral coreceptor usage biotype was determined for each isolate by measuring viral replication in U87.CD4 cells transfected with either CCR5 or CXCR4 coreceptors independently. The U87.CD4.CCR5 and U87.CD4.CXCR4 cell lines were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Hong Kui Deng and Dr. Dan R. Littman [227]. These cells were maintained in supplemented DMEM growth medium [Dulbecco's Modified Eagle's medium (DMEM) (Sigma, Steinheim, Germany), 10% fetal calf serum (FCS) (Gibco, Grand Island, USA), L-Glutamine (2 mM) (Gibco, Grand Island, USA) and penicillin (0.5 U/ml)/streptomycin (0.5 U/ml) antibiotics (Gibco, Grand Island, USA) additionally supplemented with G418 (500 µg/ml) and puromycin (1 µg/ml) (Sigma, Steinheim, Germany)] in a 37°C, 5% $C0_2$ incubator.

When the cells reached 100% confluency (a monolayer of cells within the flask), a 1% solution of 0.5 M EDTA (SIGMA, Steinheim, Germany) in 1 X Phosphate Buffered Saline (PBS) (SIGMA, Steinheim, Germany) solution was used to disrupt the adherent cells for cell splitting purposes. The cells were split in a 1:8 ratio twice per week and were only maintained for a maximum of 20 passages. Thereafter, a fresh vial of cells from the liquid nitrogen storage freezer (-130°C) was used to establish a new cell line. Cell lines were stored in a freezing mix at -130°C (see section 2.3.2).

The same positive controls used in the MT-2 assay were also used in the U87 assay. The cells were seeded at 5 x 10^4 cells/ml in 24 well NUNC culture plates (AEC Amersham, Freiburg, Germany), and left to adhere overnight. Coreceptor utilization was determined by adding 100 µl of each of the P2 infectious primary viral stocks to corresponding wells the following day and left to infect the cells

overnight for 16-18 hours. The infected cells were then washed four times with 1 x PBS (Sigma, Steinheim, Germany) and 1 ml of the supplemented DMEM growth medium was added to each well. Infections were incubated at 37°C in a 5% $C0_2$ incubator. Each infected well was visually monitored daily for CPEs and viral supernatant samples were harvested on days 3, 5, 7, 9, 11, 13, 15 and 17 post-infection and stored at -70°C for subsequent p24 antigen determinations. Isolates that induced syncytia formation and yielded an increase in p24 Ag concentration were considered positive for viral growth.

2.3.7 Proviral DNA Extraction from PBMCs

DNA was extracted from the HIV-1 infected primary co-cultured PBMCs (P2) using a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to manufacturer's instructions. Firstly, 200 µl of primary cultured PBMCs were added to 200 µl of binding buffer and 40 µl of Proteinase K to induce lysis of the cultures cells and the sample mixture was incubated at 72ºC for 10 minutes. One hundred microlitres of isopropanol (Merck, Merck Chemicals and Laboratory Supplies, South Africa) was then added to the samples post incubation, samples were mixed well and the entire mixture transferred to the High Pure Filter Tubes and centrifuged for 1 minute at 8000 rpm in an Eppendorf Centrifuge 5810 R (Eppendorf, Hamburg, Germany). The nucleic acids bind to the glass fibers packed into the High Pure Filter Tubes. After centrifugation, the flow through solution was discarded, a new collection tube was attached and 500 µl of inhibitor removal buffer was added and centrifuged under the same conditions. The inhibitor removal buffer eliminates any Polymerase Chain Reaction (PCR) inhibitory contaminants. After centrifugation the flow through solution was again discarded and a new tube attached. The nucleic acids bound to the glass fibers were washed and purified from any cellular impurities as 500 µl wash buffer was added, and centrifuged under the same conditions. The wash step was repeated twice to ensure complete removal of all residual wash buffer, the tube was further centrifuged at

13000 rpm for 10 seconds. After each centrifugation step the flow through tube was discarded and replaced with a clean collection tube. Lastly, the column was inserted into a sterile 1.5ml reaction tube and 200 µl of the pre-warmed (70°C) low salt elution buffer was added to recover the nucleic acids from the column and centrifuged at 8000 rpm for 1 minute. The isolated proviral DNA was stored at -20ºC until required.

2.3.8 Nested Conventional PCR

The extracted proviral DNA from Section 2.3.7 was amplified by a nested PCR reaction to obtain the full-length viral gp160 gene using the Expand High Fidelity^{PLUS} PCR System (Roche Diagnostics, Mannheim, Germany). Subtype C *env* specific primers were designed using *env* primers previously described in Gao *et al*., [395] by comparing them to 52 complete HIV-1 Subtype C genomic sequences (K. Michler, pers. comm.). The sequences were obtained from the NLM nucleotide database [396]. The primer concentrations were adjusted to final concentrations of 0.4 µM (10 ρmol/µl) and the deoxy Nucleotide Triphosphate (dNTP) final concentrations were 200 µM. Each 50 µl PCR Reaction contained 5 µl of 100 fold diluted proviral DNA template, 2.5 U Expand High Fidelity^{PLUS} Taq DNA Polymerase with appropriate 5 X Expand High Fidelity^{PLUS} Reaction Buffer, 1.5 mM $MgCl_2$, DNase-free and RNase-free water up to the final volume. Twenty full-length *env* genes were successfully amplified by nested PCR performed on proviral DNA, using outer (*env* A-1 and *env* N-1) and inner primer sets (*env* B-1 and *env* M-1). The first round of outer primers, *env* A-1 (5'- GGC TTA GGC ATT TCC TAT GGC AGG AAG AA -3') and *env* N-1 (5'- TTG CCA ATC AGG GAA GAA GCC TTG TGT -3') were used to amplify a 3.2 kb fragment. Amplification for the first round had the following cycling conditions; 94°C for 3 minutes, 30 cycles of 94°C for 15 seconds, 61°C for 30 seconds and 72°C for 3 minutes. A final extension was performed at 72°C for 10 minutes followed by a final storage at 4°C.

The same concentration of primers were used for the second round, as in the first round of the nested amplification, using inner primer sequences *env* B-1 (5'- GGA AAG AGC AGA AGA CAG TGG CAA TGA -3') *env* M-1 (5'- TAA CCC ATC CAG TCC CCC CTT TTC TTT TA –3') to amplify an approximately 2.7 kb fragment. The thermal cycling conditions for the second round were the same as for the first round except the denaturation time was increased to 30 seconds and the annealing temperature was decreased to 55°C for 45 seconds. All PCR reactions were performed on a Bio-Rad MyCycler™ (Bio-Rad, CA). PCR products were pooled and purified using a High Pure PCR Product Purification Kit (Roche Diagnostics, Penzberg, Germany) as per manufacturers' instructions (see section 2.3.10).

2.3.9 Agarose Gel Electrophoresis

The PCR products were resolved on a low density 0,8 % TAE agarose (Sigma, St Louis, MO) gel (Appendix D). The solution was heated until the agarose beads were fully dissolved and allowed to cool before 5 µl/100 ml solution (EtBr – 0.5 µg/mL– Promega, WI, USA) was added. Five - 10 µl of each sample was mixed with loading dye (see Appendix D) and was loaded into wells in an agarose gel and the DNA fragments were separated using electrophoresis for approximately 45 minutes – 1 hour at 60-80 V and then subjected to UV transillumination to visualize the DNA bands. The band size yielded approximately 2.7 - 3 kilobases (kb) in length, when compared to the Quick-Load™ 1kb DNA ladder (New England Biolabs, Ipswitch, MA) and samples were subsequently used for sequencing.

2.3.10 PCR Product Purification

Successfully amplified gp160 templates were cleaned using the HighPure PCR Product Purification Kit, according to the manufacture's recommended protocol

(Roche Diagnostics, Mannheim, Germany). Firstly, 500 µl of Binding Buffer was added to each 100 µl PCR reaction tube containing the gp160 PCR product from all 20 samples, and mixed well. This product was gently added into the upper reservoir of a HighPure filter tube resting within a collection tube and centrifuged for 1 minute at 13 000 rpm at room temperature. The flow through solution was discarded and the same collection tube was re-connected. Five hundred microlitres of the Wash Solution was added into the HighPure upper reservoir and centrifuged under the same conditions. The flow through solution was again discarded and the tube was re-attached to the High Pure filter tube. A further 200 µl of the wash buffer was added to the upper reservoir and centrifuged under the same conditions so as to ensure that all impurities and wash buffer have been removed from the glass fibers in the filter. The collection tube was finally discarded with the flow through solution and a new collection tube was attached onto the High Pure filter tube. Elution Buffer (100 µl) was added to the empty upper reservoir and centrifuged under the same conditions, which yielded 100 µl of purified DNA, stored at -20°C until further use.

2.3.11 Sequencing

2.3.11.1 Thermal Cycle Sequencing of the gp160 region

V3 loop specific population based sequencing of the purified second round gp160 amplicons of 20 HIV-1 viral isolates was performed using 5 primers in order to obtain double-stranded sequence in both forward and reverse directions for all amplicons. The following sequencing primers spanning the V3 loop region (~700 bp) were: AA1570 (5'-GGA GCA GCA GGA AGC ACT ATG GGC-3'), AES6 (5'-GGA CAA GCA TTC TAT GCA ACA GGT G-3'), Env 1f (5'-CCA TAA CAC AAG CCT GTC CAA AGG-5'), Env f (5'-CTG TAG AAA TTG TGT GTA CAAGAC CC-3'), and E16 (5'-CCA ATT CCC ATA CAT TAT TGT G-3'). Sequencing reactions were set up in 21 µl and each reaction contained 20-50 ng of

DNA, 4 µl Big Dye ® Terminator Version 3.1 Cycle Sequencing kit, 1x BigDye Sequencing Buffer, 1 µl of sequencing primer (3.2 ρmol/µl) and the reaction was made up with dH_2O to the final volume (Applied BioSystems, Foster City, CA). The sequencing reactions were amplified in the Bio-Rad MyCycler™ thermocycler machine (Bio-Rad, Hercules, CA), cycling for 25 cycles for 10 seconds at 96°C , 5 seconds at 50°C and 4 minutes at 60°C. The final incubation was set at 4°C. Sequencing reactions were cleaned immediately.

2.3.11.2 Isopropanol Purification

All sequencing reaction extension products were purified after the cycle sequencing reaction using Isopropanol precipitation (Merck, Merck Chemicals and Laboratory Supplies, South Africa). Each sample was carefully transferred to a 96 well optical sequencing reaction plate and unincorporated ddNTPs were removed by adding 80 µl of 80% Isopropanol to each well in preparation for capillary electrophoresis. The sequencing plate was incubated in the dark for 15 minutes prior to centrifugation at 3 200 rpm at 4°C for 45 minutes to pellet the sequence amplicon. Immediately after centrifugation, the sequencing plate was carefully inverted onto a sterile pad of paper towelling and centrifuged in the inverted position for 1 minute at 1 900 rpm, so as to remove residual isopropanol. The plate was allowed to air dry for two minutes, after which the pellet was re-suspended in 10 µl of Hi-Di™ Formamide (Applied Biosystems, Warrington, UK).

2.3.11.3 Sequencing Electrophoresis

Capillary Electrophoresis was performed on all the 20 amplicons on the ABI PRISM 3100 automated Genetic Analyzer (Applied BioSystems, Foster City, CA) in MicroAmp 96 well optical reaction plates. The sequence data was edited using the Sequence Analysis V3.3 program (Applied Biosystems) and the V3 sequences were assembled using Sequencher V4.5 (Genecodes, Ann Arbor, MI).

2.3.12 Subtyping and amino acid sequence analysis

A phylogenetic tree was constructed by performing a Clustal X multiple alignment of the nucleotide V3 loop regions with several subtype C reference sequences and sequences from HIV-1 subtype A-K from the Los Alamos HIV Sequence database [46]. Phylogenetic reconstruction of the nucleotide sequences was also performed by Neighbour-Joining using the Kimura two-parameter distance matrix. Data was converted to the MEGA V3.0 format, compatible for phylogenetic and molecular evolutionary relationship analyses. The reliability of the branching order was estimated from 100 bootstrap replicates with values > 70% considered significant [397].

The predicted amino acid sequences were aligned with a consensus C sequence and extensively analyzed. The C-PSSM bioinformatic phenotype prediction software was used to analyze all novel 20 HIV-1 Subtype C V3 sequences to determine the accuracy of the prediction method to determine viral phenotype. The algorithm was created by Mark Jenson [270] and the software is available online [398]. Finally, the N-linked glycosylation sites within the V3 loop of the dual tropic primary isolates were compared to dual tropic reference sequences from the Los Alamos sequence database of varying clades [46].

2.4 Results

2.4.1 Clinical baseline profiles of South African ARV drug-naïve AIDS patients

Epidemiological data of all ARV drug-naïve AIDS patients recruited over six months for the purposes of this study are shown in (Table 2.1). Due to poorly documented original patient files, not all of the clinical data from these patients was available.

Table 2.1 Epidemiological data of 31 AIDS patients attending a clinic in Johannesburg, South Africa.

Isolate ID	Age (years)	Gender	CD4 count (cells/ µl)	Viral Load (RNA copies/ ml)	Clinical status	Primary Isolate (Y/N)
05ZAFV01	34	F	464	NA	NA	N
05ZAFV02	46	M	159	22 999	Asymptomatic	Y
05ZAFV03	28	F	101	NA	Oral Candidiasis; HSV; retinal necrosis	Y
05ZAFV04	26	F	274	75 000	NA	N
05ZAFV05	43	M	133	>750 000	Syphilis (Previously TB)	Y
05ZAFV06	28	M	187	179 000	Asymptomatic	Y
05ZAFV07	41	F	144	598 000	Asymptomatic	Y
05ZAFV08	44	F	130	660 000	Asymptomatic	Y
05ZAFV09	34	M	NA	736 000	NA	Y
05ZAFV10	32	M	6	>750 000	On TB Treatment	Y
05ZAFV11	38	M	124	>750 000	Previously TB	Y
05ZAFV12	45	M	141	92 400	Previously TB	Y
05ZAFV13	32	F	85	378 000	Asymptomatic	Y
05ZAFV14	24	F	16	NA	NA	Y
05ZAFV15	33	F	122	343 000	Vaginal Candidiasis; Previously TB	Y
05ZAFV16	33	M	295	>750 000	NA	N
05ZAFV17	43	M	183	16 400	NA	N
05ZAFV18	31	F	119	551 000	NA	N
05ZAFV19	62	F	153	133 000	NA	N
05ZAFV20	29	M	110	>750 000	NA	Y
05ZAFV21	42	F	NA	19 500	NA	N
05ZAFV22	34	F	NA	NA	Asymptomatic; Previously TB	Y
05ZAFV23	33	F	158	37 000	Asymptomatic	Y
05ZAFV24	31	F	219	52 300	NA	N
05ZAFV25	50	F	31	>750 000	Syphilis; Previously TB	Y
05ZAFV26	49	F	NA	NA	Asymptomatic	Y
05ZAFV27	29	F	11	158 000	NA	Y
05ZAFV28	39	M	45	>750 000	On TB Treatment; PCP	Y
05ZAFV29	32	M	110	110 000	NA	N
05ZAFV30	31	F	145	NA	NA	N
05ZAFV31	60	M	138	>750 000	NA	N

Abbreviations: TB, Tuberculosis; PCP, Pneumocytis carinii pneumonia; NA, Not available (due to poorly documented original patient files); M, male; F, female, Y, yes; N, no

2.4.2 HIV-1 primary virus Isolation

HIV-1 was successfully isolated from the infected patient PBMCs using standard co-culture techniques with two different PHA-stimulated, HIV-1 negative donor PBMCs. Of the 31 patients analyzed within the cohort, 24 yielded successful primary viral isolates (isolation rate of 80%). Primary cultures were obtained for all 24 isolates within 28 days of infection. All the isolates, except 05ZAFV16 and 05ZAFV18, reached the log growth phase within 14 days of co-culture, these two isolates were only considered positive for successful viral growth after four weeks of co-culture. Twenty isolates were randomly selected for further studies.

2.4.3 Phenotyping of HIV-1 primary virus isolates

The phenotype of each of the 20 selected viral isolates was determined using the MT-2 phenotypic assay. Of the 20 isolates, 14 isolates utilized CCR5 and exhibited the NSI phenotype in the MT-2 cell line. Six (30%) primary isolates were able to successfully grow in the MT-2 cells and formed syncytia, displaying the SI phenotype within 5 days post infection (Figure 2.1, Table 2.2).

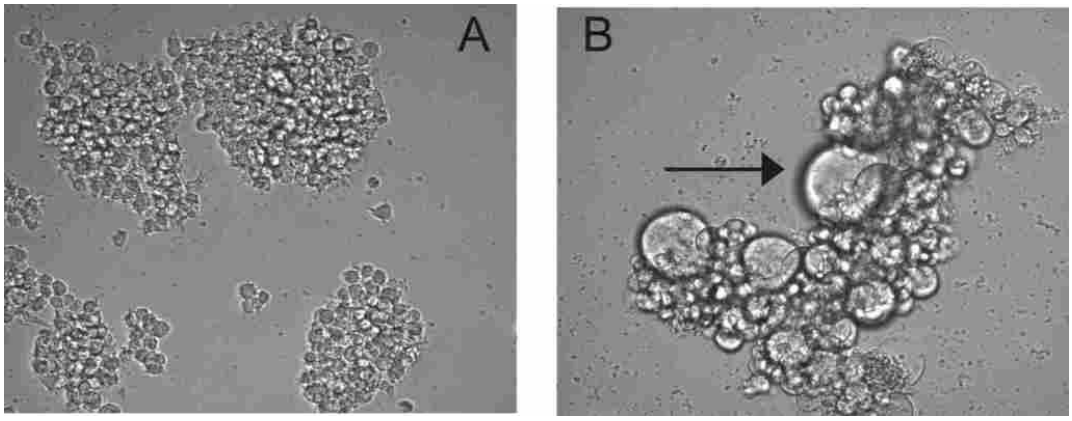


Figure 2.1 Cytopathic effects of the 05ZAFV03 isolate in MT-2 cells lines; (A) uninfected and (B) infected showing extensive formation of syncytia (see arrow), with marked ballooning at the edge of the clumps (200 X).

Table 2.2 Phenotype and biotype of the 20 HIV-1 ARV drug-naïve primary isolates.

Isolate ID	MT-2 Assay/Phenotype	U87.CD4 Assay/Bio-type
05ZAFV02	NSI	R5
05ZAFV03	SI	X4
05ZAFV05	NSI	R5
05ZAFV06	NSI	R5
05ZAFV07	NSI	R5
05ZAFV08	NSI	R5
05ZAFV09	NSI	R5
05ZAFV10	SI	X4
05ZAFV11	NSI	R5
05ZAFV12	NSI	R5
05ZAFV13	NSI	R5
05ZAFV14	SI	X4
05ZAFV15	SI	R5X4
05ZAFV20	NSI	R5
05ZAFV22	NSI	R5
05ZAFV23	NSI	R5
05ZAFV25	NSI	R5
05ZAFV26	SI	X4
05ZAFV27	SI	R5X4
05ZAFV28	NSI	R5

Abbreviations: SI – Syncytium Inducing; NSI – Non-syncytium inducing; R5 – CCR5 utilizing isolate; X4 – CXCR4 utilizing isolate

2.4.4 Coreceptor Usage of HIV-1 primary virus isolates

The results from the MT-2 assay were further confirmed with the U87.CD4.CCR5/CXCR4 assay. There was an absolute correlation between the NSI and SI phenotype of the isolates and their ability to grow in U87.CD4.CCR5 or U87.CD4.CXCR4 cells, respectively (Table 2.2). The 14 NSI isolates replicated in the U87.CD4.CCR5 expressing cells and showed zero viral p24 antigen levels in the U87.CD4.CXCR4 cells; hence, they were designated as R5 viruses. All six SI isolates managed to successfully replicate in the U87.CD4.CXCR4 expressing cells (designated as X4 viruses). Interestingly, two (ZA05FV15 and ZA05FV27) SI isolates also successfully infected the U87.CD4.CCR5 cell line, and therefore were designated as R5X4/dual tropic viruses.

2.4.5 Full Length gp160 Amplification

Proviral DNA from all 20 HIV-1 infected PBMCs from the co-culture experiments was successfully isolated and used to PCR amplify the gp160 region (~2.7 kb; Figure 2.2).

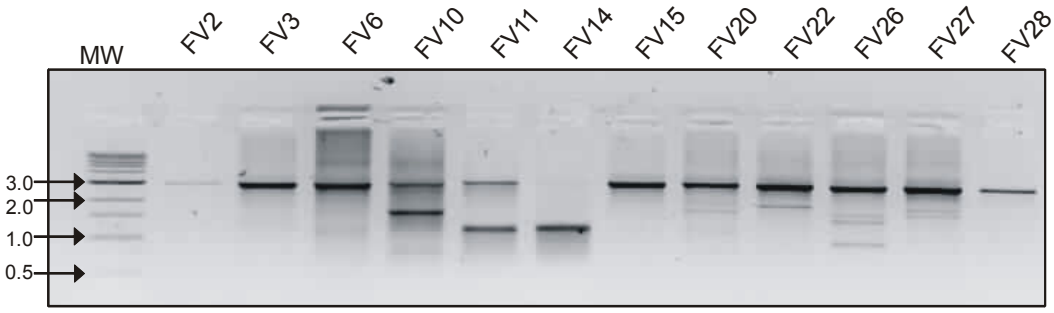


Figure 2.2 Representative electrophoresis gel of the PCR amplification of the envelope region (~2.7kb) from the HIV-1 subtype C isolates. Ten µl of second round PCR product was loaded onto a 0.8% Agarose gel. The sizes of the molecular weight marker (MW; 1kb Ladder, New England Biolabs) are indicated on the left.

Initially, some samples failed to amplify the full gp160 fragment due to primers annealing at incorrect positions and sub-stringent PCR annealing temperatures etc. However, after subsequent repeated amplification, all the full-length gp160 2.7 kb fragments were obtained (results not shown).

2.4.6 Genotypic characterization of primary virus isolates

The V3 loop regions were successfully sequenced for all 20 isolates, and the nucleotide and amino acid sequences were extensively analyzed. Phylogenetic tree analysis confirmed that 19/20 of the newly characterized isolates clustered within HIV-1 subtype C (K. Michler, pers. comm.) (Figure 2.3). However, one isolate (ZA05FV10) clustered with subtype D reference sequences.

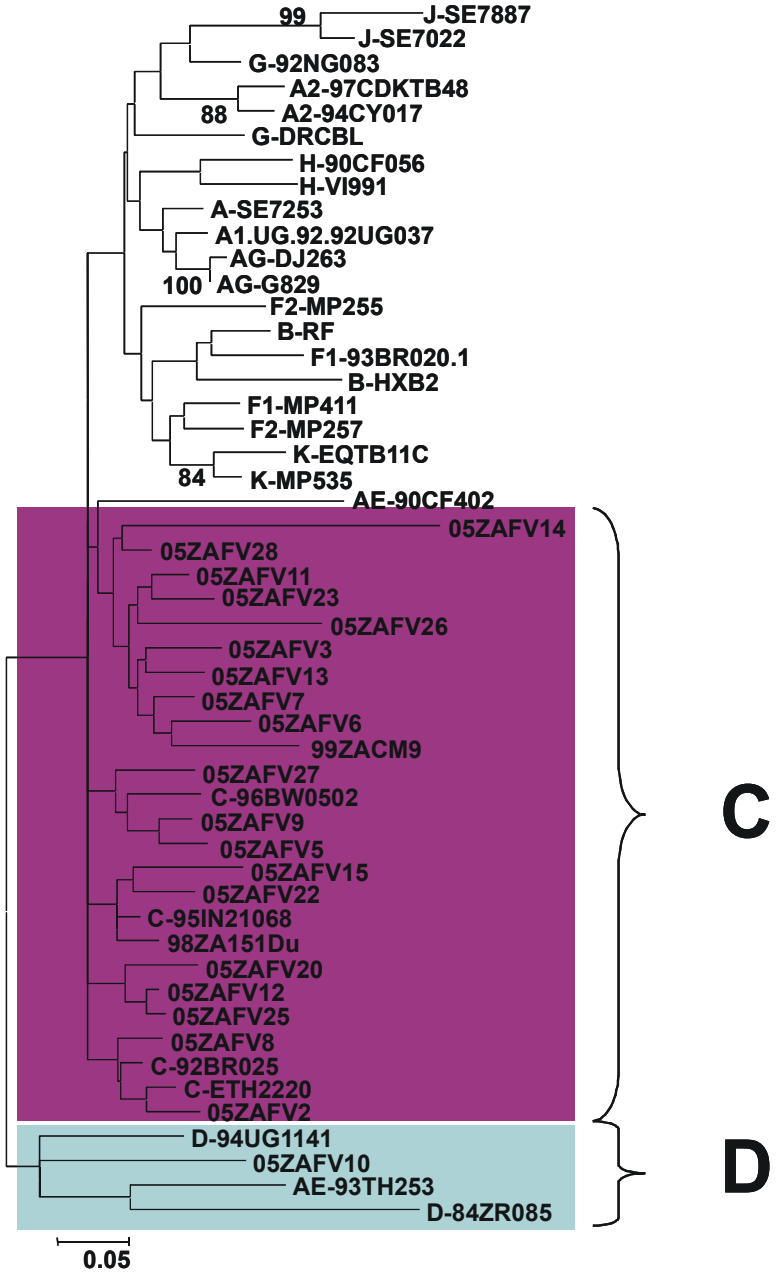


Figure 2.3 Phylogenetic relationships of the V3 loop regions (~100 bp) of Env from the 20 newly characterized HIV-1 primary viral isolates. Subtype reference sequences from the Los Alamos database are shown. Nineteen out of 20 of the isolates clustered distinctly with the subtype C reference sequences and one isolate, 05ZAFV10, was confirmed subtype D within the V3 loop. The phylogenetic tree was constructed from nucleotide sequences, using the neighbour-joining method and bootstrap values were used to estimate the stability of the nodes. Only bootstrap values of 70% or higher are shown. The phylogenetic analysis does not show all significant associations due to the relatively small fragment size.

The predicted V3 amino acid sequences were extensively analyzed and compared to determine differences between CCR5 and CXCR4 (Figure 2.4). The 20 *env* sequences (K. Michler, pers. comm.) were submitted to GenBank using Sequin V5.35 [396] and are available under the accession numbers DQ382361 – DQ382380 (see Appendix D).

Isolate	V3 loop sequence	# aa	Charge	Phenotype	Biotype
	11 25				
Cons_C	CTRPNNNTRKSIRI--GPGQTFYATGDIIGDIRQAHC	35	+5	NSI	R5
05ZAFV2	CTRPNNNTRKSVRI--GPGQTFYATGDIIGDIRQAYC	35	+1	NSI	R5
05ZAFV5	CTRPNNNTRKSVRI--GPGQTFYATGEIIGNIRQAHC	35	+4.5	NSI	R5
05ZAFV6	CARPGNNTRKSVRI--GPGQAFFATGDIIGNIRKAHC	35	+5.5	NSI	R5
05ZAFV7	CTRPGNNTRKSVRL--GPGQAFFATGDILGNIRQAHC	35	+4.5	NSI	R5
05ZAFV8	CTRHNNNTRKSVRI--GPGQTFYATGEIIGNIRQAHF	35	+5	NSI	R5
05ZAFV9	CTRPNNNTRKSMRI--GPGQTFFATGEIIGNIRQAHC	35	+4.5	NSI	R5
05ZAFV11	CIRPGNNTRKSIRI--GPGQAFYATGDIIGDIRQAHC	35	+3.5	NSI	R5
05ZAFV12	CTRPNNNTRKSVRI--GPGQTFYATGDIIGNIRQAHC	35	+4.5	NSI	R5
05ZAFV13	CARPGNNTRPSVRI--GPGQAFYATGEIIGNIRKAHC	35	+5.5	NSI	R5
05ZAFV20	CTRPNNNTRKSMRI--GPGQAFYAVGDIIGNIRQAHC	35	+4.5	NSI	R5
05ZAFV22	CTRPNNNTRRSIRI--GPGQTFYTN-DIIGNIRQAYC	34	+4	NSI	R5
05ZAFV23	CTRPGNNTRKSVRI--GPGQAFYATGDIIGNIREAHC	35	+3.5	NSI	R5
05ZAFV25	CTRPNNNTRKSVKI--GPGQTFYATGDIIGNIRQAHC	35	+4.5	NSI	R5
05ZAFV28	CTRPNNNTRTSVRI--GPGQAFYATNGIIGDIRQAHC	35	+3.5	NSI	R5
05ZAFV03	CPRPGNNTRKSVRIGIGPGLSFYATGKVLGNIRQARC	37	+8	SI	X4
05ZAFV10	CTRPYKSSRRRTHI--GTGQAWYTT-NIEGDIRKAHC	34	+6	SI	X4
05ZAFV14	CTRPANTKIKTLGI--GPGQTFRTVKKIIGNIRQSHC	35	+6.5	SI	X4
05ZAFV15	CARPNNNTRIHVRIGIGPGQTFYATGAIIGNIRQSHC	37	+5	SI	R5/X4
05ZAFV26	CTRPGKGKRTGVKI--GPGRTFYATGAVTGNIRKAHC	35	+8.5	SI	X4
05ZAFV27	CTRPSNNTRKRIRV--GPGQTVYATDAIIGDIRQAHC	35	+4.5	SI	R5/X4

Figure 2.4 Alignment of the predicted V3 amino acid sequences of 20 newly characterized South African HIV-1 subtype C and D (05ZAFV10) isolates (14 R5 and 6 R5X4/X4). The sequences are compared to a HIV-1 subtype C consensus sequence. The overall positive charge, number of amino acids and phenotype of each isolate is shown on the right. Dashes (-) indicate deletions/insertions, and positions 11 and 25 and the V3 crown/tip are highlighted.

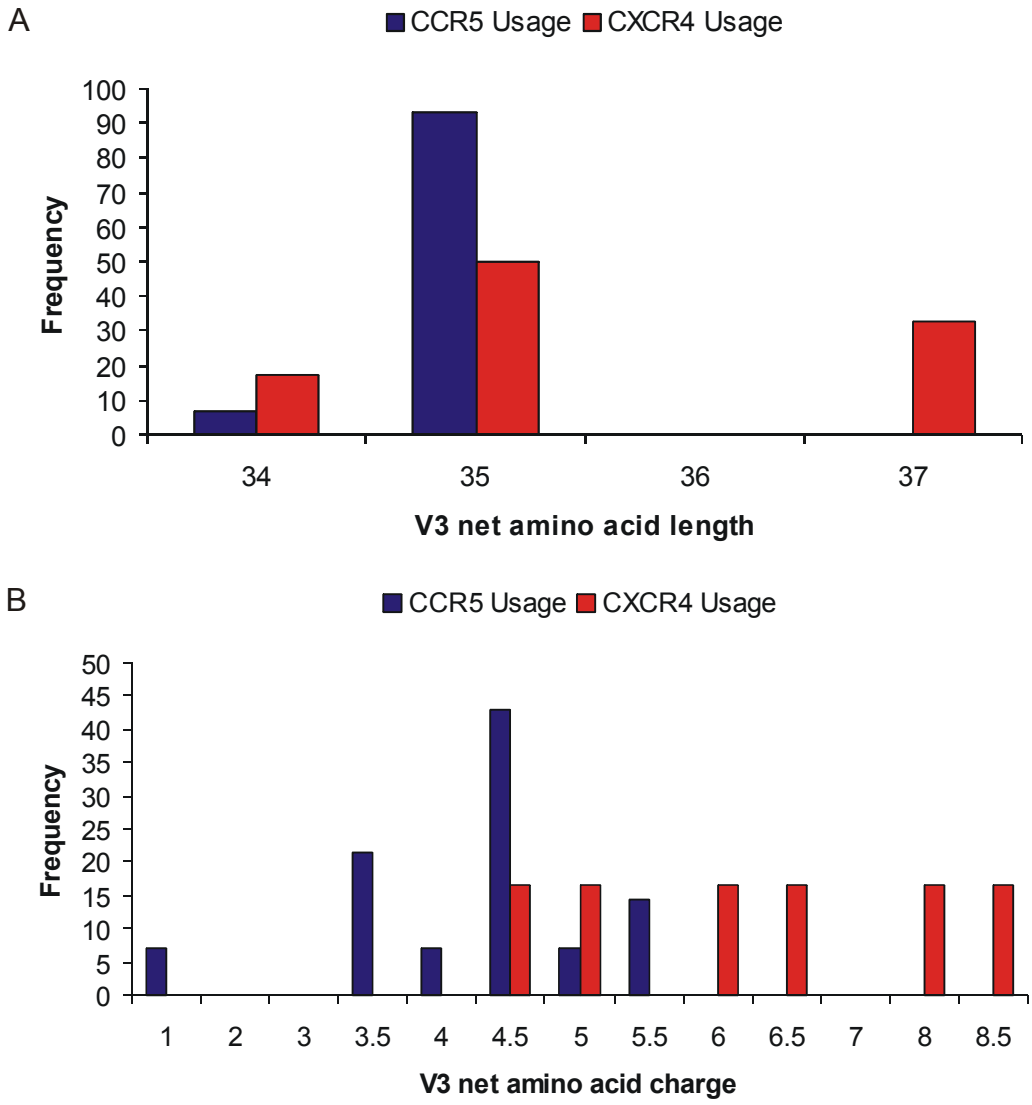


Figure 2.5 Comparison of the (A) V3 loop net amino acid length and (B) net amino acid charge of the 20 HIV-1 subtype C CCR5 and CXCR4 utilizing isolates from this study.

The predicted N-linked glycosylation sites (at position 6-8 within the V3 loop) were conserved for all 14 CCR5 utilizing HIV-1 subtype C primary isolates, and in three of the six CXCR4 utilizing isolates (05ZAFV03, 05ZAFV15 and 05ZAFV27).

Isolate name/Reference sequence name	V3 loop sequence	Subtype	Isolation Year	Area
	6-8 25			
05ZAFV15	CARPNNNTRIHVRIGIGPGQTFYATGAIIGNIRQSHC	C	2005	ZA
05ZAFV27	CTRPSNNTRKRIRV--GPGQTVYATDAIIGDIRQAHC	C	2005	ZA
ACH16810_P23	CTRPNNNIRKRIHI--GPGRAFYTTGQIIGNIRQAHC	B	NA	NL
ACH168_10P5	CTRPNNNIRKRIHI--GPGRAFYTTGQIIGNIRQAHC	B	NA	NL
DH12_3	CTRPNNNTRKGITL--GPGRVFYTTGEIVGDIRKAHC	B	1991	US
SC24_QH1520c_2	CTRPNNNTRKSIRM--GIRRAFYTREIIGDIRQAHC	B	NA	TT
SF2	CTRPNNNTRKSIYI--GPGRAFHTTGRIIGDIRKAHC	B	1983	US
RF	CTRPNNNTRKSITK--GPGRVIYATGQIIGDIRKAHC	B	1983	US
P896	CTRPNNNTRRRLSI--GPGRAFYARRNIIGDIRQAHC	B	1983	US
99ZACM9	CARPGNNTIKRIRI--GPRYAFYAKETIIGDIRQAHC	C	1999	ZA
DU179	CTRPGNNTRKSIRI--GPGQAFYTNH-IIGDIRQAYC	C	1999	ZA
99ZASW20	CTRPNNNTRKSIRTGIGRGQTFYVTGQIIGDVRQAHC	C	1999	ZA
99ZASW30_b	CTRPNNNTRKSVRIGIGRGHAFYTTGKVIGNIRQAHC	C	1999	ZA
99ZASW30_cl	CTRPNNNTRKSVRIGIGRGLSFYTTGKVLGNIRQAHC	C	1999	ZA
92UG001	CTRPYYNQIRQ-RTSIGQGQALYTTRVTGDIRKAYC	D	1992	UG

(-) Indicates amino acids not present, NA – Not available; ZA – South Africa; NL – Netherland; TT – Trinidad and Tobago; US – United States; UG - Uganda

Figure 2.6 Alignment of the predicted amino acid sequences of the V3 loop region of the two dual tropic primary isolates from this study and dual tropic reference sequences obtained from the Los Alamos database [47].

The length and charge of the V3 loops of all isolates in the study were compared (Figure 2.5) and CXCR4 usage was found to associate an increased overall positive charge as well as an increased length. The two dual tropic primary isolates showed similar motifs within their V3 loop regions when compared to some dual tropic reference sequences (Figure 2.6).

2.4.7 Prediction of phenotype using C-PSSM

The V3 loop sequences of all 20 isolates were analyzed using the C-PSSM algorithm scores [270] to predict the viral phenotype, and confirm the data obtained from the MT-2 and U87.CD4 biological assays. The C-PSSM bioinformatic algorithm predicted all of the six SI HIV-1 isolates with 100% accuracy. Only 12 of 14 (85.71%) NSI isolates were correctly predicted.

Table 2.3 Raw data generated from the 20 HIV-1 subtype C isolates in this study from the C-PSSM phenotype prediction algorithm web site.

Name	Score[a]	Pred[b]	Geno[c]	Pos Charge[d]	Net Charge[e]	Percentile[f]
Consensus	-29.93	0	S/D	6	4	0.48
05ZAFV02	-27.84	0	S/D	5	3	0.79
05ZAFV05	-28.06	0	S/E	6	5	0.53
05ZAFV06	-23.54	0	S/D	7	6	0.84
05ZAFV07 [§]	-20.03	1	S/D	6	5	0.85
05ZAFV08	-23.29	0	S/E	7	6	0.76
05ZAFV09	-27.96	0	SE	6	5	0.63
05ZAFV11	-26.17	0	S/D	6	4	0.6
05ZAFV12	-28.94	0	S/D	6	5	0.061
05ZAFV13 [§]	-20.97	1	S/E	6	5	0.81
05ZAFV20	-25.28	0	S/D	6	5	0.67
05ZAFV22	-26.78	0	S/D	5	4	0.94
05ZAFV23	-27.07	0	S/D	6	4	0.74
05ZAFV25	-25.77	0	S/D	6	5	0.62
05ZAFV28	-25.11	0	S/G	5	5	0.68
05ZAFV03	-7.51	1	S/K	7	7	1
05ZAFV10	8.61	1	R/N	9	7	1
05ZAFV14	10.18	1	T/K	8	8	1
05ZAFV15	-13.6	1	R/A	6	6	0.97
05ZAFV26	-2.1	1	G/A	9	9	0.96
05ZAFV27	-19.19	1	R/A	7	5	0.83

[a]C-PSSM score, [b]1 = CXCR4 use predicted; 0 = CCR5 use only predicted, [c]Amino acid residues at V3 sites 11 and 25, [d]Total number of positively charged (R/K/H) amino acid residues, [e]Number of positively charged (R/K/H) amino acid residues, minus number of negatively charged (D/E) residues, [f]If this number is over 0.95 this sequence is most likely not a V3.
[§] Incorrect tropism predicted by the PSSM-C bioinformatic scoring system

2.5 Discussion

HIV-1 subtype C viruses circulating worldwide have previously been shown to almost exclusively utilize CCR5 as a coreceptor for cellular entry throughout all stages of disease progression, including early and late stage AIDS. Collectively, studies reporting these data were performed in a wide range of geographical areas predominantly driven by the HIV-1 subtype C epidemic, namely Malawi, Ethiopia, Zambia, Botswana, South Africa, Brazil, China, India, Israel and Thailand and Ghana which experience lower scale subtype C epidemics [200, 232, 239, 249-261, 388, 389, 399]. This study reports the highest reported proportion and potential emergence/evolution of CXCR4 utilizing HIV-1 subtype C isolates in a South African ARV drug-naïve AIDS patient cohort.

