Characterization of genotypic and phenotypic properties of transmitted Human Immunodeficiency Virus type 1 variants circulating in Mbeya Tanzania

ANDILE NOFEMELA

Uni of Cape Town

Thesis presented for the degree of DOCTOR OF PHILOSOPY

in the Division of Medical Virology, Institute of Infectious Diseases and Molecular Medicine, Faculty of Health Sciences,

University of Cape Town

MARCH 2013

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

Table of Contents

University of Cape Town

University of Cape Town

Declaration

I, ***Andile Nofemela*** , hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

I empower the university to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signature: …Signed by candidate…

Date: 13 March 2013

University of Cape Town

Abstract

Sexual transmission accounts for the majority of HIV infections, with women bearing the brunt of the epidemic. A detailed understanding of early events during HIV transmission and the nature of the incoming virus will aid the design of vaccines and microbicides. The aim of this dissertation was three-fold: firstly, to characterize the genetic bottleneck associated with transmission in participants with acute infection in the Mbeya region of Tanzania; secondly, to investigate the biological features of transmitted/founder (T/F) viruses; and lastly to determine how these properties impact on subsequent disease progression.

The Mbeya region of Tanzania has a genetically complex HIV epidemic with multiple subtypes and recombinant forms circulating, together with a high frequency of dual infections with more than one subtype. A total of 212 *env* sequences from 22 participants were generated from recently infected women from Mbeya using the single genome amplification (SGA) approach. Participants were infected with subtypes C (n=9), A (n=4), D (n=1); or recombinants AC (n=4), CD (n=2), AD (n=1), or ACD (n=1). In sixteen participants (73%) clinical infection was the result of a single variant; whereas in five participants (23%) there was evidence of multiple variant infections with more than one closely related quasispecies; and one (4%) participant was identified to be dually infected. This individual was believed to be simultaneously infected with two phylogenetically distinct viruses prior to seroconversion, as opposed to superinfection, in which the second infection occurs after seroconversion. Thus the frequency of single variant infections was similar to cohorts located in genetically restricted subtype B or C epidemics, suggesting that multiple circulating subtypes and unique recombinant forms do not have a significant impact on the transmission bottleneck.

University of Cape Town

In order to characterize the Envelope function of T/F viruses, *env* sequences that matched the consensus of SGA - derived sequences sampled in very early infection, were cloned and used to generate pseudoviruses. There was a wide variation in the host cell entry efficiency between the T/F Envelope clones, which suggests that the genetic bottleneck does not select for viruses with equal ability to infect cells. The viruses with high entry efficiency had significantly higher viral loads at 3 and 12 months post infection ($p = 0.0022$; $p = 0.0347$, respectively), suggesting that viruses with high entry efficiency at transmission may predict disease outcome. The entry efficiency of the Env pseudovirions was measure using dual inducible HEK293 Affinofile cells, which were induced to express different levels of CD4 and CCR5. While all viruses could infect cells with high CD4 and CCR5, only 30% of the viruses were able to mediate entry efficiency at low levels of CD4, suggesting that high levels of CD4 are critical for entry of these viruses. The ability of the pseudoviruses to enter cells with low CD4 and CCR5 levels was significantly associated with the IC_{50} values for soluble CD4 ($p = 0.0438$) and CCR5 antagonist, TAK779 ($p = 0.0138$), suggesting that receptor affinity contributed to these differences in entry efficiency. The level of Envelope processing/incorporation and fusion capacity was more efficient in viruses with high entry efficiency, although this was not consistent for all the Envelope clones. Therefore, our study shows that viruses with low entry efficiency and binding affinity to CD4 and CCR5 can still be transmitted. However, high levels of entry efficiency seem to provide an advantage to the virus, leading to increased viral loads that may enhance disease progression.

In order to determine the relationship between entry efficiency *in vitro* and *in vivo* replicative capacity between the T/F viruses, recombinant viruses were generated using the transmitted Envelope clones derived from ten participants. This involved shuttling the Envelope clones into a NL4-3 backbone using a yeast gap repair method, and the replication capacity of the

University of Cape Town

recombinant viruses was measured in TZM-bl cells, PBMCs, and monocyte-derived macrophages. There was variability in replication capacity of the recombinant viruses in different donors. However, the recombinant viruses replicated much more efficiently in $CD4^+$ T lymphocytes than in monocyte-derived macrophages. This suggests that macrophages may not be the ideal target cells used by T/F viruses during transmission. The preferential replication in T-cells and not macrophages was observed irrespective of the infecting subtype or recombinant form. Although the pseudovirion assays showed an association between high *in vitro* entry efficiency and high viral loads *in vivo*, we could not confirm this finding in PBMCs due to host to host variation. However, the PBMC assay did support the findings in the 293Affinofile pseudovirion assay that transmitted viruses do not infect macrophages, which have low CD4 expression levels, very efficiently.

Overall, this study shows that the presence of multiple subtypes and recombinants in a region does not affect the transmission genetic bottleneck. Also, the ability of the viruses to mediate host cell entry effectively during transmission is dependent on both viral and host factors. Viral factors such as CD4 and CCR5 binding affinity and, to some extent, Envelope incorporation/processing and cell-cell fusion capacity; and host factors such CD4 and CCR5 expression levels can impact entry efficiency; and collectively these properties could influence disease progression in the infected individuals.

University of Cape Town

This study has resulted in the following publication:

Nofemela A, Bandawe G, Thebus R, Marais J, Wood N, Maboko L, Hoelscher M, Woodman Z, and Carolyn Williamson. Defining the Human Immunodeficiency Virus Type 1 transmission genetic bottleneck in a region with multiple circulating subtypes and recombinant forms. Virology 2011. Jul 5; 415(2):107-13

Abbreviations

5- FOA	5 – flouro – 1, 2, 3, 6 – tetrahydro – 2, 6 – dioxo – 4 – pyrimidine carboxylic acid
AIDS	Acquired Immune Deficiency Syndrome
bp	Base pairs
B-gal	Beta-galactosidase
^oC	Degrees Celsius
C1-C5	Conserved regions 1-5
CCR5	Chemokine coreceptor
cDNA	Copy DNA
CI	Confidence Interval
CMV	Cytomegalovirus
CRF	Circular Recombinant Form
CO_2	Carbon dioxide
CTL	Cytotoxic T Lymphocytes
CXCR4	CXC chemokine coreceptor
DC-SIGN	Dendritic Cell-Specific Intracellular adhesion molecule-3-Grabbing Non-Intergrin
DNA	Deoxyribose nucleic acid
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunodeficiency Assay
Env	Envelope

University of Cape Town

FACS	Fluorescence-Activated Cell Sorting
GALT	Gut Associated Lymphocyte Tissue
GT	Generation time
gp120	120kDa Envelope Glycoprotein
gp41	41kDa Envelope Glycoprotein
HCl	Hydrochloric acid
HEK	Human Embryonic Kidney
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
HR	Heptad Repeat
HTA	Heteroduplex Tracking Assay
HRP	Horseradish Peroxidase
IMC	Infectious Molecular Clone
IL-2	Interleukin 2
LTR	Long Tandem Repeat
KDa	Kilodaltons
LB	Luria-Bertani
Leu	Leucin
LLP	Lentiviral Lytic Peptides
M	Molar
mA	Milliamps
MA	Massachusetts
ml	Milliliters
mM	Millimolar
MPER	Membrane Proximal External Region
mRNA	Messenger Ribonucleic acid
MRCA	Most Recent Common Ancestor
Nef	Negative Regulatory Factor
ng	Nanogram

University of Cape Town

NICD	National Institute of Communicable Diseases
NIH	National Institute of Health
nM	Nanomolar
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Phytoerythrin
PEG	Polyethylene Glycol
pH	Hydrogen potential
PHA	Phytohaemogglutinin
RDP	Recombination Detection Program
RLU	Relative Luminescence Units
RNA	Ribonucleic acid
rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SGA	Single Genome Amplification
TBS	Tris Buffered Saline
TCR	T cell Receptor
T/F	Transmitted/founder
Tris -	Tris(hydroxymethyl)aminomethane
UAB	University of Alabama at Birmingham
UCLA	University of California, Long Angeles
UCT	University of Cape Town
ul	Microliter
UNAIDS	Joint United Nations Programme on HIV/AIDS
ug	Micrograms
uM	Micromolar
USA	United States of America

University of Cape Town

UV	Ultraviolet
Vif	Virion Infectivity Factor
V1-V5	Variable regions 1-5
V	Volts

Acknowledgements

This PhD project has been a long and fruiting academic and life journey, and would certainly not be possible without the following people/institutions:

- My project supervisors Professor Carolyn Williamson and Dr Zenda Woodman. I have been under your guidance and mentorship since I first arrived at UCT. Thank you for the support, and all the opportunities that you have given me. None of this would be not be possible without you! I will take everything that you taught me wherever I go..
- My entire family, especially my parents (Mr. Phillip and Mrs. Mirriam Ntengento) and my younger sister Lihle. Thank you very much for the loving support and encouragement for all these years. You are the inspiration behind everything that I do in my life, and I hope that I have made you proud...
- Dr. Manish Sagar. Thank you very much for giving me the opportunity to visit your laboratory, and also the wonderful hospitality that you gave me. I learnt a lot in your laboratory. I wish you nothing but success and I certainly hope our paths will cross one day in the future..
- Dr Behzad Etemad, Dr Nikolaos Chadziandreou, and Ms. Phyu Hninn Nyein. Thank you for the training and the support that you gave me while I was in the Sagar Laboratory. I will never forget you and I hope we will meet once again..
- Mrs. Debbie Stewart. Thank you for providing personal and logisitical support over the years.
- My colleagues (both past and present) at the HIV Diversity and Pathogenesis Group. Your support have meant a lot to me over the years. A special mention to Mr. Gama Bandawe, Mrs. Ruwayhida Thebus, and Mrs Jinny Marais who also contributed to part of this work. Thank you to Mr. Craig Adams and Mrs Desiree Bowers (UCT Vaccine Research Group) for your assistance with some of the experiments.
- Members of of the Woodman Laboratory at Department of Molecular and Cell Biology, UCT.
- The post-doctoral fellows, Dr. Emma Sherratt (Harvard University) and Dr. Philippe Selhorst (University of Cape Town). Thank you for your critical review of the thesis.

University of Cape Town

- The instutitions that provided funding to the project: Global HIV Vaccine Enterprise (GHAVE) and Poliomyelitis Research Foundation.
- The fellowships that I have received during the tenure of my PhD, with include the Poliomyelitis Reserch Foundation, David and Elaine Potter Fellowship, and also the Fogarty AIDS and TB Training Reserach Fellowship for affording me the opportunity to do part of this work at Harvard University Medical School.
- My IIDMM friends and colleagues. You provided 'sanity' throughout the process. Thank you for your support!
- Our collaborators Dr Micheal Hoelscher and Dr Leonard Maboko at the Mbeya Medical Research Center in Tanzania. Last but not least, none of this work would be possible without the participants of the HIV Superinfection Study (HISIS) cohort. Thank you very much!

University of Cape Town

Acknowlegement of various contributions

Chapter 2

- This chapter was published: Nofemela A, Bandawe G, Thebus R, Marais J, Wood N, Maboko L, Hoelscher M, Woodman Z, and Carolyn Williamson. Defining the Human Immunodeficiency Virus Type 1 transmission genetic bottleneck in a region with multiple circulating subtypes and recombinant forms. Virology 2011. Jul 5; 415(2):107-13
- Of the 210 sequences, the doctoral cnadidate optimized the SGA and sequencing and generated 122 sequences, and was responsible for writing the paper.
- G. Bandawe, co-first author, generated some of the sequences and assisted with the BEAST analysis and phylogenetic analysis. R. Thebus and J. Marais assisted with generating sequences, N wood with BEAST analysis, L Maboko and M Hoelscher provided samples and Z. Woodman co-supervised the project.

Chapter 3, and 4

- The doctoral candiate (Mr. Andile Nofemela) is the sole contributor to all of this work, and the work has not been published.
- The FACS analysis was done together with the assistance of Mrs. Desiree Bowers.