Primary virus was successfully isolated from 24 of 31 patient samples using standard co-culture techniques, yielding a viral isolation success rate of 80% (Table 2.1). This is a significantly high viral isolation rate of HIV-1 subtype C and greater isolation efficiency compared to other studies [251, 400]. This may be due to the fact that freshly isolated patient PBMCs were immediately used in infections. Possible reasons as to why seven isolates did not grow may be due to intrinsic characteristics of the patient virus, or an artefact of the randomly selected donor PBMCs; for instance, $CD4^+$ and $CD8^+$ T cell percentages within the donor PBMCs were not normalized, high $CD8^+$ T cell counts may have inhibited isolation or suppressed viral replication kinetics [401]. Inhibitory cytokines and/or chemokines may have also contributed to the 20% of viral isolates that were not successfully isolated. Possible contribution of these factors was beyond the scope of our study.

The 20 isolates selected for the purposes of this study were obtained from an AIDS patient cohort with nine male and eleven females, ranging from 24 to 50 years of age (Table 2.1). The CD4 counts ranged from six to 187 cells/µl, and viral loads from 22900 to >750 000 RNA copies/ml. When analyzing the opportunistic infections (OIs) found within our cohort, eight of the patients either currently had tuberculosis (TB) or had previously experienced TB infection, two patients

presented with oral and vaginal candiditis, one patient presented with syphilis and one with *Pneumocytis carinii* pneumonia (PCP). These data confirm that all the patients had AIDS, qualifying for the South African government ARV rollout program [16].

Determination of the phenotype and coreceptor usage of the 20 primary virus isolates yielded unexpected findings in light of previously published data [164, 232, 239, 249-261, 388-390]. Interestingly, 30% (6/20) of the isolates utilized CXCR4 for cell entry, while two of the six isolates were confirmed dual tropic, showing efficient replication in both the U87.CD4.CCR5 and U87.CD4.CXCR4 cell lines. The remaining 14 (70%) isolates utilized the CCR5 coreceptor, as was expected from previously published data [232, 249-260, 388, 389]. This is the highest percentage of X4 usage in any described HIV-1 subtype C cohort worldwide.

Since the primary isolates in this study were obtained from patient samples collected over a relatively short time period of 6 months, these data may suggest an on-going dynamic evolution of the *env* gene and the maturing of the HIV -1 subtype C epidemic in South Africa. A striking contrast emerges as other studies have reported on HIV-1 subtype C coreceptor usage from samples collected over longer periods of time, decreasing the impact and relevance of their findings.

McCormack *et al.,* [239] reported that in the rural Karonga district of Malawi in Central Africa, 100% of the 152 HIV-1 positive subtype C samples, were identified as NSI, carrying the characteristic GPGQ crown motif in the V3 region. This study was performed over a period of 9 years (1981–1989), where the earliest subtype C sequence came from a sample collected in 1983 in the area [239]. Only 5/29 (17%) of late stage AIDS patients yielded SI phenotypes in a recent study performed in South Africa [258]. This percentage of CXCR4 usage in this cohort is higher than other similar studies to date, however samples were collected over a period of two years (1998-2000) [258]. Another study performed on a cohort of 40 perinatally infected children in South Africa found only 10% (4/40) of the isolates to be SI in the paediatric cohort, observed over four years (1998-2002) [252].

Contrary to other studies that have observed a high frequency of HIV-1 CXCR4 variants, this study population had not received ARV drugs. Interestingly Johnston *et al.,* [264] observed a high frequency [50% (10/20)] of SI CXCR4-utilizing HIV-1 subtype C isolates in patients receiving ARV therapy [264].

This novel and significant finding prompted further comparative analysis of the baseline patient profiles. To establish whether or not the increased CXCR4 usage was due to patient disease status, the $CD4^+$ T cell counts and viral loads corresponding to the different viral tropisms, were compared. The six patients that displayed the SI phenotype in the MT-2 cell line, had low $CD4^+$ T cell counts (ranging from 6 – 122 cells/µl; mean of 51.2 cells/µl). However these values were not significantly lower than the patients $CD4^+$ T cell counts displaying NSI phenotypes (ranging from 31 – 187 cells/ µl; mean of 131.5 cells/µl).

Similarly, viral loads among the patients with SI viruses (ranged from 158 000 – 750 000 RNA copies/ml; mean of 417 000 RNA copies/ml) were not particularly higher in comparison to the patients with NSI viruses (ranged from 22 900 – 750 000 RNA copies/ml; mean of 496 407 RNA copies/ml). Three of the patients harbouring SI viruses presented with clinical symptoms of HIV infection (AIDS), and one patient presented as an asymptomatic AIDS patient with no recorded OIs recorded at the time (Table 2.1). These findings are consistent with late stage HIV-1 disease, and comparable to the clinical profiles of patients from which X4 variants were isolated in previous studies. Thus, the detected X4 emergence/evolution is not an artefact of the relatively low $CD4^+$ T cell counts harboured by these patients.

Overall, there does not seem to be any significant clinical differences between patients harbouring NSI or SI viruses, since no significant difference could be noted in patient age, gender, clinical presentation, $CD4^+$ T cell count or viral load. Similar patient clinical baseline profiles were described in various studies investigating coreceptor utilization and disease progression, [249-256]. However, data from these studies on HIV-1 subtype C infected individuals, yielded almost

exclusive usage of CCR5 (under representation of CXCR4 usage) by the isolates, irrespective of HIV-1 disease status, $CD4^+$ T cell count and viral load. Cecilia *et al.,* [249] demonstrated that there was an exclusive prevalence of CCR5 usage by all the viral isolates at both ends of the disease spectrum, namely symptomatic (~33 $CD4^+$ T cells/ml) and asymptomatic (~905 $CD4^+$ T cells/ml). These findings support the argument that the evolution/emergence of CXCR4 utilizing HIV-1 subtype C isolates and expansion of coreceptor usage, is neither associated with nor a prerequisite for progression of disease pathogenesis to AIDS.

Phylogenetic analysis of the 20 HIV-1 V3 loop sequences revealed that 19 of the 20 isolates clustered within HIV-1 subtype C reference, while 05ZAFV10 clustered within subtype D (Figure 2.3). Further analysis of 05ZAFV10 in the *vif, vpr* and *vpu* [402] and *pol* and full-length gp160 (K. Michler, pers. comm.) revealed that 05ZAFV10 is a unique CD recombinant. There are two recombination breakpoints in the *env* region at nucleotide positions of 160 and 1850 (results not shown). The nucleotide segments from 1 – 160 and 1850 – 2559 were identified as subtype C and the middle segment from nucleotides 160 – 1850 was classified as subtype D. Therefore, 05ZAFV10 appears to be an interesting recombinant strain, combining segments of subtype D that is typically found in West/West Central Africa, and subtype C, which is more common in Southern Africa [403]. Thus, this strain could have originated in West Central Africa, or it could represent a recombination event between a West Central African and South African strain.

To establish whether we could distinguish between CCR5 and CXCR4 usage at the amino acid level, the tetramer crown motifs, positions 11 and 25 and N-linked glycosylation sites for each V3 loop of each isolate were extensively analyzed (Figure 2.4) and length and charge were compared (Figure 2.5). CXCR4 usage is associated with nucleotide sequence changes within the V3 loop region of *env* resulting in an increased overall positive charge as well as an increased length [201, 202, 229, 234, 404].

The majority of the CCR5 utilizing variants contained V3 loops of 35 amino acids in length, with the exception of 05ZAFV22 which had a single amino acid deletion at position 24 (Figure 2.5 (A)). However, the CXCR4 utilizing sequences consisted of 34-37 amino acids in length due to insertions and deletions (Figure 2.5 (A)). Two of the X4 isolates had extended V3 loop regions of 37 amino acids in length (05ZAFV03 and 05ZAFV15) and one had a shortened V3 loop of 34 amino acids (05ZAFV10). Interestingly, variations in the length of the V3 loop are generally considered to be associated with CXCR4 usage [236, 405].

The V3 net charge for the CCR5 and CXCR4 utilizing isolates within this study were distinct with slight overlap (Figure 2.5 (B)). The six CXCR4 tropic viral isolates contained V3 loop sequences with a higher overall positive charge (≥ 4.5) and all of the X4 utilizing isolates had positively charged amino acids at position 11 of the V3 loop, except for 05ZAFV03, possessing a serine (S). Basic/positively or neutral charged amino acid residue substitutions were detected at position 25 of all the X4 utilizing isolates, except 05ZAFV10 which had an asparagine (N).

A comparison of the amino acids at position 25 of subtypes C and D in the databases confirm that N is rarely present at this site [51]. The CCR5 variants harboured amino acid charges ranging from +1 → +5.5, with the majority of the isolates having a charge between +3.5 and +4.5. The CXCR4 variants harboured charges ranging from +4.5 → +8.5, with an equal distribution over the range of charges (Figure 2.5 (B)). Interestingly, most of the R5X4 and X4 isolates had either a neutral or a positively charged amino acid residue at position 25.

The amino acid position 11 and/or 25 within the V3 loop of gp120 are of particular importance in the coreceptor usage determination [201, 202, 229, 231, 234]. All 12 R5 isolates possessed the characteristic GPGQ crown/tip motif and all R5 viruses had a neutral serine residue present at position 11, and amino acid residues with a negatively charged side chains (D or E) were present at position 25, except for 05ZAFV28. Interestingly, four out of six X4 isolates exhibited changes in the V3 tip tetramer motif, and two of the six varied from the consensus sequence due to

two amino acid insertions. One X4 isolate had a GRGL crown (05ZAFV03), one had GTGQ (05ZAFV10), one had GRGQ (05ZAFV15) and one had a GPGR motif (05ZAFV26).

The N-linked glycosylation epitope (NNT) at positions 6 - 8 (corresponding to positions 300 to 303 in the prototypic HXBc2 gp120) within the V3 loop of Env in R5 HIV-1 variants is highly conserved, and shown to result in a decreased sensitivity of the virus to neutralizing antibodies [241, 242]. Many CCR5 utilizing viruses detected early in infection, contain this N-linked glycan, suggesting that it may be a pre-requisite for CCR5 usage [243]. During disease progression, the immune pressure against the HIV-1 virus decreases and CXCR4 variants have been shown to emerge, lacking this N-linked glycan [243, 406]. Mutational changes within this epitope are detected with a high frequency in SI variants, especially among the CXCR4-only utilizing variants. Thus, lack of a glycan at the N-terminal of the SI V3 loop, may alter the conformation of the envelope, increasing efficiency of the CXCR4 coreceptor usage and contribute to coreceptor switching from CXCR4 to dual/R5X4 usage [243, 279].

All the R5 isolates from this study contained the highly conserved N-linked glycosylation motif, NNT, at positions 6 – 8 within the V3 loop. Polzer *et al.,* [243] suggested that the presence of this NNT glycan in the V3 loop of R5 HIV-1 isolates is involved with the interaction with the CCR5 coreceptor [243]. This is consistent with recent findings, which highlight the functional importance of this site for CCR5 usage, through masking the surrounding positively charged amino acids at the N-terminus of the V3 region [222, 245, 407]. However, 50% of the subtype C CXCR4 utilizing isolates in this study did not contain the consensus V3 N-liked glycan. The loss of this glycan in the V3 loop of the SI/X4 utilizing viruses may cause conformational changes that may further expose the V3 loop and therefore increase sensitivity of the isolate to neutralizing antibodies. Similar results were obtained in a previous report showing altered N-linked glycosylation sites in SI subtype C isolates [245, 257]. However, contrasting findings were made

by van Rensburg *et al.,* [408] who found intact N-linked glycosylation sites within the V3 loops of their subtype C SI isolates [408].

The mutational changes required for the loss of this N-linked glycan may further expose the coreceptor binding, expanding and increasing viral tropism and viral neutralizing sensitivity, respectively. Thus, as an infected individual experiences HIV-1 disease progression, immune pressure may subsequently decrease and allow for the emergence of isolates lacking the N-linked glycan and therefore able to utilize the CXCR4 coreceptor. A viral isolate lacking this V3 loop glycan may conformationally assist the steric binding of CXCR4 and thus contribute to the tropism switch from R5 to X4 virus [243, 279, 406].

The fact that the two dual tropic isolates, ZA05FV15 and ZA05FV26, retained the N-linked glycan in the V3 loop structure may reinforce the hypothesis that the presence of the glycan constrains the envelope protein, promoting CCR5 binding. When comparing the two dual tropic isolate's predicted V3 loop amino acid sequences from this study, to various V3 loop sequences of dual tropic isolates from the Los Alamos database, a striking similarity was found [47]. Ten out of thirteen (77%) of the subtype B, C and D dual tropic sequences retained the NNT N-linked glycosylation site within their V3 loop sequences (Figure 2.6).

In summary, the HIV-1 subtype C CXCR4 utilizing variants showed an increased number of positively charged amino acids in their V3 loop and hypervariable V3 loop length and a different tetrapeptide motif at the tip of the V3 loop, as has been described previously [236]. Interestingly, there were no striking sequence motifs within the V3 loop that could distinguish between dualtropic and CXCR4 utilizing viruses.

The phenotypic coreceptor utilization assays (MT-2 and U87 cell line assays) initially flagged the CXCR4 using isolates from the cohort. Subsequent sequence analysis of the sequence data confirmed that the X4 isolates were both genotypically and phenotypically CXCR4 utilizing. One of the software-based phenotypic predictor packages (C-PSSM) was used to confirm the phenotypic

classification of the isolates [271]. To test the predictability and accuracy of a bioinformatic algorithm C-PSSM, the 20 HIV-1 V3 sequences were analyzed with the software. All the SI utilizing isolates were identified correctly; however, two R5 utilizing isolates were incorrectly identified as SI isolates (05ZAFV07 and 05ZAFV13). A possible reason for the incorrect phenotype prediction of these two CCR5 utilizing isolates may be due to the insufficient data set available at the time of the development of this bioinformatic tool. It is possible that once more subtype C sequences and their corresponding tropisms are available in the C-PSSM analysis software, the prediction capability of this bioinformatics tool will improve. This implies that there is no current, infallible, suitable substitute for coreceptor determination, other than *in vitro* phenotypic determination assays.

There was an incongruity between the overall charges of the V3 loop regions calculated in Figure 2.4 and Table 2.3. The C-PSSM software calculates the overall charge by minusing the number of positively charged (R/K/H) amino acid residues (each +1 in charge) from the number of negatively charged (D/E) residues (each –1 in charge) (Table 2.3). However, in Figure 2.4, the overall V3 loop charge was calculated as follows; D and E were each assigned a -1 charge, R and K were each assigned a +1 charge and H was assigned a +½ charge. Thus, the different V3 loop charge outputs have different values yet reflect the same overall output/predicted phenotype.

These data prompt many speculative rationales as to why this detected evolution/emergence of HIV-1 subtype C is noticeable in South Africa. The driving force for this emergence/evolution may be host or virus-dependent, or a complex interplay between the two. The genotypic and consequently phenotypic changes that are necessary for HIV-1 subtype C viruses to "switch" from CCR5 to CXCR4 tropic may only be a few amino acid changes within the V3 loop, as seen in HIV-1 and 2 [405, 409]. Considering the high mutation rate along the entire length of the genome being 3×10^{-5}/base/replication cycle and the high replication rate [17, 21, 22, 26, 410], it is surprising that CXCR4 coreceptor binding preference is not detected earlier. Thus, the role of neutralizing antibodies and

other cellular host factors in preventing the emergence/evolution of X4 variants requires further investigation.

Overall, these results imply that the frequency of HIV-1 subtype C CXCR4 utilizing viruses may be increasing with time, as was previously documented in the CRF01_AE epidemic [411]. CXCR4 utilizing viruses began to appear with increased frequency within the HIV-1 subtype E (CRF01_AE) epidemic in Thailand that was initially defined by NSI/R5 isolates. Since the current study was cross-sectional, no conclusions about whether HIV-1 subtype C viruses can evolve from CCR5 to CXCR4 usage can be made. Future work should focus on longitudinal follow-up studies to determine whether *env* is capable of mutating/evolving from CCR5 usage to CXCR4 usage within the same drug-naïve patient over time, or whether CXCR4 utilizing viruses are inherently present from early infection. Furthermore, findings from this study need to be confirmed in a larger cohort. These results emphasize the importance for ongoing investigation of coreceptor usage of HIV-1 subtype C isolates particularly since it is likely that the next FDA approved antiviral drug will be the CCR5 antagonist, Maraviroc [265, 272, 412, 413].

Chapter 3 *IN VITRO* ADAPTATION OF AN HIV-1 SUBTYPE C CXCR4-UTILIZING ISOLATE TO CD4-INDEPENDENCE

3.1 Abstract

The HIV-1 subtype C is the most prevalent subtype in the world and there is currently no preventative vaccine or curative drug against it. The HIV-1 envelope glycoprotein (Env) exists as a trimer on the viral surface and upon binding to CD4 on the host cell, a cascade of conformational changes occur within Env, exposing the conserved, vulnerable coreceptor binding site. These sites are the main targets for host induced neutralizing antibodies and are constitutively exposed in CD4-independent HIV-1 isolates. A CXCR4 utilizing HIV-1 subtype C primary isolate was obtained from co-culture of Peripheral Blood Mononuclear Cells (PBMCs) from an HIV-1 infected treatment-naïve AIDS patient. The isolate (05ZAFV03) was serially passaged 12 times in the Cf2Th cell line with an aim to gradually decrease numbers of CD4 expressing cells over time until the virus was fully adapted to CD4-independence. The virus successfully adapted and grew in Cf2Th cell cultures where only 10% of the cells co-expressed CD4 and CXCR4. However, despite several attempts, growth in Cf2Th cells lacking CD4 was not achieved. Nucleotide and predicted amino acid sequence analysis was performed on the full-length *env* (gp160) from viruses for each of the serial passages selected for analysis. The sequences were compared with each other and to the parental primary sequence using phylogenetic methods. After serial *in vitro* viral passage, a bottleneck occurs and the *env* region remains relatively conserved, contrary to what was expected due to the high mutation and replication rate of HIV-1. A single non-synonymous nucleotide mutation was detected in the V1 loop region (E152K according to Consensus_C numbering) at 50% CD4 expression after four viral passages. All subsequent viral passages down to 10% CD4 expression retained the mutation. The E152K mutation may have conformational and steric implications for CD4 binding. Further adaptation towards 100% CD4-independence may depend on this mutation and possibly others that may expose the conserved coreceptor binding domain. Elucidating these conserved coreceptor binding domains may have profound implications for vaccine and novel drug target design.

3.2 Introduction

HIV entry into the host cell requires the initial interaction of viral gp120 with the host cellular primary receptor, CD4, and successive binding to the coreceptors CCR5 and/or CXCR4, respectively [56, 57, 66, 69-71, 77, 80, 164, 227, 228]. CD4 binding is the first step in the entry process, and is necessary for HIV systemic infection in the peripheral cells of the host. Once gp120 interacts with the host CD4 receptor, a series of conformational changes within the heavily glycosylated gp120 are induced, exposing the conserved, high-affinity coreceptor binding sites thereby allowing CCR5 and/or CXCR4 coreceptor engagement [56, 71, 167, 175].

Crystallographic structures of the gp120 core, the two amino-terminal domains (D1D2) of the CD4 and the antigen-binding fragment of the human neutralizing antibody 17b, have elucidated the interaction between gp120 and CD4 [66, 176]. The gp120 core consists of three major units, the inner and outer domains and the intervening bridging sheet, a four stranded β sheet comprised of conserved residues that links the two domains (Figure 3.1) [66]. CD4 is bound into a depression formed by all three of these units, particularly the V1 and V2 loops which contribute two loops of the bridging sheet upon direct interaction with CD4 (Figure 1.5) [64, 66, 171, 414].

Recently, the crystal structure of an unliganded, pre-CD4 fusion SIV gp120 core has been described (Figure 3.1) [82]. The inner domain of the heavily glycosylated gp120 undergoes extensive conformational rearrangements upon CD4 receptor binding. Extensive structural characterization and comparison between the unliganded pre-CD4 fusion SIV gp120 and the CD4-bound HIV-1 gp120 structure revealed four conserved inner-domain disulphide bonds that are critical in positioning structural elements involved in CD4 binding [82]. Despite the conserved coreceptor binding epitopes being hidden in the unliganded gp120 state, there are distinct antigenic surfaces on the gp120 that may also be targeted with entry inhibitors.

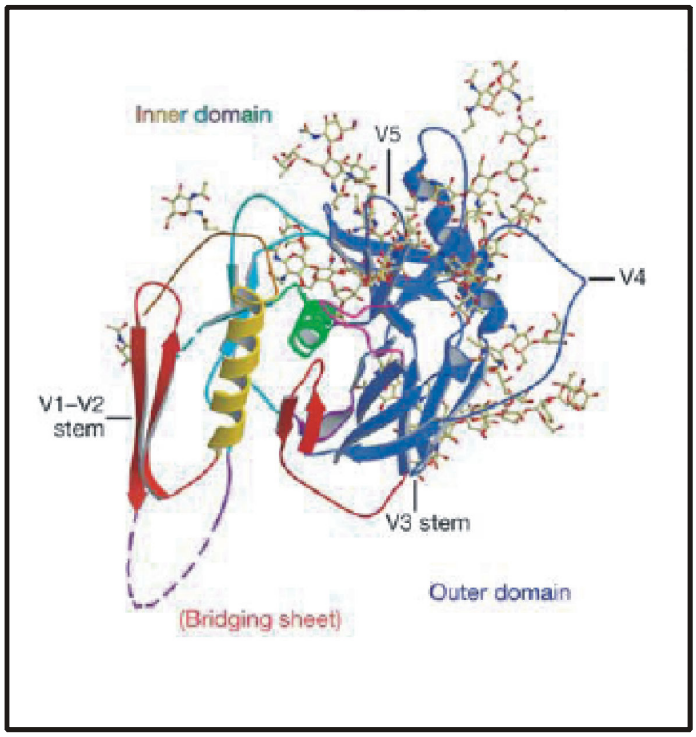


Figure 3.1 Unliganded SIV gp120 core structure. All carbohydrates are depicted as stick models and polypeptides as ribbons. The outer domain (in blue) and inner domain is coloured according to substructure (N terminus is orange; α1 is yellow; three-strand sheet is cyan; outer/inner domain transition is purple; α5 is green). The four strands that form the bridging sheet in the CD4-bound conformation are depicted (in red). Disordered residues are shown as dashed lines and the stumps of truncated variable loops V1, V2 and V3 are indicated and the intact variable loops V4 and V5 are also labelled. Figure adapted from [82].

Within Env there are three distinct changes that take place during gp120-CD4 binding; initially, the conserved bridging sheet pre-folds to expose/create the conserved coreceptor binding site [64, 66]. Next, the inner and outer domains are pulled towards each other. Finally the V1-V2 loops conformationally adjust to uncover the coreceptor binding site residues, increasing the affinity of the viral gp120 for the downstream binding of the CCR5/CXCR4 coreceptor [64, 66, 67, 171, 414]. This conformational shift is fundamental in the determination and specificity of coreceptor utilization [163, 164, 167, 175, 415-419].

Crystallographic modelling of the gp120-CD4 interaction shows that only a small number of residues are actually involved in direct interatomic binding between the

two proteins [66]. The CD4 binding site in this model, is devoid of carbohydrate residues and consists of 26 specific residues within HIV-1 subtype B (HXBc2) Env, contacting 22 CD4 residues [66]. Six segments of gp120 are integrally involved in the interaction with the CD4 binding pocket. The representative contact residues are L125 (from the V1/V2 stem), N280 (from the ℒD), D368 (from β15 to α3), W427 (from β20 – β21), D457 (from β23) and G473 (from β24 – α5) [66].

While the CD4 and coreceptor binding sites within gp120 are not accessible and only exposed for very brief moments during viral entry, they are highly sensitive to neutralization by monoclonal antibodies [68, 283, 354, 414, 420, 421]. The envelope escapes this immune pressure through acquiring mutations, due to its highly error prone reverse transcriptase and high replication rate [22, 27]. The genetic diversity within the HIV Env allows adaptation to evade the immune system through changes in the variable loops, changes in tropism and acquisition of N-linked glycosylation sites. The glycans conceal the conserved CD4 binding and coreceptor binding epitopes, also contributing to the decreased viral susceptibility to host neutralizing antibodies [167, 169, 171, 176, 177, 422]. Thus, the neutralization-resistant state in wild type (wt) HIV-1 is maintained by CD4 engagement prior to coreceptor utilization [355].

The 35 amino acid V3 loop also plays an important role in chemokine receptor binding and viral tropism; coreceptor specificity is largely determined by its sequence [164, 167, 414, 415, 417]. Huang *et al.*, [174] recently revealed the crystal structure of gp120 with an intact V3 loop extending out from the envelope like a “molecular hook” towards the N-terminus of the CCR5 coreceptor. These crystallographic structures of gp120 also suggests that the CD4 and coreceptor binding domains are strategically hidden and poorly accessible within the Env trimer [66, 174].

Few primary HIV-2, SIV and some HIV-1 isolates naturally infect a variety of different cell types with little to no CD4 expression by interacting directly with the

coreceptor (Figure 3.2) [344-347]. These viruses are termed CD4-independent. This naturally occurring phenomenon is rare, however arises due to viral envelopes being "partially triggered", allowing direct binding of the coreceptor to initiate host cell infection. Thus, the normal need for CD4 binding to induce the conformational changes in Env is avoided (Figure 3.2) [345, 346, 348-350].

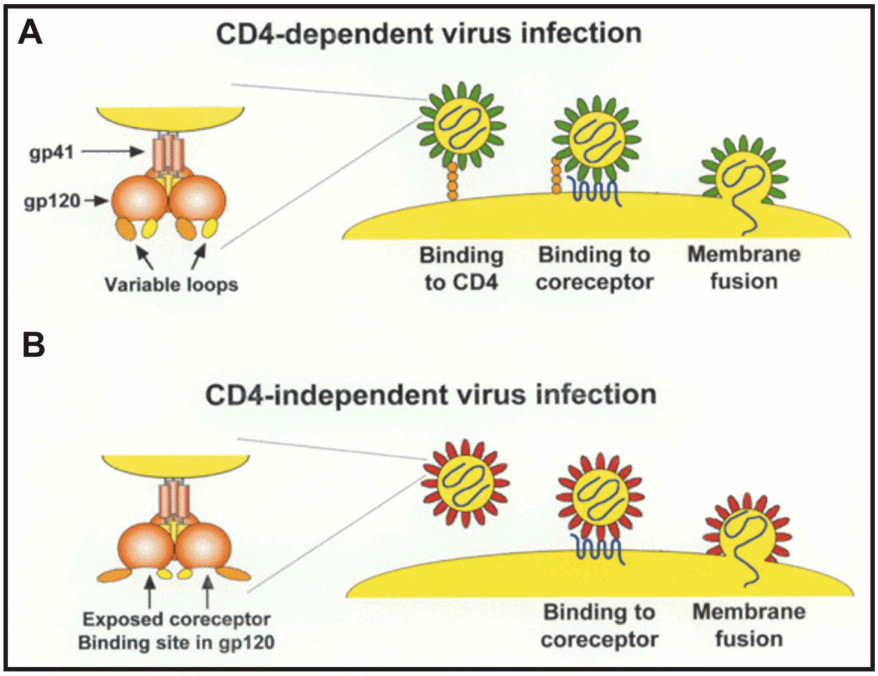


Figure 3.2 Schematic illustration of the molecular arrangement of the gp120 proteins on the surface of the HIV-1 virion during CD dependent virus infection (A) and CD4-independent Viral infection (B). Figure adapted from **[423]**.

There are low/negligible levels of CD4 expression on cell types such as macaque and primate macrophages, microglia, oligodendrites, brain microvascular endothelial cells and perivascular macrophages found in the brain. Despite decreased CD4 expression, these cells are all permissive to SIV/HIV infection [308, 358-360, 424-429]. HIV-1 naturally enters the Central Nervous System (CNS) early after systemic infection, via crossing the blood-brain barrier, where antibodies are excluded, and infects cells in this immune privileged area. This results in neurological disease, ranging from minor cognitive/motor function

disorder, to severe HIV dementia (HIVD) and HIV-1 Associated Dementia Complex (HADC) [430, 431].

Consequently, these CD4-independent viruses infect target cells with a much higher efficiency compared to wt viruses [363]. This is due to expression of gp120 in a 'pre-triggered' state that results in a conformation where the coreceptor binding site is exposed, allowing a high affinity and direct interaction between the gp120 and the coreceptor. However, CD4-independent viruses with exposed, conserved coreceptor binding sites, exhibit heightened sensitivity to neutralizing antibodies and neutralization by HIV-1 positive sera, that recognize conserved epitopes which are essential for coreceptor engagement [351, 354-357, 432]. This occurrence suggests that the immune system may place a powerful selective pressure against the emergence/evolution of such viruses, which may be an explanation for the rarity of the CD4-independent phenotype *in vivo*, in the periphery [354].

CD4-independent Envs are thought to possess multiple amino acid substitutions and/or deletions compared to the CD4-dependent wt viruses. These mutations map over broad regions in *env*, encompassing areas required for coreceptor specificity. These changes ultimately lead to the exposure of conserved epitopes that mediate direct binding to the CCR5 and/or CXCR4 coreceptors [351]. However, the extent to which CD4-independent viruses enter cells via a CD4-independent mechanism and have an expanded tropism for CD4-negative cells *in vivo* is currently unclear.

Several CD4-independent SIV, HIV-2 and HIV-1 isolates have been characterized *in vivo*. CD4-independent SIVs have a reduced affinity for CD4 and have been previously isolated from rapid progressor rhesus macaques [364]. Macrophages from these macaques express relatively low levels of CD4, and thus CD4-independent SIV isolates have an increased capacity to infect CD4-negative, CCR5-positive macrophages [356, 357]. CD4-independent CCR5 chemokine utilization *in vivo* is relatively common in SIV and HIV-2 strains [345, 362, 363]. This has led to the speculation that CCR5 usage may have been the initial

prehistoric coreceptor used by primate lentiviruses [354] and the expanded tropism for CXCR4 usage may have evolved later, in humans.

To date, the only HIV-1 primary isolate which has been shown to inefficiently infect CD4-negative, CCR5-positive cells, is R2, which exhibits a single rare mutation in the V3 loop of gp120 [365]. The CD4-independent phenotype was mapped to a single proline (P) substitution in the proximal limb of the V3 loop, (313-4 PM) [365]. Studies concerning CD4-independent HIV-2 confirm that there is also a direct interaction between CXCR4 and gp120, which allows for subsequent membrane fusion; this phenotype is correlated with mutations throughout *env* [346, 372].

There have also been several reports of *in vitro* studies showing that strains of SIV, HIV-2 and laboratory adapted HIV-1 strains can bind either CXCR4 or CCR5 coreceptors in the absence of CD4 [344-346, 349, 350, 367-371]. Zerhouni *et al.,* [367] hypothesized that these CD4-independent quasispecies would exist at low concentrations (in primary culture) compared to the more abundant CD4-dependent strains, and that they may be enriched through serial propagation [367]. However, infection of T cell lines lacking CD4 expression is much less efficient.

CD4-independent HIV-1 (subtype B) and HIV-2 variants that utilize CXCR4 for cell entry have been previously derived *in vitro* by serial passage through T cells lacking CD4 [344, 350, 351]. The HIV-1 HXBc2 *env* clone (8x) variant was serially passaged and shown to successfully infect CD4-negative, CXCR4-positive BC7 cells [350, 351]. Sequence analysis of 8x revealed that gp120 was lacking 5 glycosylation sites and harboured 18 mutations, resulting in a stably exposed coreceptor binding site [346, 350].

Another CD4-independent HIV-1/IIIB variant was selected through repeated *in vitro* passage in CD4 negative T cell lines and utilized the CXCR4 coreceptor [369]. In general, few changes in the V3 loop sequence of gp120, around the base of the V4 and in the transmembrane gp41 were required to expose the coreceptor binding site [350, 373]. The literature reports that high affinity CXCR4 coreceptor

usage was also shown in CD4-independent HIV-2 variants, ROD/B and VCP [346, 347, 369, 370, 372].

In contrast, CD4-independence in the HIV-1 m7NDK variant correlated with the crucial acquisition of two glycosylation sites in V1/V2 and 7 amino acid changes within the C2, V3 and C3 regions of *env* [344]. These changes are likely to be responsible for the spontaneous exposure and increased flexibility of the V3 loop that allows for direct interaction with CXCR4. A study was performed on a CXCR4 tropic HIV-1 iNDK recombinant clone in intestinal cells, derived from the same parental virus as the m7NDK variant. The clone showed the acquisition of two N-linked glycosylation sites in the V1/V2 region, 3 amino acid changes (^{296}KYT → ^{296}NNI) in the V3 loop and the loss of one N-linked glycan from the C2 region [374]. The loss of the positive charge (K296N) within the V3 loop might have increased the Env hydrophobicity, and contributed to the direct binding of gp120 to the coreceptor. In all these cases, CD4-independence has been associated with the alteration of the N-linked glycosylation profile.

CCR5 tropic, CD4-independent HIV-1, HIV-2 and SIV have also been reported. Kolchinsky *et al.*, [349] found two sets of genotypic changes within *env* upon CD4-independent adaptation. The intact whole virus was not used for infections, rather the HIV-1 *env* was cloned into a pNL4-3 plasmid, to produce an infectious provirus [349]. The first change detected was a 10 residue sequence from the C-terminal of the V2 loop into the V1/V2 stem (positions 188 – 197 in the HXBc2 strain) and the second change was a conserved pair of adjacent residues (positions 539 – 540 in the HXBc2 strain) in the gp41 ectodomain [349]. Here, the repositioning of the V2 loop is largely responsible for the major gp120 conformational change in response to CD4 binding, and unmasking of the coreceptor binding site.

An HIV-1 ADA variant, also described by Kolchinsky *et al.,* [366], could infect cells via CCR5 in the absence of CD4 [366]. Removal of a single N-linked glycosylation site (N197) at the base of the V1/V2 region was found to be

responsible for this CD4-independent phenotype [366]. Structural models of gp120 suggest that removal of this glycan probably results in the repositioning of the V1/V2 loops so as to expose the highly conserved coreceptor binding site [366]. This was supported by studies which showed that HIV-1 ADA and SIV variants with deleted V1/V2 loops, could infect CD4-negative CCR5 positive cells, and bind CCR5 directly [366].

CD4-independent usage of the CCR5 coreceptor by an HIV-2/vcpm variant mapped to a positively charged amino acid in the C4 region of *env* [375]. There may be an electrostatic interaction between the negatively charged CCR5 amino acid and the positive residue within the C4 region [375]. The critical amino acid substitutions in *env* that were responsible for the CD4-independent phenotype in HIV-2 were shown to partially expose the coreceptor binding domains.

CD4-independent infection of host cells, and infection with a reduced requirement for CD4 have been shown in SIV, and the most common utilized coreceptor is CCR5 [345, 357, 376-378]. Puffer *et al.,* [379] determined that two amino acid changes are responsible for the CD4-independent phenotypic effects on the SIVmac239 Env [379]. These two changes include substitutions at M165I within the V1/V2 loop and K573T of gp41 that supposedly enhance coreceptor binding [379].

The determinants for the CD4-independent phenotype are mapped to *env*, however the underlying mechanisms of this phenomenon are largely unknown. Taken together, the changes acquired by different HIV-1 *env* clones post CD4-independent adaptation vary considerably between isolates. There does not seem to be a common, standard motif associated with *env* that is required for CD4-independence. The changes observed *in vitro* may not be a direct reflection of the exact changes that are taking place *in vivo,* due to the lack of immune pressure *in vitro*.

In general, mutations acquired by CD4-independent isolates *in vitro* can be divided into two distinct groups: those that harbor mutations (deletions/changes) in/around

the V1/V2 region causing downstream unmasking of the of the coreceptor binding site, and isolates which harbor mutations elsewhere in the envelope (gp41, V3, V4 etc). The elucidation of this complex process and the many determinants for CD4-independence may provide useful information pertaining to the exact step-wise process of CD4 binding, viral tropism and fusion. However, the clinical and pathogenic significance of HIV-1 CD4-independent infection is largely unknown due to their rarity and difficulty of isolating such viruses from primary culture.

Many biochemical and crystallographic studies over the past few decades have provided detailed descriptions of the multifactorial complexity of viral entry into host cells. However, there are still many unanswered questions relating to the structural moieties and details of the dynamic conformational changes involved in the viral entry process into the host cell. An in depth understanding of the mechanism and specific resides involved in HIV-1 entry into host cells is paramount in developing immunological and pharmacological strategies to prevent infection.

The evolution and occurrence of CD4-independent HIV-1 variants within infected individuals, ultimately expands viral tropism and target cell range *in vivo*. The mutations within the gp160 of the CD4-independent envelope allow exposure of the conserved coreceptor binding domains, and enhance fusogenicity with the host cell membrane. It is expected that the exposed conserved coreceptor binding sites of CD4-independent viruses may allow targeting of these immunogenically enhanced conserved neutralization epitopes.

The CD4-independent, CCR5/CXCR4 dependent phenotype in HIV and SIV can thus be attributed to one of two changes in gp120; Firstly, insertions and/or deletions can cause repositioning of the variable V1/V2 loops so as to unmask the coreceptor binding site. Secondly, an accumulation of successive mutations in V3 and the base of V4 might increase envelope affinity for coreceptor binding (or pre-folding of the gp120 bridging sheet). Mutational changes in gp41 may also trigger conformational rearrangements and fusion to occur after a weaker gp120-

coreceptor interaction [348]. In general, HIV-1 envelopes with decreased dependence on the primary CD4 receptor harbour altered glycosylation patterns. Thus, such viruses have partially triggered envelopes as a result of their adaptation to growth in cells of the CNS environment with decreased CD4 expression profiles.

A number of potential sites may be identified/determined from neutralization-sensitive Envs that can be used to generate effective immunogens. These prospective immunogens may be used for future vaccination studies and therapeutic intervention strategies that elicit broadly neutralizing antibodies. Small molecule inhibitors/compounds may also be designed to potently block the conserved coreceptor binding domains. To this end, the purpose of this study was to adapt an HIV-1 subtype C primary isolate/s to CD4-independence and to elucidate the residues that are responsible for this phenotype in the envelope glycoprotein/s.

3.2.1 Objectives

The overall aim of this study was to adapt HIV-1 subtype C primary isolates to CD4-independence *in vitro*.