University of Cape Town

Chapter 1: Introduction and Literature review

University of Cape Town

Human Immunodeficiency Virus (HIV), the causative agent of Acquired Immunodeficiency Syndrome (AIDS), has had a devastating effect worldwide with an estimated 34 million people infected (UNAIDS, 2012). High levels of HIV diversity remains one of the major obstacles in the development of an efficacious vaccine. An understanding of the extent of viral genotypic and phenotypic diversity in different geographical regions will help in designing a globally efficacious vaccine. Following transmission, the virus is initially contained locally in the mucosa for a few days before susbsequent systemic infection (Li et al., 2009a), and the limited inoculum following transmission and low diversity during early infection provides a 'window of opportunity' for vaccines to act. Thus a major focus in the field is to understand the characteristics of the incoming transmitted or founder viruses that establish infection, as these are the precise targets of vaccines and microbicides. The aim of this dissertation is three-fold: firstly, to characterize the extent of the diversity of the transmitted/founder virus population during acute infection in the Mbeya region of Tanzania; secondly, to investigate the biological features of transmitted viruses; and lastly to determine how these properties impact on subsequent disease progression.

This study forms part of the Mbeya Medical Research Programme, and was conducted using plasma samples from recently infected women from the Mbeya region of Tanzania, which is a region that has number of subtypes co-circulating. This cohort comprised of 600 HIV positive and HIV negative female bar workers who were at high risk of infection, and were followed up every three months over 3 years as part of HIV Superinfection Study (HISIS) cohort (Hoelscher *et al.*, 1998; Riedner *et al.*, 2006).

University of Cape Town

This literature review will provide an overview of the extent of HIV-1 diversity both within Tanzania and rest of the world, the current thinking in this field with regard to the early events following transmission, and what is known about the characteristics of the incoming transmitted/founder virus population. This will give a broader understanding of both host and viral factors that affect transmission.

1.1 HIV diversity

HIV is classified into two genetically distinct types, type 1 (HIV-1) and type 2 (HIV-2) which are then further subdivided into a hierarchy of groups and subtypes based on genetic variation. This thesis focuses only on HIV-1, which is classified into four Groups (M, major; O, outlier; N, non-M, non- O; and P) and Group M further divided into 9 subtypes (A1, A2, B, C, D, F1, G, H, J, and K) (Robertson *et al*., 2000); HIV Sequence Database. http://www.hiv.lanl.gov) (**Fig. 1.1**). In regions of the world where various HIV subtypes co-mingle, recombinant viruses have emerged resulting in the formation of circulating recombinant forms (CRFs), complex CRFs (CRFcpx), and unique recombinant strains (URFs).Circulating recombinant forms are intersubtype recombinant viruses that are transmitted to many people and as a result become one of the circulating strains in the HIV epidemic. For example, the CRFO7_BC consists of regions of both subtypes B and C. Unique recombinant viruses are recombinant viruses with unique breakpoints and have limited transmission in the human population. These URFs are usually produced in a dually infected or multiply infected individual (Robertson *et al.,* 2000; Los Alamos, 2007). If the recombinant infects many people and becomes a circulating strain in an HIV epidemic, it becomes a CRF. CRFs are recombinant viruses that can infect three or more

University of Cape Town

individuals that are not epidemiologically related, and therefore can be assumed to have to have a significant contribution on the HIV epidemic. When these viruses are created, they circulate in regions independent of their parental subtype. Currently, there are 48 CRFs that have been identified (see Los Alamos National Lab (LANL)-database; http://www.hiv.lanl.gov).

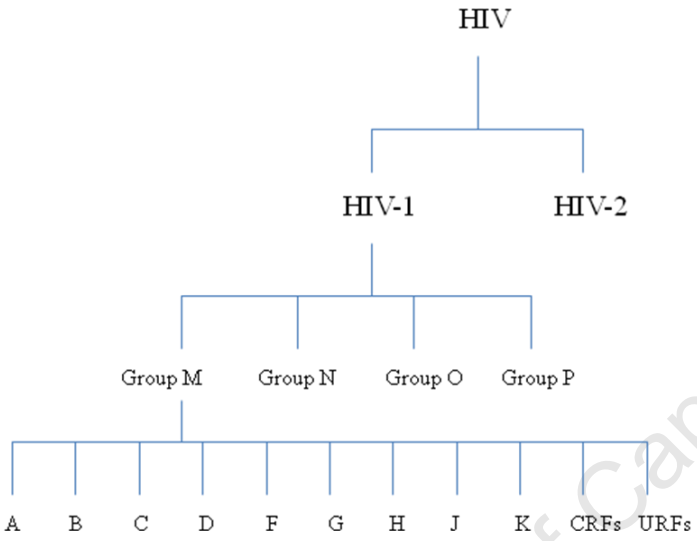


Figure 1.1. **Classification of HIV into HIV-1 and HIV-2 with the former divided into 4 groups with the Group M comprising of 9 subtypes, 48 circulating recombinant forms (CRFs), and unique recombinant forms (URFs)** (modified from www.avert.org).

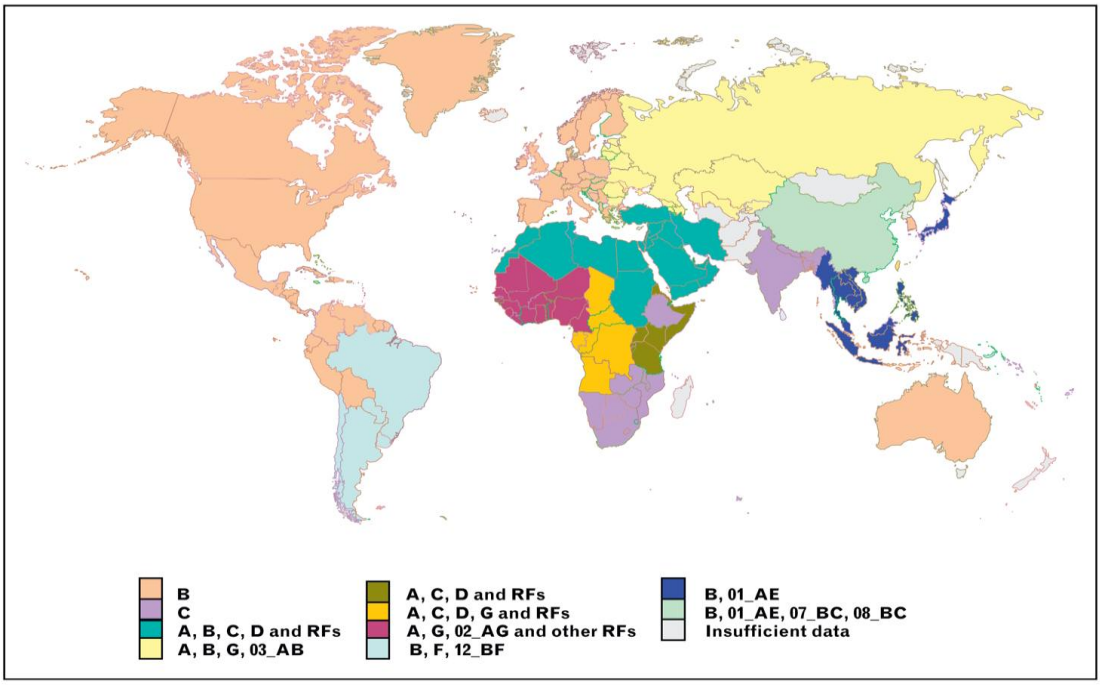
University of Cape Town

1.2 Worldwide distribution of HIV subtypes and circular recombinant forms

HIV originated in equatorial central Africa, and diversity in this region can be attributed to viral evolution (Corbet *et al.*, 2000; Gao *et al.*, 1999; Keele *et al.*, 2006; Santiago *et al.*, 2002). However outside of this region, the global distribution of subtypes and CRFs largely reflects the historical spread of the virus. There is an uneven distribution of HIV subtypes and circulating recombinant forms (CRFs) throughout the world, with some genetic forms becoming dominant

in certain geographic regions. This uneven distribution can be largely attributed to founder effects, although differences in biological properties of the virus may also play a role (**see 1.9.2**).

Founder effects result when a single virus is introduced into a transmission network, resulting in the spread of that subtype in the region (Rambaut *et al.*, 2004). There are numerous examples of founder effects: the Subtype C epidemics in India, Ethiopia and South America (Fontella *et al.*, 2008); the subtype B epidemic in Haiti and North America (Gilbert *et al.*, 2007) and CRF strains CRF01_AE and CRF02_AG in Thailand and West/ Central Africa, respectively (McCutchan *et al.*, 1992; Montavon *et al.*, 2000).



Modified and updated from Hemelaar *et al.* [6]. 03_AB = CRF03_AB; 12_BF = CRF12_BF; 01_AE = CRF01_AE; 07_BC = CRF07_BC; 08_BC = CRF08_BC; RF = unique recombinant form.

Figure 1.2. **Worldwide distribution of HIV subtypes and recombinants.** from (Woodman & Williamson, 2012)

1.3 Mechanisms of HIV diversification

HIV evolves more rapidly than other known organism (Rambaut *et al.,* 2004) enabling the virus to rapidly adapt to the host, and avoid detection by the host's immune system. Mechanisms of HIV diversification include recombination and a high mutation rate due to the error-prone reverse transcriptase enzyme. The error-prone nature of reverse transcriptase causes a mutation rate of 0.3 nucleotide changes per genome per replication cycle, resulting in the emergence of a cloud of highly related but distinct viruses (quasispecies) within an infected individual (Mansky, 1995). Recombination occurs when the reverse transcriptase switches RNA templates during replication, thereby forming progeny viruses that are representative of different parent viruses (Wooley *et al.*, 1997) . HIV has one of the highest recombination rates compared to other organisms, with approximately 2.8 recombination events occurring per genome per replication cycle (Zhuang *et al.*, 2002). The recombination rate, therefore, exceeds the mutation rate per replication cycle. Recombination events can occur between viruses from different subtypes (intersubype recombination) and also viruses derived from the same subtype (intrasubtype recombination) (Takehisa *et al.*, 1999).

HIV sequence evolution is also affected by the host innate restriction factor, such as APOBEC3, which blocks retroviral infection. The two main cellular proteins APOBEC3F and APOBEC3G deaminate deoxycytidine to deoxyuridine on the first retroviral DNA strand (minus strand) during reverse transcription of RNA into cDNA. If APOBEC is incorporated into the virion, it will result in the deamination of cytosine and also the accumulation of uracil residues, resulting in guanosine-to-adenosine (G-to-A) hypermutations which are detrimental to the viral replication (Harris *et al.,* 2004). HIV counters the action of APOBEC by producing the viral infectivity

University of Cape Town

factor (Vif) protein which acts by binding to the APOBEC and targets it for proteosomal degradation (Sheehy *et al.*, 2002; Yu *et al.*, 2003). There have been conflicting reports on the effect of hypermutation on disease progression. A study by Pace *et al.*, (2006) found a reduction in viral load in individuals with detectable hypermutation in the peripheral blood mononuclear cells, and (Land *et al.*, 2008) found an increase in the amount of CD4 cells when hypermutation of HIV was detected. Conversely, others studies have not found any correlation between hypermutation and viral load (Piantadosi *et al.*, 2009a; Ulenga *et al.*, 2008). A study by Wood *et al.*, (2009) found that transmitted/ founder viral sequences in recently infected participants with subtype B contain rapidly evolving sites that are embedded in an APOBEC motif and in a CTL epitope, suggesting that APOBEC - induced mutations may facilitate viral escape.

University of Cape Town

1.4 HIV diversity and subtype distribution in Tanzania

This thesis characterized viruses from high risk women from the Mbeya region of Tanzania. The number of people that are infected with HIV in Tanzania is estimated to be at 1.5 million (UNAIDS report, 2012). HIV subtypes A, C, and D were identified as predominant subtypes since the early 1990s (Hoelscher *et al.*, 1998), and the distribution of the subtypes varies depending on the geographic region sampled. HIV subtype A and D were the predominant subtypes in the northern parts of Tanzania, with subtypes A, C, D co-circulating in the Kilimanjaro region (Nyombi *et al.*, 2008), together with a number of intersubtype recombinants (Arroyo *et al.*, 2005; Herbinger *et al.*, 2006) (**Fig 1.3**).

The HIV-1 prevalence in adults of 8 % in Mbeya is higher than in the rest of the country (UNAIDS report, 2010), with the epidemic driven by genetically complex viruses, with subtype C identified as the predominant subtype in the southwestern region of Mbeya. Using the multi-region hybridization assay, the subtype distribution in this region was found to be subtype A (8.5 %), subtype C (40.8 %), D (3.8 %), along with a number of recombinants AC (25.4 %), AD (5.4 %), CD (8.8 %), and ACD (7.3 %) (Arroyo *et al.*, 2005; Herbinger *et al.*, 2006).