This was attempted by the following three procedures:

1. Adaptation of CCR5 and CXCR4 utilizing primary HIV-1 subtype C to the Cf2Th.CD4.CCR5 or Cf2Th.CD4.CXCR4 expressing cell lines respectively, as measured by p24 antigen ELISA.

2. Adaptation of cell-line adapted viruses to decreasing concentrations of cellular expressed CD4 by serial passaging in cell cultures containing mixtures of Cf2Th.CD4.CCR5/CXCR4 and Cf2Th.CCR5/CXCR4, as determined by p24 antigen ELISA.

3. Proviral DNA sequencing and extensive analysis of full-length Env nucleotide and amino acid sequences, from each successful serial passage to determine the changes associated with decreased dependence of CD4 and/or CD4-independence.

3.3 Materials and Methods

3.3.1 Primary virus Selection

Five CCR5 utilizing and five CXCR4 utilizing primary isolates were randomly selected for the purposes of this study (see Table 2.3) from the cohort (described in Section 2.3.1). These included 05ZAFV02, 05ZAFV03, 05ZAFV06, 05ZAFV10, 05ZAFV11, 05ZAFV14, 05ZAFV15, 05ZAFV20, 05ZAFV22, 05ZAFV26, 05ZAFV27 and 05ZAFV28. The passage 2 (P2) primary viral isolates were used in all experiments and each viral supernatant (1.5 ml) was added to 1.5 ml of sterile supplemented DMEM (Sigma, Steinheim, Germany) growth medium (described below) and filter sterilized through a 0.2 μm filter (Acrodisc PF Syringe Filter, Pall Corporation, UK) before infection.

3.3.2 Thymocyte Cell Lines and Growth Conditions

The Canine Thymocyte cell lines [433] (Cf2Th.CD4.CCR5 ; Cf2Th.CD4.CXCR4; Cf2Th.CCR5 and Cf2Th.CXCR4) were generous gifts from Joseph G. Sodroski, (Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts). These cells were maintained in supplemented DMEM growth medium [DMEM (Sigma, Steinheim, Germany) supplemented with 10% FCS, L-Glutamine (2 mM) (Gibco, Grand Island, USA) and penicillin (0.5 U/ml)/streptomycin (0.5 U/ml) antibiotics (Gibco, Grand Island, USA)]. The supplemented DMEM growth medium for the Cf2Th.CD4.CCR5 expressing cell lines, was additionally supplemented with G418 (500 μg/ml) (Calbiochem, Canada) and Hygromycin (150 μg/ml) (Calbiochem, Canada). The Cf2Th.CD4.CXCR4 expressing cell lines, were also grown in supplemented DMEM growth medium, additionally supplemented with Zeocin (300 μg/ml) (Invitrogen) and Hygromycin (150 μg/ml) (Calbiochem, Canada). The Cf2Th.CCR5 and Cf2Th.CXCR4 cell lines, were grown in supplemented DMEM growth medium and additionally supplemented with only G418 (500 μg/ml) (Calbiochem, Canada).

3.3.2.1 Basic Cell Growth and Storage

All the cell lines were maintained in the supplemented DMEM growth medium with appropriate antibiotic supplements in T25 NUNC culture flasks (AEC Amersham, Denmark) in a 37°C, 5% $C0_2$ incubator. When the cells reached 100% confluency, they were split as described for the U87.CD4 cell line in section 2.3.6. A 1% solution of 0.5 M EDTA (SIGMA, Steinheim, Germany) in 1 X Phosphate Buffered Saline (PBS) (SIGMA, Steinheim, Germany) was also used to disrupt cell line adherent properties and cells were split in a 1:8 ratio twice per week, and where only maintained for a maximum of 20 passages. Cell lines were stored in a freezing mix in liquid nitrogen (see 2.3.3.2 in Chapter 2).

3.3.3 Viral Infection of Thymocyte Cells

Cf2Th cells were seeded either in T25 NUNC culture flasks (AEC Amersham, Denmark), or in 24 well flat-bottomed NUNC culture plates (AEC Amersham, Denmark). Cells for all infection experiments were plated at 5 x 10^4 cells/ml in 2 ml of supplemented DMEM growth medium. The supplemented DMEM growth medium was additionally supplemented with the appropriate antibiotics. The following day, the supplemented DMEM growth medium was removed and between 70 - 500 µl of P2 viral supernatant, was added to the adherent cells with 500 µl of supplemented DMEM growth medium, to prevent the cells from drying out, during infection. A maximum of ten wells were seeded and infected in the same way as in the T25 culture flasks, so as to allow for sampling each day post infection. The number of wells that were infected was limited by the volume of infectious viral supernatant that was available from the previous round of infections. Viral infections were incubated in a 37°C, in a 5% $C0_2$ incubator between 12-18 hours (overnight). The input virus was subsequently washed off gently four times with 1 x PBS (SIGMA, Steinheim, Germany) to remove unbound virus. Both the input virus and the 4^{th} wash with 1 x PBS (SIGMA, Steinheim,

Germany) were stored at -80°C for further analysis by p24 Ag ELISA (Abbot Murex, Dartford, UK). Infections in the T25 culture flasks were split as required or when cells were confluent.

Fresh supplemented DMEM growth medium (2 ml) was added to each well post washing with PBS. The wells were visually monitored for Cytopathic Effects (CPEs) daily. Viral supernatant (2 ml) and adherent cells were harvested from different wells on days 0, 3, 5, 7, 10, 11, 13, 15 and 17 post-infection and were stored at -80°C for subsequent p24 Antigen (Ag) ELISA analysis (Abbot Murex, Dartford, UK). However, for certain infections viral supernatant was sampled on different days.

3.3.4 Murex p24 HIV-1 Antigen ELISA

All culture supernatants (including input virus and the 4th PBS wash (controls)) were monitored by p24 Ag ELISA (Abbot Murex, Dartford, UK) measured by the plate reader (ELX 808, Bio-tek Instruments, South Africa) as described in Chapter 2, section 2.3.4. Negative control wells were not infected with virus and performed for each separate infection. A positive control from the kit was also performed for every p24 ELISA.

3.3.5 Adaptation to CD4-independence

The experimental design described here was based on a previously published protocol [349] and initially a pilot study was performed to establish the most effective protocol for infection of the Canine Thymocyte (Cf2Th) cell lines. Initially 30 µl, 50 µl, 70 µl, 100 µl and 200 µl of filter sterilized infectious P2 viral supernatant was used to infect the cell lines in T25 NUNC culture flasks (AEC Amersham, Denmark). Each day post infection, 200 µl of supernatant was harvested for p24 Ag ELISA from the same flask. This method did not yield an

accurate reflection of the viral growth curve, because infectious virus was constantly being removed from the total viral population in the T25 flask. Thus, 24 well NUNC culture plates (AEC Amersham, Denmark) were used for infections and viral supernatant and infected cells were removed from a separate well for each harvest on separate days. An infectious viral input volume of 70 - 200 µl was sufficient for successful infection.

Infection of a particular cell line (e.g. Cf2Th.CD4.CXCR4) was repeated between 2 and 4 passages to achieve stable infection in that cell line before infecting the next lowest concentration of CD4 expressing cells. Viral supernatants from infected wells, yielding absorbances reading "out of detection range" of the instrument ($A_{450/630}$), were considered to be replication positive and capable of successfully infecting the next passage of the same cell line or the next cell line. Once stable infection within a certain cell line had been reached, viral supernatant yielding a high p24 Ag absorbance reading was then used to infect another passage of the same cell population as well as a population of cells expressing decreased levels of CD4, in parallel. Throughout the adaptation process, the concentration of cellular expressed CD4 was incrementally decreased within each different cell line i.e. ratios of Cf2Th.CD4.CXCR4 : Cf2Th.CXCR4 expression decreased from 100:0 to 50:50 to 25:75 to 10:90 to 1:99 and finally down to 0:100.

3.3.6 Proviral DNA Isolation

Proviral DNA isolation was performed on all infected cell culture samples from each of the sequential adaptation stages, i.e. P2 100% Cf2Th.CD4.CXCR4, P2 50% Cf2Th.CD4.CXCR4 : 50% Cf2Th.CXCR4 (50%:50%), P3 and P4 from 25% Cf2Th.CD4.CXCR4 : 75% Cf2Th.CXCR4 (25%:75%) and P1 and P4 from 10% Cf2Th.CD4.CXCR4 : 90% Cf2Th.CXCR4 (10%:90%). The High Pure PCR Template Kit (Roche, Mannheim, Germany) was used as per manufacturer's instructions (see Chapter 2, section 2.3.7). All the samples were eluted in 200 µl pre-warmed (70°C) elution buffer and stored at -20°C until further use.

3.3.7 Amplification and Sequencing of Full-length gp160

The gp160 coding region was successfully amplified from all the extracted proviral DNA samples, using the same PCR primers and protocol as described in Chapter 2, section 2.3.8. The PCR products were resolved on a 0.8% agarose gel (Sigma, St Louis, MO) to confirm the amplicon size, as described previously (Chapter 2, section 2.3.9). All the successfully amplified p160 amplicons from each cultured sample were immediately purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Penzberg, Germany) as per manufacturer's instructions (see Chapter 2, section 2.3.10).

Sequencing reactions were performed in MicroAmp 96 well optical reaction plates using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (V3.1), according to manufacturer's instructions (Applied Biosystems, Foster City, CA). Sixteen primers encompassing both forward and reverse strands were used to sequence the ~2.7 kb fragment in both directions (Table 3.1). Sequences were resolved on the ABI 3100-*Avant* Automated Genetic Analyzer (Applied Biosystems, Foster City, CA) as previously described (Chapter 2, section 2.3.11). Sequence data editing was performed using the Sequencing Analysis V3.3 programs (Applied Biosystems) and assembled using Sequencher V4.5 (Genecodes, Ann Arbor, MI). A nucleotide and amino acid alignment with the primary proviral 05ZAFV03 gp160 sequence was performed using CLUSTAL X V1.81 and analyzed in Genedoc and for any amino acid sequence changes acquired during adaptation.

Table 3.1 Primers used for gp160 cycle sequencing.

Primer	Primer Sequence
5R(3)C[φ]	5'-CAT TGC TCG TCC TAC CCC CTG CCA C-3'
A590[*]	5'-AAT CGC GAA ACC AGC CGG CGC ACA AT-3'
AA1570 [§]	5'-GGA GCA GCA GGA AGC ACT ATG GGC-3'
AES6[§]	5'-GGA CAA GCA TTC TAT GCA ACA GGT G-3'
DR7C[φ]	5'-TCA ACT CAA CTA CTG TTA AAT GGT AGC CTA GC-3'
E16[*]	5'-CCA ATT CCC ATA CAT TAT TGT G-3'
Env1f [*]	5'-CCA TAA CAC AAG CCT GTC CAA AGG-5'
Env3f [*]	5'-GGA AGC ACT ATG GGC GCG GC-3'
Env4f [*]	5'-GAG TTA GGC AGG GAT ACT CAC C-3'
ES21 [§]	5'-ACA CAT GCC TGT GTA CCC ACA G-3'
Gp120 5' [§]	5'-AGA GCA GAA GAC AGT GGC AAT GA-3'
JL103 [§]	5'-TAA CAA ATT GGC TGT GGT ATA TAA-3'
JL74 [§]	5'-ACA TGT GGA AAA ATA ACA TGG TAG AAC AG-3'
SQ15FC[φ]	5'-GAG AGC GGT GGA ACT TCT GG-3'
TU-J [§]	5'-GTT AGG CAG GGA TAC TCA CC-3'
ZM184D [§]	5'-CCA CTC AGC TAC TGC TAT TGC TAT GGT-3'

[§] F. McCutchan
***** Personal communication with Dr. Maria Papathanasopolous
[φ] Taken from Rousseau *et al*., [390]

3.4 Results

3.4.1 Adaptation of Primary CCR5 and CXCR4 utilizing viruses to Cf2Th.CD4.CXCR4/CCR5 cell lines

Despite repeated attempts, stable, sustainable infection with 05ZAFV22 (and four other R5 primary isolates; 05ZAFV02, 05ZAFV06, 05ZAFV11 and 05ZAFV20) was not successful in the Cff2Th.CD4.CCR5 cell line. A very slight increase in p24 antigen was detected on day 7 for the 05ZAFV22 infection (Figure 3.3 (A) Blue curve), however, this result may be background absorbance and/or very low/slow inefficient replication of the R5 virus in this particular cell line. The R5 viruses were thus no longer incorporated in any of the cell line adaptation experiments.

Stable infection of the 100% Cf2Th.CD4.CXCR4 cell line was achieved with one out of the five selected CXCR4 utilizing primary isolates, 05ZAFV03. By day 7 post P1 of the 100% Cf2Th.CD4.CXCR4 infection, the cells in the 24 well NUNC culture plates were fully confluent and required a larger surface area in which to grow. Thus, the infections (viral supernatant and cells of the controls and infections) were carefully transferred into the larger wells of the 6 well NUNC culture plates, to allow for further cell growth and to prevent over-growing and premature cell death caused by cell crowding. The viral growth curve over 13 days is shown with the viral growth peak detectable by day 10 (Figure 3.3 (A) red curve).

To strengthen the viral growth of 05ZAFV03 in the Cf2Th.CD4.CXCR4 cell line, high titer virus was taken from the P1 generation, into a second generation of Cf2Th.CD4.CXCR4 expressing cells (P2). Here, 70 µl of viral supernatant from day 10 of the P1 generation was used to infect each of the 14 wells of pre-seeded Cf2Th.CD4.CXCR4 cells. The 05ZAFV03 virus had reached the log phase of viral infectivity for the P2 by day 3 and had reached out of range p24 Ag by day 5 (Figure 3.3 (B)).

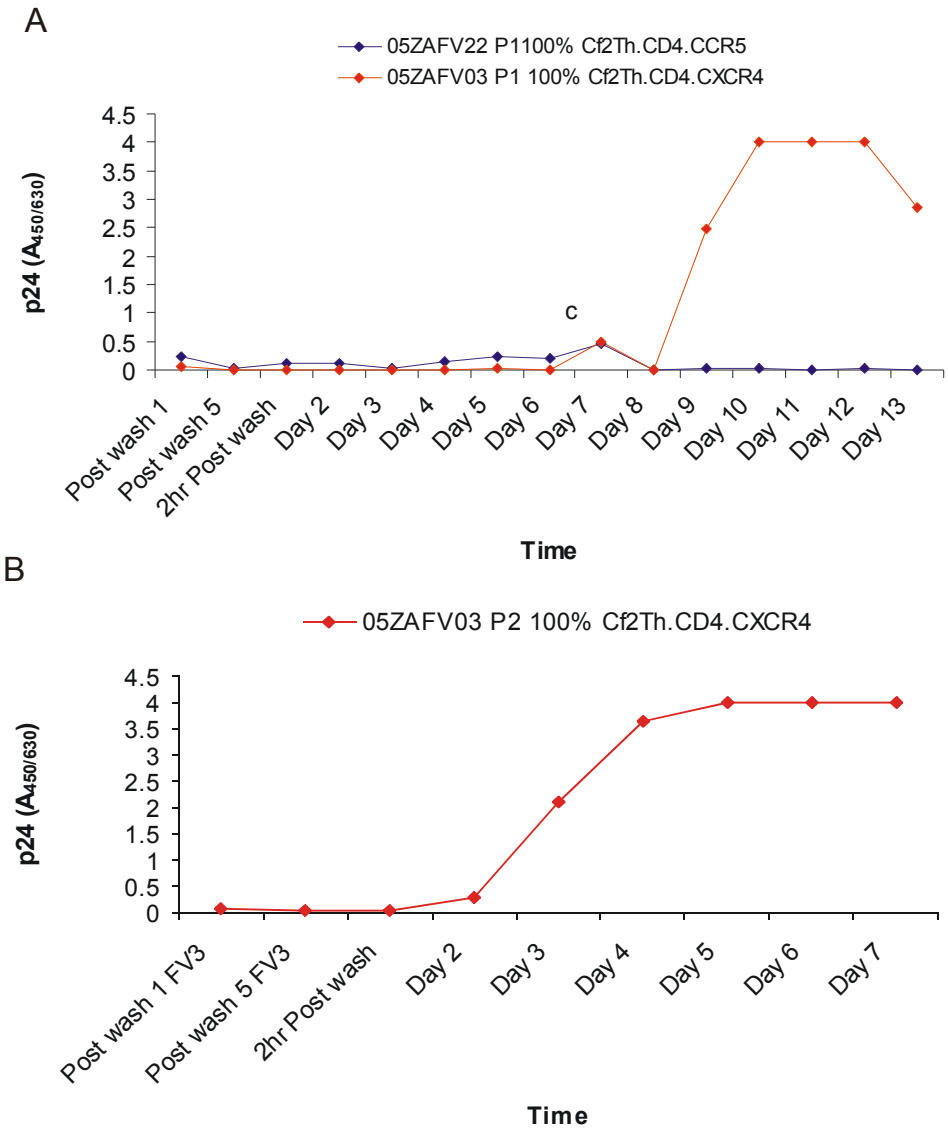


Figure 3.3 Graphical representation of successful infection of the P1 Cf2Th.CD4.CXCR4 cell line with 05ZAFV03 by day 9 post infection (red graph) and unsuccessful infection of the Cf2Th.CD4.CCR5 cell line with 05ZAFV22 (blue graph) (A). Graph representing successful infection of the second generation (P2) of Cf2Th.CD4.CXCR4 cells with 05ZAFV03 by day 3. Infection had reached a plateau by day 4-5 (B).

3.4.2 Adaptation of 05ZAFV03 towards CD4-Independence

The aim to achieve CD4-independent infection of the Cf2Th cell line by 05ZAFV03 was executed using the following methodical process (Figure 3.4).

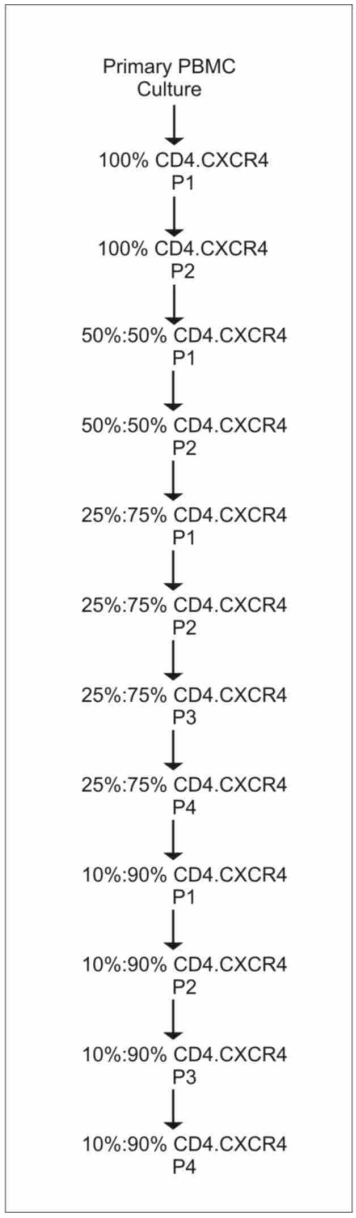


Figure 3.4 Schematic overview of the serial Cf2Th cell line passages of 05ZAFV03 grown in decreasing amounts of CD4 cell surface expression. As described in the Section **3.3**, there were sequential passages were performed with a volume of infectious cell-free viral supernatant from the preceding passage.

Infections performed with viral supernatant were either in duplicate, triplicate or quadruplicate, with most of the infections performed once, due to limiting volumes of infectious supernatant (Table 3.2). For all the infections performed, p24 Ag levels were over the detectable range between 5 - 12 days post infection. A total of

12 serial viral passages were performed in this experiment, towards viral growth in cells only expressing 10% cellular CD4 (Table 3.2).

In parallel with each viral passage, the same starting volume of input virus was used to directly infect the Cf2Th.CXCR4 (CD4 negative) cell line, however, this approach of achieving CD4-independent infection was not successful.

Table 3.2 Cell culture data for 05ZAFV03 growth as the CD4 cell surface concentration decreased towards CD4-independence.

Cell Culture Composition (CD4 percentage Expression %)	Number of Cell line passages		Number of culture replicates [c]	Culture time in days until OUT of range viral p24 detection [d]
	Sequential [a]	Total [b]		
100	1	1	1	10
100	2	2	1	5
50	1	3	1	6
50	2	4	1	12
25	1	5	2	7
25	2	6	3	10
25	3	7	3	10
25	4	8	4	5
10	1	9	1	9
10	2	10	1	10
10	3	11	1	11
10	4	12	1	12

[a] p24 positive sequential passages that were transferred to the same CD4 percentage expression uninfected fresh Cf2Th cells (see figure 3.3)
[b] Cumulative number of passages from first infectious viral supernatant.
[c] Most of the cell culture infections were performed in single wells. Only the 25%:75% CD4.CXCR4 infections were performed in replicates.
[d] All cell cultures reached log phase viral growth before two weeks.

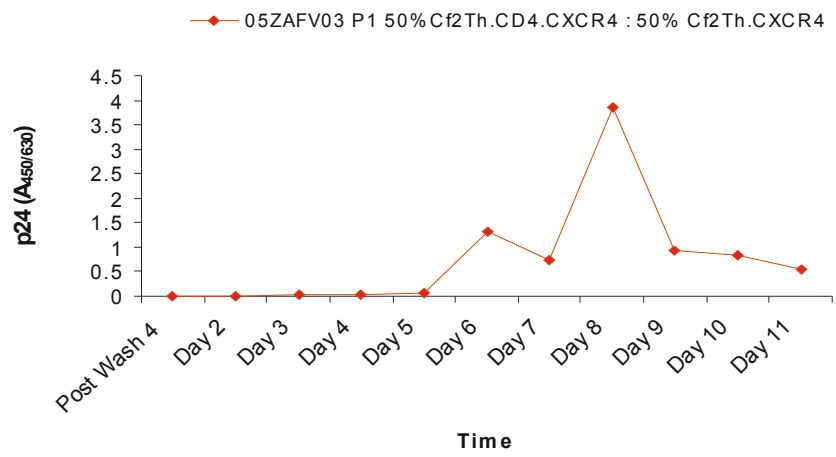
3.4.3 Passage 1 (P1) an P2 50% Cf2Th.CD4.CXCR4 : 50% Cf2Th.CXCR4

One hundred and fifty microlitres of the highest viral titer supernatant obtained from Day 5 in the P2 100% Cf2Th.CD4.CXCR4 infection, was used to infect the next generation of cells in the CD4-independent adaptation process; 50%:50%

expressing cells. As in the P1 100% Cf2Th.CD4.CXCR4 infection, the cells reached confluency by day 7, and infected cells and supernatants (and negative controls), were transferred into the 6 well NUNC culture plates.

Similarly, the P2 50%:50% infection was performed. Interestingly, viral supernatant from day 6 of the P1 50%:50% cell infection passage, yielded a higher infectious viral titer compared to viral supernatant from day 8, when used to infect the P2, despite the lower absorbance reading detected on day 6 (Figure 3.5).The two passages within the 50% CD4 expressing cell line population were successful. Two distinct peaks in infectious virus are visible, on days 6 and 8 in P1 and days 9 and 10 in P2.

A



B

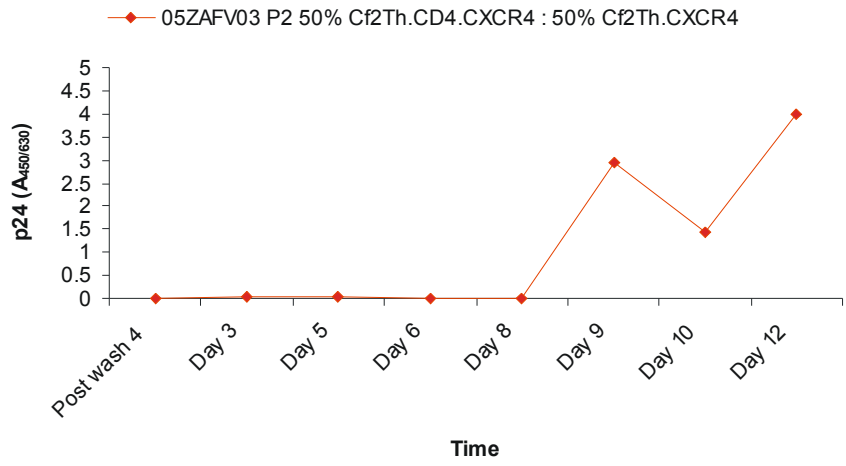


Figure 3.5 Graphical representation of successful P1 infection within the cell population comprising 50% Cf2Th.CD4.CXCR4 expressing cells and 50% Cf2Th.CXCR4 expressing cells (A). Graphical representation of the successful P2 50% Cf2Th.CD4.CXCR4 : 50% Cf2Th.CXCR4 infection (B).

3.4.4 P1, P2 and P3 25% Cf2Th.CD4.CXCR4 : 75% Cf2Th.CXCR4

The highest viral titer supernatant from P2 50%:50% day 12, was diluted two fold with supplemented DMEM growth medium and 200 µl viral supernatant was used

to infect each pre-seeded well of P1 25%:75% expressing cells. The highest viral titer supernatant from P1 (day 7) and P2 (day 10), were used to infect the second and third passages, respectively (Figure 3.6). Three passages of 05ZAFV03 within this ratio of cell lines, was performed to reinforce the evolving/adapting population of CD4-independent isolates. Each successive passage depicts a different viral growth curve from the previous passage.

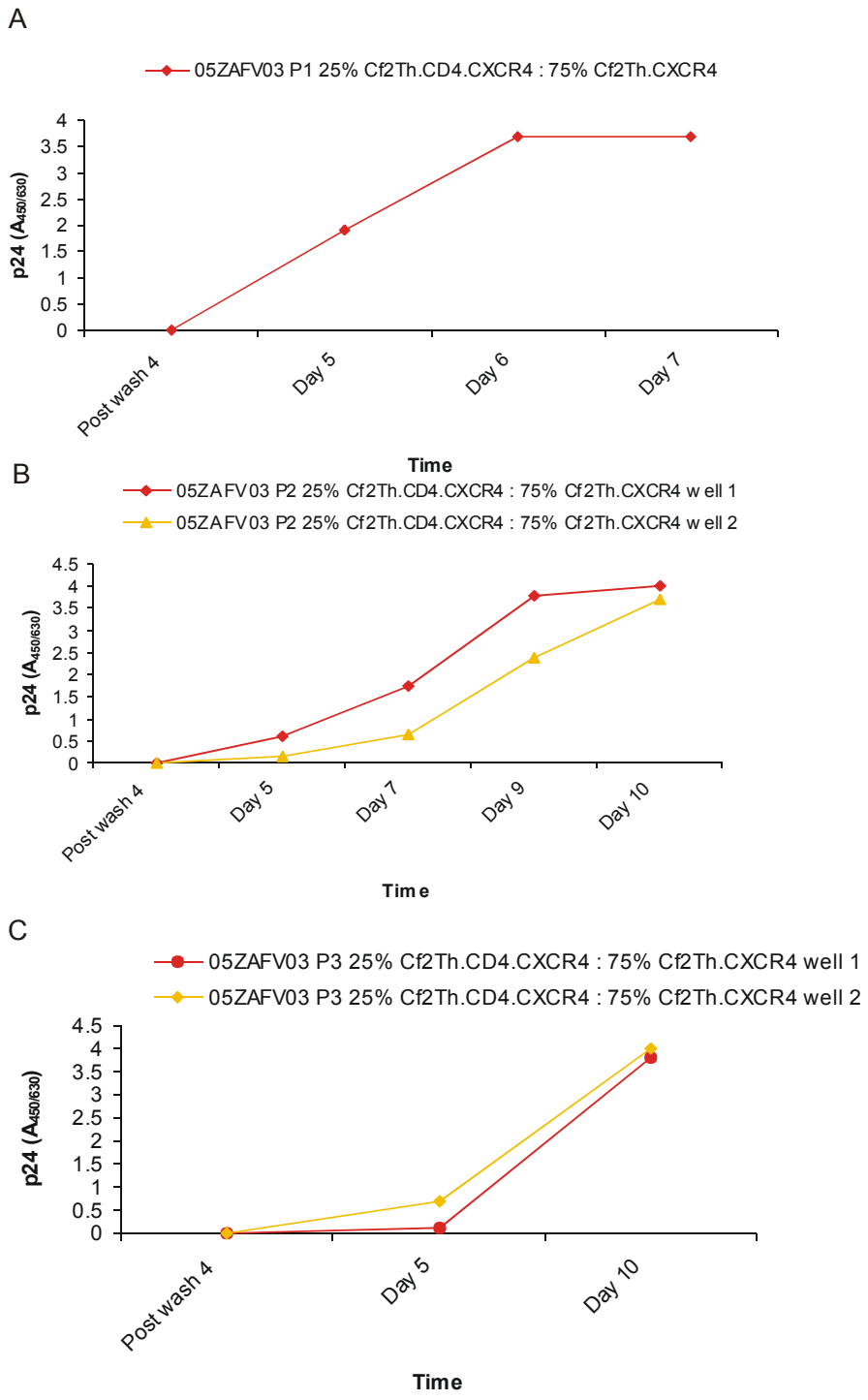


Figure 3.6 Successful infection by day 7 of the first passage of 25% Cf2Th.CD4.CXCR4 : 75% Cf2Th.CXCR4 (A). The viral supernatant was then used to infect the second passage, in parallel (B) and to further strengthen the viral infectivity of this percentage cell line, the viral supernatant was taken through a passage 3, again in parallel (C).

3.4.5 Cell-Cell Infection

Fifty thousand infected cells from Day 10 of the P3 25%:75% were successfully co-cultured with 7 x 10^5 uninfected Cf2Th.CXCR4 expressing cells in T25 flasks (AEC Amersham, Denmark), in a total culture volume of 8 ml supplemented DMEM growth medium. Two separate infections were performed from the two separate successful P3 25%:75% adaptations (Figure 3.7).

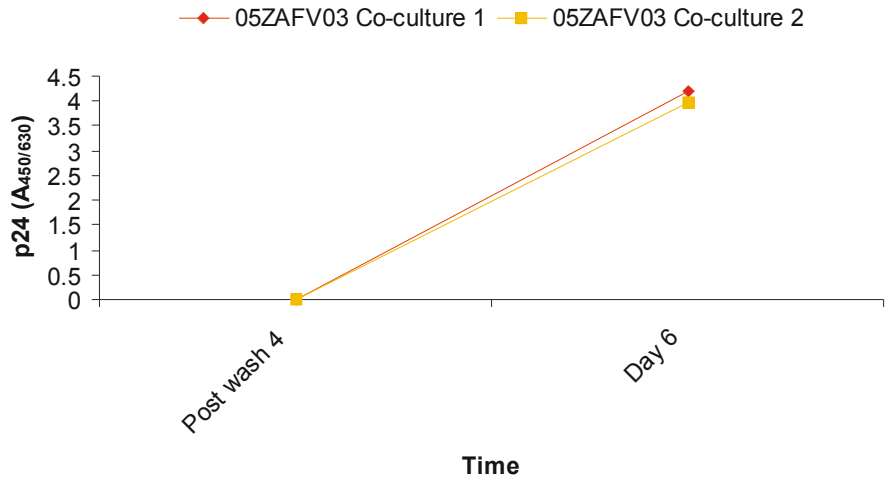


Figure 3.7 Co-culture of infected cells from P3 25% Cf2Th.CD4.CXCR4 : 75% Cf2Th.CXCR4 with uninfected CXCR4 expressing cell in a ratio of 1:1400.

3.4.6 P1, P2, P3 and P4 10% Cf2Th.CD4.CXC4 : 90% Cf2Th.CXCR4

The P1 10%:90% was infected with 410 µl of the day 6 viral supernatant from the highest viral titer obtained in the co-cultured infection. The viral supernatant was harvested on day 7 and 500 µl of P1 was used to infect P2. Similarly, 400 µl of the highest viral titer from day 7 of P2 was used to infect P3 and finally, 300 µl from the peak of the viral growth curve on day 7, was used to infect P4 (Figure 3.8). Infection of all four passages was successful.

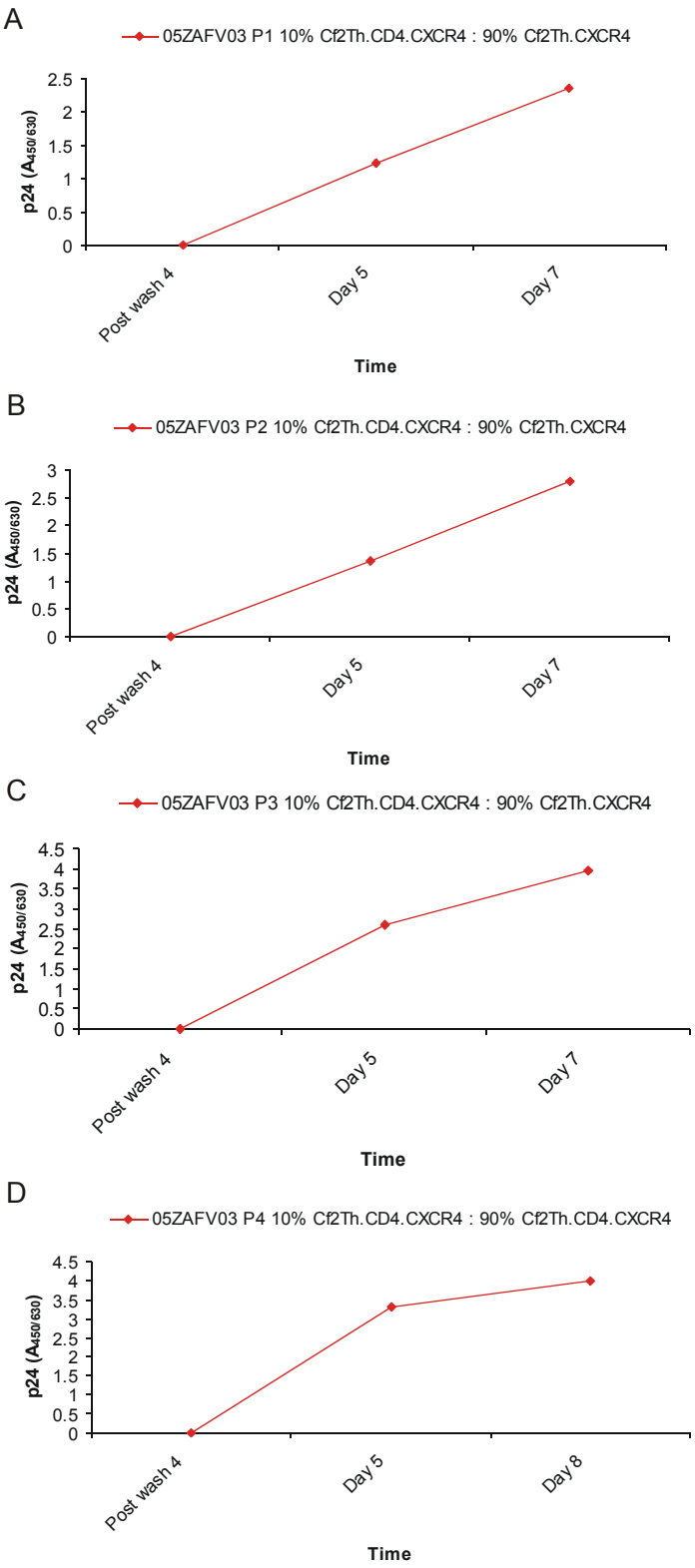


Figure 3.8 Graphical representations of the P1 – P4 of 10% Cf2Th.CD4.CXCR4 : 90% Cf2Th.CXCR4 viral growth over 7 – 8 days represented by graphs A – D respectively.

3.4.7 P1 1% Cf2Th.CD4.CXC4 : 99% Cf2Th.CXCR4 and P1 100% Cf2Th.CXCR4

From day 8 of the P4 10%:90%, 300 µl viral supernatant was used to infect the final passage containing only 1% of the Cf2Th.CD4.CXCR4 expressing cells (1%:99%). The viral growth curve peaked at day 6 and the culture was subsequently expanded into a larger volume, to accommodate the rapid cell growth. The numbering of the days was re-started from 1 after the culture was expanded. However, the infectious viral titer was diluted after expansion and the p24 Ag levels in the culture were unrecoverable (Figure 3.9 (A)). This experiment was not repeated due to insufficient starting volume of infectious virus and insufficient volume of virus from day 6.

Seven hundred microlitres of viral supernatant from the expanded P1 1% Cf2Th.CD4.CXCR4 : 99% Cf2Th.CXCR4 day 4 culture was used to infect the P1 100% Cf2Th.CXCR4 expressing cells. No growth was detected here (Figure 3.9 (B)).

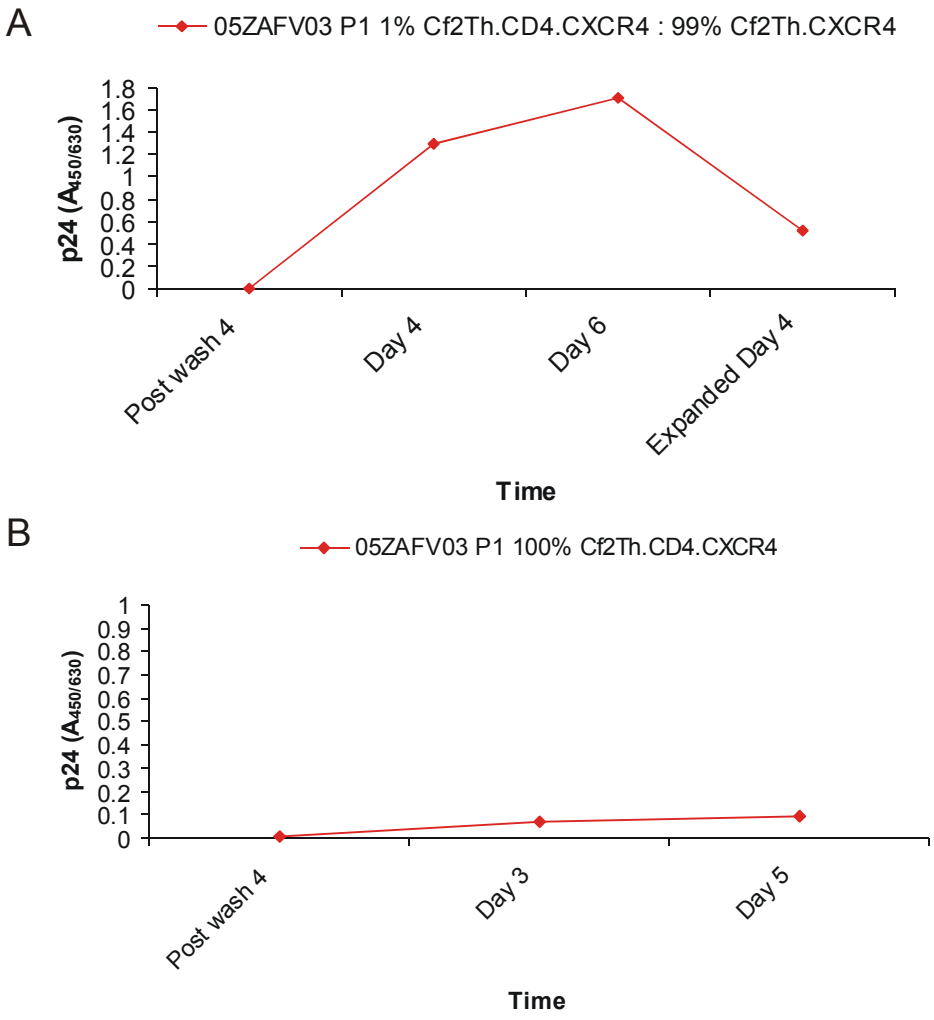


Figure 3.9 (A) Graphical representation of the P1 1% Cf2Th.CD4.CXCR4 : 99% Cf2Th.CXCR4 viral growth curve over almost 2 weeks. The viral culture was expanded on day 6 and the days were continually number from one, one day post expansion. (B) Graphical representation of the minimal 05ZAFV03 viral growth in the 100% Cf2Th.CXCR4 expressing cell line by day 5.

3.4.8 Uninfected and Infected Cf2Th cells

No CPEs were ever detected when viral replication in these cell lines was present.

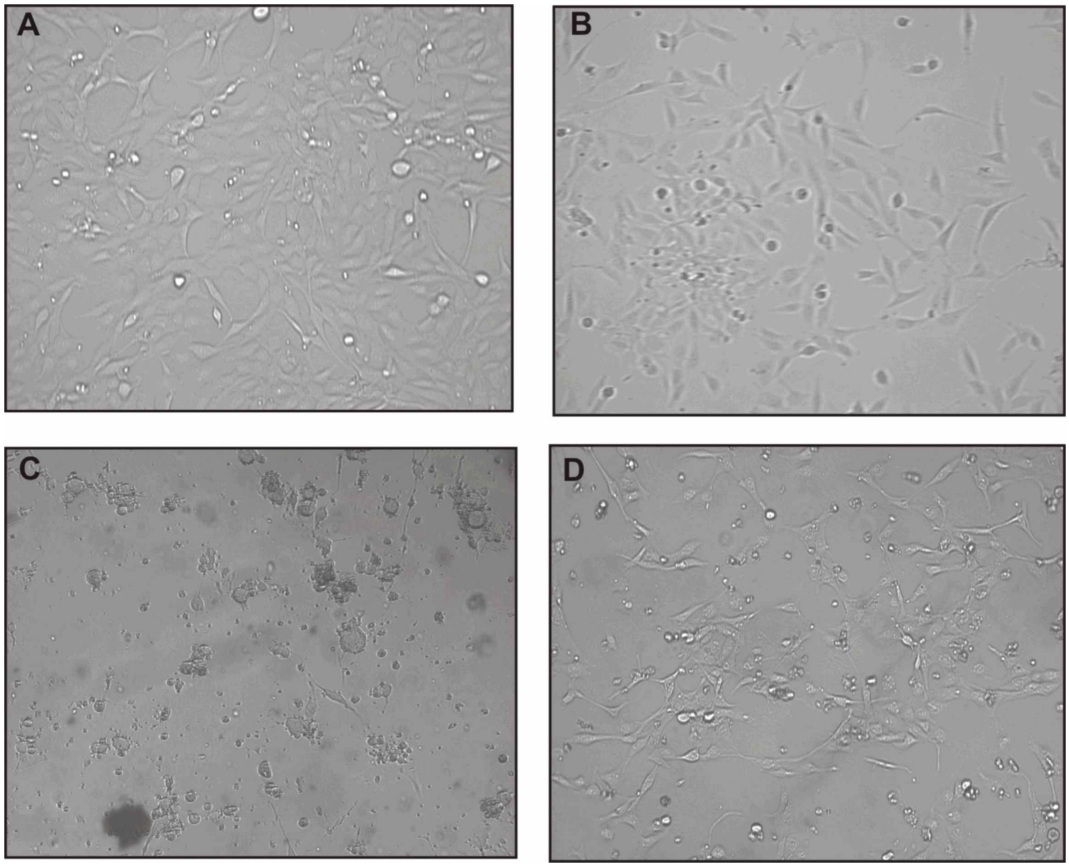


Figure 3.10 Uninfected Cf2Th cells (A) Cf2Th.CD4.CXCR4 expressing and (B) Cf2Th.CXCR4 expressing, respectively, and infected Cf2Th cells with 05ZAFV03 (C) Cf2Th.CD4.CXCR4 expressing and (D) Cf2Th.CXCR4 expressing, respectively. Cytopathic effects and cell death can be seen in Cf2Th.CD4.CXCR4 expressing cells, panel C. However, there are negligible, if any, cytopathic effects in infected Cf2Th.CXCR4 expressing cells, panel D. All images were acquired at 10 X magnification on the Zeiss Axiovert 100 M microscope (Zeiss, Göttingen, Germany).

3.4.9 Amplification and Sequencing of Full-Length gp160

The amino acid changes arising within *env* responsible for the adaptation of 05ZAFV03 to cell culture conditions of low CD4 expression were identified by sequencing the gp160 regions. Cleaned PCR amplified gp160 regions are shown (Figure 3.11).

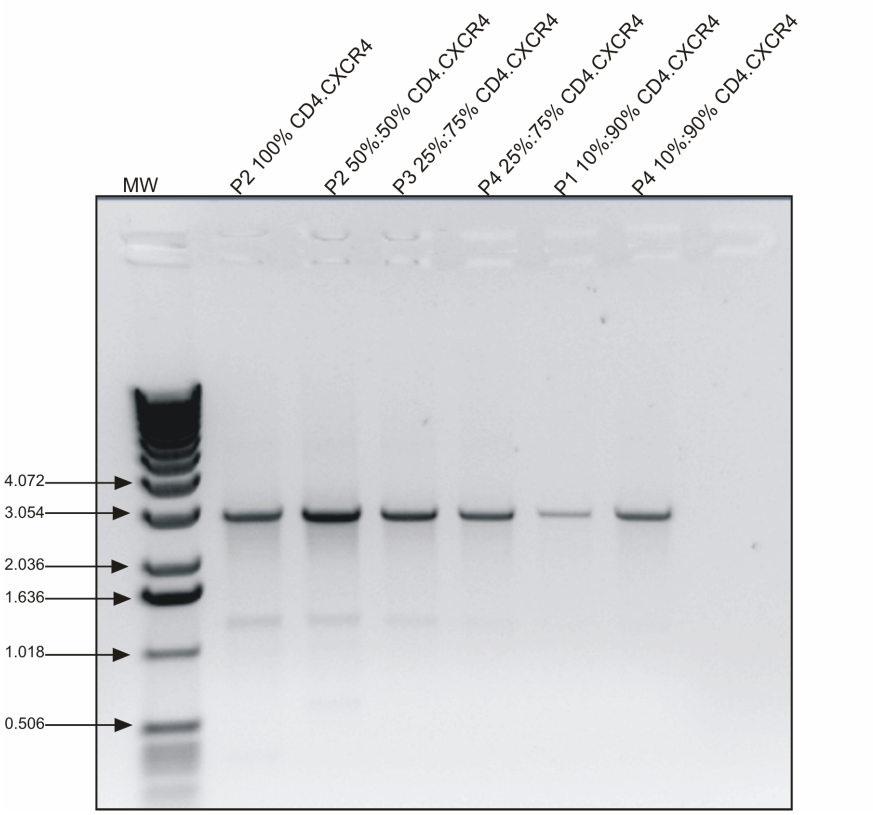
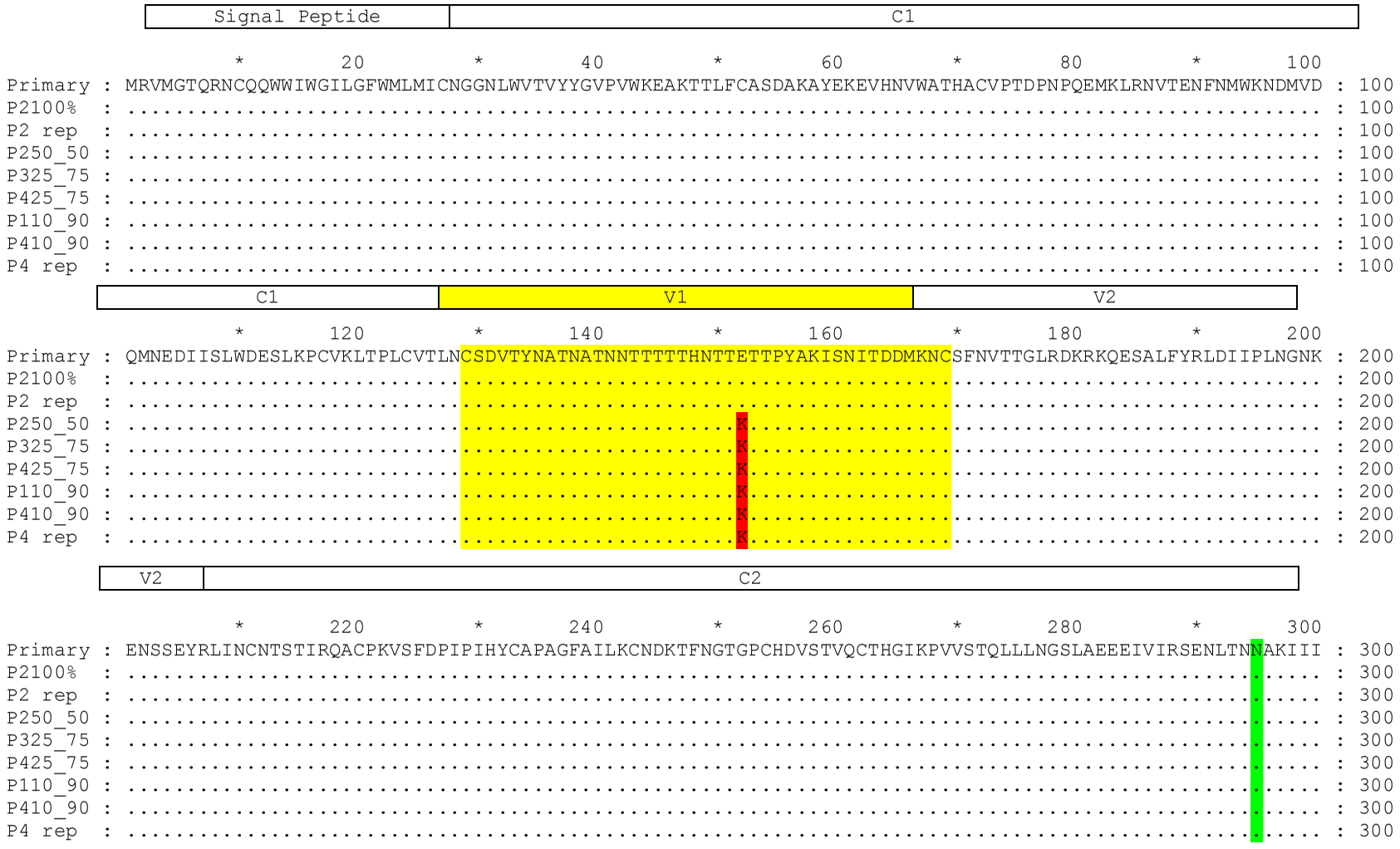
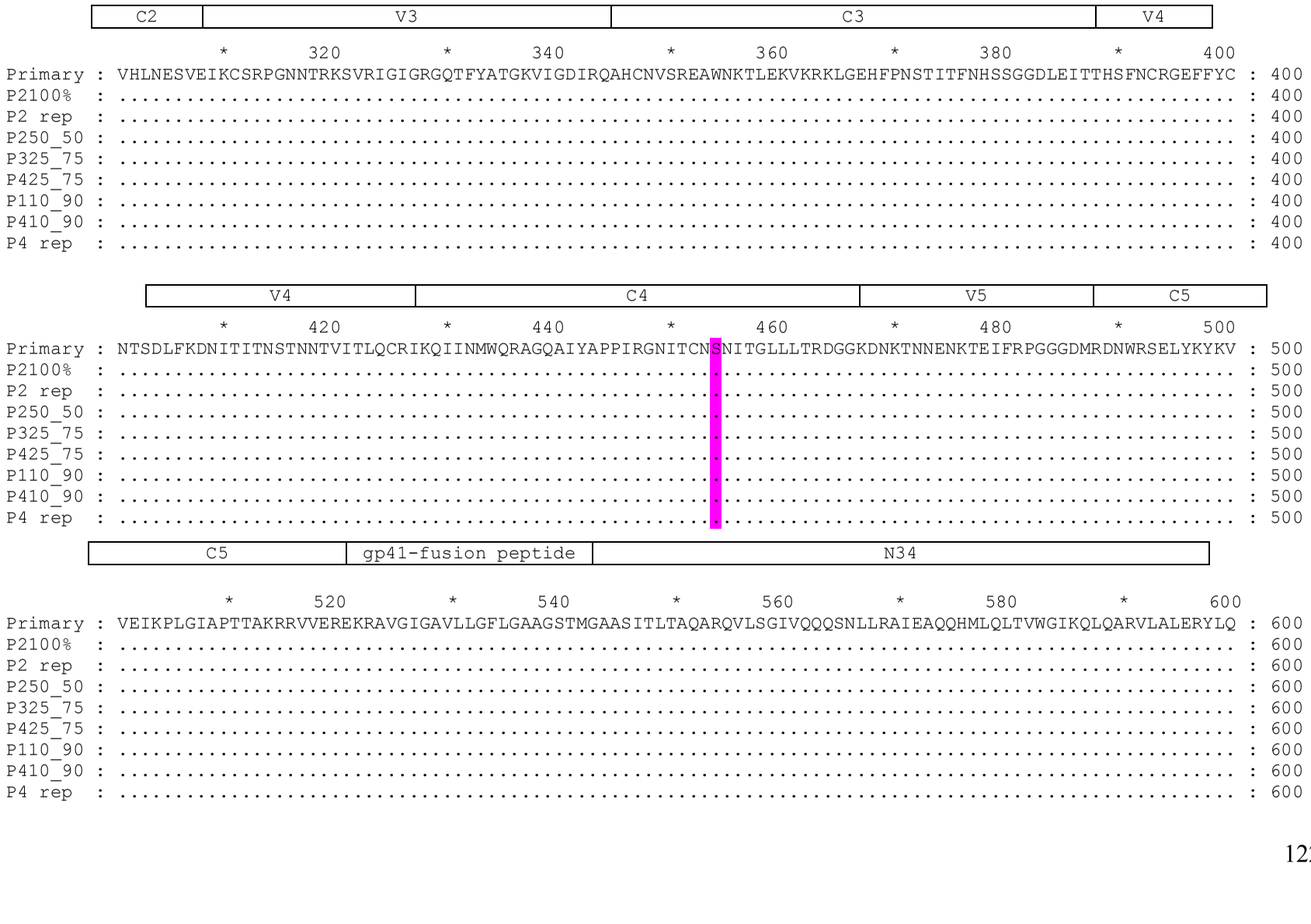


Figure 3.11 Cleaned gp160 PCR Products (~ 2.7 kb) of all the isolates chosen for sequence analysis for the adaptation towards CD4-independence. The DNA Molecular Weight Marker (MW) X (Roche, Mannheim, Germany) was used and 10 µl of each PCR product was electrophoresed in a 0.8% Agarose gel (Sigma, St Louis, MO).

The following cultured passages were used to amplify proviral gp160 sequences; P2 from 100% Cf2Th.CD4.CXCR4, P2 from 50%:50%, P3 and P4 from 25%:75% and finally P1 and P4 from 10%:90%.

To reveal molecular changes within the gp160 region that associated with cell line adaptation and growth in low levels of CD4 expression, detailed analysis of the sequences electropherograms was performed. Overall, there were 3 nucleotide differences between all the cultured gp160 sequences and the primary gp160 parental sequence. These differences resulted in two synonymous mutations and one non-synonymous mutation (Figure 3.12). PCR amplification of the P2 100% Cf2Th.CD4.CXCR4 and the P4 10%:90% was repeated twice on different days, to confirm the results and prove the absence of contamination.


Signal Peptide
C1
C1
V1
V2
V2
C2
Primary : MRVMGTQRNCQQWWIWGILGFWMLMICNGGNLWVTVYYGVPVWKEAKTTLFCASDAKAYEKEVHNVWATHACVPTDPNPQEMKLRNVTENFNMWKNDMVD : 100
P2100% : ..... : 100
P2 rep : ..... : 100
P250_50 : ..... : 100
P325_75 : ..... : 100
P425_75 : ..... : 100
P110_90 : ..... : 100
P410_90 : ..... : 100
P4 rep : ..... : 100
Primary : QMNEDIISLWDESLKPCVKLTPLCVTLNCSDVTYNATNATNNTTTTTHNTTETTPYAKISNITDDMKNCSFNVTTGLRDKRKQESALFYRLDIIPLNGNK : 200
P2100% : ..... : 200
P2 rep : ..... : 200
P250_50 : ..... : 200
P325_75 : ..... : 200
P425_75 : ..... : 200
P110_90 : ..... : 200
P410_90 : ..... : 200
P4 rep : ..... : 200
Primary : ENSSEYRLINCNTSTIRQACPKVSFDPIPIHYCAPAGFAILKCNDKTFNGTGPCHDVSTVQCTHGIKPVVSTQLLLNGSLAEEEIVIRSENLTNNAKIII : 300
P2100% : ..... : 300
P2 rep : ..... : 300
P250_50 : ..... : 300
P325_75 : ..... : 300
P425_75 : ..... : 300
P110_90 : ..... : 300
P410_90 : ..... : 300
P4 rep : ..... : 300


C2
V3
C3
V4
* 320 * 340 * 360 * 380 * 400
Primary : VHLNESVEIKCSRPGNNTRKSVRIGIGRGQTFYATGKVIGDIRQAHCNVSREAWNKTLEKVKRKLGEHFPNSTITFNHSSGGDLEITTHSFNCRGEFFYC : 400
P2100% : 400
P2 rep : 400
P250_50 : 400
P325_75 : 400
P425_75 : 400
P110_90 : 400
P410_90 : 400
P4 rep : 400
V4
C4
V5
C5
* 420 * 440 * 460 * 480 * 500
Primary : NTSDLFKDNITITNSTNNTVITLQCRIKQIINMWQRAGQAIYAPPIRGNITCNSNITGLLLTRDGGKDNKTNNENKTEIFRPGGGDMRDNWRSELYKYKV : 500
P2100% : 500
P2 rep : 500
P250_50 : 500
P325_75 : 500
P425_75 : 500
P110_90 : 500
P410_90 : 500
P4 rep : 500
C5
gp41-fusion peptide
N34
* 520 * 540 * 560 * 580 * 600
Primary : VEIKPLGIAPTTAKRRVVEREKRAVGIGAVLLGFLGAAGSTMGAASITLTAQARQVLSGIVQQQSNLLRAIEAQQHMLQLTVWGIKQLQARVLALERYLQ : 600
P2100% : 600
P2 rep : 600
P250_50 : 600
P325_75 : 600
P425_75 : 600
P110_90 : 600
P410_90 : 600
P4 rep : 600

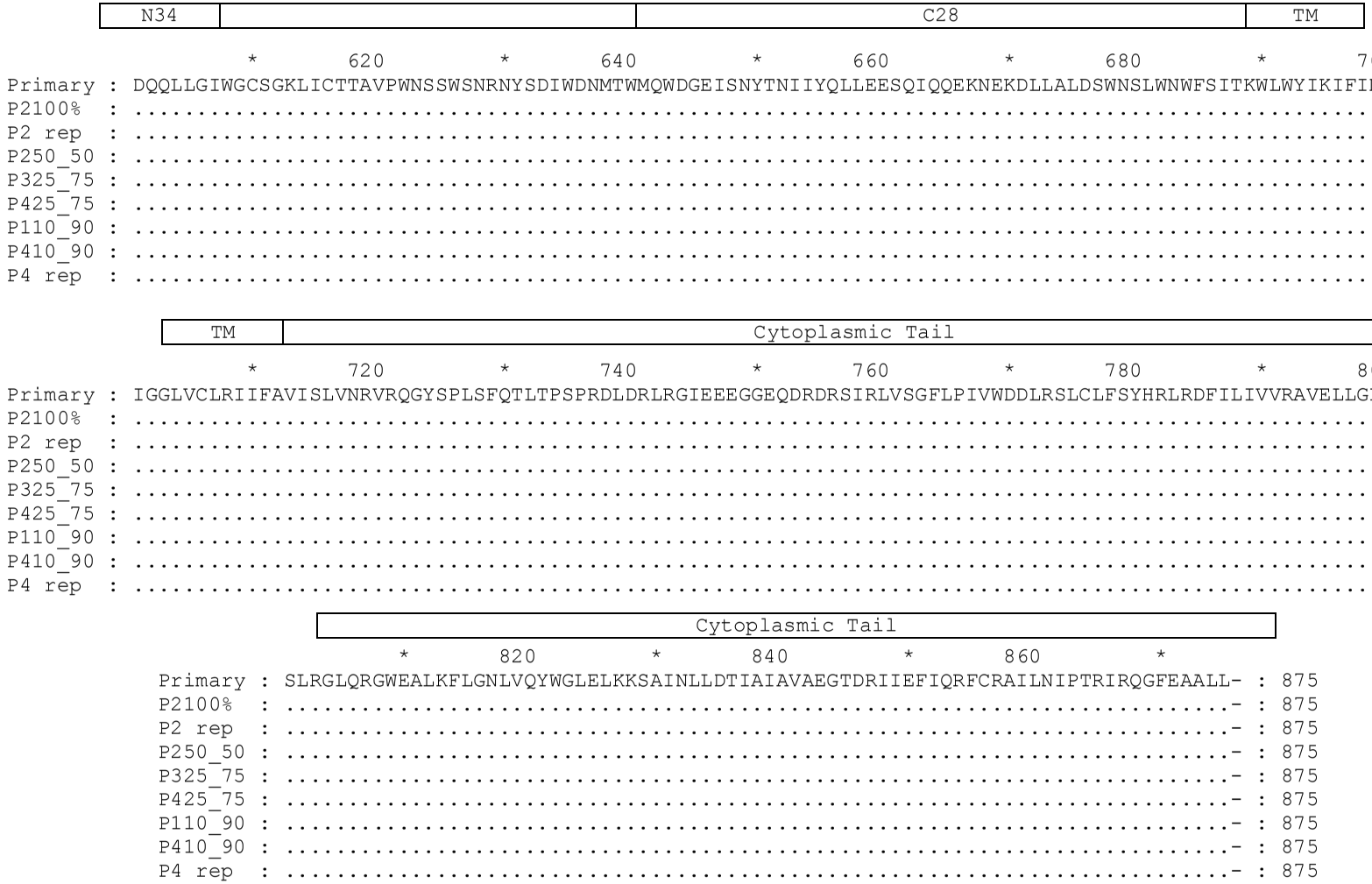

N34
C28
TM
620
640
660
680
700
Primary : DQQLLGIWGCSGKLICTTAVPWNSSWSNRNYSDIWDNMTWMQWDGEISNYTNIIYQLLEESQIQQEKNEKDLLALDSWNSLWNWFSITKWLWYIKIFIMI : 700
P2100% : ..... : 700
P2 rep : ..... : 700
P250_50 : ..... : 700
P325_75 : ..... : 700
P425_75 : ..... : 700
P110_90 : ..... : 700
P410_90 : ..... : 700
P4 rep : ..... : 700
TM
Cytoplasmic Tail
720
740
760
780
800
Primary : IGGLVCLRIIFAVISLVNRVRQGYSPLSFQTLTPSPRDLDRLRGIEEEGGEQDRDRSIRLVSGFLPIVWDDLRSLCLFSYHRLRDFILIVVRAVELLGRS : 800
P2100% : ..... : 800
P2 rep : ..... : 800
P250_50 : ..... : 800
P325_75 : ..... : 800
P425_75 : ..... : 800
P110_90 : ..... : 800
P410_90 : ..... : 800
P4 rep : ..... : 800
Cytoplasmic Tail
820
840
860
Primary : SLRGLQRGWEALKFLGNLVQYWGLELKKSAINLLDTIAIAVAEGTDRIIEFIQRFCRAILNIPTRIRQGFEAALL- : 875
P2100% : .....- : 875
P2 rep : .....- : 875
P250_50 : .....- : 875
P325_75 : .....- : 875
P425_75 : .....- : 875
P110_90 : .....- : 875
P410_90 : .....- : 875
P4 rep : .....- : 875

Figure 3.12 Alignment of the predicted amino acid sequences of the parental (primary) sequence with the sequences obtained from the Cf2Th cell line serial passage variants with decreasing amounts of CD4 expression. Variable (V), constant (C) and other regions of the gp160 region are indicated. Synonymous mutations are indicated at amino acids 295 (in green) and at 454 (in pink). The non-synonymous mutation within the V1 loop (in yellow) is indicated by the E to K mutation (in red) shown at position 152. Primary refers to the primary 05ZAFV03 gp160 sequence (section 2.4.5); P2 100% refers to gp160 sequence from the P2 100% Cf2Th.CD4.CXCR4; P2 rep refers to the repeated P2 100% sequence; P2 50%50% refers to gp160 sequence from the P2 50% Cf2Th.CD4.CXCR4 : 50% Cf2Th.CXCR4; P3 25%:75% refers to gp160 sequence P3 25% Cf2Th.CD4.CXCR4 : 75% Cf2Th.CXCR4; P4 25%:75% refers to gp160 sequence from the co-culture; P1 10:90% refers to gp160 sequence from the P1 10% Cf2Th.CD4.CXCR4 : 90% Cf2Th.CXCR4; P4 refers to gp160 sequence from the P4 10% Cf2Th.CD4.CXCR4 : 90% Cf2Th.CXCR4 and P4 rep refers to the repeated P4 10%:90% sequence. The dots represent identical amino acids.

3.5 Discussion

HIV-1 is currently the most thoroughly researched human pathogen and there is a wealth of knowledge pertaining to its *in vivo* evolution, however, *in vitro* HIV-1 evolution is less widely researched. To date, HIV-1 Subtype C isolates have never been adapted to CD4-independence. The present study aimed to adapt an HIV-1 isolate towards CD4-independence and analyze the genetic changes that accompanied serial passage of a CXCR4 utilizing HIV-1 isolate in the Cf2Th cell line with decreasing concentration of CD4 expressing cells over time. The gp160 sequence analysis showed that *env* is relatively conserved throughout serial *in vitro* passage with decreasing cellular CD4 expression, and a single non-synonymous nucleotide mutation (E152K) was detected within the V1 loop compared to the parental sequence.

Here, a CXCR4 utilizing HIV-1 subtype C isolate, 05ZAFV03, was adapted and passaged 12 times through the Cf2Th cell line. An attempt was made to adapt the isolate towards CD4-independence and the isolate was adapted to grow in cell cultures where only 10% of the cells expressed CD4. All the viral infections throughout this study were either performed in duplicate, triplicate or quadruplicate to increase sample volume and to ensure reproducibility of each passage. The 05ZAFV03 isolate did require serial *in vitro* passages within cell populations of gradually decreasing amounts of CD4 expression, as the primary isolate was unable to directly infect cell populations with no CD4 expression (data not shown). The rapid detection of viral p24 Ag post infection *in vitro* may be a reflection of the aggressive and highly pathogenic nature of the 05ZAFV03 isolate.

Despite several attempts to adapt CCR5 utilizing HIV-1 subtype C isolates to *in vitro* growth in the Cf2Th cell lines, successful T cell line infection for this viral tropic variant was not established. Reasons for this are speculative at this stage. There may be some subtype C specific inhibitory mechanism that resists HIV-1 CCR5 utilizing variants from adapting to the Cf2Th cell line.

The first passage (P1) of the CXCR4 utilizing HIV-1 05ZAFV03 isolate, adapted relatively rapidly to *in vitro* growth in the Cf2Th.CD4.CXCR4 cell line. By day 10

of P1, the viral titer had reached the log phase and the absorbance was out of the detection range of the spectrophotometer (Figure 3.3 (A)). Two stable rounds of infection were performed for the 05ZAFV03 isolate within the 100% Cf2Th.CD4.CXCR4 cell line.

Similarly, two successful rounds of viral passage were achieved in the 50%:50% cell line population. The repeated passage (P2) was necessary for the reinforcement of any mutational derived genotypic and phenotypic adaptive changes that may have occurred within the *env* (Figure 3.5 (B)). There are two distinct viral infectious peaks at days 6 and 8 in P1 and days 9 and 12 in P2, with a drastic decrease in infection on day 7 and 10, respectively (Figure 3.5). There may be two explanations for such a phenomenon.

Firstly, due to the fact that infections were performed in 24 NUNC culture plates, for each successive day post infection a single well was harvested each day, achieving the effect of following the infection day by day, all wells were treated equally. Here, there may have been well-to-well variation of viral p24 Ag titer and this may have contributed to the perceived "dip" in infectivity. It is unlikely that such well-to-well variation would occur twice in such a similar pattern.

Another explanation might be that each peak in viral infectivity may be a consequence of two separate phenotypic viral populations within the viral supernatant (Figure 3.5). One population of viruses may have budded from the Cf2Th.CD4.CXCR4 expressing cell population and another viral population may have emerged from the Cf2Th.CXCR4 expressing cells. Both populations of target cells (CD4 positive and CD4 negative) were present in equal proportions in this infection and it is reasonable to assume that the first peak in viral growth may have come from the Cf2Th.CD4.CXCR4 cell population due to the viral selective advantage and preference for this cell line. However, there is more "conformational stress" on the Env to enter CD4 negative cells. This may be the reason for a second peak being observed two days later - CD4-independent viral production emerging from Cf2Th.CXCR4 expressing cells. Thus two different

homogenous viral populations may have existed one after the other. However, this hypothesis is unlikely as the virus harvested from either peaks, was incapable of growth in 100% CD4 negative Cf2Th cells.

Each viral growth curve differs from the previous curve in the 25%:75% cell line infections. The first passage, (Figure 3.6 (A)), shows a relatively rapid adaptation to the cell line with the virus reaching log growth phase growth levels by day 5. However, in P2 and P3, (Figure 3.6 (B) and (C), respectively), the virus had established stable infection within culture in a slightly longer time period. This might be due to the decrease in actively replicating CD4.CXCR4 tropic virions due to their overall reduced target cell count. This may then lead to the gradual increase in the population of viruses that are evolving/adapting towards the CD4-independent phenotype.

Relatively rapid viral growth was detected in co-culture, as within 6 days of infection, the viral p24 Ag levels were out of range of detection. This high viral growth rate may indicate the viral adaptation to the changing target cell range. All four passages of the 10%:90% cell line ratio, were successful. Here, the minority (10%) of cells in culture expressed cellular CD4, thus increasing the probability of viruses infecting the 90% of the remaining CD4 negative cells. In theory, the experimental process was forcing/favouring the adapted, pre-triggered Env conformational state. A population of the virions in culture may have been under selective pressure to enter the Cf2Th cells via CXCR4 coreceptor binding alone.

A single round of infection within the P1 1%:99% cell line was successful. Viral titer reached a peak by day 6 and the cells had over-grown and reached confluency within the culture well. The infection was expanded into a larger volume and the expansion was not successful. This passage was the final viral passage reached due to time being a limiting factor as well as the depletion of stored viral supernatant in repeated infections and sequencing. The P1 100% CXCR4 cell line infection was unsuccessful and yielded low viral titers that were detected day 5. Due to an

insufficient volume of stored viral supernatant this experiment was not able to be repeated.

At each stage of the viral adaptation process, viral supernatant was used in parallel to directly infect CD4 negative Cf2Th cells, which did not result in infection. This implies that the necessary changes in the Env of 05ZAFV03 required to achieve CD4-independence have not been achieved in this study, and supports continued passage of the virus with sequential decreases in the proportion of CD4 expressing Cf2Th cells in cell culture.

Overall, the CXCR4-utilizing HIV-1 subtype C 05ZAFV03 isolate grew in cell cultures where 10% of the cells expressed CD4 however, adaptation towards CD4-independence was unsuccessful. Since this strategy was previously successful in adapting a CCR5 utilizing HIV isolate to CD4-independence [349], it raises the possibility that it is more difficult/not possible to adapt CXCR4 utilizing isolates to CD4-independence or the 05ZAFV03 isolate needs to be passaged for longer to achieve CD4-independence.

The full-length gp160 sequence data from all six stages of the serial *in vitro* passage adaptation experiments towards decreased CD4 expression levels, were analyzed and compared to the parental primary gp160 sequence (see section 2.4.5). The HIV-1 virus that was passaged in the 100% Cf2Th.CD4.CXCR4 expressing cell line showed no mutations compared to the parental strain. Upon genotypic analysis, three mutations were detected within the *env* region in all the passages from the P2 50%:50% through till the final P4 1%:99% adapted isolate.

A single non-synonymous nucleotide mutation was detected at nucleotide position 454 (G to A) between the primary sequence, P2 100% Cf2Th.CD4.CXCR4, and the rest of the cultured sequences. This translated to an amino acid change at position 152 (according to the numbering of Consensus_C), between the N (position 125) and C (position 194) termini within the V1/V2 loop region, specifically at the mid-point of the V1 loop. The E152K mutation was detected in every *env* gene obtained from the samples that were grown in Cf2Th cell

concentrations with less than 100% CD4 expression (Figure 3.12). The single amino acid change that was detected in 5 out of 6 *env* sequences resulted from a negatively charged Glutamate (G) to a highly positive Lysine (K) at position 152 within the V1 region of gp160 (Figure 3.12), altering the size and charge of the the residue within the V1 loop.

The other two mutations were synonymous nucleotide differences detected at position 885 (C to T) between the primary sequence and all the sequences derived from culture and at position 1362 (A to T) between the primary, P2 100% Cf2Th.CD4.CXCR4, P2 50%:50% and the remaining sequences derived from culture. These synonymous mutations did not result in amino acid changes at positions 295 and 454, within the C2 and C4 regions of gp160 respectively (according to the numbering of Consensus_C) (Figure 3.12).

The acquisition of a single highly positive Lysine within the V1/V2 loop, may also increase the overall electrostatic attraction of the Env for the highly negatively charged CXCR4 chemokine coreceptor [434].

The CD4 epitope that sterically interacts with HIV-1 Env harbours a highly positive residue [66] which is supposedly electrostatically attracted to the negatively charged CD4 binding pocket within the V1/V2 loops. The non-synonymous E152K mutation detected in this study may electrostatically repel the positive epitope of the CD4 molecule, therefore decreasing frequency of the initial binding of CD4 by the 05ZAFV03 HIV-1 Env. A sequence alignment was performed in GeneDoc (V2.6.003) between the gp160 gene obtained in this study harbouring the E152K mutation, and the full spectrum of HIV-1 *env* sequences in the Los Alamos database [46]. About 0.6% (6/1000) of the HIV-1 *env* sequences from the database that were aligned to the gp160 region of interest, harboured positive amino acid residues at this position (152 according to Consensus_C numbering) within the V1 loop, however, the positive lysine was not a rare amino acid within the V1 loops compared.

The V1/V2 loop structure is in direct contact with the CD4 receptor and this loop contributes two strands to the bridging sheet of gp120, which is intimately involved in coreceptor binding [66, 414]. Therefore, mutational changes in this region may disrupt the normal binding process that takes place between CD4 and gp120, leading to the CD4-independent conformational state. There are eight potential N-linked glycosylation sites within the primary 05ZAFV03 V1 region and an extension of the V1 region by 15 amino acids was noted, when compared to the HXBc2 gp160 reference sequence. Changes in the length and N-linked glycosylation profile of the V1/V2 loops in gp120 are generally necessary for the CD4-independent phenotype in some HIV and SIV isolates. Further studies are necessary to determine exactly which changes mediate the decreased CD4-dependent phenotype in HIV-1 subtype C.

Of the CD4-independent HIV and SIV isolates characterized thus far, five research groups have also detected amino acid substitutions in the V1/V2 region of gp160 [350, 355, 374, 379, 435]. La Branche *et al*., [350] characterized an HIV-1 IIIBx CXCR4 tropic CD4-independent isolate which harboured mutations in the trans membrane domain, V1/V2, V3 and C4 regions of gp160. Of the mutations within the V1/V2 loop, I165K and Q170K substitutions, conferred two positive lysine residues in the V1 region that were previously absent in the CD4-dependent HIV-1 IIIB variant. Kolchinsky *et al*., [355] also reported on the acquisition of a lysine residue within the V1/V2 region (N197K) of the CD4-independent HIV-1 ADA variant. Gorry *et al*., [435] and Chenine *et al*., [374] showed extensions of the V1/V2 regions by 12 and 5 amino acids, respectively, in the CD4-independent isolates they derived.

The single non-synonymous nucleotide mutation, resulting after four serial *in vitro* passages obtained in this study, was not expected and it is in contrast to the large number of mutations observed in HIV-1 populations *in vivo* [76, 230, 231, 436-438]. Many mutations after serial passages *in vitro* with decreasing concentrations of CD4 expression were expected, due to the rapid replication rate of HIV-1 and the low fidelity of the reverse transcriptase enzyme [22, 27]. The gp160 gene is the

most variable and highly diverse gene within the HIV-1 virion and sequence variation within this gene increases at a rate of about 1-2% per year [15, 439-441]. This phenomenon is utilized by HIV-1 to adapt and avoid many selective pressures such as host neutralizing antibodies, cytotoxic T lymphocytes and antiretroviral drugs *in vivo* [176, 177, 442-444]. However, these selective pressures are not present *in vitro* and the HIV-1 *env* experiences less pressure to adapt and escape.

Due to the low fidelity of the HIV-1 genome replication system, a population of related, yet genetically heterogeneous circulating HIV-1 viral variants exist within an infected individual [445-447]. The population of related, yet distinct genomes, is referred to as the quasispecies of circulating HIV-1 [446, 448]. During *in vitro* culture, pre-existing quasispecies may emerge from the background parental population and reach lower levels of genetic diversity. The adaptation of primary HIV-1 to growth in T cell lines generally selects for and encourages the emergence of pre-existing variants from the quasispecies which have a heightened sensitivity to neutralization [449-452].

The chromatograms obtained in this study underwent extensive sequence analysis and assembly in Sequencher V4.5 and no quasispecies were detected in proportions >10%. This is an unusual result when analyzing population based *env* sequences and may suggest that each population was almost 100% homogeneous and that the predominating quasispecies *in vivo* may have been selected for *in vitro*. These data imply that it may be valuable to further investigate and compare the quasispecies present within the patient at the time of viral isolation.

To exclude the possibility of PCR cross-contamination, the proviral DNA extraction (using a brand new High Pure PCR Template Preparation Kit (Roche, Penzberg, Germany)), PCR experiments and sequencing reactions were performed (and repeated) on separate days. The same sequencing results were obtained each time and all the negative controls for all experiments were confirmed negative.