Full-length sequencing and recombination analysis identified the intersubtype recombinants as unique, as they did not share recombination breakpoints with any previously identified recombinant strains (Hoelscher *et al.*, 2001). In this region, there was a higher frequency of recombinant strains (54 % vs 40 %) and infection with more than one subtype (dual infections) (19 % vs 9 %) in the high risk population compared to the normal risk populations.

University of Cape Town

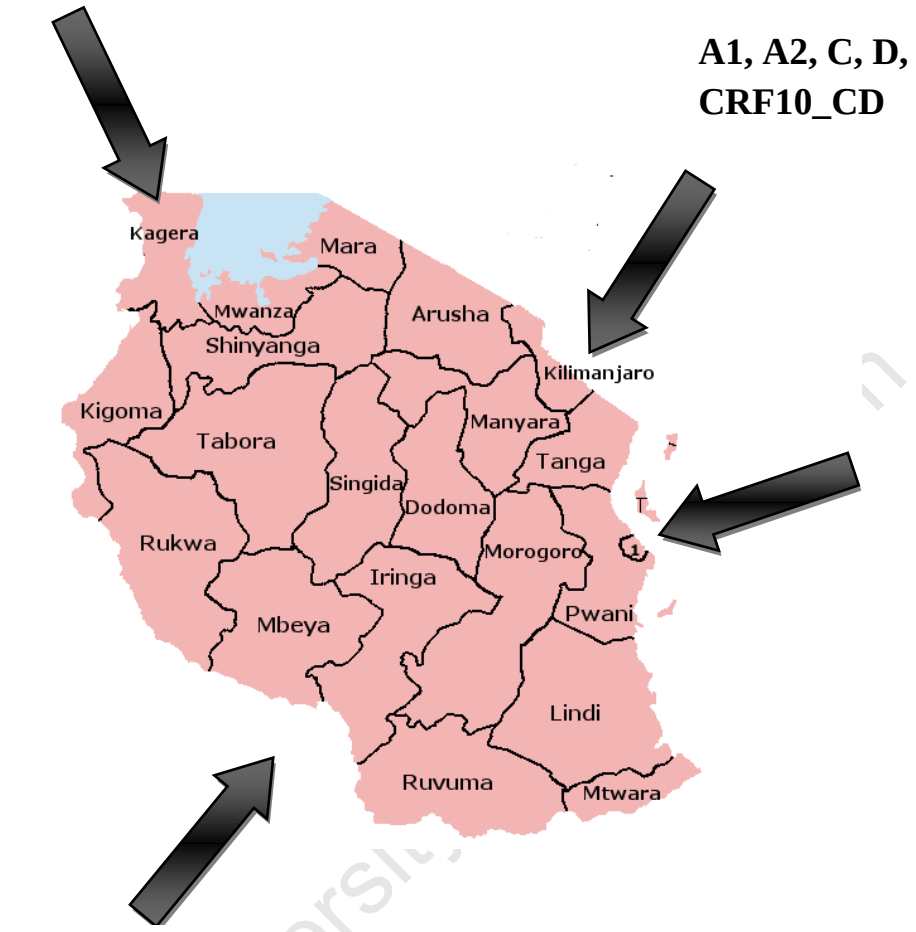


Figure 1.3. Distribution of HIV subtypes in Tanzania. (Adapted from http://en.wikipedia.org/wiki/File:Tanzania_Regions.png).

1.5 Dual infection

Dual infection occurs when an individual is infected with two phylogenetically distinct and epidemiologically unlinked viruses, which ultimately results in the generation of recombinant viruses. These viruses can either be derived from the same subtype (intrasubtype) or from different subtypes (intersubype). Dual infections can occur either by coinfection or by superinfection. Coinfection is defined as infection with two strains, either simultaneously or within rapid succession of one another prior to seroconversion. Superinfection occurs when an HIV infected individual becomes infected with a second strain after an immune response to the initial strain has been established (Gottlieb *et al.*, 2004; Grobler *et al.*, 2004; Smith *et al.*, 2005; Zhu *et al.*, 1995). Dual infection has been shown to affect disease progression with an association identified between dual infection and high viral load set point (Grobler *et al.*, 2004) as well as rapid disease progression where individuals who were dually infected with two HIV subtype B viruses, and progressed to AIDS within 1.5 years of seroconversion, compared to single infected individuals who progress to AIDS within 8-10 years (Gottlieb *et al.*, 2004). However, a recent study by Woodman *et al.*, (2011) did not find an association between dual infection and disease progression. It has been suggested that dual infection, which is common in the Mbeya region of Tanzania, will enable rapid recombination which might allow viruses to adapt faster and exploit cellular niches more efficiently than single infections with low diversity (Gottlieb *et al.*, 2004; Herbinger *et al.*, 2006).

Recent studies have investigated factors that make individuals susceptible to superinfection. It has been shown that superinfected individuals mount a low autologous neutralizing antibody response prior to superinfection, which may increase their susceptibility to superinfection (Basu

University of Cape Town

et al., 2012; Smith *et al.*, 2006). Following superinfection however the neutralizing antibody response appears to broaden as a result of antigenic stimulation by a second distinct virus (Cortez *et al.*, 2012). Although individuals that are superinfected with discordant HIV subtypes have an effective neutralizing antibody response against heterologous viruses, their potency against autologous viruses is not different compared to singly infected individuals (Mayr *et al.*, 2012).

The role of HIV-1 CTL specific immune response in providing protection to superinfection is not clear. The HIV-1 CTL immune response, both in human and primate studies, does not seem to be effective in providing protection to superinfection, and it is likely that protection from superinfection requires a much higher frequency of T cells with a different specificity than those present during chronic infection (Blish *et al.*, 2012; Weinfurter *et al.*, 2011).