In general, when RNA viruses undergo repeated bottleneck passages or multiplication of their genetic material from a small/one viral genome *in vitro* the

average fitness of the viral population decreases [78, 453, 454]. The phenomenon of an observed decrease in fitness caused by repeated bottlenecks is known as the Muller's ratchet effect [454]. According to this model, small asexual populations will incorporate deleterious mutations, unless recombination events can restore the functionality of their genome [454]. This effect has been seen in many viruses such as the bacteriophage φ6 [78], vesicular stomatitis virus [453], foot-and-mouth disease virus [455] and was first detected for HIV-1 in MT-4 plaque-to-plaque passages [456].

Yuste *et al.*, [456] and colleagues performed full-length HIV-1 genome sequencing and revealed that *gag* had a three-fold higher mutation frequency compared to *env,* and the V3 loop remained conserved, after fifteen passages. The amino acid changes that had accumulated throughout the passages were uncharacteristic and several had not been previously recorded in natural occurring HIV-1 isolates [456].

Yuste *et al.*, [457] also detected few mutations throughout the HIV-1 viral genome which were associated with serial bottleneck passages *in vitro*. Fifteen clones from four different HIV-1 viruses underwent serial (15) viral passages in the MT-4 T cell line. The *env* was found to be the most conserved genomic region in all the clones examined in the assay, yet this phenomenon was unexplained [457]. Fifty percent of the acquired mutations were detected within the V1/V2 region (between positions 130 – 232 according to the numbering of HIV-1 HXBc2).

Another study by Yuste *et al.*, [458] compared the HIV-1 genomic nucleotide parental strain sequences to those of viruses that had been passaged between 5 and 10 times in an MT-4 T cell line [458]. Most of the identified mutations that had accumulated along the genomic length were synonymous mutations and predominantly found in the 5' un-translated leader region [458]. Only a quarter of the HIV-1 clones acquired a single amino acid mutation in the *env* region, the others were 100% conserved from the parental strain [458].

A study by Kusumi *et al.*, [459] commented on the loss of HIV diversity that results from co-culture. They showed that the mean diversity between individual

gp120 coding regions in PBMCs was five-fold greater (3.24%) than the diversity after co-culture (0.65%) [459].

Zhang *et al.*, [450] performed multiple passages of HIV-1 clones on different T cell lines (MT-2, H9 and CEM) and showed that two out of three viruses did not accumulate any mutations within the V3 region of gp120 after three passages [450]. Similar findings were also shown when multiple passages of a primary CXCR4 utilizing HIV-1 virus was performed in H9 cells and the resulting viruses all retained the genotypic characteristics of the parental strain [460].

Quakkelaar *et al.*, [449] showed that a pre-existing neutralization sensitive HIV-1 variant may emerge from a quasispecies during T cell line adaptation. After two serial passages within the H9 T cell line, 50% of the H9 T cell passaged variants showed only one and/or two amino acid changes within the V1-V5 regions [449].

These studies are strongly supportive of the data in this study. A single non-synonymous mutation within *env* detected in this study, does concur with previously published data on serial *in vitro* passages of HIV-1 primary virus in T cell lines and the low genetic diversity that is observed, especially in *env* [456-458]. The selective pressure *in vitro* on the HIV-1 isolate in this study was relatively low, thus the 05ZAFV03 isolate underwent minimal mutational changes when adapting to the altered, low CD4 expression environment. However the current literature reporting on mutational changes in cloned *envs* that are responsible for the phenotypic conversion from CD4 dependence to CD4-independence, are contrary to the findings in this study. Generally, more than a single non-synonymous mutation within gp160 is responsible for CD4-independent phenotypes [344, 349, 350, 364, 366, 367, 374, 379, 461-463].

The 05ZAFV03 viral isolate may have been isolated from a highly immunocompromised HIV-1 infected AIDS patient. If so, at the time of viral isolation, this relatively ‘fit’ pre-selected virus within the patient would display rapid growth *in vitro* and its sequence would not change drastically through serial

in vitro passage. The high pathogenicity and conserved nature of the 05ZAFV03 *env* may support this theory.

The study of CD4-independent envelopes provides valuable insights into the different conformational states of gp120 during the host entry process. CD4-independent virus are detected *in vivo* in the immune privileged sites of the body, where antibodies are excluded by the blood-brain barrier, or in individuals with severely compromised immune systems. However, the precise mechanisms as to how and why CD4-independent envelopes display an increased sensitivity to humoral neutralizing antibodies *in vivo*, is still unclear. The genotypic and structural determinants of CD4-independence vary considerably among CD4-independent HIV and SIV isolates, and therefore the structure/function relationships may also differ. The relevance of these changes and their impact on pathogenesis and neuropathogenesis is also not yet clear.

This is the first study that has successfully passaged an HIV-1 subtype C CXCR4-utilizing isolate in the Cf2Th cell line and serially passaged the intact virus towards a final concentration of 10% CD4 expressing cells. The isolate adapted towards decreased CD4-dependence in this study, originated from a parental CXCR4 utilizing isolate, similar to other studies [367, 464, 465].

Future studies aimed at achieving 100% CD4-independence should re-grow the virus from the 10%:90% adaptation stage and generate large amounts of viral stocks before progressing and infecting the 1%:99% cell mixture and similarly with viral growth in the CD4 negative cells. Additionally, future studies should determine whether this E152K mutated envelope is intrinsically less dependent upon CD4 for cellular entry. This may be achieved by testing the 10%:90% virus in a PBMC based inhibition assay to determine the extent of inhibition by soluble CD4 verses the 100%:0% virus. Alternatively, the pseudovirion assay [466] can be used to test the 10%:90% envelope, where the E152K mutated envelope can be cloned and co-transfected with a backbone virus into a CD4-negative cell line.

This assay is important because if there is not enough virus stock available from the 10%:90% passage, a clone of the envelope can still be used in the system.

Many studies demonstrate that in some cases, CD4 is dispensable as the primary receptor and that CXCR4/CCR5 is essential for cellular entry. The availability of modified Env proteins will also be a powerful tool for analyzing gp120-coreceptor interactions and possibilities for inhibiting these interactions. As the functionally relevant coreceptor binding sites are elucidated and correlated with pathogenicity, we can begin to gain comprehensive understandings of the relationship between viral phenotype and disease progression and perhaps an evolution thereof. The impact of this knowledge extends to the preventative and clinical treatment implications for vaccine design and drug development respectively.

From this study, there is strong evidence for the presence of a highly positive lysine residue at position 152 within the V1/V2 loop region that may contribute to the evolution and/or adaptation to CD4-independence of a CXCR4 utilizing HIV-1 subtype C isolate in the Cf2Th cell line. Further experimentation and possibly repeats of these experiments with different HIV-1 subtype C isolates (both R5 and dual tropic R5X4 variants) and possibly in different T cell lines are required to verify these data. As can be seen, the extent of *env* diversity *in vitro* is clearly a complex multifactorial interplay of HIV-1 isolate- and cell-type-specific factors.

Overall, the 05ZAFV03 CXCR4-utilizing HIV-1 subtype C isolate was serially passaged 12 times through the Cf2Th cell line. After the fourth passage with 50% of the cells expressing CD4, a single non-synonymous mutation (E152K) was detected in V1 loop region of the gp160 sequence. This change did not support CD4-independence and future work should continue to serially passage the virus for longer periods while sequentially decreasing numbers of CD4 expressing cells in culture until CD4-independence is reached. Once the CD4-independent determinants are elucidated, rational drug design techniques can be employed to target the vulnerable, exposed and conserved viral binding epitopes on such CD4-independent isolates.

Chapter 4 SUMMARIZING DISCUSSION AND CONCLUSION

At the end of 2006 there was an estimated 39.5 million people worldwide living with HIV-1 and an estimated 2.9 million people lost their lives to AIDS [16]. These figures constitute one of the largest medical crises the human race has ever experienced.

Entry of HIV-1 into host $CD4^+$ T lymphocytes is mediated by the HIV-1 envelope glycoprotein (e*nv*/gp160), which is initially synthesized as a precursor and subsequently cleaved to yield gp120 and gp41, the surface and transmembrane subunits of the envelope respectively [59, 467-471]. Classical HIV-1 entry requires the gp120 to bind both the host surface receptor CD4 [56, 57] and a chemokine receptor, either CXCR4 and/or CCR5 [69-71, 77, 80, 164]. Interestingly, many primary HIV-2, SIV and some HIV-1 isolates can, to some extent, enter cells *in vivo* without the requirement for CD4, and bind directly to a coreceptor to initiate infection. However, these studies have only investigated HIV-1 subtype B isolates [344-348].

CD4-independent viruses are rare, yet are a natural phenomenon and found in immune privileged sites within the host. Previous studies have suggested that the HIV-1 subtype B Env from such viruses are "partially triggered", thus invariably exposing their conserved coreceptor binding domains [345, 346, 348-350, 472]. Therefore, CD4-independent Envs exhibit heightened sensitivity to neutralizing antibodies and neutralization by HIV-1 positive sera [351, 354-357, 432]. These vulnerable epitopes are usually shielded from the surveillance by the immune system *in vivo* in a CD4-dependent HIV-1 Env, however if this functionally important site is targeted, HIV-1 may be effectively neutralized.

According to the 2006 WHO/UNAIDS statistics [16, 52], Subtype C was the most prevalent HIV-1 subtype accounting for 55% of all HIV-1 infections worldwide, yet this clade is less widely studied. Thus, this study aimed to successfully adapt and genotypically characterize an HIV-1 subtype C isolate/s to CD4-independence *in vitro*.

In order to obtain a sample size of primary isolates for the present study, the genotypic and phenotypic properties of 20 primary isolates from antiretroviral (ARV) naïve advanced AIDS patients attending an HIV/AIDS clinic in Johannesburg were investigated. Primary viruses were isolated through standard co-culture techniques and subsequent *in vitro* viral tropism determination assays were performed. Phenotype and biotype were elucidated by means of the MT-2 and U87.CD4 cellular-based assays, respectively. The full-length *env* and V3 loop genes were amplified from proviral DNA, sequenced and extensively analyzed (K. Michler, pers. comm.).

Fourteen primary isolates utilized the CCR5 coreceptor exclusively and exhibited the Non-Syncytium Inducing (NSI) phenotype and the remaining six (30%) utilized the CXCR4 coreceptor for cell entry and exhibited the Syncytium Inducing (SI) phenotype. Phylogenetic analysis of *env* sequences from 19/20 isolates confirmed they were HIV-1 subtype C and one isolate was a CD recombinant. Two dual-tropic isolates were identified and they were both X4 utilizing isolates that retained the N-Linked glycosylation site within the V3 loop. Overall, the CXCR4 utilizing amino acid sequences showed a higher positive charge and increased variability of V3 loop length, compared to those of the CCR5 utilizing regions.

HIV-1 variants may emerge during the late phases of disease pathogenesis, which correlates with the accelerated loss of naïve and resting $CD4^+$ T cells, broadening of viral tropism and rapid progression to AIDS. Disease progression as just described is observed in at least 50%-60% of HIV-1 subtype B patients [73, 193, 194, 196, 199-207]. To date however, only a few CXCR4 utilizing HIV-1 subtype C viruses have been identified, and the majority of HIV-1 subtype C viruses have shown almost exclusive CCR5 usage, even in late stage disease [164, 249, 251-253, 257-260, 388, 389]. The present study reports the highest ever-detected percentage of CXCR4 utilizing/SI variants within a cohort of similar clinical background. Future follow-on studies will investigate HIV-1 subtype C tropism in longitudinal follow-up cohorts of larger sample size.

To facilitate the adaptation of an HIV-1 subtype C primary isolate towards CD4-independence, an isolate (05ZAFV03) was subject to serial viral passage in the Cf2Th T cell line co-expressing cellular receptors CD4 and CXCR4. Over time, the concentration of cellular CD4 expression was incrementally decreased from passage to passage. After the *in vitro* culturing, the proviral gp160 regions from isolates at each subsequent adaptation step were sequenced and analyzed. Due to serial *in vitro* bottle-neck passage of 05ZAFV03, the *env* region remained relatively conserved as observed in similar experiments [456-458]. After four serial passages, a single non-synonymous mutation, E152K, occurred within the V1 loop, in the gp160 sequences obtained from the proviral DNA of cultured cells with 50% CD4 expression. Throughout the 12 serial passages in the Cf2Th T cell line, with decreasing concentration of cellular CD4 expression, two synonymous mutations were detected at positions 295 and 454, respectively (according to the numbering of Consensus_C).

For the first time, an HIV-1 subtype C isolate has been successfully passaged in the Cf2Th cell-line and has successfully grown *in vitro* towards a final concentration of 10% CD4 expressing cells. The single non-synonymous mutation within the V1 loop of gp160, may contribute to the conformational changes that are required to expose the coreceptor binding domain in CD4-independent isolates. The repeated infection of a cell line may enhance the selection of the mutations throughout the HIV-1 genome acquired during the *in vitro* adaptation process. Additionally, the experimental approach of serial HIV-1 viral passage in T cell lines in the absence of host immune pressure, adds to the complexity of studying the behaviour of HIV-1 *in vitro*. Further experimentation on larger sample sizes, differing viral tropisms and different T cell lines is required to obtain comprehensive understanding of the CD4-independent HIV-1 subtype C genotype and phenotype.

Ultimately, after 12 serial passages the X4 HIV-1 subtype C isolate successfully infected Cf2Th cell cultures where only 10% of the cell population expressed CD4. The viral adaptation process was performed as described previously [349] and all

infections were performed in duplicate, triplicate or quadruplicate to ensure reproducibility and increased sample volume for further use. Viral infection was achieved with 1% CD4 expression however, due to time constraints and low volumes of viral sample, continuation of the assay was not possible. In future work continuing towards 100% CD4-independence, larger volumes of viral samples should be stored at the 10%:90% stage before progressing to the next stage.

Overall results from this study imply that the frequency of CXCR4-utilizing HIV-1 subtype C viruses may be increasing with time. This detected emergence/evolution of CXCR4 usage amongst HIV-1 subtype C isolates may have profound implications for viral pathogenesis, disease progression and future use of CCR5 antagonists as ARVs. Hence, the search for targeting conserved/vulnerable sites on the HIV-1 envelope as drug targets and vaccine candidates is ever more crucial. Adaptation of a primary HIV-1 subtype C isolate towards CD4-independence via serial *in vitro* passage, may expose these vulnerable epitopes that could potentially be drug targets/vaccine immunogens.

REFERENCES

1. *Pneumocystis pneumonia--Los Angeles.* MMWR Morb Mortal Wkly Rep, 1981. **30**(21): p. 250-2.
2. Gottlieb, M.S., R. Schroff, H.M. Schanker, J.D. Weisman, P.T. Fan, R.A. Wolf and A. Saxon, *Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency.* N Engl J Med, 1981. **305**(24): p. 1425-31.
3. Hymes, K.B., T. Cheung, J.B. Greene, N.S. Prose, A. Marcus, H. Ballard, D.C. William and L.J. Laubenstein, *Kaposi's sarcoma in homosexual men-a report of eight cases.* Lancet, 1981. **2**(8247): p. 598-600.
4. Masur, H., M.A. Michelis, J.B. Greene, I. Onorato, R.A. Stouwe, R.S. Holzman, G. Wormser, L. Brettman, M. Lange, H.W. Murray and S. Cunningham-Rundles, *An outbreak of community-acquired Pneumocystis carinii pneumonia: initial manifestation of cellular immune dysfunction.* N Engl J Med, 1981. **305**(24): p. 1431-8.
5. Siegal, F.P., C. Lopez, G.S. Hammer, A.E. Brown, S.J. Kornfeld, J. Gold, J. Hassett, S.Z. Hirschman, C. Cunningham-Rundles, B.R. Adelsberg and et al., *Severe acquired immunodeficiency in male homosexuals, manifested by chronic perianal ulcerative herpes simplex lesions.* N Engl J Med, 1981. **305**(24): p. 1439-44.
6. *Update on acquired immune deficiency syndrome (AIDS)--United States.* MMWR Morb Mortal Wkly Rep, 1982. **31**(37): p. 507-8, 513-4.
7. Harris, C., C.B. Small, R.S. Klein, G.H. Friedland, B. Moll, E.E. Emeson, I. Spigland and N.H. Steigbigel, *Immunodeficiency in female sexual partners of men with the acquired immunodeficiency syndrome.* N Engl J Med, 1983. **308**(20): p. 1181-4.
8. Barre-Sinoussi, F., J.C. Chermann, F. Rey, M.T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum and L. Montagnier, *Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS).* Science, 1983. **220**(4599): p. 868-71.
9. Gallo, R.C., S.Z. Salahuddin, M. Popovic, G.M. Shearer, M. Kaplan, B.F. Haynes, T.J. Palker, R. Redfield, J. Oleske, B. Safai and et al., *Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS.* Science, 1984. **224**(4648): p. 500-3.
10. Gallo, R.C. and L. Montagnier, *The discovery of HIV as the cause of AIDS.* N Engl J Med, 2003. **349**(24): p. 2283-5.
11. Chakrabarti, L., M. Guyader, M. Alizon, M.D. Daniel, R.C. Desrosiers, P. Tiollais and P. Sonigo, *Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses.* Nature, 1987. **328**(6130): p. 543-7.
12. Desrosiers, R.C. and D.J. Ringler, *Use of simian immunodeficiency viruses for AIDS research.* Intervirology, 1989. **30**(6): p. 301-12.
13. Gao, F., E. Bailes, D.L. Robertson, Y. Chen, C.M. Rodenburg, S.F. Michael, L.B. Cummins, L.O. Arthur, M. Peeters, G.M. Shaw, P.M. Sharp

and B.H. Hahn, *Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes.* Nature, 1999. **397**(6718): p. 436-41.
14. Hillis, D.M., *AIDS. Origins of HIV*. Science, 2000. **288**(5472): p. 1757-9.
15. Korber, B., M. Muldoon, J. Theiler, F. Gao, R. Gupta, A. Lapedes, B.H. Hahn, S. Wolinsky and T. Bhattacharya, *Timing the ancestor of the HIV-1 pandemic strains.* Science, 2000. **288**(5472): p. 1789-96.
16. 2007 [cited 25 February 2007]; Available from: www.unaids.org.
17. Roberts, J.D., K. Bebenek and T.A. Kunkel, *The accuracy of reverse transcriptase from HIV-1.* Science, 1988. **242**(4882): p. 1171-3.
18. Ho, D.D., A.U. Neumann, A.S. Perelson, W. Chen, J.M. Leonard and M. Markowitz, *Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection.* Nature, 1995. **373**(6510): p. 123-6.
19. Wei, X., S.K. Ghosh, M.E. Taylor, V.A. Johnson, E.A. Emini, P. Deutsch, J.D. Lifson, S. Bonhoeffer, M.A. Nowak, B.H. Hahn and et al., *Viral dynamics in human immunodeficiency virus type 1 infection.* Nature, 1995. **373**(6510): p. 117-22.
20. Mansky, L.M., *Retrovirus mutation rates and their role in genetic variation.* J Gen Virol, 1998. **79 (Pt 6)**: p. 1337-45.
21. Mansky, L.M. and H.M. Temin, *Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase.* J Virol, 1995. **69**(8): p. 5087-94.
22. Preston, B.D., B.J. Poiesz and L.A. Loeb, *Fidelity of HIV-1 reverse transcriptase.* Science, 1988. **242**(4882): p. 1168-71.
23. Nowak, M., *HIV mutation rate.* Nature, 1990. **347**(6293): p. 522.
24. Pathak, V.K. and H.M. Temin, *Broad spectrum of in vivo forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: substitutions, frameshifts, and hypermutations.* Proc Natl Acad Sci U S A, 1990. **87**(16): p. 6019-23.
25. Zhang, Z., T. Schuler, M. Zupancic, S. Wietgrefe, K.A. Staskus, K.A. Reimann, T.A. Reinhart, M. Rogan, W. Cavert, C.J. Miller, R.S. Veazey, D. Notermans, S. Little, S.A. Danner, D.D. Richman, D. Havlir, J. Wong, H.L. Jordan, T.W. Schacker, P. Racz, K. Tenner-Racz, N.L. Letvin, S. Wolinsky and A.T. Haase, *Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells.* Science, 1999. **286**(5443): p. 1353-7.
26. Coffin, J.M., *HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy.* Science, 1995. **267**(5197): p. 483-9.
27. Perelson, A.S., A.U. Neumann, M. Markowitz, J.M. Leonard and D.D. Ho, *HIV-1 dynamics in vivo: virion clearance rate, infected cell life-span, and viral generation time.* Science, 1996. **271**(5255): p. 1582-6.
28. Jung, A., R. Maier, J.P. Vartanian, G. Bocharov, V. Jung, U. Fischer, E. Meese, S. Wain-Hobson and A. Meyerhans, *Multiply infected spleen cells in HIV patients.* Nature, 2002. **418**(6894): p. 144.
29. Levy, D.N., G.M. Aldrovandi, O. Kutsch and G.M. Shaw, *Dynamics of HIV-1 recombination in its natural target cells.* Proc Natl Acad Sci U S A, 2004. **101**(12): p. 4204-9.

30. Zhuang, J., A.E. Jetzt, G. Sun, H. Yu, G. Klarmann, Y. Ron, B.D. Preston and J.P. Dougherty, *Human immunodeficiency virus type 1 recombination: rate, fidelity, and putative hot spots.* J Virol, 2002. **76**(22): p. 11273-82.
31. Santiago, M.L., C.M. Rodenburg, S. Kamenya, F. Bibollet-Ruche, F. Gao, E. Bailes, S. Meleth, S.J. Soong, J.M. Kilby, Z. Moldoveanu, B. Fahey, M.N. Muller, A. Ayouba, E. Nerrienet, H.M. McClure, J.L. Heeney, A.E. Pusey, D.A. Collins, C. Boesch, R.W. Wrangham, J. Goodall, P.M. Sharp, G.M. Shaw and B.H. Hahn, *SIVcpz in wild chimpanzees.* Science, 2002. **295**(5554): p. 465.
32. Gao, F., L. Yue, A.T. White, P.G. Pappas, J. Barchue, A.P. Hanson, B.M. Greene, P.M. Sharp, G.M. Shaw and B.H. Hahn, *Human infection by genetically diverse SIVSM-related HIV-2 in west Africa.* Nature, 1992. **358**(6386): p. 495-9.
33. Rambaut, A., D. Posada, K.A. Crandall and E.C. Holmes, *The causes and consequences of HIV evolution.* Nat Rev Genet, 2004. **5**(1): p. 52-61.
34. Marlink, R., P. Kanki, I. Thior, K. Travers, G. Eisen, T. Siby, I. Traore, C.C. Hsieh, M.C. Dia, E.H. Gueye and et al., *Reduced rate of disease development after HIV-2 infection as compared to HIV-1.* Science, 1994. **265**(5178): p. 1587-90.
35. Pepin, J., G. Morgan, D. Dunn, S. Gevao, M. Mendy, I. Gaye, N. Scollen, R. Tedder and H. Whittle, *HIV-2-induced immunosuppression among asymptomatic West African prostitutes: evidence that HIV-2 is pathogenic, but less so than HIV-1.* Aids, 1991. **5**(10): p. 1165-72.
36. Babu, P.G., N.K. Saraswathi, F. Devapriya and T.J. John, *The detection of HIV-2 infection in southern India.* Indian J Med Res, 1993. **97**: p. 49-52.
37. Rubsamen-Waigmann, H., H.V. Briesen, J.K. Maniar, P.K. Rao, C. Scholz and A. Pfutzner, *Spread of HIV-2 in India.* Lancet, 1991. **337**(8740): p. 550-1.
38. Whittle, H., J. Morris, J. Todd, T. Corrah, S. Sabally, J. Bangali, P.T. Ngom, M. Rolfe and A. Wilkins, *HIV-2-infected patients survive longer than HIV-1-infected patients.* Aids, 1994. **8**(11): p. 1617-20.
39. Charneau, P., A.M. Borman, C. Quillent, D. Guetard, S. Chamaret, J. Cohen, G. Remy, L. Montagnier and F. Clavel, *Isolation and envelope sequence of a highly divergent HIV-1 isolate: definition of a new HIV-1 group.* Virology, 1994. **205**(1): p. 247-53.
40. Mauclere, P., I. Loussert-Ajaka, F. Damond, P. Fagot, S. Souquieres, M. Monny Lobe, F.X. Mbopi Keou, F. Barre-Sinoussi, S. Saragosti, F. Brun-Vezinet and F. Simon, *Serological and virological characterization of HIV-1 group O infection in Cameroon.* Aids, 1997. **11**(4): p. 445-53.
41. Peeters, M., A. Gueye, S. Mboup, F. Bibollet-Ruche, E. Ekaza, C. Mulanga, R. Ouedrago, R. Gandji, P. Mpele, G. Dibanga, B. Koumare, M. Saidou, E. Esu-Williams, J.P. Lombart, W. Badombena, N. Luo, M. Vanden Haesevelde and E. Delaporte, *Geographical distribution of HIV-1 group O viruses in Africa.* Aids, 1997. **11**(4): p. 493-8.
42. Simon, F., P. Mauclere, P. Roques, I. Loussert-Ajaka, M.C. Muller-Trutwin, S. Saragosti, M.C. Georges-Courbot, F. Barre-Sinoussi and F.

Brun-Vezinet, *Identification of a new human immunodeficiency virus type 1 distinct from group M and group O.* Nat Med, 1998. **4**(9): p. 1032-7.
43. Louwagie, J., F.E. McCutchan, M. Peeters, T.P. Brennan, E. Sanders-Buell, G.A. Eddy, G. van der Groen, K. Fransen, G.M. Gershy-Damet, R. Deleys and et al., *Phylogenetic analysis of gag genes from 70 international HIV-1 isolates provides evidence for multiple genotypes.* Aids, 1993. **7**(6): p. 769-80.
44. Robertson, D.L., J.P. Anderson, J.A. Bradac, J.K. Carr, B. Foley, R.K. Funkhouser, F. Gao, B.H. Hahn, M.L. Kalish, C. Kuiken, G.H. Learn, T. Leitner, F. McCutchan, S. Osmanov, M. Peeters, D. Pieniazek, M. Salminen, P.M. Sharp, S. Wolinsky and B. Korber, *HIV-1 nomenclature proposal.* Science, 2000. **288**(5463): p. 55-6.
45. Casado, G., M.M. Thomson, M. Sierra and R. Najera, *Identification of a novel HIV-1 circulating ADG intersubtype recombinant form (CRF19_cpx) in Cuba.* J Acquir Immune Defic Syndr, 2005. **40**(5): p. 532-7.
46. 2007 [cited 14 February 2007]; Available from: www.hiv.lanl.gov.
47. 2007 [cited 8 April 2007]; Available from: http://www.hiv.lanl.org.
48. Robertson, D.L., J.P. Anderson, J.A. Bradac, J.K. Carr, B. Foley, R.K. Funkhouser, F. Gao, B.H. Hahn, M.L. Kalish, C. Kuiken, G.H. Learn, T. Leitner, F. McCutchan, S. Osmanov, M. Peeters, D. Pieniazek, M. Salminen, P.M. Sharp, S. Wolinsky and B. Korber, *HIV-1 Nomenclature Proposal: A Reference Guide to HIV-1 Classification* Los Alamos National Laboratory, Los Alamos, NM 1999.
49. Gaschen, B., J. Taylor, K. Yusim, B. Foley, F. Gao, D. Lang, V. Novitsky, B. Haynes, B.H. Hahn, T. Bhattacharya and B. Korber, *Diversity considerations in HIV-1 vaccine selection.* Science, 2002. **296**(5577): p. 2354-60.
50. Korber, B., B. Gaschen, K. Yusim, R. Thakallapally, C. Kesmir and V. Detours, *Evolutionary and immunological implications of contemporary HIV-1 variation.* Br Med Bull, 2001. **58**: p. 19-42.
51. 2007 [cited 16 April 2007]; Available from: http://www.hiv.lanl.gov/content/hiv-db/.
52. Hemelaar, J., E. Gouws, P.D. Ghys and S. Osmanov, *Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004.* Aids, 2006. **20**(16): p. W13-23.
53. Esparza, J. and N. Bhamarapravati, *Accelerating the development and future availability of HIV-1 vaccines: why, when, where, and how?* Lancet, 2000. **355**(9220): p. 2061-6.
54. Frankel, A.D. and J.A. Young, *HIV-1: fifteen proteins and an RNA.* Annu Rev Biochem, 1998. **67**: p. 1-25.
55. Turner, B.G. and M.F. Summers, *Structural biology of HIV.* J Mol Biol, 1999. **285**(1): p. 1-32.
56. Dalgleish, A.G., P.C. Beverley, P.R. Clapham, D.H. Crawford, M.F. Greaves and R.A. Weiss, *The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus.* Nature, 1984. **312**(5996): p. 763-7.

57. Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J.C. Gluckman and L. Montagnier, *T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV*. Nature, 1984. **312**(5996): p. 767-8.
58. Helenius, A. and M. Aebi, *Intracellular functions of N-linked glycans.* Science, 2001. **291**(5512): p. 2364-9.
59. Veronese, F.D., A.L. DeVico, T.D. Copeland, S. Oroszlan, R.C. Gallo and M.G. Sarngadharan, *Characterization of gp41 as the transmembrane protein coded by the HTLV-III/LAV envelope gene.* Science, 1985. **229**(4720): p. 1402-5.
60. Franzusoff, A., A.M. Volpe, D. Josse, S. Pichuantes and J.R. Wolf, *Biochemical and genetic definition of the cellular protease required for HIV-1 gp160 processing.* J Biol Chem, 1995. **270**(7): p. 3154-9.
61. Starcich, B.R., B.H. Hahn, G.M. Shaw, P.D. McNeely, S. Modrow, H. Wolf, E.S. Parks, W.P. Parks, S.F. Josephs, R.C. Gallo and et al., *Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS.* Cell, 1986. **45**(5): p. 637-48.
62. Center, R.J., R.D. Leapman, J. Lebowitz, L.O. Arthur, P.L. Earl and B. Moss, *Oligomeric structure of the human immunodeficiency virus type 1 envelope protein on the virion surface.* J Virol, 2002. **76**(15): p. 7863-7.
63. Chan, D.C., D. Fass, J.M. Berger and P.S. Kim, *Core structure of gp41 from the HIV envelope glycoprotein.* Cell, 1997. **89**(2): p. 263-73.
64. Wyatt, R., P.D. Kwong, E. Desjardins, R.W. Sweet, J. Robinson, W.A. Hendrickson and J.G. Sodroski, *The antigenic structure of the HIV gp120 envelope glycoprotein.* Nature, 1998. **393**(6686): p. 705-11.
65. Maddon, P.J., A.G. Dalgleish, J.S. McDougal, P.R. Clapham, R.A. Weiss and R. Axel, *The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain.* Cell, 1986. **47**(3): p. 333-48.
66. Kwong, P.D., R. Wyatt, J. Robinson, R.W. Sweet, J. Sodroski and W.A. Hendrickson, *Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody.* Nature, 1998. **393**(6686): p. 648-59.
67. Myszka, D.G., R.W. Sweet, P. Hensley, M. Brigham-Burke, P.D. Kwong, W.A. Hendrickson, R. Wyatt, J. Sodroski and M.L. Doyle, *Energetics of the HIV gp120-CD4 binding reaction.* Proc Natl Acad Sci U S A, 2000. **97**(16): p. 9026-31.
68. Thali, M., J.P. Moore, C. Furman, M. Charles, D.D. Ho, J. Robinson and J. Sodroski, *Characterization of conserved human immunodeficiency virus type 1 gp120 neutralization epitopes exposed upon gp120-CD4 binding.* J Virol, 1993. **67**(7): p. 3978-88.
69. Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R.E. Sutton, C.M. Hill, C.B. Davis, S.C. Peiper, T.J. Schall, D.R. Littman and N.R. Landau, *Identification of a major co-receptor for primary isolates of HIV-1.* Nature, 1996. **381**(6584): p. 661-6.

70. Dragic, T., V. Litwin, G.P. Allaway, S.R. Martin, Y. Huang, K.A. Nagashima, C. Cayanan, P.J. Maddon, R.A. Koup, J.P. Moore and W.A. Paxton, *HIV-1 entry into CD4+ cells is mediated by the chemokine receptor CC-CKR-5.* Nature, 1996. **381**(6584): p. 667-73.
71. Feng, Y., C.C. Broder, P.E. Kennedy and E.A. Berger, *HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor.* Science, 1996. **272**(5263): p. 872-7.
72. Michael, N.L., L.G. Louie, A.L. Rohrbaugh, K.A. Schultz, D.E. Dayhoff, C.E. Wang and H.W. Sheppard, *The role of CCR5 and CCR2 polymorphisms in HIV-1 transmission and disease progression.* Nat Med, 1997. **3**(10): p. 1160-2.
73. Koot, M., I.P. Keet, A.H. Vos, R.E. de Goede, M.T. Roos, R.A. Coutinho, F. Miedema, P.T. Schellekens and M. Tersmette, *Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4+ cell depletion and progression to AIDS.* Ann Intern Med, 1993. **118**(9): p. 681-8.
74. Koot, M., R. van Leeuwen, R.E. de Goede, I.P. Keet, S. Danner, J.K. Eeftinck Schattenkerk, P. Reiss, M. Tersmette, J.M. Lange and H. Schuitemaker, *Conversion rate towards a syncytium-inducing (SI) phenotype during different stages of human immunodeficiency virus type 1 infection and prognostic value of SI phenotype for survival after AIDS diagnosis.* J Infect Dis, 1999. **179**(1): p. 254-8.
75. Philpott, S., B. Weiser, K. Anastos, C.M. Kitchen, E. Robison, W.A. Meyer, 3rd, H.S. Sacks, U. Mathur-Wagh, C. Brunner and H. Burger, *Preferential suppression of CXCR4-specific strains of HIV-1 by antiviral therapy.* J Clin Invest, 2001. **107**(4): p. 431-8.
76. Shankarappa, R., J.B. Margolick, S.J. Gange, A.G. Rodrigo, D. Upchurch, H. Farzadegan, P. Gupta, C.R. Rinaldo, G.H. Learn, X. He, X.L. Huang and J.I. Mullins, *Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection.* J Virol, 1999. **73**(12): p. 10489-502.
77. Alkhatib, G., C. Combadiere, C.C. Broder, Y. Feng, P.E. Kennedy, P.M. Murphy and E.A. Berger, *CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1.* Science, 1996. **272**(5270): p. 1955-8.
78. Chao, L., *Fitness of RNA virus decreased by Muller's ratchet.* Nature, 1990. **348**(6300): p. 454-5.
79. Doms, R.W. and S.C. Peiper, *Unwelcomed guests with master keys: how HIV uses chemokine receptors for cellular entry.* Virology, 1997. **235**(2): p. 179-90.
80. Doranz, B.J., J. Rucker, Y. Yi, R.J. Smyth, M. Samson, S.C. Peiper, M. Parmentier, R.G. Collman and R.W. Doms, *A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors.* Cell, 1996. **85**(7): p. 1149-58.
81. Lusso, P., *HIV and the chemokine system: 10 years later.* Embo J, 2006. **25**(3): p. 447-56.

82. Chen, B., E.M. Vogan, H. Gong, J.J. Skehel, D.C. Wiley and S.C. Harrison, *Structure of an unliganded simian immunodeficiency virus gp120 core.* Nature, 2005. **433**(7028): p. 834-41.
83. Gallo, S.A., C.M. Finnegan, M. Viard, Y. Raviv, A. Dimitrov, S.S. Rawat, A. Puri, S. Durell and R. Blumenthal, *The HIV Env-mediated fusion reaction.* Biochim Biophys Acta, 2003. **1614**(1): p. 36-50.
84. Pierson, T.C. and R.W. Doms, *HIV-1 entry and its inhibition.* Curr Top Microbiol Immunol, 2003. **281**: p. 1-27.
85. Sattentau, Q.J., S. Zolla-Pazner and P. Poignard, *Epitope exposure on functional, oligomeric HIV-1 gp41 molecules.* Virology, 1995. **206**(1): p. 713-7.
86. Weissenhorn, W., A. Dessen, S.C. Harrison, J.J. Skehel and D.C. Wiley, *Atomic structure of the ectodomain from HIV-1 gp41.* Nature, 1997. **387**(6631): p. 426-30.
87. Lu, M., S.C. Blacklow and P.S. Kim, *A trimeric structural domain of the HIV-1 transmembrane glycoprotein.* Nat Struct Biol, 1995. **2**(12): p. 1075-82.
88. Phillips, D.M. and A.S. Bourinbaiar, *Mechanism of HIV spread from lymphocytes to epithelia.* Virology, 1992. **186**(1): p. 261-73.
89. Fassati, A. and S.P. Goff, *Characterization of intracellular reverse transcription complexes of human immunodeficiency virus type 1.* J Virol, 2001. **75**(8): p. 3626-35.
90. Grewe, C., A. Beck and H.R. Gelderblom, *HIV: early virus-cell interactions.* J Acquir Immune Defic Syndr, 1990. **3**(10): p. 965-74.
91. Erikson, E. and R.L. Erikson, *Association of 4S ribonucleic acid with oncornavirus ribonucleic acids.* J Virol, 1971. **8**(2): p. 254-6.
92. Marquet, R., C. Isel, C. Ehresmann and B. Ehresmann, *tRNAs as primer of reverse transcriptases.* Biochimie, 1995. **77**(1-2): p. 113-24.
93. Sawyer, R.C., F. Harada and J.E. Dahlberg, *Virion-associated RNA primer for Rous sarcoma virus DNA synthesis: isolation from uninfected cells.* J Virol, 1974. **13**(6): p. 1302-11.
94. Depienne, C., A. Mousnier, H. Leh, E. Le Rouzic, D. Dormont, S. Benichou and C. Dargemont, *Characterization of the nuclear import pathway for HIV-1 integrase.* J Biol Chem, 2001. **276**(21): p. 18102-7.
95. Fouchier, R.A., B.E. Meyer, J.H. Simon, U. Fischer, A.V. Albright, F. Gonzalez-Scarano and M.H. Malim, *Interaction of the human immunodeficiency virus type 1 Vpr protein with the nuclear pore complex.* J Virol, 1998. **72**(7): p. 6004-13.
96. Gallay, P., S. Swingler, J. Song, F. Bushman and D. Trono, *HIV nuclear import is governed by the phosphotyrosine-mediated binding of matrix to the core domain of integrase.* Cell, 1995. **83**(4): p. 569-76.
97. Bouyac-Bertoia, M., J.D. Dvorin, R.A. Fouchier, Y. Jenkins, B.E. Meyer, L.I. Wu, M. Emerman and M.H. Malim, *HIV-1 infection requires a functional integrase NLS.* Mol Cell, 2001. **7**(5): p. 1025-35.
98. Sanchez-Pescador, R., M.D. Power, P.J. Barr, K.S. Steimer, M.M. Stempien, S.L. Brown-Shimer, W.W. Gee, A. Renard, A. Randolph, J.A.