University of Cape Town

1.6 Immune responses impacting on viral genotype and phenotype following infection.

1.6.1 Cell-mediated immunity to HIV-1 infection

Cytotoxic T-lymphocytes (CTLs) play an important role in suppressing viral replication, both during acute and chronic infection. After infection, the viral peptides become processed inside the endoplasmic reticulum, before being transported to the cell membrane for presentation by the Major Histocompatibility Complex (MHC) class I molecules. The presentation of the viral peptide on the surface of the cell triggers a response by the CTLs. The T cell receptors (TCR) that are expressed on the surface of CTLs bind to the MHC class I molecules and its peptide on the surface of the virus-infected cells, and induces the release of perforins and proteases that lyse the infected cells (Janeway, CA & Travers, 1994). HIV has evolved both mutational and non-

mutational mechanisms to avoid recognition by CTLs. Mutational escape mechanisms are amino acid changes in the viral peptides or epitopes that reduce binding to the MHC class I molecules and/or alter the interaction with the TCR. These changes result in a loss of recognition by CTLs (Le Gall *et al.*, 2007; Goulder *et al.*, 2004; Klenerman *et al*., 1994; Tenzer *et al.*, 2009). Another possible mutational escape by HIV-1 involves interfering with the intracellular processing of the viral peptides, which prevents these epitopes from being presented at the cell surface in the context of MHC class I molecules for recognition by CTLs.

The escape of CTLs by HIV is also achieved by nonmutational mechanisms such as the downregulation of the MHC class I molecules by the HIV infected cells (McMichael, 1998). This function is mediated by the Nef protein of HIV, which causes the MHC class 1 molecules to accumulate in clathrin coated vesicles in the Golgi apparatus resulting in the lack of recognition of the infected cell by the CTLs (Tomiyama *et al.*, 2002).

There has been overwhelming evidence over the years that support the role of human leukocyte antigen (HLA) molecules, and by implication CTLs, in determining the rate at which individuals progress to disease after infection (Fellay *et al.*, 2007). A genome wide association study identified single nucleotide polymorphisms (SNPs) in HLA region of chromosome 6 associated with viral control. Further analysis in the HLA B gene found five amino acids that are critical for the binding of the viral peptide to the HLA binding groove. There was also a single polymorphism that was found in the HLA- C gene, which is 35kb away from the transcription initiation site. This allele has been involved in the high expression of the HLA-C gene, which can influence viral loads (Fellay *et al.*, 2007).

University of Cape Town

The HLA molecules determine how different individuals can respond to specific immunodominant epitopes and certain CTL response are more effective at controlling viral replication than others. HLA alleles such as HLA B*27, HLA B*57, HLA B*58, and HLA B*63 have been strongly associated with immune control of HIV, resulting in slower disease progression (Gao *et al.*, 2001; Kaslow *et al.*, 1996 Kiepiela *et al.*, 2004; Migueles *et al.*, 2000). On the other hand, individuals with HLA alleles such HIV B*35 and HLA B*53 has been associated with rapid disease progression (Carrington & O'Brien, 2003; Carrington, 1999; Itescu *et al.*, 1992).

CTL have been shown to play a role in suppressing viral replication during acute infection until the viral set point is achieved, and responses during acute infection appears to be very narrow (Borrow *et al.*, 2007). CTL infection occurs as early as two to three weeks after infection (Goonetilleke *et al.*, 2009a; Lichterfeld *et al.*, 2004; Mlotshwa *et al.*, 2010), and Nef, Gag, and Pol are regions that are mostly targeted during the first 6 months of infection (Goonetilleke *et al.*, 2009b; Gray *et al.*, 2009a; Mlotshwa *et al.*, 2010). During chronic infection, the Gag and Envelope-specific CTL response have been found to be associated with low and high viral loads respectively, suggesting that Gag, and not Envelope, is critical in CTL-mediated viral control (Geldmacher *et al.*, 2007; Kiepiela *et al.*, 2007). Also, the most protective HLA alleles such as HLA B14, B27, B57, B58, B81 target very conserved regions in Gag, resulting in lower viral loads.

1.6.2 Humoral immunity to HIV-1 infection

The first antibody response produced by the B cells are in the form of virion - antibody immune complexes, which is followed by free IgM plasma antibodies that are specific for gp41 and

University of Cape Town

usually emerge 2 to 3 weeks post-infection (Tomaras *et al.*, 2008). The anti-gp41 antibodies are followed by the anti-gp120 antibodies a few weeks later, and these antibodies target the V3 loop. These antibodies do not have an effect on viremia (Tomaras *et al.*, 2008). Antibodies that are able to prevent infection are called neutralizing antibodies and these usually appear a few months post-infection (Gray *et al.*, 2007; Li *et al.*, 2006; Wei *et al.*, 2003) (**Fig. 1.4**). These early antibodies are effective against the autologous virus but are usually unable to neutralize divergent viruses from other hosts (Moore *et al.*, 1994). The plasticity of the HIV genome, and the narrow specificity of the antibody response, allow the virus to easily escape these responses (Moore *et al.*, 2009; Rong *et al.*, 2009; Wei *et al.*, 2003). Viruses are able to evade antibody responses through disruption of epitopes by means of single amino acid substitutions, insertions and deletions, as well as by structural shielding which can occur through shifting N-glycosylation sites that prevent access of the antibodies to the viral epitopes or by elongation of variable envelope domains (Bunnik *et al.*, 2008; Herrera *et al.*, 2003; Overbaugh & Rudensey, 1992; Richman *et al.*, 2003).