Levy and et al., *Nucleotide sequence and expression of an AIDS-associated retrovirus (ARV-2).* Science, 1985. **227**(4686): p. 484-92.
99. Mitchell, R.S., B.F. Beitzel, A.R. Schroder, P. Shinn, H. Chen, C.C. Berry, J.R. Ecker and F.D. Bushman, *Retroviral DNA integration: ASLV, HIV, and MLV show distinct target site preferences.* PLoS Biol, 2004. **2**(8): p. E234.
100. Schroder, A.R., P. Shinn, H. Chen, C. Berry, J.R. Ecker and F. Bushman, *HIV-1 integration in the human genome favors active genes and local hotspots.* Cell, 2002. **110**(4): p. 521-9.
101. Kim, S.Y., R. Byrn, J. Groopman and D. Baltimore, *Temporal aspects of DNA and RNA synthesis during human immunodeficiency virus infection: evidence for differential gene expression.* J Virol, 1989. **63**(9): p. 3708-13.
102. Klotman, M.E., S. Kim, A. Buchbinder, A. DeRossi, D. Baltimore and F. Wong-Staal, *Kinetics of expression of multiply spliced RNA in early human immunodeficiency virus type 1 infection of lymphocytes and monocytes.* Proc Natl Acad Sci U S A, 1991. **88**(11): p. 5011-5.
103. Greene, W.C., *AIDS and the immune system.* Sci Am, 1993. **269**(3): p. 98-105.
104. Sleasman, J.W. and M.M. Goodenow, *Pathogenesis and natural history of HIV infection.* J Fla Med Assoc, 1991. **78**(10): p. 678-81.
105. Chun, T.W., L. Carruth, D. Finzi, X. Shen, J.A. DiGiuseppe, H. Taylor, M. Hermankova, K. Chadwick, J. Margolick, T.C. Quinn, Y.H. Kuo, R. Brookmeyer, M.A. Zeiger, P. Barditch-Crovo and R.F. Siliciano, *Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection.* Nature, 1997. **387**(6629): p. 183-8.
106. Pomerantz, R.J., *Reservoirs of human immunodeficiency virus type 1: the main obstacles to viral eradication.* Clin Infect Dis, 2002. **34**(1): p. 91-7.
107. Bryant, M. and L. Ratner, *Myristoylation-dependent replication and assembly of human immunodeficiency virus 1.* Proc Natl Acad Sci U S A, 1990. **87**(2): p. 523-7.
108. Hauber, J., M. Bouvier, M.H. Malim and B.R. Cullen, *Phosphorylation of the rev gene product of human immunodeficiency virus type 1.* J Virol, 1988. **62**(12): p. 4801-4.
109. Kozarsky, K., M. Penman, L. Basiripour, W. Haseltine, J. Sodroski and M. Krieger, *Glycosylation and processing of the human immunodeficiency virus type 1 envelope protein.* J Acquir Immune Defic Syndr, 1989. **2**(2): p. 163-9.
110. Liao, Z., L.M. Cimakasky, R. Hampton, D.H. Nguyen and J.E. Hildreth, *Lipid rafts and HIV pathogenesis: host membrane cholesterol is required for infection by HIV type 1.* AIDS Res Hum Retroviruses, 2001. **17**(11): p. 1009-19.
111. Nguyen, D.H. and J.E. Hildreth, *Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts.* J Virol, 2000. **74**(7): p. 3264-72.
112. Gottlinger, H.G., *The HIV-1 assembly machine.* Aids, 2001. **15 Suppl 5**: p. S13-20.

113. Sakalian, M. and E. Hunter, *Molecular events in the assembly of retrovirus particles.* Adv Exp Med Biol, 1998. **440**: p. 329-39.
114. Gelderblom, H.R., *Assembly and morphology of HIV: potential effect of structure on viral function.* Aids, 1991. **5**(6): p. 617-37.
115. Orenstein, J.M., M.S. Meltzer, T. Phipps and H.E. Gendelman, *Cytoplasmic assembly and accumulation of human immunodeficiency virus types 1 and 2 in recombinant human colony-stimulating factor-1-treated human monocytes: an ultrastructural study.* J Virol, 1988. **62**(8): p. 2578-86.
116. Pelchen-Matthews, A., B. Kramer and M. Marsh, *Infectious HIV-1 assembles in late endosomes in primary macrophages.* J Cell Biol, 2003. **162**(3): p. 443-55.
117. Pelchen-Matthews, A., G. Raposo and M. Marsh, *Endosomes, exosomes and Trojan viruses.* Trends Microbiol, 2004. **12**(7): p. 310-6.
118. Krausslich, H.G., M. Facke, A.M. Heuser, J. Konvalinka and H. Zentgraf, *The spacer peptide between human immunodeficiency virus capsid and nucleocapsid proteins is essential for ordered assembly and viral infectivity.* J Virol, 1995. **69**(6): p. 3407-19.
119. Nowak, M.A., S. Bonhoeffer, A.M. Hill, R. Boehme, H.C. Thomas and H. McDade, *Viral dynamics in hepatitis B virus infection.* Proc Natl Acad Sci U S A, 1996. **93**(9): p. 4398-402.
120. 2006 [cited 20 November 2006]; Available from: http://www.wrongdiagnosis.com/h/hiv_aids/wiki.htm.
121. Lehner, T., L. Hussain, J. Wilson and M. Chapman, *Mucosal transmission of HIV.* Nature, 1991. **353**(6346): p. 709.
122. Hussain, L.A. and T. Lehner, *Comparative investigation of Langerhans' cells and potential receptors for HIV in oral, genitourinary and rectal epithelia.* Immunology, 1995. **85**(3): p. 475-84.
123. Igarashi, T., C.R. Brown, R.A. Byrum, Y. Nishimura, Y. Endo, R.J. Plishka, C. Buckler, A. Buckler-White, G. Miller, V.M. Hirsch and M.A. Martin, *Rapid and irreversible CD4+ T-cell depletion induced by the highly pathogenic simian/human immunodeficiency virus SHIV(DH12R) is systemic and synchronous.* J Virol, 2002. **76**(1): p. 379-91.
124. Miyake, A., K. Ibuki, Y. Enose, H. Suzuki, R. Horiuchi, M. Motohara, N. Saito, T. Nakasone, M. Honda, T. Watanabe, T. Miura and M. Hayami, *Rapid dissemination of a pathogenic simian/human immunodeficiency virus to systemic organs and active replication in lymphoid tissues following intrarectal infection.* J Gen Virol, 2006. **87**(Pt 5): p. 1311-20.
125. Korber, B., S. Wolinsky, B. Haynes, K. Kunstman, R. Levy, M. Furtado, P. Otto and G. Myers, *HIV-1 intrapatient sequence diversity in the immunogenic V3 region.* AIDS Res Hum Retroviruses, 1992. **8**(8): p. 1461-5.
126. McNearney, T., Z. Hornickova, R. Markham, A. Birdwell, M. Arens, A. Saah and L. Ratner, *Relationship of human immunodeficiency virus type 1 sequence heterogeneity to stage of disease.* Proc Natl Acad Sci U S A, 1992. **89**(21): p. 10247-51.

127. Zhang, L.Q., P. MacKenzie, A. Cleland, E.C. Holmes, A.J. Brown and P. Simmonds, *Selection for specific sequences in the external envelope protein of human immunodeficiency virus type 1 upon primary infection.* J Virol, 1993. **67**(6): p. 3345-56.
128. Cooper, D.A., B. Tindall, E.J. Wilson, A.A. Imrie and R. Penny, *Characterization of T lymphocyte responses during primary infection with human immunodeficiency virus.* J Infect Dis, 1988. **157**(5): p. 889-96.
129. Daar, E.S., T. Moudgil, R.D. Meyer and D.D. Ho, *Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection.* N Engl J Med, 1991. **324**(14): p. 961-4.
130. Mellors, J.W., C.R. Rinaldo, Jr., P. Gupta, R.M. White, J.A. Todd and L.A. Kingsley, *Prognosis in HIV-1 infection predicted by the quantity of virus in plasma.* Science, 1996. **272**(5265): p. 1167-70.
131. Koup, R.A., J.T. Safrit, Y. Cao, C.A. Andrews, G. McLeod, W. Borkowsky, C. Farthing and D.D. Ho, *Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome.* J Virol, 1994. **68**(7): p. 4650-5.
132. Moore, J.P., Q.J. Sattentau, R. Wyatt and J. Sodroski, *Probing the structure of the human immunodeficiency virus surface glycoprotein gp120 with a panel of monoclonal antibodies.* J Virol, 1994. **68**(1): p. 469-84.
133. Moore, J.P. and J. Sodroski, *Antibody cross-competition analysis of the human immunodeficiency virus type 1 gp120 exterior envelope glycoprotein.* J Virol, 1996. **70**(3): p. 1863-72.
134. Vanhems, P., J. Hughes, A.C. Collier, J. Vizzard, L. Perrin, D.A. Cooper, B. Hirschel and L. Corey, *Comparison of clinical features, CD4 and CD8 responses among patients with acute HIV-1 infection from Geneva, Seattle and Sydney.* Aids, 2000. **14**(4): p. 375-81.
135. Wawer, M.J., D. Serwadda and X. Li. *HIV-1 transmission per coital act, by stage of HIV infection in the HIV+ index partner, in discordant couples*. in *Tenth Conference on Retroviruses and Opportunistic Infections*. 2003. Boston.
136. Bukrinsky, M.I., T.L. Stanwick, M.P. Dempsey and M. Stevenson, *Quiescent T lymphocytes as an inducible virus reservoir in HIV-1 infection.* Science, 1991. **254**(5030): p. 423-7.
137. McElrath, M.J., J.E. Pruett and Z.A. Cohn, *Mononuclear phagocytes of blood and bone marrow: comparative roles as viral reservoirs in human immunodeficiency virus type 1 infections.* Proc Natl Acad Sci U S A, 1989. **86**(2): p. 675-9.
138. Schnittman, S.M., M.C. Psallidopoulos, H.C. Lane, L. Thompson, M. Baseler, F. Massari, C.H. Fox, N.P. Salzman and A.S. Fauci, *The reservoir for HIV-1 in human peripheral blood is a T cell that maintains expression of CD4.* Science, 1989. **245**(4915): p. 305-8.
139. Tornatore, C., R. Chandra, J.R. Berger and E.O. Major, *HIV-1 infection of subcortical astrocytes in the pediatric central nervous system.* Neurology, 1994. **44**(3 Pt 1): p. 481-7.

140. von Laer, D., F.T. Hufert, T.E. Fenner, S. Schwander, M. Dietrich, H. Schmitz and P. Kern, *CD34+ hematopoietic progenitor cells are not a major reservoir of the human immunodeficiency virus.* Blood, 1990. **76**(7): p. 1281-6.
141. Berger, E.A., R.W. Doms, E.M. Fenyo, B.T. Korber, D.R. Littman, J.P. Moore, Q.J. Sattentau, H. Schuitemaker, J. Sodroski and R.A. Weiss, *A new classification for HIV-1.* Nature, 1998. **391**(6664): p. 240.
142. Dittmar, M.T., A. McKnight, G. Simmons, P.R. Clapham, R.A. Weiss and P. Simmonds, *HIV-1 tropism and co-receptor use.* Nature, 1997. **385**(6616): p. 495-6.
143. Pantaleo, G., C. Graziosi, J.F. Demarest, L. Butini, M. Montroni, C.H. Fox, J.M. Orenstein, D.P. Kotler and A.S. Fauci, *HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease.* Nature, 1993. **362**(6418): p. 355-8.
144. Masur, H., F.P. Ognibene, R. Yarchoan, J.H. Shelhamer, B.F. Baird, W. Travis, A.F. Suffredini, L. Deyton, J.A. Kovacs, J. Falloon and et al., *CD4 counts as predictors of opportunistic pneumonias in human immunodeficiency virus (HIV) infection.* Ann Intern Med, 1989. **111**(3): p. 223-31.
145. Fauci, A.S., G. Pantaleo, S. Stanley and D. Weissman, *Immunopathogenic mechanisms of HIV infection.* Ann Intern Med, 1996. **124**(7): p. 654-63.
146. WHO/UNAIDS, *Progress in scaling up access to HIV treatment in low and middle-income countries*. WHO/UNAIDS, Fact Sheet. August. Geneva, 2006.
147. Chaisson, R.E., R.D. Moore, D.D. Richman, J. Keruly and T. Creagh, *Incidence and natural history of Mycobacterium avium-complex infections in patients with advanced human immunodeficiency virus disease treated with zidovudine. The Zidovudine Epidemiology Study Group.* Am Rev Respir Dis, 1992. **146**(2): p. 285-9.
148. Gallant, J.E., R.D. Moore, D.D. Richman, J. Keruly and R.E. Chaisson, *Incidence and natural history of cytomegalovirus disease in patients with advanced human immunodeficiency virus disease treated with zidovudine. The Zidovudine Epidemiology Study Group.* J Infect Dis, 1992. **166**(6): p. 1223-7.
149. Moore, R.D., H. Kessler, D.D. Richman, C. Flexner and R.E. Chaisson, *Non-Hodgkin's lymphoma in patients with advanced HIV infection treated with zidovudine.* Jama, 1991. **265**(17): p. 2208-11.
150. Phair, J., A. Munoz, R. Detels, R. Kaslow, C. Rinaldo and A. Saah, *The risk of Pneumocystis carinii pneumonia among men infected with human immunodeficiency virus type 1. Multicenter AIDS Cohort Study Group.* N Engl J Med, 1990. **322**(3): p. 161-5.
151. Salman, M.D., J.M. Hutchison and M. Smith, *Re: "The spectrum of medical conditions and symptoms before acquired immunodeficiency syndrome in homosexual and bisexual men infected with the human immunodeficiency virus".* Am J Epidemiol, 1996. **143**(1): p. 102-3.

152. Sackoff, J.E., D.B. Hanna, M.R. Pfeiffer and L.V. Torian, *Causes of death among persons with AIDS in the era of highly active antiretroviral therapy: New York City.* Ann Intern Med, 2006. **145**(6): p. 397-406.
153. Kiepiela, P., A.N. Smith and E. Rosenberg, *Laboratory markers associated with progression of HIV infection.* Best Pract Res Clin Obstet Gynaecol, 2005. **19**(2): p. 243-54.
154. 2006 [cited 21 December 2006]; Available from: http://www.doh.gov.za/.
155. WHO. *Scaling up Antiretroviral in resource- limited settings: Treatment guidelines for a public health approach*. 2003 [cited 10 January 2007]; Available from: http://www.wpro.who.int/NR/rdonlyres/BA347BAA-EDBD-42BF-9788-9C4F9F09534B/0/ART_Newsletter_Issue_7.pdf.
156. Leitner, T., B. Foley and B. Hahn. *The 2003 HIV Sequence Compendium*. 2003 [cited 19 January 2006]; Available from: http://hiv-web.lanl.gov/content/hiv-db/HTML/2003compendium.html.
157. Clevenbergh, P., S. Mouly, P. Sellier, E. Badsi, J. Cervoni, V. Vincent, H. Trout and J.F. Bergmann, *Improving HIV infection management using antiretroviral plasma drug levels monitoring: a clinician's point of view.* Curr HIV Res, 2004. **2**(4): p. 309-21.
158. Wyatt, R., P. Kwong, W. Hendrickson and J. Sodroski, *Structure of the Core of the HIV-1 gp120 Exterior Envelope Glycoprotein*. Analyses. 1998. 9.
159. Chen, B., E.M. Vogan, H. Gong, J.J. Skehel, D.C. Wiley and S.C. Harrison, *Determining the structure of an unliganded and fully glycosylated SIV gp120 envelope glycoprotein.* Structure, 2005. **13**(2): p. 197-211.
160. Kwong, P.D., R. Wyatt, S. Majeed, J. Robinson, R.W. Sweet, J. Sodroski and W.A. Hendrickson, *Structures of HIV-1 gp120 envelope glycoproteins from laboratory-adapted and primary isolates.* Structure, 2000. **8**(12): p. 1329-39.
161. Wyatt, R., J. Moore, M. Accola, E. Desjardin, J. Robinson and J. Sodroski, *Involvement of the V1/V2 variable loop structure in the exposure of human immunodeficiency virus type 1 gp120 epitopes induced by receptor binding.* J Virol, 1995. **69**(9): p. 5723-33.
162. Boyd, M.T., G.R. Simpson, A.J. Cann, M.A. Johnson and R.A. Weiss, *A single amino acid substitution in the V1 loop of human immunodeficiency virus type 1 gp120 alters cellular tropism.* J Virol, 1993. **67**(6): p. 3649-52.
163. Cho, M.W., M.K. Lee, M.C. Carney, J.F. Berson, R.W. Doms and M.A. Martin, *Identification of determinants on a dualtropic human immunodeficiency virus type 1 envelope glycoprotein that confer usage of CXCR4.* J Virol, 1998. **72**(3): p. 2509-15.
164. Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P.D. Ponath, L. Wu, C.R. Mackay, G. LaRosa, W. Newman, N. Gerard, C. Gerard and J. Sodroski, *The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates.* Cell, 1996. **85**(7): p. 1135-48.

165. Kim, F.M., D.L. Kolson, J.W. Balliet, A. Srinivasan and R.G. Collman, *V3-independent determinants of macrophage tropism in a primary human immunodeficiency virus type 1 isolate.* J Virol, 1995. **69**(3): p. 1755-61.
166. Koito, A., G. Harrowe, J.A. Levy and C. Cheng-Mayer, *Functional role of the V1/V2 region of human immunodeficiency virus type 1 envelope glycoprotein gp120 in infection of primary macrophages and soluble CD4 neutralization.* J Virol, 1994. **68**(4): p. 2253-9.
167. Wu, L., N.P. Gerard, R. Wyatt, H. Choe, C. Parolin, N. Ruffing, A. Borsetti, A.A. Cardoso, E. Desjardin, W. Newman, C. Gerard and J. Sodroski, *CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5.* Nature, 1996. **384**(6605): p. 179-83.
168. Cao, J., L. Bergeron, E. Helseth, M. Thali, H. Repke and J. Sodroski, *Effects of amino acid changes in the extracellular domain of the human immunodeficiency virus type 1 gp41 envelope glycoprotein.* J Virol, 1993. **67**(5): p. 2747-55.
169. Leonard, C.K., M.W. Spellman, L. Riddle, R.J. Harris, J.N. Thomas and T.J. Gregory, *Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells.* J Biol Chem, 1990. **265**(18): p. 10373-82.
170. Modrow, S., B.H. Hahn, G.M. Shaw, R.C. Gallo, F. Wong-Staal and H. Wolf, *Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions.* J Virol, 1987. **61**(2): p. 570-8.
171. Wyatt, R. and J. Sodroski, *The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens.* Science, 1998. **280**(5371): p. 1884-8.
172. Lamers, S.L., J.W. Sleasman, J.X. She, K.A. Barrie, S.M. Pomeroy, D.J. Barrett and M.M. Goodenow, *Independent variation and positive selection in env V1 and V2 domains within maternal-infant strains of human immunodeficiency virus type 1 in vivo.* J Virol, 1993. **67**(7): p. 3951-60.
173. Helseth, E., U. Olshevsky, C. Furman and J. Sodroski, *Human immunodeficiency virus type 1 gp120 envelope glycoprotein regions important for association with the gp41 transmembrane glycoprotein.* J Virol, 1991. **65**(4): p. 2119-23.
174. Huang, C.C., M. Tang, M.Y. Zhang, S. Majeed, E. Montabana, R.L. Stanfield, D.S. Dimitrov, B. Korber, J. Sodroski, I.A. Wilson, R. Wyatt and P.D. Kwong, *Structure of a V3-containing HIV-1 gp120 core.* Science, 2005. **310**(5750): p. 1025-8.
175. Trkola, A., T. Dragic, J. Arthos, J.M. Binley, W.C. Olson, G.P. Allaway, C. Cheng-Mayer, J. Robinson, P.J. Maddon and J.P. Moore, *CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5.* Nature, 1996. **384**(6605): p. 184-7.
176. Kwong, P.D., M.L. Doyle, D.J. Casper, C. Cicala, S.A. Leavitt, S. Majeed, T.D. Steenbeke, M. Venturi, I. Chaiken, M. Fung, H. Katinger, P.W. Parren, J. Robinson, D. Van Ryk, L. Wang, D.R. Burton, E. Freire, R. Wyatt, J. Sodroski, W.A. Hendrickson and J. Arthos, *HIV-1 evades*

antibody-mediated neutralization through conformational masking of receptor-binding sites. Nature, 2002. **420**(6916): p. 678-82.
177. Wei, X., J.M. Decker, S. Wang, H. Hui, J.C. Kappes, X. Wu, J.F. Salazar-Gonzalez, M.G. Salazar, J.M. Kilby, M.S. Saag, N.L. Komarova, M.A. Nowak, B.H. Hahn, P.D. Kwong and G.M. Shaw, *Antibody neutralization and escape by HIV-1.* Nature, 2003. **422**(6929): p. 307-12.
178. Zhou, T., L. Xu, B. Dey, A.J. Hessell, D. Van Ryk, S.H. Xiang, X. Yang, M.Y. Zhang, M.B. Zwick, J. Arthos, D.R. Burton, D.S. Dimitrov, J. Sodroski, R. Wyatt, G.J. Nabel and P.D. Kwong, *Structural definition of a conserved neutralization epitope on HIV-1 gp120.* Nature, 2007. **445**(7129): p. 732-7.
179. Gavel, Y. and G. von Heijne, *Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites: implications for protein engineering.* Protein Eng, 1990. **3**(5): p. 433-42.
180. Zhu, X., C. Borchers, R.J. Bienstock and K.B. Tomer, *Mass spectrometric characterization of the glycosylation pattern of HIV-gp120 expressed in CHO cells.* Biochemistry, 2000. **39**(37): p. 11194-204.
181. Allan, J.S., J.E. Coligan, F. Barin, M.F. McLane, J.G. Sodroski, C.A. Rosen, W.A. Haseltine, T.H. Lee and M. Essex, *Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III.* Science, 1985. **228**(4703): p. 1091-4.
182. Land, A. and I. Braakman, *Folding of the human immunodeficiency virus type 1 envelope glycoprotein in the endoplasmic reticulum.* Biochimie, 2001. **83**(8): p. 783-90.
183. Sullivan, N., Y. Sun, J. Li, W. Hofmann and J. Sodroski, *Replicative function and neutralization sensitivity of envelope glycoproteins from primary and T-cell line-passaged human immunodeficiency virus type 1 isolates.* J Virol, 1995. **69**(7): p. 4413-22.
184. Sagar, M., X. Wu, S. Lee and J. Overbaugh, *Human immunodeficiency virus type 1 V1-V2 envelope loop sequences expand and add glycosylation sites over the course of infection, and these modifications affect antibody neutralization sensitivity.* J Virol, 2006. **80**(19): p. 9586-98.
185. Zolla-Pazner, S., *Identifying epitopes of HIV-1 that induce protective antibodies.* Nat Rev Immunol, 2004. **4**(3): p. 199-210.
186. Goudsmit, J., C. Debouck, R.H. Meloen, L. Smit, M. Bakker, D.M. Asher, A.V. Wolff, C.J. Gibbs, Jr. and D.C. Gajdusek, *Human immunodeficiency virus type 1 neutralization epitope with conserved architecture elicits early type-specific antibodies in experimentally infected chimpanzees.* Proc Natl Acad Sci U S A, 1988. **85**(12): p. 4478-82.
187. Safrit, J.T., A.Y. Lee, C.A. Andrews and R.A. Koup, *A region of the third variable loop of HIV-1 gp120 is recognized by HLA-B7-restricted CTLs from two acute seroconversion patients.* J Immunol, 1994. **153**(8): p. 3822-30.
188. Delwart, E.L., H. Pan, H.W. Sheppard, D. Wolpert, A.U. Neumann, B. Korber and J.I. Mullins, *Slower evolution of human immunodeficiency*

virus type 1 quasispecies during progression to AIDS. J Virol, 1997. **71**(10): p. 7498-508.
189. Kalpana, A., T. Srikanth, J. Abhay, J. Sushama, K. Swarali and P. Ramesh, *gp120 sequences from HIV type 1 subtype C early seroconverters in India.* AIDS Res Hum Retroviruses, 2004. **20**(8): p. 889-94.
190. Lukashov, V.V. and J. Goudsmit, *HIV heterogeneity and disease progression in AIDS: a model of continuous virus adaptation.* Aids, 1998. **12 Suppl A**: p. S43-52.
191. Paxton, W.A., T. Dragic, R.A. Koup and J.P. Moore, *The beta-chemokines, HIV type 1 second receptors, and exposed uninfected persons.* AIDS Res Hum Retroviruses, 1996. **12**(13): p. 1203-7.
192. Berger, E.A., *HIV entry and tropism: the chemokine receptor connection.* Aids, 1997. **11 Suppl A**: p. S3-16.
193. Connor, R.I. and D.D. Ho, *Human immunodeficiency virus type 1 variants with increased replicative capacity develop during the asymptomatic stage before disease progression.* J Virol, 1994. **68**(7): p. 4400-8.
194. Scarlatti, G., E. Tresoldi, A. Bjorndal, R. Fredriksson, C. Colognesi, H.K. Deng, M.S. Malnati, A. Plebani, A.G. Siccardi, D.R. Littman, E.M. Fenyo and P. Lusso, *In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression.* Nat Med, 1997. **3**(11): p. 1259-65.
195. Zhu, T., H. Mo, N. Wang, D.S. Nam, Y. Cao, R.A. Koup and D.D. Ho, *Genotypic and phenotypic characterization of HIV-1 patients with primary infection.* Science, 1993. **261**(5125): p. 1179-81.
196. Schuitemaker, H., M. Koot, N.A. Kootstra, M.W. Dercksen, R.E. de Goede, R.P. van Steenwijk, J.M. Lange, J.K. Schattenkerk, F. Miedema and M. Tersmette, *Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population.* J Virol, 1992. **66**(3): p. 1354-60.
197. Schuitemaker, H., N.A. Kootstra, R.E. de Goede, F. de Wolf, F. Miedema and M. Tersmette, *Monocytotropic human immunodeficiency virus type 1 (HIV-1) variants detectable in all stages of HIV-1 infection lack T-cell line tropism and syncytium-inducing ability in primary T-cell culture.* J Virol, 1991. **65**(1): p. 356-63.
198. van't Wout, A.B., N.A. Kootstra, G.A. Mulder-Kampinga, N. Albrecht-van Lent, H.J. Scherpbier, J. Veenstra, K. Boer, R.A. Coutinho, F. Miedema and H. Schuitemaker, *Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission.* J Clin Invest, 1994. **94**(5): p. 2060-7.
199. Berger, E.A., P.M. Murphy and J.M. Farber, *Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease.* Annu Rev Immunol, 1999. **17**: p. 657-700.
200. Connor, R.I., K.E. Sheridan, D. Ceradini, S. Choe and N.R. Landau, *Change in coreceptor use coreceptor use correlates with disease progression in HIV-1--infected individuals.* J Exp Med, 1997. **185**(4): p. 621-8.

201. De Jong, J.J., A. De Ronde, W. Keulen, M. Tersmette and J. Goudsmit, *Minimal requirements for the human immunodeficiency virus type 1 V3 domain to support the syncytium-inducing phenotype: analysis by single amino acid substitution.* J Virol, 1992. **66**(11): p. 6777-80.
202. Fouchier, R.A., M. Groenink, N.A. Kootstra, M. Tersmette, H.G. Huisman, F. Miedema and H. Schuitemaker, *Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule.* J Virol, 1992. **66**(5): p. 3183-7.
203. Hoffman, T.L., E.B. Stephens, O. Narayan and R.W. Doms, *HIV type I envelope determinants for use of the CCR2b, CCR3, STRL33, and APJ coreceptors.* Proc Natl Acad Sci U S A, 1998. **95**(19): p. 11360-5.
204. Maas, J.J., S.J. Gange, H. Schuitemaker, R.A. Coutinho, R. van Leeuwen and J.B. Margolick, *Strong association between failure of T cell homeostasis and the syncytium-inducing phenotype among HIV-1-infected men in the Amsterdam Cohort Study.* Aids, 2000. **14**(9): p. 1155-61.
205. Tersmette, M., R.E. de Goede, B.J. Al, I.N. Winkel, R.A. Gruters, H.T. Cuypers, H.G. Huisman and F. Miedema, *Differential syncytium-inducing capacity of human immunodeficiency virus isolates: frequent detection of syncytium-inducing isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex.* J Virol, 1988. **62**(6): p. 2026-32.
206. Tersmette, M., J.M. Lange, R.E. de Goede, F. de Wolf, J.K. Eeftink-Schattenkerk, P.T. Schellekens, R.A. Coutinho, J.G. Huisman, J. Goudsmit and F. Miedema, *Association between biological properties of human immunodeficiency virus variants and risk for AIDS and AIDS mortality.* Lancet, 1989. **1**(8645): p. 983-5.
207. Richman, D.D. and S.A. Bozzette, *The impact of the syncytium-inducing phenotype of human immunodeficiency virus on disease progression.* J Infect Dis, 1994. **169**(5): p. 968-74.
208. Glushakova, S., Y. Yi, J.C. Grivel, A. Singh, D. Schols, E. De Clercq, R.G. Collman and L. Margolis, *Preferential coreceptor utilization and cytopathicity by dual-tropic HIV-1 in human lymphoid tissue ex vivo.* J Clin Invest, 1999. **104**(5): p. R7-R11.
209. Moriuchi, M., H. Moriuchi, W. Turner and A.S. Fauci, *Cloning and analysis of the promoter region of CXCR4, a coreceptor for HIV-1 entry.* J Immunol, 1997. **159**(9): p. 4322-9.
210. Schacker, T., S. Little, E. Connick, K. Gebhard, Z.Q. Zhang, J. Krieger, J. Pryor, D. Havlir, J.K. Wong, R.T. Schooley, D. Richman, L. Corey and A.T. Haase, *Productive infection of T cells in lymphoid tissues during primary and early human immunodeficiency virus infection.* J Infect Dis, 2001. **183**(4): p. 555-62.
211. van der Ende, M.E., M. Schutten, B. Raschdorff, G. Grossschupff, P. Racz, A.D. Osterhaus and K. Tenner-Racz, *CD4 T cells remain the major source of HIV-1 during end stage disease.* Aids, 1999. **13**(9): p. 1015-9.
212. Kaneshima, H., L. Su, M.L. Bonyhadi, R.I. Connor, D.D. Ho and J.M. McCune, *Rapid-high, syncytium-inducing isolates of human*

immunodeficiency virus type 1 induce cytopathicity in the human thymus of the SCID-hu mouse. J Virol, 1994. **68**(12): p. 8188-92.
213. Kreisberg, J.F., D. Kwa, B. Schramm, V. Trautner, R. Connor, H. Schuitemaker, J.I. Mullins, A.B. van't Wout and M.A. Goldsmith, *Cytopathicity of human immunodeficiency virus type 1 primary isolates depends on coreceptor usage and not patient disease status.* J Virol, 2001. **75**(18): p. 8842-7.
214. Moore, J.P. and M. Stevenson, *New targets for inhibitors of HIV-1 replication.* Nat Rev Mol Cell Biol, 2000. **1**(1): p. 40-9.
215. Simmons, G., D. Wilkinson, J.D. Reeves, M.T. Dittmar, S. Beddows, J. Weber, G. Carnegie, U. Desselberger, P.W. Gray, R.A. Weiss and P.R. Clapham, *Primary, syncytium-inducing human immunodeficiency virus type 1 isolates are dual-tropic and most can use either Lestr or CCR5 as coreceptors for virus entry.* J Virol, 1996. **70**(12): p. 8355-60.
216. Berson, J.F., D. Long, B.J. Doranz, J. Rucker, F.R. Jirik and R.W. Doms, *A seven-transmembrane domain receptor involved in fusion and entry of T-cell-tropic human immunodeficiency virus type 1 strains.* J Virol, 1996. **70**(9): p. 6288-95.
217. Kajumo, F., D.A. Thompson, Y. Guo and T. Dragic, *Entry of R5X4 and X4 human immunodeficiency virus type 1 strains is mediated by negatively charged and tyrosine residues in the amino-terminal domain and the second extracellular loop of CXCR4.* Virology, 2000. **271**(2): p. 240-7.
218. Brelot, A., N. Heveker, O. Pleskoff, N. Sol and M. Alizon, *Role of the first and third extracellular domains of CXCR-4 in human immunodeficiency virus coreceptor activity.* J Virol, 1997. **71**(6): p. 4744-51.
219. Lu, Z., J.F. Berson, Y. Chen, J.D. Turner, T. Zhang, M. Sharron, M.H. Jenks, Z. Wang, J. Kim, J. Rucker, J.A. Hoxie, S.C. Peiper and R.W. Doms, *Evolution of HIV-1 coreceptor usage through interactions with distinct CCR5 and CXCR4 domains.* Proc Natl Acad Sci U S A, 1997. **94**(12): p. 6426-31.
220. Chan, S.Y., R.F. Speck, C. Power, S.L. Gaffen, B. Chesebro and M.A. Goldsmith, *V3 recombinants indicate a central role for CCR5 as a coreceptor in tissue infection by human immunodeficiency virus type 1.* J Virol, 1999. **73**(3): p. 2350-8.
221. Shimizu, N., Y. Haraguchi, Y. Takeuchi, Y. Soda, K. Kanbe and H. Hoshino, *Changes in and discrepancies between cell tropisms and coreceptor uses of human immunodeficiency virus type 1 induced by single point mutations at the V3 tip of the env protein.* Virology, 1999. **259**(2): p. 324-33.
222. Hartley, O., P.J. Klasse, Q.J. Sattentau and J.P. Moore, *V3: HIV's switch-hitter.* AIDS Res Hum Retroviruses, 2005. **21**(2): p. 171-89.
223. Parren, P.W., J.P. Moore, D.R. Burton and Q.J. Sattentau, *The neutralizing antibody response to HIV-1: viral evasion and escape from humoral immunity.* Aids, 1999. **13 Suppl A**: p. S137-62.

224. Rizzuto, C. and J. Sodroski, *Fine definition of a conserved CCR5-binding region on the human immunodeficiency virus type 1 glycoprotein 120.* AIDS Res Hum Retroviruses, 2000. **16**(8): p. 741-9.
225. Saphire, E.O., P.W. Parren, R. Pantophlet, M.B. Zwick, G.M. Morris, P.M. Rudd, R.A. Dwek, R.L. Stanfield, D.R. Burton and I.A. Wilson, *Crystal structure of a neutralizing human IGG against HIV-1: a template for vaccine design.* Science, 2001. **293**(5532): p. 1155-9.
226. Stanfield, R.L., M.K. Gorny, S. Zolla-Pazner and I.A. Wilson, *Crystal structures of human immunodeficiency virus type 1 (HIV-1) neutralizing antibody 2219 in complex with three different V3 peptides reveal a new binding mode for HIV-1 cross-reactivity.* J Virol, 2006. **80**(12): p. 6093-105.
227. Bjorndal, A., H. Deng, M. Jansson, J.R. Fiore, C. Colognesi, A. Karlsson, J. Albert, G. Scarlatti, D.R. Littman and E.M. Fenyo, *Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype.* J Virol, 1997. **71**(10): p. 7478-87.
228. de Roda Husman, A.M., R.P. van Rij, H. Blaak, S. Broersen and H. Schuitemaker, *Adaptation to promiscuous usage of chemokine receptors is not a prerequisite for human immunodeficiency virus type 1 disease progression.* J Infect Dis, 1999. **180**(4): p. 1106-15.
229. Fouchier, R.A., M. Brouwer, S.M. Broersen and H. Schuitemaker, *Simple determination of human immunodeficiency virus type 1 syncytium-inducing V3 genotype by PCR.* J Clin Microbiol, 1995. **33**(4): p. 906-11.
230. Milich, L., B. Margolin and R. Swanstrom, *V3 loop of the human immunodeficiency virus type 1 Env protein: interpreting sequence variability.* J Virol, 1993. **67**(9): p. 5623-34.
231. Hoffman, N.G., F. Seillier-Moiseiwitsch, J. Ahn, J.M. Walker and R. Swanstrom, *Variability in the human immunodeficiency virus type 1 gp120 Env protein linked to phenotype-associated changes in the V3 loop.* J Virol, 2002. **76**(8): p. 3852-64.
232. Xiao, L., S.M. Owen, I. Goldman, A.A. Lal, J.J. deJong, J. Goudsmit and R.B. Lal, *CCR5 coreceptor usage of non-syncytium-inducing primary HIV-1 is independent of phylogenetically distinct global HIV-1 isolates: delineation of consensus motif in the V3 domain that predicts CCR-5 usage.* Virology, 1998. **240**(1): p. 83-92.
233. Brumme, Z.L., J. Goodrich, H.B. Mayer, C.J. Brumme, B.M. Henrick, B. Wynhoven, J.J. Asselin, P.K. Cheung, R.S. Hogg, J.S. Montaner and P.R. Harrigan, *Molecular and clinical epidemiology of CXCR4-using HIV-1 in a large population of antiretroviral-naive individuals.* J Infect Dis, 2005. **192**(3): p. 466-74.
234. Milich, L., B.H. Margolin and R. Swanstrom, *Patterns of amino acid variability in NSI-like and SI-like V3 sequences and a linked change in the CD4-binding domain of the HIV-1 Env protein.* Virology, 1997. **239**(1): p. 108-18.
235. Brumme, Z.L., W.W. Dong, B. Yip, B. Wynhoven, N.G. Hoffman, R. Swanstrom, M.A. Jensen, J.I. Mullins, R.S. Hogg, J.S. Montaner and P.R.

Harrigan, *Clinical and immunological impact of HIV envelope V3 sequence variation after starting initial triple antiretroviral therapy.* Aids, 2004. **18**(4): p. F1-9.
236. Coetzer, M., T. Cilliers, M. Papathanasopoulos, G. Ramjee, S.A. Karim, C. Williamson and L. Morris, *Longitudinal analysis of HIV type 1 subtype C envelope sequences from South Africa.* AIDS Res Hum Retroviruses, 2007. **23**(2): p. 316-21.
237. Gorry, P.R., R.L. Dunfee, M.E. Mefford, K. Kunstman, T. Morgan, J.P. Moore, J.R. Mascola, K. Agopian, G.H. Holm, A. Mehle, J. Taylor, M. Farzan, H. Wang, P. Ellery, S.J. Willey, P.R. Clapham, S.M. Wolinsky, S.M. Crowe and D. Gabuzda, *Changes in the V3 region of gp120 contribute to unusually broad coreceptor usage of an HIV-1 isolate from a CCR5 Delta32 heterozygote.* Virology, 2007. **362**(1): p. 163-78.
238. Engelbrecht, S., T. de Villiers, C.C. Sampson, J. zur Megede, S.W. Barnett and E.J. van Rensburg, *Genetic analysis of the complete gag and env genes of HIV type 1 subtype C primary isolates from South Africa.* AIDS Res Hum Retroviruses, 2001. **17**(16): p. 1533-47.
239. McCormack, G.P., J.R. Glynn, A.C. Crampin, F. Sibande, D. Mulawa, L. Bliss, P. Broadbent, K. Abarca, J.M. Ponnighaus, P.E. Fine and J.P. Clewley, *Early evolution of the human immunodeficiency virus type 1 subtype C epidemic in rural Malawi.* J Virol, 2002. **76**(24): p. 12890-9.
240. Van Harmelen, J.H., E. Van der Ryst, A.S. Loubser, D. York, S. Madurai, S. Lyons, R. Wood and C. Williamson, *A predominantly HIV type 1 subtype C-restricted epidemic in South African urban populations.* AIDS Res Hum Retroviruses, 1999. **15**(4): p. 395-8.
241. Back, N.K., L. Smit, J.J. De Jong, W. Keulen, M. Schutten, J. Goudsmit and M. Tersmette, *An N-glycan within the human immunodeficiency virus type 1 gp120 V3 loop affects virus neutralization.* Virology, 1994. **199**(2): p. 431-8.
242. Schonning, K., B. Jansson, S. Olofsson, J.O. Nielsen and J.S. Hansen, *Resistance to V3-directed neutralization caused by an N-linked oligosaccharide depends on the quaternary structure of the HIV-1 envelope oligomer.* Virology, 1996. **218**(1): p. 134-40.
243. Polzer, S., M.T. Dittmar, H. Schmitz and M. Schreiber, *The N-linked glycan g15 within the V3 loop of the HIV-1 external glycoprotein gp120 affects coreceptor usage, cellular tropism, and neutralization.* Virology, 2002. **304**(1): p. 70-80.
244. Ogert, R.A., M.K. Lee, W. Ross, A. Buckler-White, M.A. Martin and M.W. Cho, *N-linked glycosylation sites adjacent to and within the V1/V2 and the V3 loops of dualtropic human immunodeficiency virus type 1 isolate DH12 gp120 affect coreceptor usage and cellular tropism.* J Virol, 2001. **75**(13): p. 5998-6006.
245. Coetzer, M., T. Cilliers, L.H. Ping, R. Swanstrom and L. Morris, *Genetic characteristics of the V3 region associated with CXCR4 usage in HIV-1 subtype C isolates.* Virology, 2006. **356**(1-2): p. 95-105.