University of Cape Town

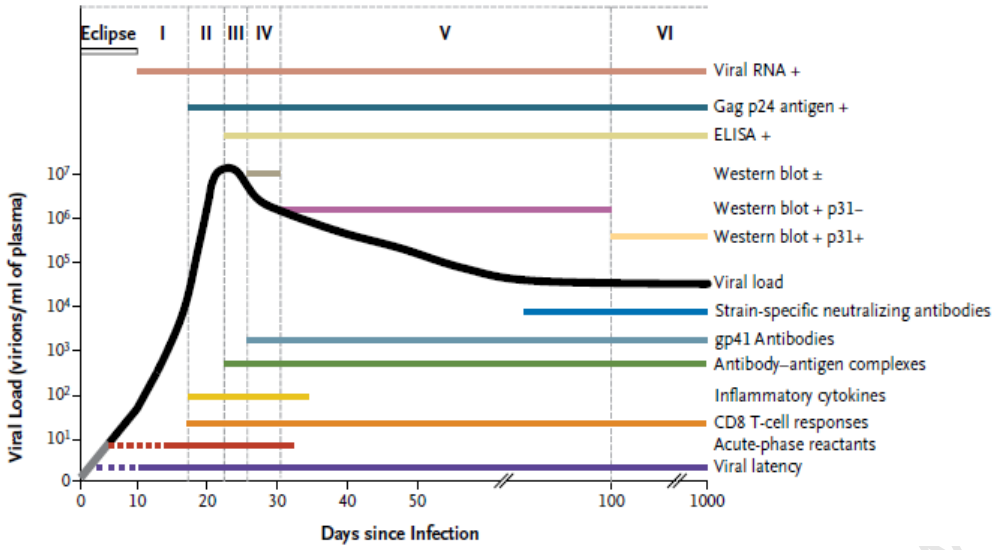


Figure 1.4. **The timing of CTL and antibody responses during acute stage of HIV infection** (Cohen *et al.*, 2011). The laboratory staging indicated in Roman numerals with the detection of viral RNA, and viral antigens indicated (Fiebig *et al.*, 2003). Individuals classified as being in Stage I/II were viral RNA positive, ELISA antibody negative; those in stage III were ELISA antibody positive but negative by Western blot; those in stage IV had an indeterminate Western blot; those in stage V were Western blot positive but without reactivity to the p31 integrase band; and those in stage VI were Western blot positive with a p31 band present.

Autologous neutralizing antibodies preferentially target certain regions in the viral envelope protein, particularly the V1V2 region because it is on the outer surface of the virus and is also extensively N-glycosylated (Overbaugh *et al.*, 1991). V1V2 length was correlated with the levels of N-linked glycosylation, with both mechanisms thought to be important for the viral evasion of the humoral immune response (Curlin *et al.*, 2010). The C3-V4 region has also been shown to be a target for neutralization during early subtype C infection (Moore *et al.*, 2008). This target is more common in subtype C due to the variability of the alpha-2 helix in the C3 region and a shorter V4 region in subtype C viruses compared to subtype B viruses (Gnanakaran *et al.*, 2007). V3-V5 region, and the V1V2 region in association with the gp41 region or V3 loop have also been implicated as targets for neutralizing antibodies (Rong *et al.*, 2009; Tang *et al.*, 2011).

University of Cape Town

Antibodies that are able to neutralize a range of viruses are called broadly neutralizing antibodies, and these have been identified in approximately 10 – 30 % of individuals after three years of infection (Doria-Rose *et al.*, 2009; Gray *et al.*, 2011a; Sather *et al.*, 2009; Simek *et al.*, 2009). It is still unclear why certain individuals develop these antibodies and others do not. It has been shown that longer periods of infection and high levels of viremia (causing persistent viral stimulation) are associated with the development of neutralization breadth (Euler *et al.*, 2010; Gray *et al.*, 2011b; Piantadosi *et al.*, 2009b; Sather *et al.*, 2009). Several studies have reported that infection with a subtype A and C viruses results in greater breadth and potency than subtype B viruses (Brown *et al.*, 2008; Bures *et al.*, 2002; Dreja *et al.*, 2010; Li *et al.*, 2006). A number of cross-reactive monoclonal neutralizing antibodies, and the epitopes that they target, have been identified. These include the IgGb12 (CD4 binding site target), 4E10 (carboxy terminal MPER target), 2F5 (amino-terminal MPER target), 2G12 (high mannose region of gp120), and the more recently discovered PG9 and PG16 (V1/V2 target), VRC01 (CD4 target), and PGT 127 and 128 antibodies (gp120 V3 loop) (Binley, 2009; Gray *et al.*, 2009a, b; Li *et al.*, 2009a; Pejchal *et al.*, 2011).

University of Cape Town

1.7 Structure and function of HIV Envelope (Env)

The HIV Envelope is the target of neutralizing antibodies, mediates viral tropism, and is a major determinant of viral fitness (Marozsan *et al.*, 2005). The HIV Envelope is a heterodimer comprised of gp120, which is involved in the attachment of the virus to the target host cell, and gp41, which is involved in the fusion between the viral and host membrane resulting in the entry of the viral material inside the host cell. These dimers are structurally organized as trimers which are presented at the surface of viral membrane (Berger *et al.*, 1999; Wyatt, 1998). The

Envelope protein is initially synthesized as a 160 KDa single polyprotein precursor in the endoplasmic reticulum (Freed & Martin, 1995; Wyatt, 1998). The protein subsequently undergoes several post-translational modifications, which include the addition of N-linked high mannose oligosaccharides resulting in an extensively glycosylated protein (Bernstein *et al.*, 1994; Leonard *et al.*, 1990). This process is essential for protein folding and conformational stability of the protein. The glycan sugars on the protein become trimmed and modified by cellular enzymes as it is transported through the Golgi network (Fenouillet *et al.*, 1994; Wyatt, 1998). Within the trans-Golgi network, the 160 kDa becomes cleaved by the cellular enzyme furin into gp120 and gp41 at a K/R-X-K/R-R motif (Freed *et al.*, 1989; McCune *et al.*, 1988). The cleavage is critical for fusion efficiency and also the infectivity of the virus. The three molecules of gp120 and gp41 combine to form a heterotrimer Envelope spike, and the two subunits are joined by noncovalent interactions (Egan *et al.*, 1996; Rowell *et al.*, 1995). The Envelope is transported to the plasma membrane and can be recycled by endocytosis. As the viral budding occurs, the gp120-gp41 is incorporated in the virus and thereafter is displayed as viral spikes on the surface of the virion (Wyatt, 1998). The gp120 and gp41 subunits form a cage-like structure which has a void surrounding its axis, and this architecture could be important in restricting antibody access to the virus (Mao *et al.*, 2012).

The gp120 component consists of five conserved (C1- C5) and five variable (V1- V5) regions (Modrow *et al.*, 1987). The conserved regions are important in the interaction with gp41. These regions are accessible to antibodies that bind to monomeric gp120, but not to gp120-gp41 complexes (Helseth *et al.*, 1991; Moore *et al.*, 1994; Olshevsky *et al.*, 1990). The C2, C3, and C4 regions of the protein are buried and inaccessible, and form a hydrophobic core of the protein

University of Cape Town

(Moore *et al.*, 1994). The conserved regions is critical to CD4 binding (Olshevsky *et al.*, 1990), and certain mutations in the C2 and C3 region affect the binding of gp120 to CD4 (Douagi *et al.*, 2010; Wyatt, 1998). The conserved region C1 is involved in the processing of the furin recognition site which is found in the C5 region, and also important in the interaction between gp120 and gp41 (Wang *et al.*, 2008).