246. Shioda, T., J.A. Levy and C. Cheng-Mayer, *Small amino acid changes in the V3 hypervariable region of gp120 can affect the T-cell-line and macrophage tropism of human immunodeficiency virus type 1.* Proc Natl Acad Sci U S A, 1992. **89**(20): p. 9434-8.
247. LaRosa, G.J., J.P. Davide, K. Weinhold, J.A. Waterbury, A.T. Profy, J.A. Lewis, A.J. Langlois, G.R. Dreesman, R.N. Boswell, P. Shadduck and et al., *Conserved sequence and structural elements in the HIV-1 principal neutralizing determinant.* Science, 1990. **249**(4971): p. 932-5.
248. Jansson, M., B. Wahren, J. Albert, G. Scarlatti, L. Franzen, P.K. Kataaha, E. Katabira, F. Mubiro, H. Wigzell and P. Rossi, *Peptide serology for analysis of the inter- and intra-individual variation in the HIV-1 V3 domain.* Aids, 1994. **8**(4): p. 413-21.
249. Cecilia, D., S.S. Kulkarni, S.P. Tripathy, R.R. Gangakhedkar, R.S. Paranjape and D.A. Gadkari, *Absence of coreceptor switch with disease progression in human immunodeficiency virus infections in India.* Virology, 2000. **271**(2): p. 253-8.
250. Neilson, J.R., G.C. John, J.K. Carr, P. Lewis, J.K. Kreiss, S. Jackson, R.W. Nduati, D. Mbori-Ngacha, D.D. Panteleeff, S. Bodrug, C. Giachetti, M.A. Bott, B.A. Richardson, J. Bwayo, J. Ndinya-Achola and J. Overbaugh, *Subtypes of human immunodeficiency virus type 1 and disease stage among women in Nairobi, Kenya.* J Virol, 1999. **73**(5): p. 4393-403.
251. Ndung'u, T., E. Sepako, M.F. McLane, F. Chand, K. Bedi, S. Gaseitsiwe, F. Doualla-Bell, T. Peter, I. Thior, S.M. Moyo, P.B. Gilbert, V.A. Novitsky and M. Essex, *HIV-1 subtype C in vitro growth and coreceptor utilization.* Virology, 2006. **347**(2): p. 247-60.
252. Choge, I., T. Cilliers, P. Walker, N. Taylor, M. Phoswa, T. Meyers, J. Viljoen, A. Violari, G. Gray, P.L. Moore, M. Papathanosopoulos and L. Morris, *Genotypic and phenotypic characterization of viral isolates from HIV-1 subtype C-infected children with slow and rapid disease progression.* AIDS Res Hum Retroviruses, 2006. **22**(5): p. 458-65.
253. Bjorndal, A., A. Sonnerborg, C. Tscherning, J. Albert and E.M. Fenyo, *Phenotypic characteristics of human immunodeficiency virus type 1 subtype C isolates of Ethiopian AIDS patients.* AIDS Res Hum Retroviruses, 1999. **15**(7): p. 647-53.
254. Morris, L., T. Cilliers, H. Bredell, M. Phoswa and D.J. Martin, *CCR5 is the major coreceptor used by HIV-1 subtype C isolates from patients with active tuberculosis.* AIDS Res Hum Retroviruses, 2001. **17**(8): p. 697-701.
255. Peeters, M., R. Vincent, J.L. Perret, M. Lasky, D. Patrel, F. Liegeois, V. Courgnaud, R. Seng, T. Matton, S. Molinier and E. Delaporte, *Evidence for differences in MT2 cell tropism according to genetic subtypes of HIV-1: syncytium-inducing variants seem rare among subtype C HIV-1 viruses.* J Acquir Immune Defic Syndr Hum Retrovirol, 1999. **20**(2): p. 115-21.
256. Ping, L.H., J.A. Nelson, I.F. Hoffman, J. Schock, S.L. Lamers, M. Goodman, P. Vernazza, P. Kazembe, M. Maida, D. Zimba, M.M. Goodenow, J.J. Eron, Jr., S.A. Fiscus, M.S. Cohen and R. Swanstrom, *Characterization of V3 sequence heterogeneity in subtype C human*

immunodeficiency virus type 1 isolates from Malawi: underrepresentation of X4 variants. J Virol, 1999. **73**(8): p. 6271-81.
257. Abebe, A., D. Demissie, J. Goudsmit, M. Brouwer, C.L. Kuiken, G. Pollakis, H. Schuitemaker, A.L. Fontanet and T.F. Rinke de Wit, *HIV-1 subtype C syncytium- and non-syncytium-inducing phenotypes and coreceptor usage among Ethiopian patients with AIDS.* Aids, 1999. **13**(11): p. 1305-11.
258. Cilliers, T., J. Nhlapo, M. Coetzer, D. Orlovic, T. Ketas, W.C. Olson, J.P. Moore, A. Trkola and L. Morris, *The CCR5 and CXCR4 coreceptors are both used by human immunodeficiency virus type 1 primary isolates from subtype C.* J Virol, 2003. **77**(7): p. 4449-56.
259. Batra, M., P.C. Tien, R.W. Shafer, C.H. Contag and D.A. Katzenstein, *HIV type 1 envelope subtype C sequences from recent seroconverters in Zimbabwe.* AIDS Res Hum Retroviruses, 2000. **16**(10): p. 973-9.
260. Tscherning, C., A. Alaeus, R. Fredriksson, A. Bjorndal, H. Deng, D.R. Littman, E.M. Fenyo and J. Albert, *Differences in chemokine coreceptor usage between genetic subtypes of HIV-1.* Virology, 1998. **241**(2): p. 181-8.
261. Sanchez-Merino, V., C.C. Herrero, A. Amorin-Nink, H. von Briesen and C. Lopez-Galindez, *Genetic analysis of culture-negative UNAIDS subtype C samples.* AIDS Res Hum Retroviruses, 2003. **19**(1): p. 49-55.
262. Papathanasopoulos, M.A., T. Cilliers, L. Morris, J.L. Mokili, W. Dowling, D.L. Birx and F.E. McCutchan, *Full-length genome analysis of HIV-1 subtype C utilizing CXCR4 and intersubtype recombinants isolated in South Africa.* AIDS Res Hum Retroviruses, 2002. **18**(12): p. 879-86.
263. Rodenburg, C.M., Y. Li, S.A. Trask, Y. Chen, J. Decker, D.L. Robertson, M.L. Kalish, G.M. Shaw, S. Allen, B.H. Hahn and F. Gao, *Near full-length clones and reference sequences for subtype C isolates of HIV type 1 from three different continents.* AIDS Res Hum Retroviruses, 2001. **17**(2): p. 161-8.
264. Johnston, E.R., L.S. Zijenah, S. Mutetwa, R. Kantor, C. Kittinunvorakoon and D.A. Katzenstein, *High frequency of syncytium-inducing and CXCR4-tropic viruses among human immunodeficiency virus type 1 subtype C-infected patients receiving antiretroviral treatment.* J Virol, 2003. **77**(13): p. 7682-8.
265. Westby, M., M. Lewis, J. Whitcomb, M. Youle, A.L. Pozniak, I.T. James, T.M. Jenkins, M. Perros and E. van der Ryst, *Emergence of CXCR4-Using Human Immunodeficiency Virus Type 1 (HIV-1) Variants in a Minority of HIV-1-Infected Patients following Treatment with the CCR5 Antagonist Maraviroc Is from a Pretreatment CXCR4-Using Virus Reservoir.* J Virol, 2006. **80**(10): p. 4909-20.
266. van Rij, R.P., J.A. Visser, R.M. van Praag, R. Rientsma, J.M. Prins, J.M. Lange and H. Schuitemaker, *Both R5 and X4 human immunodeficiency virus type 1 variants persist during prolonged therapy with five antiretroviral drugs.* J Virol, 2002. **76**(6): p. 3054-8.
267. Asjo, B., L. Morfeldt-Manson, J. Albert, G. Biberfeld, A. Karlsson, K. Lidman and E.M. Fenyo, *Replicative capacity of human immunodeficiency*

virus from patients with varying severity of HIV infection. Lancet, 1986. **2**(8508): p. 660-2.
268. Koot, M., A.H. Vos, R.P. Keet, R.E. de Goede, M.W. Dercksen, F.G. Terpstra, R.A. Coutinho, F. Miedema and M. Tersmette, *HIV-1 biological phenotype in long-term infected individuals evaluated with an MT-2 cocultivation assay.* Aids, 1992. **6**(1): p. 49-54.
269. Vodicka, M.A., W.C. Goh, L.I. Wu, M.E. Rogel, S.R. Bartz, V.L. Schweickart, C.J. Raport and M. Emerman, *Indicator cell lines for detection of primary strains of human and simian immunodeficiency viruses.* Virology, 1997. **233**(1): p. 193-8.
270. Jensen, M.A., M. Coetzer, A.B. van 't Wout, L. Morris and J.I. Mullins, *A reliable phenotype predictor for human immunodeficiency virus type 1 subtype C based on envelope V3 sequences.* J Virol, 2006. **80**(10): p. 4698-704.
271. Jensen, M.A., F.S. Li, A.B. van 't Wout, D.C. Nickle, D. Shriner, H.X. He, S. McLaughlin, R. Shankarappa, J.B. Margolick and J.I. Mullins, *Improved coreceptor usage prediction and genotypic monitoring of R5-to-X4 transition by motif analysis of human immunodeficiency virus type 1 env V3 loop sequences.* 2003. **77**(24): p. 13376-88.
272. Dorr, P., M. Westby, S. Dobbs, P. Griffin, B. Irvine, M. Macartney, J. Mori, G. Rickett, C. Smith-Burchnell, C. Napier, R. Webster, D. Armour, D. Price, B. Stammen, A. Wood and M. Perros, *Maraviroc (UK-427,857), a potent, orally bioavailable, and selective small-molecule inhibitor of chemokine receptor CCR5 with broad-spectrum anti-human immunodeficiency virus type 1 activity.* Antimicrob Agents Chemother, 2005. **49**(11): p. 4721-32.
273. Fatkenheuer, G., A.L. Pozniak, M.A. Johnson, A. Plettenberg, S. Staszewski, A.I. Hoepelman, M.S. Saag, F.D. Goebel, J.K. Rockstroh, B.J. Dezube, T.M. Jenkins, C. Medhurst, J.F. Sullivan, C. Ridgway, S. Abel, I.T. James, M. Youle and E. van der Ryst, *Efficacy of short-term monotherapy with maraviroc, a new CCR5 antagonist, in patients infected with HIV-1.* Nat Med, 2005. **11**(11): p. 1170-2.
274. Johnson, B.M., I.H. Song, K.K. Adkison, J. Borland, L. Fang, Y. Lou, M.M. Berrey, A.N. Nafziger, S.C. Piscitelli and J.S. Bertino, Jr., *Evaluation of the drug interaction potential of aplaviroc, a novel human immunodeficiency virus entry inhibitor, using a modified cooperstown 5 + 1 cocktail.* J Clin Pharmacol, 2006. **46**(5): p. 577-87.
275. Strizki, J.M., C. Tremblay, S. Xu, L. Wojcik, N. Wagner, W. Gonsiorek, R.W. Hipkin, C.C. Chou, C. Pugliese-Sivo, Y. Xiao, J.R. Tagat, K. Cox, T. Priestley, S. Sorota, W. Huang, M. Hirsch, G.R. Reyes and B.M. Baroudy, *Discovery and characterization of vicriviroc (SCH 417690), a CCR5 antagonist with potent activity against human immunodeficiency virus type 1.* Antimicrob Agents Chemother, 2005. **49**(12): p. 4911-9.
276. Profy, A.T., P.A. Salinas, L.I. Eckler, N.M. Dunlop, P.L. Nara and S.D. Putney, *Epitopes recognized by the neutralizing antibodies of an HIV-1-infected individual.* J Immunol, 1990. **144**(12): p. 4641-7.

277. Moulard, M., S.K. Phogat, Y. Shu, A.F. Labrijn, X. Xiao, J.M. Binley, M.Y. Zhang, I.A. Sidorov, C.C. Broder, J. Robinson, P.W. Parren, D.R. Burton and D.S. Dimitrov, *Broadly cross-reactive HIV-1-neutralizing human monoclonal Fab selected for binding to gp120-CD4-CCR5 complexes.* Proc Natl Acad Sci U S A, 2002. **99**(10): p. 6913-8.
278. Muster, T., F. Steindl, M. Purtscher, A. Trkola, A. Klima, G. Himmler, F. Ruker and H. Katinger, *A conserved neutralizing epitope on gp41 of human immunodeficiency virus type 1.* J Virol, 1993. **67**(11): p. 6642-7.
279. Nabatov, A.A., G. Pollakis, T. Linnemann, A. Kliphius, M.I. Chalaby and W.A. Paxton, *Intrapatient alterations in the human immunodeficiency virus type 1 gp120 V1V2 and V3 regions differentially modulate coreceptor usage, virus inhibition by CC/CXC chemokines, soluble CD4, and the b12 and 2G12 monoclonal antibodies.* J Virol, 2004. **78**(1): p. 524-30.
280. Nelson, J.D., F.M. Brunel, R. Jensen, E.T. Crooks, R.M. Cardoso, M. Wang, A. Hessell, I.A. Wilson, J.M. Binley, P.E. Dawson, D.R. Burton and M.B. Zwick, *An Affinity-Enhanced Neutralizing Antibody against the Membrane-Proximal External Region of Human Immunodeficiency Virus Type 1 gp41 Recognizes an Epitope between Those of 2F5 and 4E10.* J Virol, 2007. **81**(8): p. 4033-43.
281. Scanlan, C.N., R. Pantophlet, M.R. Wormald, E. Ollmann Saphire, R. Stanfield, I.A. Wilson, H. Katinger, R.A. Dwek, P.M. Rudd and D.R. Burton, *The broadly neutralizing anti-human immunodeficiency virus type 1 antibody 2G12 recognizes a cluster of alpha1-->2 mannose residues on the outer face of gp120.* J Virol, 2002. **76**(14): p. 7306-21.
282. Stiegler, G., R. Kunert, M. Purtscher, S. Wolbank, R. Voglauer, F. Steindl and H. Katinger, *A potent cross-clade neutralizing human monoclonal antibody against a novel epitope on gp41 of human immunodeficiency virus type 1.* AIDS Res Hum Retroviruses, 2001. **17**(18): p. 1757-65.
283. Trkola, A., M. Purtscher, T. Muster, C. Ballaun, A. Buchacher, N. Sullivan, K. Srinivasan, J. Sodroski, J.P. Moore and H. Katinger, *Human monoclonal antibody 2G12 defines a distinctive neutralization epitope on the gp120 glycoprotein of human immunodeficiency virus type 1.* J Virol, 1996. **70**(2): p. 1100-8.
284. Zwick, M.B., R. Jensen, S. Church, M. Wang, G. Stiegler, R. Kunert, H. Katinger and D.R. Burton, *Anti-human immunodeficiency virus type 1 (HIV-1) antibodies 2F5 and 4E10 require surprisingly few crucial residues in the membrane-proximal external region of glycoprotein gp41 to neutralize HIV-1.* J Virol, 2005. **79**(2): p. 1252-61.
285. Burton, D.R., J. Pyati, R. Koduri, S.J. Sharp, G.B. Thornton, P.W. Parren, L.S. Sawyer, R.M. Hendry, N. Dunlop, P.L. Nara and et al., *Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody.* Science, 1994. **266**(5187): p. 1024-7.
286. Trkola, A., A.B. Pomales, H. Yuan, B. Korber, P.J. Maddon, G.P. Allaway, H. Katinger, C.F. Barbas, 3rd, D.R. Burton, D.D. Ho and et al., *Cross-clade neutralization of primary isolates of human immunodeficiency virus type 1*

by human monoclonal antibodies and tetrameric CD4-IgG. J Virol, 1995. **69**(11): p. 6609-17.
287. Sanders, R.W., M. Venturi, L. Schiffner, R. Kalyanaraman, H. Katinger, K.O. Lloyd, P.D. Kwong and J.P. Moore, *The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120.* J Virol, 2002. **76**(14): p. 7293-305.
288. Bou-Habib, D.C., G. Roderiquez, T. Oravecz, P.W. Berman, P. Lusso and M.A. Norcross, *Cryptic nature of envelope V3 region epitopes protects primary monocytotropic human immunodeficiency virus type 1 from antibody neutralization.* J Virol, 1994. **68**(9): p. 6006-13.
289. Cheng-Mayer, C., J. Homsy, L.A. Evans and J.A. Levy, *Identification of human immunodeficiency virus subtypes with distinct patterns of sensitivity to serum neutralization.* Proc Natl Acad Sci U S A, 1988. **85**(8): p. 2815-9.
290. Javaherian, K., A.J. Langlois, C. McDanal, K.L. Ross, L.I. Eckler, C.L. Jellis, A.T. Profy, J.R. Rusche, D.P. Bolognesi, S.D. Putney and et al., *Principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein.* Proc Natl Acad Sci U S A, 1989. **86**(17): p. 6768-72.
291. Vancott, T.C., V.R. Polonis, L.D. Loomis, N.L. Michael, P.L. Nara and D.L. Birx, *Differential role of V3-specific antibodies in neutralization assays involving primary and laboratory-adapted isolates of HIV type 1.* AIDS Res Hum Retroviruses, 1995. **11**(11): p. 1379-91.
292. Vogel, T., R. Kurth and S. Norley, *The majority of neutralizing Abs in HIV-1-infected patients recognize linear V3 loop sequences. Studies using HIV-1MN multiple antigenic peptides.* J Immunol, 1994. **153**(4): p. 1895-904.
293. Ebenbichler, C., P. Westervelt, A. Carrillo, T. Henkel, D. Johnson and L. Ratner, *Structure-function relationships of the HIV-1 envelope V3 loop tropism determinant: evidence for two distinct conformations.* Aids, 1993. **7**(5): p. 639-46.
294. Fauci, A.S., *HIV and AIDS: 20 years of science.* Nat Med, 2003. **9**(7): p. 839-43.
295. Daelemans, D., R. Lu, E. De Clercq and A. Engelman, *Characterization of a Replication-Competent, Integrase-Defective Human Immunodeficiency Virus (HIV)/Simian Virus 40 Chimera as a Powerful Tool for the Discovery and Validation of HIV Integrase Inhibitors.* J Virol, 2007. **81**(8): p. 4381-5.
296. Liu, S., S. Wu and S. Jiang, *HIV entry inhibitors targeting gp41: from polypeptides to small-molecule compounds.* Curr Pharm Des, 2007. **13**(2): p. 143-62.
297. Zhou, J., C.H. Chen and C. Aiken, *Human immunodeficiency virus type 1 resistance to the small molecule maturation inhibitor 3-O-(3',3'-dimethylsuccinyl)-betulinic acid is conferred by a variety of single amino acid substitutions at the CA-SP1 cleavage site in Gag.* J Virol, 2006. **80**(24): p. 12095-101.
298. Huang, M., A. Maynard, J.A. Turpin, L. Graham, G.M. Janini, D.G. Covell and W.G. Rice, *Anti-HIV agents that selectively target retroviral nucleocapsid protein zinc fingers without affecting cellular zinc finger proteins.* J Med Chem, 1998. **41**(9): p. 1371-81.

299. Unwalla, H., S. Chakraborti, V. Sood, N. Gupta and A.C. Banerjea, *Potent inhibition of HIV-1 gene expression and TAT-mediated apoptosis in human T cells by novel mono- and multitarget anti-TAT/Rev/Env ribozymes and a general purpose RNA-cleaving DNA-enzyme.* Antiviral Res, 2006. **72**(2): p. 134-44.
300. Kilby, J.M., J.P. Lalezari, J.J. Eron, M. Carlson, C. Cohen, R.C. Arduino, J.C. Goodgame, J.E. Gallant, P. Volberding, R.L. Murphy, F. Valentine, M.S. Saag, E.L. Nelson, P.R. Sista and A. Dusek, *The safety, plasma pharmacokinetics, and antiviral activity of subcutaneous enfuvirtide (T-20), a peptide inhibitor of gp41-mediated virus fusion, in HIV-infected adults.* AIDS Res Hum Retroviruses, 2002. **18**(10): p. 685-93.
301. Wild, C.T., D.C. Shugars, T.K. Greenwell, C.B. McDanal and T.J. Matthews, *Peptides corresponding to a predictive alpha-helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection.* Proc Natl Acad Sci U S A, 1994. **91**(21): p. 9770-4.
302. Deeks, S.G., J. Lu, R. Hoh, T.B. Neilands, G. Beatty, W. Huang, T. Liegler, P. Hunt, J.N. Martin and D.R. Kuritzkes, *Interruption of Enfuvirtide in HIV-1-Infected Adults with Incomplete Viral Suppression on an Enfuvirtide-Based Regimen.* J Infect Dis, 2007. **195**(3): p. 387-91.
303. Allaway, G.P., K.L. Davis-Bruno, G.A. Beaudry, E.B. Garcia, E.L. Wong, A.M. Ryder, K.W. Hasel, M.C. Gauduin, R.A. Koup, J.S. McDougal and et al., *Expression and characterization of CD4-IgG2, a novel heterotetramer that neutralizes primary HIV type 1 isolates.* AIDS Res Hum Retroviruses, 1995. **11**(5): p. 533-9.
304. Guo, Q., H.T. Ho, I. Dicker, L. Fan, N. Zhou, J. Friborg, T. Wang, B.V. McAuliffe, H.G. Wang, R.E. Rose, H. Fang, H.T. Scarnati, D.R. Langley, N.A. Meanwell, R. Abraham, R.J. Colonno and P.F. Lin, *Biochemical and genetic characterizations of a novel human immunodeficiency virus type 1 inhibitor that blocks gp120-CD4 interactions.* J Virol, 2003. **77**(19): p. 10528-36.
305. Lin, P.F., W. Blair, T. Wang, T. Spicer, Q. Guo, N. Zhou, Y.F. Gong, H.G. Wang, R. Rose, G. Yamanaka, B. Robinson, C.B. Li, R. Fridell, C. Deminie, G. Demers, Z. Yang, L. Zadjura, N. Meanwell and R. Colonno, *A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding.* Proc Natl Acad Sci U S A, 2003. **100**(19): p. 11013-8.
306. Boyd, M.R., K.R. Gustafson, J.B. McMahon, R.H. Shoemaker, B.R. O'Keefe, T. Mori, R.J. Gulakowski, L. Wu, M.I. Rivera, C.M. Laurencot, M.J. Currens, J.H. Cardellina, 2nd, R.W. Buckheit, Jr., P.L. Nara, L.K. Pannell, R.C. Sowder, 2nd and L.E. Henderson, *Discovery of cyanovirin-N, a novel human immunodeficiency virus-inactivating protein that binds viral surface envelope glycoprotein gp120: potential applications to microbicide development.* Antimicrob Agents Chemother, 1997. **41**(7): p. 1521-30.
307. Dey, B., D.L. Lerner, P. Lusso, M.R. Boyd, J.H. Elder and E.A. Berger, *Multiple antiviral activities of cyanovirin-N: blocking of human immunodeficiency virus type 1 gp120 interaction with CD4 and coreceptor*

and inhibition of diverse enveloped viruses. J Virol, 2000. **74**(10): p. 4562-9.
308. Simmons, G., P.R. Clapham, L. Picard, R.E. Offord, M.M. Rosenkilde, T.W. Schwartz, R. Buser, T.N. Wells and A.E. Proudfoot, *Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist.* Science, 1997. **276**(5310): p. 276-9.
309. Baba, M., O. Nishimura, N. Kanzaki, M. Okamoto, H. Sawada, Y. Iizawa, M. Shiraishi, Y. Aramaki, K. Okonogi, Y. Ogawa, K. Meguro and M. Fujino, *A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity.* Proc Natl Acad Sci U S A, 1999. **96**(10): p. 5698-703.
310. Dragic, T., A. Trkola, D.A. Thompson, E.G. Cormier, F.A. Kajumo, E. Maxwell, S.W. Lin, W. Ying, S.O. Smith, T.P. Sakmar and J.P. Moore, *A binding pocket for a small molecule inhibitor of HIV-1 entry within the transmembrane helices of CCR5.* Proc Natl Acad Sci U S A, 2000. **97**(10): p. 5639-44.
311. Takashima, K., H. Miyake, N. Kanzaki, Y. Tagawa, X. Wang, Y. Sugihara, Y. Iizawa and M. Baba, *Highly potent inhibition of human immunodeficiency virus type 1 replication by TAK-220, an orally bioavailable small-molecule CCR5 antagonist.* Antimicrob Agents Chemother, 2005. **49**(8): p. 3474-82.
312. Fernandez, E.J. and E. Lolis, *Structural studies of chemokines that inhibit HIV-1 entry.* Antivir Chem Chemother, 2001. **12 Suppl 1**: p. 43-9.
313. Bleul, C.C., M. Farzan, H. Choe, C. Parolin, I. Clark-Lewis, J. Sodroski and T.A. Springer, *The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry.* Nature, 1996. **382**(6594): p. 829-33.
314. Labrosse, B., A. Brelot, N. Heveker, N. Sol, D. Schols, E. De Clercq and M. Alizon, *Determinants for sensitivity of human immunodeficiency virus coreceptor CXCR4 to the bicyclam AMD3100.* J Virol, 1998. **72**(8): p. 6381-8.
315. Schols, D., S. Struyf, J. Van Damme, J.A. Este, G. Henson and E. De Clercq, *Inhibition of T-tropic HIV strains by selective antagonization of the chemokine receptor CXCR4.* J Exp Med, 1997. **186**(8): p. 1383-8.
316. O'Brien, W.A., M. Sumner-Smith, S.H. Mao, S. Sadeghi, J.Q. Zhao and I.S. Chen, *Anti-human immunodeficiency virus type 1 activity of an oligocationic compound mediated via gp120 V3 interactions.* J Virol, 1996. **70**(5): p. 2825-31.
317. Tamamura, H., A. Otaka, T. Murakami, T. Ishihara, T. Ibuka, M. Waki, A. Matsumoto, N. Yamamoto and N. Fujii, *Interaction of an anti-HIV peptide, T22, with gp120 and CD4.* Biochem Biophys Res Commun, 1996. **219**(2): p. 555-9.
318. Castagna, A., P. Biswas, A. Beretta and A. Lazzarin, *The appealing story of HIV entry inhibitors : from discovery of biological mechanisms to drug development.* Drugs, 2005. **65**(7): p. 879-904.
319. Reeves, J.D. and A.J. Piefer, *Emerging drug targets for antiretroviral therapy.* Drugs, 2005. **65**(13): p. 1747-66.

320. Maeda, K., H. Nakata, Y. Koh, T. Miyakawa, H. Ogata, Y. Takaoka, S. Shibayama, K. Sagawa, D. Fukushima, J. Moravek, Y. Koyanagi and H. Mitsuya, *Spirodiketopiperazine-based CCR5 inhibitor which preserves CC-chemokine/CCR5 interactions and exerts potent activity against R5 human immunodeficiency virus type 1 in vitro.* J Virol, 2004. **78**(16): p. 8654-62.
321. Schols, D., *HIV co-receptors as targets for antiviral therapy.* Curr Top Med Chem, 2004. **4**(9): p. 883-93.
322. Lalezari, J., M. Lederman, G. Yadavalli, M. Para, G. Richmond, E. DeJesus, J. Searle, W. Cai, V. Roschke, J. Zhong, C. Hicks, W. Freimuth and M. Subramanian. *New CCR5 Drug HGS004 Phase 1 Study Results*. in *46th Annual ICAAC Interscience Conference on Antimicrobial Agents and Chemotherapy*. 2006. San Francisco.
323. Olson, W.C., G.E. Rabut, K.A. Nagashima, D.N. Tran, D.J. Anselma, S.P. Monard, J.P. Segal, D.A. Thompson, F. Kajumo, Y. Guo, J.P. Moore, P.J. Maddon and T. Dragic, *Differential inhibition of human immunodeficiency virus type 1 fusion, gp120 binding, and CC-chemokine activity by monoclonal antibodies to CCR5.* J Virol, 1999. **73**(5): p. 4145-55.
324. Strizki, J.M., S. Xu, N.E. Wagner, L. Wojcik, J. Liu, Y. Hou, M. Endres, A. Palani, S. Shapiro, J.W. Clader, W.J. Greenlee, J.R. Tagat, S. McCombie, K. Cox, A.B. Fawzi, C.C. Chou, C. Pugliese-Sivo, L. Davies, M.E. Moreno, D.D. Ho, A. Trkola, C.A. Stoddart, J.P. Moore, G.R. Reyes and B.M. Baroudy, *SCH-C (SCH 351125), an orally bioavailable, small molecule antagonist of the chemokine receptor CCR5, is a potent inhibitor of HIV-1 infection in vitro and in vivo.* Proc Natl Acad Sci U S A, 2001. **98**(22): p. 12718-23.
325. Tagat, J.R., S.W. McCombie, D. Nazareno, M.A. Labroli, Y. Xiao, R.W. Steensma, J.M. Strizki, B.M. Baroudy, K. Cox, J. Lachowicz, G. Varty and R. Watkins, *Piperazine-based CCR5 antagonists as HIV-1 inhibitors. IV. Discovery of 1-[(4,6-dimethyl-5-pyrimidinyl)carbonyl]- 4-[4-[2-methoxy-1(R)-4-(trifluoromethyl)phenyl]ethyl-3(S)-methyl-1-piperaz inyl]- 4-methylpiperidine (Sch-417690/Sch-D), a potent, highly selective, and orally bioavailable CCR5 antagonist.* J Med Chem, 2004. **47**(10): p. 2405-8.
326. *Schering-Plough discontinues Phase II study of vicriviroc in treatment-naive HIV patients, continues Phase II study in treatment-experienced patients*. [News Release] 2005 [cited 01 October 2007]; Available from: <http://www.scheringplough.com/schering_plough/news/release.jsp?releaseID=774673>.
327. Wallis, C., *Evaluation and Design of Affordable and Novel HIV-1 Drug Resistance Assays: with reference to mutations arising from 2 nucleoside reverse transcriptase inhibitors, Stavudine & Didanosine, & one non-nucleoside reverse transcriptase inhibitor, Efavirenz.*, in *Department of Molecular Medicine and Haematology*. 2005, University of the Witwatersrand: Johannesburg. p. 168.
328. Lazzari, S., A. de Felici, H. Sobel and S. Bertagnolio, *HIV drug resistance surveillance: summary of an April 2003 WHO consultation.* Aids, 2004. **18 Suppl 3**: p. S49-53.

329. Kijak, G.H. and F.E. McCutchan, *HIV diversity, molecular epidemiology, and the role of recombination.* Curr Infect Dis Rep, 2005. **7**(6): p. 480-8.
330. Steain, M.C., B. Wang, D.E. Dwyer and N.K. Saksena, *HIV-1 co-infection, superinfection and recombination.* Sex Health, 2004. **1**(4): p. 239-50.
331. Poignard, P., E.O. Saphire, P.W. Parren and D.R. Burton, *gp120: Biologic aspects of structural features.* Annu Rev Immunol, 2001. **19**: p. 253-74.
332. Burton, D.R., R.L. Stanfield and I.A. Wilson, *Antibody vs. HIV in a clash of evolutionary titans.* Proc Natl Acad Sci U S A, 2005. **102**(42): p. 14943-8.
333. Huang, H., R. Chopra, G.L. Verdine and S.C. Harrison, *Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance.* Science, 1998. **282**(5394): p. 1669-75.
334. Larder, B.A. and D.K. Stammers, *Closing in on HIV drug resistance.* Nat Struct Biol, 1999. **6**(2): p. 103-6.
335. Shafer, R.W. *'HIV in Site'- Genotypic Testing for HIV-1 drug resistance.* 2004 [cited 21 Febuary 2005]; Available from: http://hivinsite.ucsf.edu/InSite?page=kb-03-02-07.
336. Jackson, J.B., G. Becker-Pergola, L.A. Guay, P. Musoke, M. Mracna, M.G. Fowler, L.M. Mofenson, M. Mirochnick, F. Mmiro and S.H. Eshleman, *Identification of the K103N resistance mutation in Ugandan women receiving nevirapine to prevent HIV-1 vertical transmission.* Aids, 2000. **14**(11): p. F111-5.
337. Condra, J.H., *Resistance to HIV protease inhibitors.* Haemophilia, 1998. **4**(4): p. 610-5.
338. Miller, V., *International perspectives on antiretroviral resistance. Resistance to protease inhibitors.* J Acquir Immune Defic Syndr, 2001. **26 Suppl 1**: p. S34-50.
339. O'Meara, D., K. Wilbe, T. Leitner, B. Hejdeman, J. Albert and J. Lundeberg, *Monitoring resistance to human immunodeficiency virus type 1 protease inhibitors by pyrosequencing.* J Clin Microbiol, 2001. **39**(2): p. 464-73.
340. Chinnadurai, R., D. Rajan, J. Munch and F. Kirchhoff, *HIV-1 Variants Resistant against 1st and 2nd Generation Fusion Inhibitors and Cytopathic in ex vivo Human Lymphoid Tissue.* J Virol, 2007.
341. Greenberg, M.L. and N. Cammack, *Resistance to enfuvirtide, the first HIV fusion inhibitor.* J Antimicrob Chemother, 2004. **54**(2): p. 333-40.
342. Reeves, J.D., F.H. Lee, J.L. Miamidian, C.B. Jabara, M.M. Juntilla and R.W. Doms, *Enfuvirtide resistance mutations: impact on human immunodeficiency virus envelope function, entry inhibitor sensitivity, and virus neutralization.* J Virol, 2005. **79**(8): p. 4991-9.
343. Westby, M., C. Smith-Burchnell, J. Mori, M. Lewis, M. Mosley, M. Stockdale, P. Dorr, G. Ciaramella and M. Perros, *Reduced maximal inhibition in phenotypic susceptibility assays indicates that viral strains resistant to the CCR5 antagonist maraviroc utilize inhibitor-bound receptor for entry.* J Virol, 2007. **81**(5): p. 2359-71.

344. Dumonceaux, J., S. Nisole, C. Chanel, L. Quivet, A. Amara, F. Baleux, P. Briand and U. Hazan, *Spontaneous mutations in the env gene of the human immunodeficiency virus type 1 NDK isolate are associated with a CD4-independent entry phenotype.* J Virol, 1998. **72**(1): p. 512-9.
345. Edinger, A.L., J.L. Mankowski, B.J. Doranz, B.J. Margulies, B. Lee, J. Rucker, M. Sharron, T.L. Hoffman, J.F. Berson, M.C. Zink, V.M. Hirsch, J.E. Clements and R.W. Doms, *CD4-independent, CCR5-dependent infection of brain capillary endothelial cells by a neurovirulent simian immunodeficiency virus strain.* Proc Natl Acad Sci U S A, 1997. **94**(26): p. 14742-7.
346. Endres, M.J., P.R. Clapham, M. Marsh, M. Ahuja, J.D. Turner, A. McKnight, J.F. Thomas, B. Stoebenau-Haggarty, S. Choe, P.J. Vance, T.N. Wells, C.A. Power, S.S. Sutterwala, R.W. Doms, N.R. Landau and J.A. Hoxie, *CD4-independent infection by HIV-2 is mediated by fusin/CXCR4.* Cell, 1996. **87**(4): p. 745-56.
347. Reeves, J.D. and T.F. Schulz, *The CD4-independent tropism of human immunodeficiency virus type 2 involves several regions of the envelope protein and correlates with a reduced activation threshold for envelope-mediated fusion.* J Virol, 1997. **71**(2): p. 1453-65.
348. Bhattacharya, J., P.J. Peters and P.R. Clapham, *CD4-independent infection of HIV and SIV: implications for envelope conformation and cell tropism in vivo.* Aids, 2003. **17 Suppl 4**: p. S35-43.
349. Kolchinsky, P., T. Mirzabekov, M. Farzan, E. Kiprilov, M. Cayabyab, L.J. Mooney, H. Choe and J. Sodroski, *Adaptation of a CCR5-using, primary human immunodeficiency virus type 1 isolate for CD4-independent replication.* J Virol, 1999. **73**(10): p. 8120-6.
350. LaBranche, C.C., T.L. Hoffman, J. Romano, B.S. Haggarty, T.G. Edwards, T.J. Matthews, R.W. Doms and J.A. Hoxie, *Determinants of CD4 independence for a human immunodeficiency virus type 1 variant map outside regions required for coreceptor specificity.* J Virol, 1999. **73**(12): p. 10310-9.
351. Hoffman, T.L., C.C. LaBranche, W. Zhang, G. Canziani, J. Robinson, I. Chaiken, J.A. Hoxie and R.W. Doms, *Stable exposure of the coreceptor-binding site in a CD4-independent HIV-1 envelope protein.* Proc Natl Acad Sci U S A, 1999. **96**(11): p. 6359-64.
352. Moore, J.P. and P.L. Nara, *The role of the V3 loop of gp120 in HIV infection.* Aids, 1991. **5 Suppl 2**: p. S21-33.
353. Sattentau, Q.J. and J.P. Moore, *Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding.* J Exp Med, 1991. **174**(2): p. 407-15.
354. Edwards, T.G., T.L. Hoffman, F. Baribaud, S. Wyss, C.C. LaBranche, J. Romano, J. Adkinson, M. Sharron, J.A. Hoxie and R.W. Doms, *Relationships between CD4 independence, neutralization sensitivity, and exposure of a CD4-induced epitope in a human immunodeficiency virus type 1 envelope protein.* J Virol, 2001. **75**(11): p. 5230-9.

355. Kolchinsky, P., E. Kiprilov and J. Sodroski, *Increased neutralization sensitivity of CD4-independent human immunodeficiency virus variants.* J Virol, 2001. **75**(5): p. 2041-50.
356. Means, R.E., T. Matthews, J.A. Hoxie, M.H. Malim, T. Kodama and R.C. Desrosiers, *Ability of the V3 loop of simian immunodeficiency virus to serve as a target for antibody-mediated neutralization: correlation of neutralization sensitivity, growth in macrophages, and decreased dependence on CD4.* J Virol, 2001. **75**(8): p. 3903-15.
357. Puffer, B.A., S. Pohlmann, A.L. Edinger, D. Carlin, M.D. Sanchez, J. Reitter, D.D. Watry, H.S. Fox, R.C. Desrosiers and R.W. Doms, *CD4 independence of simian immunodeficiency virus Envs is associated with macrophage tropism, neutralization sensitivity, and attenuated pathogenicity.* J Virol, 2002. **76**(6): p. 2595-605.
358. Bannert, N., S. Craig, M. Farzan, D. Sogah, N.V. Santo, H. Choe and J. Sodroski, *Sialylated O-glycans and sulfated tyrosines in the NH2-terminal domain of CC chemokine receptor 5 contribute to high affinity binding of chemokines.* J Exp Med, 2001. **194**(11): p. 1661-73.
359. Morner, A., A. Bjorndal, J. Albert, V.N. Kewalramani, D.R. Littman, R. Inoue, R. Thorstensson, E.M. Fenyo and E. Bjorling, *Primary human immunodeficiency virus type 2 (HIV-2) isolates, like HIV-1 isolates, frequently use CCR5 but show promiscuity in coreceptor usage.* J Virol, 1999. **73**(3): p. 2343-9.
360. Gonzalez-Scarano, F. and J. Martin-Garcia, *The neuropathogenesis of AIDS.* Nat Rev Immunol, 2005. **5**(1): p. 69-81.
361. *Nomenclature and research case definitions for neurologic manifestations of human immunodeficiency virus-type 1 (HIV-1) infection. Report of a Working Group of the American Academy of Neurology AIDS Task Force.* Neurology, 1991. **41**(6): p. 778-85.
362. Edinger, A.L., C. Blanpain, K.J. Kunstman, S.M. Wolinsky, M. Parmentier and R.W. Doms, *Functional dissection of CCR5 coreceptor function through the use of CD4-independent simian immunodeficiency virus strains.* J Virol, 1999. **73**(5): p. 4062-73.
363. Reeves, J.D., S. Hibbitts, G. Simmons, A. McKnight, J.M. Azevedo-Pereira, J. Moniz-Pereira and P.R. Clapham, *Primary human immunodeficiency virus type 2 (HIV-2) isolates infect CD4-negative cells via CCR5 and CXCR4: comparison with HIV-1 and simian immunodeficiency virus and relevance to cell tropism in vivo.* J Virol, 1999. **73**(9): p. 7795-804.
364. Ryzhova, E., J.C. Whitbeck, G. Canziani, S.V. Westmoreland, G.H. Cohen, R.J. Eisenberg, A. Lackner and F. Gonzalez-Scarano, *Rapid progression to simian AIDS can be accompanied by selection of CD4-independent gp120 variants with impaired ability to bind CD4.* J Virol, 2002. **76**(15): p. 7903-9.
365. Zhang, P.F., P. Bouma, E.J. Park, J.B. Margolick, J.E. Robinson, S. Zolla-Pazner, M.N. Flora and G.V. Quinnan, Jr., *A variable region 3 (V3) mutation determines a global neutralization phenotype and CD4-*

independent infectivity of a human immunodeficiency virus type 1 envelope associated with a broadly cross-reactive, primary virus-neutralizing antibody response. J Virol, 2002. **76**(2): p. 644-55.
366. Kolchinsky, P., E. Kiprilov, P. Bartley, R. Rubinstein and J. Sodroski, *Loss of a single N-linked glycan allows CD4-independent human immunodeficiency virus type 1 infection by altering the position of the gp120 V1/V2 variable loops.* J Virol, 2001. **75**(7): p. 3435-43.
367. Zerhouni, B., J.A. Nelson and K. Saha, *Isolation of CD4-independent primary human immunodeficiency virus type 1 isolates that are syncytium inducing and acutely cytopathic for CD8+ lymphocytes.* J Virol, 2004. **78**(3): p. 1243-55.
368. Matthews, T., M. Salgo, M. Greenberg, J. Chung, R. DeMasi and D. Bolognesi, *Enfuvirtide: the first therapy to inhibit the entry of HIV-1 into host CD4 lymphocytes.* Nat Rev Drug Discov, 2004. **3**(3): p. 215-25.
369. Hoxie, J.A., C.C. LaBranche, M.J. Endres, J.D. Turner, J.F. Berson, R.W. Doms and T.J. Matthews, *CD4-independent utilization of the CXCR4 chemokine receptor by HIV-1 and HIV-2.* J Reprod Immunol, 1998. **41**(1-2): p. 197-211.
370. Lin, G., F. Baribaud, J. Romano, R.W. Doms and J.A. Hoxie, *Identification of gp120 binding sites on CXCR4 by using CD4-independent human immunodeficiency virus type 2 Env proteins.* J Virol, 2003. **77**(2): p. 931-42.
371. Liu, H.Y., Y. Soda, N. Shimizu, Y. Haraguchi, A. Jinno, Y. Takeuchi and H. Hoshino, *CD4-Dependent and CD4-independent utilization of coreceptors by human immunodeficiency viruses type 2 and simian immunodeficiency viruses.* Virology, 2000. **278**(1): p. 276-88.
372. Clapham, P.R., A. McKnight and R.A. Weiss, *Human immunodeficiency virus type 2 infection and fusion of CD4-negative human cell lines: induction and enhancement by soluble CD4.* J Virol, 1992. **66**(6): p. 3531-7.
373. Reeves, J.D., A. McKnight, S. Potempa, G. Simmons, P.W. Gray, C.A. Power, T. Wells, R.A. Weiss and S.J. Talbot, *CD4-independent infection by HIV-2 (ROD/B): use of the 7-transmembrane receptors CXCR-4, CCR-3, and V28 for entry.* Virology, 1997. **231**(1): p. 130-4.
374. Chenine, A.L., M. Pion, E. Matouskova, F. Gondois-Rey, R. Vigne and I. Hirsch, *Adaptation of a CXCR4-using human immunodeficiency type 1 NDK virus in intestinal cells is associated with CD4-independent replication.* Virology, 2002. **304**(2): p. 403-14.
375. Lin, G., B. Lee, B.S. Haggarty, R.W. Doms and J.A. Hoxie, *CD4-independent use of Rhesus CCR5 by human immunodeficiency virus Type 2 implicates an electrostatic interaction between the CCR5 N terminus and the gp120 C4 domain.* J Virol, 2001. **75**(22): p. 10766-78.
376. Edinger, A.L., A. Amedee, K. Miller, B.J. Doranz, M. Endres, M. Sharron, M. Samson, Z.H. Lu, J.E. Clements, M. Murphey-Corb, S.C. Peiper, M. Parmentier, C.C. Broder and R.W. Doms, *Differential utilization of CCR5*

by macrophage and T cell tropic simian immunodeficiency virus strains. Proc Natl Acad Sci U S A, 1997. **94**(8): p. 4005-10.
377. Sattentau, Q.J., J.P. Moore, F. Vignaux, F. Traincard and P. Poignard, *Conformational changes induced in the envelope glycoproteins of the human and simian immunodeficiency viruses by soluble receptor binding.* J Virol, 1993. **67**(12): p. 7383-93.
378. Watry, D., T.E. Lane, M. Streb and H.S. Fox, *Transfer of neuropathogenic simian immunodeficiency virus with naturally infected microglia.* Am J Pathol, 1995. **146**(4): p. 914-23.
379. Puffer, B.A., L.A. Altamura, T.C. Pierson and R.W. Doms, *Determinants within gp120 and gp41 contribute to CD4 independence of SIV Envs.* Virology, 2004. **327**(1): p. 16-25.
380. Fenyo, E.M., L. Morfeldt-Manson, F. Chiodi, B. Lind, A. von Gegerfelt, J. Albert, E. Olausson and B. Asjo, *Distinct replicative and cytopathic characteristics of human immunodeficiency virus isolates.* J Virol, 1988. **62**(11): p. 4414-9.
381. Rubsamen-Waigmann, H., W.B. Becker, E.B. Helm, R. Brodt, H. Fischer, K. Henco and H.D. Brede, *Isolation of variants of lymphocytopathic retroviruses from the peripheral blood and cerebrospinal fluid of patients with ARC or AIDS.* J Med Virol, 1986. **19**(4): p. 335-44.
382. Blaak, H., A.B. van't Wout, M. Brouwer, B. Hooibrink, E. Hovenkamp and H. Schuitemaker, *In vivo HIV-1 infection of CD45RA(+)CD4(+) T cells is established primarily by syncytium-inducing variants and correlates with the rate of CD4(+) T cell decline.* Proc Natl Acad Sci U S A, 2000. **97**(3): p. 1269-74.
383. Regoes, R.R. and S. Bonhoeffer, *The HIV coreceptor switch: a population dynamical perspective.* Trends Microbiol, 2005. **13**(6): p. 269-77.
384. Ribeiro, R.M., M.D. Hazenberg, A.S. Perelson and M.P. Davenport, *Naive and memory cell turnover as drivers of CCR5-to-CXCR4 tropism switch in human immunodeficiency virus type 1: implications for therapy.* J Virol, 2006. **80**(2): p. 802-9.
385. Mild, M., J. Esbjornsson, E.M. Fenyo and P. Medstrand, *Frequent intrapatient recombination between human immunodeficiency virus type 1 R5 and X4 envelopes: implications for coreceptor switch.* J Virol, 2007. **81**(7): p. 3369-76.
386. Cann, A.J., M.J. Churcher, M. Boyd, W. O'Brien, J.Q. Zhao, J. Zack and I.S. Chen, *The region of the envelope gene of human immunodeficiency virus type 1 responsible for determination of cell tropism.* J Virol, 1992. **66**(1): p. 305-9.
387. Briggs, D.R., D.L. Tuttle, J.W. Sleasman and M.M. Goodenow, *Envelope V3 amino acid sequence predicts HIV-1 phenotype (co-receptor usage and tropism for macrophages).* Aids, 2000. **14**(18): p. 2937-9.
388. Monteiro, J.P., G.A. Ferraro, T. Oliveira, L.Z. Goldani, S. Kashima, L.C. Alcantara, M.G. Morgado, D.C. Bou-Habib and B. Galvao-Castro, *Genetic and Biologic Characterization of HIV Type 1 Subtype C Isolates from South Brazil.* AIDS Res Hum Retroviruses, 2007. **23**(1): p. 135-43.

389. Brandful, J.A., M.E. Coetzer, T. Cilliers, M. Phoswa, M.A. Papathanasopoulos, L. Morris and P.L. Moore, *Phenotypic Characterization of HIV Type 1 Isolates from Ghana.* AIDS Res Hum Retroviruses, 2007. **23**(1): p. 144-152.
390. Rousseau, C.M., B.A. Birditt, A.R. McKay, J.N. Stoddard, T.C. Lee, S. McLaughlin, S.W. Moore, N. Shindo, G.H. Learn, B.T. Korber, C. Brander, P.J. Goulder, P. Kiepiela, B.D. Walker and J.I. Mullins, *Large-scale amplification, cloning and sequencing of near full-length HIV-1 subtype C genomes.* J Virol Methods, 2006. **136**(1-2): p. 118-25.
391. Ceuppens, J.L., M.L. Baroja, K. Lorre, J. Van Damme and A. Billiau, *Human T cell activation with phytohemagglutinin. The function of IL-6 as an accessory signal.* J Immunol, 1988. **141**(11): p. 3868-74.
392. Haertle, T., C.J. Carrera, D.B. Wasson, L.C. Sowers, D.D. Richman and D.A. Carson, *Metabolism and anti-human immunodeficiency virus-1 activity of 2-halo-2',3'-dideoxyadenosine derivatives.* J Biol Chem, 1988. **263**(12): p. 5870-5.
393. Harada, S., Y. Koyanagi and N. Yamamoto, *Infection of HTLV-III/LAV in HTLV-I-carrying cells MT-2 and MT-4 and application in a plaque assay.* Science, 1985. **229**(4713): p. 563-6.
394. Sullivan, P.S., A.N. Do, D. Ellenberger, C.P. Pau, S. Paul, K. Robbins, M. Kalish, C. Storck, C.A. Schable, H. Wise, C. Tetteh, J.L. Jones, J. McFarland, C. Yang, R.B. Lal and J.W. Ward, *Human immunodeficiency virus (HIV) subtype surveillance of African-born persons at risk for group O and group N HIV infections in the United States.* J Infect Dis, 2000. **181**(2): p. 463-9.
395. Gao, F., S.G. Morrison, D.L. Robertson, C.L. Thornton, S. Craig, G. Karlsson, J. Sodroski, M. Morgado, B. Galvao-Castro, H. von Briesen, S. Beddows, J. Weber, P.M. Sharp, G.M. Shaw and B.H. Hahn, *Molecular cloning and analysis of functional envelope genes from human immunodeficiency virus type 1 sequence subtypes A through G. The WHO and NIAID Networks for HIV Isolation and Characterization.* J Virol, 1996. **70**(3): p. 1651-67.
396. 2005 [cited 5 May 2005]; Available from: www.ncbi.nlm.nih.gov/gov/entrez/.
397. Hillis, D.M., M.W. Allard and M.M. Miyamoto, *Analysis of DNA sequence data: phylogenetic inference.* Methods Enzymol, 1993. **224**: p. 456-87.
398. 2006 [cited 2 June 2006]; Available from: http://mullinslab.microbiol.washington.edu/computing/pssm/.
399. Novitsky, V.A., M.A. Montano, M.F. McLane, B. Renjifo, F. Vannberg, B.T. Foley, T.P. Ndung'u, M. Rahman, M.J. Makhema, R. Marlink and M. Essex, *Molecular cloning and phylogenetic analysis of human immunodeficiency virus type 1 subtype C: a set of 23 full-length clones from Botswana.* J Virol, 1999. **73**(5): p. 4427-32.
400. Gorry, P.R., S. Sonza, K. Kedzierska and S.M. Crowe, *Isolation of human immunodeficiency virus type 1 from peripheral blood monocytes.* Methods Mol Biol, 2005. **304**: p. 25-33.

401. Van Baalen, C.A., M. Schutten, R.C. Huisman, P.H. Boers, R.A. Gruters and A.D. Osterhaus, *Kinetics of antiviral activity by human immunodeficiency virus type 1-specific cytotoxic T lymphocytes (CTL) and rapid selection of CTL escape virus in vitro.* J Virol, 1998. **72**(8): p. 6851-7.
402. Bell, C.M., B.J. Connell, A. Capovilla, W.D. Venter, W.S. Stevens and M.A. Papathanasopoulos, *Molecular characterization of the HIV type 1 subtype C accessory genes vif, vpr, and vpu.* AIDS Res Hum Retroviruses, 2007. **23**(2): p. 322-30.
403. Bredell, H., C. Williamson, P. Sonnenberg, D.J. Martin and L. Morris, *Genetic characterization of HIV type 1 from migrant workers in three South African gold mines.* AIDS Res Hum Retroviruses, 1998. **14**(8): p. 677-84.
404. De Wolf, F., E. Hogervorst, J. Goudsmit, E.M. Fenyo, H. Rubsamen-Waigmann, H. Holmes, B. Galvao-Castro, E. Karita, C. Wasi, S.D. Sempala and et al., *Syncytium-inducing and non-syncytium-inducing capacity of human immunodeficiency virus type 1 subtypes other than B: phenotypic and genotypic characteristics. WHO Network for HIV Isolation and Characterization.* AIDS Res Hum Retroviruses, 1994. **10**(11): p. 1387-400.
405. Gorry, P.R., R.L. Dunfee, M. Mefford, K. Kunstman, T. Morgan, J.P. Moore, J.R. Mascola, K. Agopian, G.H. Holm, A. Mehle, J. Taylor, M. Farzan, H. Wang, P. Ellery, S.J. Willey, P.R. Clapham, S.M. Wolinsky, S.M. Crowe and D. Gabuzda, *Changes in the V3 region of gp120 contribute to unusually broad coreceptor usage of an HIV-1 isolate from a CCR5 Delta32 heterozygote.* Virology, 2007. **Epub ahead of print**.
406. Pollakis, G., A. Abebe, A. Kliphuis, M.I. Chalaby, M. Bakker, Y. Mengistu, M. Brouwer, J. Goudsmit, H. Schuitemaker and W.A. Paxton, *Phenotypic and genotypic comparisons of CCR5- and CXCR4-tropic human immunodeficiency virus type 1 biological clones isolated from subtype C-infected individuals.* J Virol, 2004. **78**(6): p. 2841-52.
407. Clevestig, P., L. Pramanik, T. Leitner and A. Ehrnst, *CCR5 use by human immunodeficiency virus type 1 is associated closely with the gp120 V3 loop N-linked glycosylation site.* J Gen Virol, 2006. **87**(Pt 3): p. 607-12.
408. van Rensburg, E.J., T.L. Smith, M. Zeier, B. Robson, C. Sampson, F. Treurnicht and S. Engelbrecht, *Change in co-receptor usage of current South African HIV-1 subtype C primary isolates.* Aids, 2002. **16**(18): p. 2479-80.
409. Isaka, Y., A. Sato, S. Miki, S. Kawauchi, H. Sakaida, T. Hori, T. Uchiyama, A. Adachi, M. Hayami, T. Fujiwara and O. Yoshie, *Small amino acid changes in the V3 loop of human immunodeficiency virus type 2 determines the coreceptor usage for CXCR4 and CCR5.* Virology, 1999. **264**(1): p. 237-43.
410. Boyer, J.C., K. Bebenek and T.A. Kunkel, *Unequal human immunodeficiency virus type 1 reverse transcriptase error rates with RNA and DNA templates.* Proc Natl Acad Sci U S A, 1992. **89**(15): p. 6919-23.

411. Sutthent, R., K. Sumrangsurp, P. Wirachsilp, P. Chaisilwattana, A. Roongpisuthipong, P. Chaiyakul, P. Nooma, M. Honda and P. Warachit, *Diversity of HIV-1 subtype E in semen and cervicovaginal secretion.* J Hum Virol, 2001. **4**(5): p. 260-8.
412. *Anti-HIV agents. Maraviroc in treatment-experienced PHAs.* TreatmentUpdate, 2006. **18**(4): p. 5.
413. Ji, C., J. Zhang, M. Dioszegi, S. Chiu, E. Rao, A. Derosier, N. Cammack, M. Brandt and S. Sankuratri, *Ccr5 Small Molecule Antagonists and Monoclonal Antibodies Exert Potent Synergistic Antiviral Effects by Co-Binding to the Receptor.* Mol Pharmacol, 2007.
414. Rizzuto, C.D., R. Wyatt, N. Hernandez-Ramos, Y. Sun, P.D. Kwong, W.A. Hendrickson and J. Sodroski, *A conserved HIV gp120 glycoprotein structure involved in chemokine receptor binding.* Science, 1998. **280**(5371): p. 1949-53.
415. Cocchi, F., A.L. DeVico, A. Garzino-Demo, A. Cara, R.C. Gallo and P. Lusso, *The V3 domain of the HIV-1 gp120 envelope glycoprotein is critical for chemokine-mediated blockade of infection.* Nat Med, 1996. **2**(11): p. 1244-7.
416. Ross, T.M. and B.R. Cullen, *The ability of HIV type 1 to use CCR-3 as a coreceptor is controlled by envelope V1/V2 sequences acting in conjunction with a CCR-5 tropic V3 loop.* Proc Natl Acad Sci U S A, 1998. **95**(13): p. 7682-6.
417. Speck, R.F., K. Wehrly, E.J. Platt, R.E. Atchison, I.F. Charo, D. Kabat, B. Chesebro and M.A. Goldsmith, *Selective employment of chemokine receptors as human immunodeficiency virus type 1 coreceptors determined by individual amino acids within the envelope V3 loop.* J Virol, 1997. **71**(9): p. 7136-9.
418. Jones, P.L., T. Korte and R. Blumenthal, *Conformational changes in cell surface HIV-1 envelope glycoproteins are triggered by cooperation between cell surface CD4 and co-receptors.* J Biol Chem, 1998. **273**(1): p. 404-9.
419. Wyatt, R., M. Thali, S. Tilley, A. Pinter, M. Posner, D. Ho, J. Robinson and J. Sodroski, *Relationship of the human immunodeficiency virus type 1 gp120 third variable loop to a component of the CD4 binding site in the fourth conserved region.* J Virol, 1992. **66**(12): p. 6997-7004.
420. Hwang, S.S., T.J. Boyle, H.K. Lyerly and B.R. Cullen, *Identification of the envelope V3 loop as the primary determinant of cell tropism in HIV-1.* Science, 1991. **253**(5015): p. 71-4.
421. Thali, M., U. Olshevsky, C. Furman, D. Gabuzda, M. Posner and J. Sodroski, *Characterization of a discontinuous human immunodeficiency virus type 1 gp120 epitope recognized by a broadly reactive neutralizing human monoclonal antibody.* J Virol, 1991. **65**(11): p. 6188-93.
422. Wyatt, R., N. Sullivan, M. Thali, H. Repke, D. Ho, J. Robinson, M. Posner and J. Sodroski, *Functional and immunologic characterization of human immunodeficiency virus type 1 envelope glycoproteins containing deletions of the major variable regions.* J Virol, 1993. **67**(8): p. 4557-65.

423. Reeves, J.D. and R.W. Doms, *Human immunodeficiency virus type 2.* J Gen Virol, 2002. **83**(Pt 6): p. 1253-65.
424. Dick, A.D., M. Pell, B.J. Brew, E. Foulcher and J.D. Sedgwick, *Direct ex vivo flow cytometric analysis of human microglial cell CD4 expression: examination of central nervous system biopsy specimens from HIV-seropositive patients and patients with other neurological disease.* Aids, 1997. **11**(14): p. 1699-708.
425. Jordan, C.A., B.A. Watkins, C. Kufta and M. Dubois-Dalcq, *Infection of brain microglial cells by human immunodeficiency virus type 1 is CD4 dependent.* J Virol, 1991. **65**(2): p. 736-42.
426. Peudenier, S., C. Hery, L. Montagnier and M. Tardieu, *Human microglial cells: characterization in cerebral tissue and in primary culture, and study of their susceptibility to HIV-1 infection.* Ann Neurol, 1991. **29**(2): p. 152-61.
427. Peudenier, S., C. Hery, K.H. Ng and M. Tardieu, *HIV receptors within the brain: a study of CD4 and MHC-II on human neurons, astrocytes and microglial cells.* Res Virol, 1991. **142**(2-3): p. 145-9.
428. Gorry, P.R., G. Bristol, J.A. Zack, K. Ritola, R. Swanstrom, C.J. Birch, J.E. Bell, N. Bannert, K. Crawford, H. Wang, D. Schols, E. De Clercq, K. Kunstman, S.M. Wolinsky and D. Gabuzda, *Macrophage tropism of human immunodeficiency virus type 1 isolates from brain and lymphoid tissues predicts neurotropism independent of coreceptor specificity.* J Virol, 2001. **75**(21): p. 10073-89.
429. Saito, Y., L.R. Sharer, L.G. Epstein, J. Michaels, M. Mintz, M. Louder, K. Golding, T.A. Cvetkovich and B.M. Blumberg, *Overexpression of nef as a marker for restricted HIV-1 infection of astrocytes in postmortem pediatric central nervous tissues.* Neurology, 1994. **44**(3 Pt 1): p. 474-81.
430. McArthur, J.C., N. Haughey, S. Gartner, K. Conant, C. Pardo, A. Nath and N. Sacktor, *Human immunodeficiency virus-associated dementia: an evolving disease.* J Neurovirol, 2003. **9**(2): p. 205-21.
431. Sacktor, N., M.P. McDermott, K. Marder, G. Schifitto, O.A. Selnes, J.C. McArthur, Y. Stern, S. Albert, D. Palumbo, K. Kieburtz, J.A. De Marcaida, B. Cohen and L. Epstein, *HIV-associated cognitive impairment before and after the advent of combination therapy.* J Neurovirol, 2002. **8**(2): p. 136-42.
432. Thomas, E.R., C. Shotton, R.A. Weiss, P.R. Clapham and A. McKnight, *CD4-dependent and CD4-independent HIV-2: consequences for neutralization.* Aids, 2003. **17**(3): p. 291-300.
433. Nelson-Rees, W.A., R.B. Owens, P. Arnstein and A.J. Kniazeff, *Source, alterations, characteristics and use of a new dog cell line (Cf2Th).* In Vitro, 1976. **12**(10): p. 665-9.
434. Simmons, G., J.D. Reeves, A. McKnight, N. Dejucq, S. Hibbitts, C.A. Power, E. Aarons, D. Schols, E. De Clercq, A.E. Proudfoot and P.R. Clapham, *CXCR4 as a functional coreceptor for human immunodeficiency virus type 1 infection of primary macrophages.* J Virol, 1998. **72**(10): p. 8453-7.

435. Gorry, P.R., J. Taylor, G.H. Holm, A. Mehle, T. Morgan, M. Cayabyab, M. Farzan, H. Wang, J.E. Bell, K. Kunstman, J.P. Moore, S.M. Wolinsky and D. Gabuzda, *Increased CCR5 affinity and reduced CCR5/CD4 dependence of a neurovirulent primary human immunodeficiency virus type 1 isolate.* J Virol, 2002. **76**(12): p. 6277-92.
436. Casado, C., I. Urtasun, M.V. Martin-Walther, S. Garcia, C. Rodriguez, J. del Romero and C. Lopez-Galindez, *Genetic analysis of HIV-1 samples from Spain.* J Acquir Immune Defic Syndr, 2000. **23**(1): p. 68-74.
437. Lech, W.J., G. Wang, Y.L. Yang, Y. Chee, K. Dorman, D. McCrae, L.C. Lazzeroni, J.W. Erickson, J.S. Sinsheimer and A.H. Kaplan, *In vivo sequence diversity of the protease of human immunodeficiency virus type 1: presence of protease inhibitor-resistant variants in untreated subjects.* J Virol, 1996. **70**(3): p. 2038-43.
438. Lukashov, V.V. and J. Goudsmit, *Evolution of the human immunodeficiency virus type 1 subtype-specific V3 domain is confined to a sequence space with a fixed distance to the subtype consensus.* J Virol, 1997. **71**(9): p. 6332-8.
439. Balfe, P., P. Simmonds, C.A. Ludlam, J.O. Bishop and A.J. Brown, *Concurrent evolution of human immunodeficiency virus type 1 in patients infected from the same source: rate of sequence change and low frequency of inactivating mutations.* J Virol, 1990. **64**(12): p. 6221-33.
440. Smith, T.F., A. Srinivasan, G. Schochetman, M. Marcus and G. Myers, *The phylogenetic history of immunodeficiency viruses.* Nature, 1988. **333**(6173): p. 573-5.
441. Leitner, T., S. Kumar and J. Albert, *Tempo and mode of nucleotide substitutions in gag and env gene fragments in human immunodeficiency virus type 1 populations with a known transmission history.* J Virol, 1997. **71**(6): p. 4761-70.
442. Jones, N.A., X. Wei, D.R. Flower, M. Wong, F. Michor, M.S. Saag, B.H. Hahn, M.A. Nowak, G.M. Shaw and P. Borrow, *Determinants of human immunodeficiency virus type 1 escape from the primary CD8+ cytotoxic T lymphocyte response.* J Exp Med, 2004. **200**(10): p. 1243-56.
443. Richman, D.D., D. Havlir, J. Corbeil, D. Looney, C. Ignacio, S.A. Spector, J. Sullivan, S. Cheeseman, K. Barringer, D. Pauletti and et al., *Nevirapine resistance mutations of human immunodeficiency virus type 1 selected during therapy.* J Virol, 1994. **68**(3): p. 1660-6.
444. Richman, D.D., T. Wrin, S.J. Little and C.J. Petropoulos, *Rapid evolution of the neutralizing antibody response to HIV type 1 infection.* Proc Natl Acad Sci U S A, 2003. **100**(7): p. 4144-9.
445. Goodenow, M., T. Huet, W. Saurin, S. Kwok, J. Sninsky and S. Wain-Hobson, *HIV-1 isolates are rapidly evolving quasispecies: evidence for viral mixtures and preferred nucleotide substitutions.* J Acquir Immune Defic Syndr, 1989. **2**(4): p. 344-52.
446. Meyerhans, A., R. Cheynier, J. Albert, M. Seth, S. Kwok, J. Sninsky, L. Morfeldt-Manson, B. Asjo and S. Wain-Hobson, *Temporal fluctuations in*

HIV quasispecies in vivo are not reflected by sequential HIV isolations. Cell, 1989. **58**(5): p. 901-10.
447. Sabino, E., L.Z. Pan, C. Cheng-Mayer and A. Mayer, *Comparison of in vivo plasma and peripheral blood mononuclear cell HIV-1 quasi-species to short-term tissue culture isolates: an analysis of tat and C2-V3 env regions.* Aids, 1994. **8**(7): p. 901-9.
448. Wain-Hobson, S., *The fastest genome evolution ever described: HIV variation in situ.* Curr Opin Genet Dev, 1993. **3**(6): p. 878-83.
449. Quakkelaar, E.D., T. Beaumont, A.C. van Nuenen, F.P. van Alphen, B.D. Boeser-Nunnink, A.B. van 't Wout and H. Schuitemaker, *T cell line passage can select for pre-existing neutralization-sensitive variants from the quasispecies of primary human immunodeficiency virus type-1 isolates.* Virology, 2006.
450. Zhang, Y.J., R. Fredriksson, J.A. McKeating and E.M. Fenyo, *Passage of HIV-1 molecular clones into different cell lines confers differential sensitivity to neutralization.* Virology, 1997. **238**(2): p. 254-64.
451. Moore, J.P. and D.D. Ho, *HIV-1 neutralization: the consequences of viral adaptation to growth on transformed T cells.* Aids, 1995. **9 Suppl A**: p. S117-36.
452. Kozak, S.L., E.J. Platt, N. Madani, F.E. Ferro, Jr., K. Peden and D. Kabat, *CD4, CXCR-4, and CCR-5 dependencies for infections by primary patient and laboratory-adapted isolates of human immunodeficiency virus type 1.* J Virol, 1997. **71**(2): p. 873-82.
453. Duarte, E., D. Clarke, A. Moya, E. Domingo and J. Holland, *Rapid fitness losses in mammalian RNA virus clones due to Muller's ratchet.* Proc Natl Acad Sci U S A, 1992. **89**(13): p. 6015-9.
454. Muller, H.J., *The Relation of Recombination to Mutational Advance.* Mutat Res, 1964. **106**: p. 2-9.
455. Escarmis, C., M. Davila, N. Charpentier, A. Bracho, A. Moya and E. Domingo, *Genetic lesions associated with Muller's ratchet in an RNA virus.* J Mol Biol, 1996. **264**(2): p. 255-67.
456. Yuste, E., S. Sanchez-Palomino, C. Casado, E. Domingo and C. Lopez-Galindez, *Drastic fitness loss in human immunodeficiency virus type 1 upon serial bottleneck events.* J Virol, 1999. **73**(4): p. 2745-51.
457. Yuste, E., C. Lopez-Galindez and E. Domingo, *Unusual distribution of mutations associated with serial bottleneck passages of human immunodeficiency virus type 1.* J Virol, 2000. **74**(20): p. 9546-52.
458. Yuste, E., A.V. Borderia, E. Domingo and C. Lopez-Galindez, *Few mutations in the 5' leader region mediate fitness recovery of debilitated human immunodeficiency type 1 viruses.* J Virol, 2005. **79**(9): p. 5421-7.
459. Kusumi, K., B. Conway, S. Cunningham, A. Berson, C. Evans, A.K. Iversen, D. Colvin, M.V. Gallo, S. Coutre, E.G. Shpaer and et al., *Human immunodeficiency virus type 1 envelope gene structure and diversity in vivo and after cocultivation in vitro.* J Virol, 1992. **66**(2): p. 875-85.

460. Platt, E.J., S.L. Kozak and D. Kabat, *Critical role of enhanced CD4 affinity in laboratory adaptation of human immunodeficiency virus type 1.* AIDS Res Hum Retroviruses, 2000. **16**(9): p. 871-82.
461. Bonavia, A., B.T. Bullock, K.M. Gisselman, B.J. Margulies and J.E. Clements, *A single amino acid change and truncated TM are sufficient for simian immunodeficiency virus to enter cells using CCR5 in a CD4-independent pathway.* Virology, 2005. **341**(1): p. 12-23.
462. Dumonceaux, J., C. Chanel, S. Valente, L. Quivet, P. Briand and U. Hazan, *Mutations in the env gene of human immunodeficiency virus type 1 NDK isolates and the use of African green monkey CXCR4 as a co-receptor in COS-7 cells.* J Gen Virol, 1999. **80 (Pt 8)**: p. 1975-82.
463. Dumonceaux, J., C. Goujon, V. Joliot, P. Briand and U. Hazan, *Determination of essential amino acids involved in the CD4-independent tropism of the X4 human immunodeficiency virus type 1 m7NDK isolate: role of potential N glycosylations in the C2 and V3 regions of gp120.* J Virol, 2001. **75**(11): p. 5425-8.
464. Misse, D., M. Cerutti, N. Noraz, P. Jourdan, J. Favero, G. Devauchelle, H. Yssel, N. Taylor and F. Veas, *A CD4-independent interaction of human immunodeficiency virus-1 gp120 with CXCR4 induces their cointernalization, cell signaling, and T-cell chemotaxis.* Blood, 1999. **93**(8): p. 2454-62.
465. Misse, D., M. Cerutti, I. Schmidt, A. Jansen, G. Devauchelle, F. Jansen and F. Veas, *Dissociation of the CD4 and CXCR4 binding properties of human immunodeficiency virus type 1 gp120 by deletion of the first putative alpha-helical conserved structure.* J Virol, 1998. **72**(9): p. 7280-8.
466. Kirchherr, J.L., X. Lu, W. Kasongo, V. Chalwe, L. Mwananyanda, R.M. Musonda, S.M. Xia, R.M. Scearce, H.X. Liao, D.C. Montefiori, B.F. Haynes and F. Gao, *High throughput functional analysis of HIV-1 env genes without cloning.* J Virol Methods, 2007. **143**(1): p. 104-11.
467. Willey, R.L., J.S. Bonifacino, B.J. Potts, M.A. Martin and R.D. Klausner, *Biosynthesis, cleavage, and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160.* Proc Natl Acad Sci U S A, 1988. **85**(24): p. 9580-4.
468. Earl, P.L., B. Moss and R.W. Doms, *Folding, interaction with GRP78-BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein.* J Virol, 1991. **65**(4): p. 2047-55.
469. Stein, B.S. and E.G. Engleman, *Intracellular processing of the gp160 HIV-1 envelope precursor. Endoproteolytic cleavage occurs in a cis or medial compartment of the Golgi complex.* J Biol Chem, 1990. **265**(5): p. 2640-9.
470. Dettenhofer, M. and X.F. Yu, *Characterization of the biosynthesis of human immunodeficiency virus type 1 Env from infected T-cells and the effects of glucose trimming of Env on virion infectivity.* J Biol Chem, 2001. **276**(8): p. 5985-91.
471. McCune, J.M., L.B. Rabin, M.B. Feinberg, M. Lieberman, J.C. Kosek, G.R. Reyes and I.L. Weissman, *Endoproteolytic cleavage of gp160 is*

required for the activation of human immunodeficiency virus. Cell, 1988. **53**(1): p. 55-67.
472. Reeves, J.D., S.A. Gallo, N. Ahmad, J.L. Miamidian, P.E. Harvey, M. Sharron, S. Pohlmann, J.N. Sfakianos, C.A. Derdeyn, R. Blumenthal, E. Hunter and R.W. Doms, *Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics.* Proc Natl Acad Sci U S A, 2002. **99**(25): p. 16249-54.

Appendix A: Ethics clearance

Ethical clearance was granted by the University of the Witwatersrand Medical School Ethics Committee clearance certificate no. M060734.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Connell

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M060734**
PROJECT	In vitro Selection of CD4 Independent HIV-1 Subtype C Relevance for HIV Pathogenesis and Therapeutic Inter..
INVESTIGATOR	Ms BJ Connell
DEPARTMENT	Molecular Medicine
DATE CONSIDERED	06.07.13

DECISION OF THE COMMITTEE*

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE **CHAIRPERSON** .....
(Professor A Dhai)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr M Papathans..

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10005, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B: Amino Acid Abbreviations

Amino Acid	Abbreviation
Alanine	A
Arginine	R
Asparagine	N
Aspartic acid	D
Cysteine	C
Glutamine	Q
Glutamic acid	E
Glycine	G
Histidine	H
Isoleucine	I
Leucine	L
Lysine	K
Methionine	M
Phenylalanine	F
Proline	P
Serine	S
Threonine	T
Tryptophan	W
Tyrosine	Y
Valine	V

Appendix C: Base Pair Abbreviations

Adenine	A
Cytosine	C
Guanine	G
Thymidine	T

Appendix D: Media, Buffers and Solutions

B1 Buffers

B 1.1 0.5 M EDTA

0.5 M EDTA (186.10 g in a final volume of 1 L) pH 8

B 1.2 50 X TAE Buffer

400 mM Tris-acetate base, 50 mM Na_2EDTA pH 7.6 Adjust volume to 0.2 L with dH_2O, sterilize by autoclaving, stored at room temperature (~25°C).

B 1.3 TE buffer

10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 7.6 Adjust volume to 1 L with dH_2O, sterilize by autoclaving, stored at room temperature (~25°C).

B2 Solutions

B2.1 Loading dye

4.5 mL glycerol, 10.5 mL deionised sterile distilled water, 0.04 g bromophenol blue

Appendix E: GenBank Accession Numbers for gp160 sequences

DQ382361 (05ZAFV02)	DQ382368 (05ZAFV10)	DQ382375 (05ZAFV22)
DQ382362 (05ZAFV03)	DQ382369 (05ZAFV11)	DQ382376 (05ZAFV23)
DQ382363 (05ZAFV05)	DQ382370 (05ZAFV12)	DQ382377 (05ZAFV25)
DQ382364 (05ZAFV06)	DQ382371 (05ZAFV13)	DQ382378 (05ZAFV26)
DQ382365 (05ZAFV07)	DQ382372 (05ZAFV14)	DQ382379 (05ZAFV27)
DQ382366 (05ZAFV08)	DQ382373 (05ZAFV15)	DQ382380 (05ZAFV28)
DQ382367 (05ZAFV09)	DQ382374 (05ZAFV20)	