In comparison to the conserved regions, the variable regions (mainly the V1, V2, V4, and V5 regions) are much more exposed and extensively glycosylated (Kwong *et al.*, 1998; Kwong *et al.*, 2000; White *et al.*, 2010) thereby shielding the conserved regions from being recognized by the antibodies. Deletion of V1, V2, and V3 regions does not affect binding to the CD4 and, suggesting the variable regions are not important in CD4 binding (Cao *et al.*, 1997; Checkley *et al.*, 2011; Moore *et al.*, 1994; Wyatt *et al.*, 1993, 1995). However, the V3 region has been shown to be important for membrane fusion (Freed *et al.*, 1991) and viral tropism (Cilliers *et al.*, 2005; Shioda *et al.*, 1991). A switch from CCR5 to CXCR4 is associated with an increase in the positive charge of the V3 region, thus allowing for an interaction with the negatively charged CXCR4 coreceptor (Pollakis *et al.*, 2004). Mutations in the crown and the base of the V3 region, along with the bridging sheet of the gp120 region also affect co-receptor binding (Cormier & Dragic, 2002; Cormier *et al.*, 2001). The role of the V3 region in coreceptor binding will be discussed in more detail later in the review (see section 1.7.1). The role of V4 and V5 is less well described, however it is known that the deletion of V4 affects Envelope conformation and glycan packing (Frost *et al.*, 2005b; Teeraputon *et al.*, 2005; Wei *et al.*, 2003), thus potentially limiting neutralizing antibody access.

The gp41 region is approximately 345 amino acids in length, and consists of three domains: the extracellular domain, the transmembrane domain, and the C-terminal cytoplasmic tail. The

University of Cape Town

extracellular domain consists of the fusion peptide (which is the N-terminal hydrophobic region) (Freed & Martin, 1995; Freed *et al.*, 1990), the polar region which consists of two heptad repeats HR1 and HR2 (Chan *et al.*, 1997;Weissenhorn *et al.*, 1997), and the membrane proximal external region (MPER) (Muñoz-Barroso *et al.*, 1999). The transmembrane domain has a 25 amino acid highly conserved region that allow the envelope protein to anchor into the lipid bilayer. Mutations in this region has been shown to affect fusion, suggesting that this region plays an important role in Envelope function (Kondo *et al.*, 2010; Shang & Hunter, 2010; Shang *et al.*, 2008; Zeller *et al.*, 2001). The C-terminal cytoplasmic tail consists of three helical segments that are known as lentiviral lytic peptides (LLP1, LLP2, and LLP3) (Lee *et al.*, 2002). These peptides have been previously shown to play a role in fusogenicity (Kalia *et al.*, 2003), protein stability (Lee *et al.*, 2002) , multimerization (Lee *et al.*, 2000), and cell surface expression and incorporation (Piller *et al.*, 2000).

of Cape Town

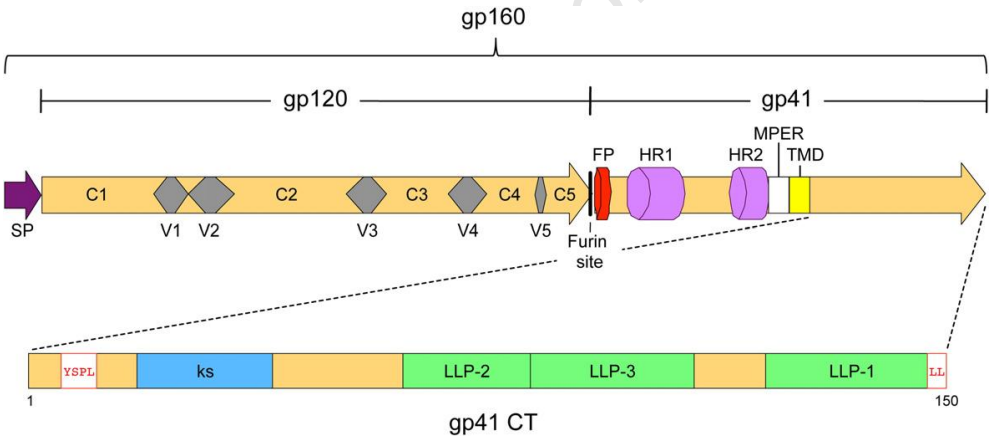


Figure 1.5. **Different regions or domains of the HIV-1 Envelope**. (from review by Checkley et al., (2011).

1.7.1 The role of HIV Envelope in host cell binding and entry

The functional Envelope trimer is involved in the binding of viral gp120 to the host cell CD4 receptor and coreceptor molecules, which ultimately results in the entry of the virus into the host cell (Berger *et al.*, 1999; Wyatt, 1998). This thesis aims to measure differences in Envelope entry efficiency between transmitted viruses, and how this impact on their overall replication capacity. An understanding of the mechanism and the efficiency at which the transmitted viruses mediate host cell entry can help in the design of novel compounds to prevent infection.

The process of host cell entry by HIV-1 involves the binding of the trimeric Envelope protein to CD4 and a chemokine coreceptor, which is usually either CCR5 or CXCR4 (Berger *et al.*, 1999). The essential role of the CCR5 coreceptor during entry was observed when individuals who were homozygous for a 32bp deletion in the CCR5 gene (Δ 32- CCR5) were largely protected against HIV-1 infection, and those that were heterozygous for the Δ 32- CCR5 allele showed delayed disease progression (Liu *et al.*, 1996). It has been well documented that viruses that utilize the CCR5 coreceptors, termed R5 viruses, are predominately found early during infection, while viruses that utilize the CXCR4 coreceptor, termed X4 viruses, are detected later during infection, and are associated with rapid CD4 decline and disease progression (Connor *et al.*, 1997; Schuitemaker *et al.*, 1992). R5 viruses predominate early during infection for all modes of transmission (Masharsky *et al.*, 2010).

Prior to CD4 binding, the first step to host cell entry is the adhesion of the virus to the host cell. This event can be facilitated by either the Envelope or membrane proteins that are incorporated into the virion with a number of cell attachment factors. The Envelope protein has been found to interact with a number of adhesion molecules, which include the cell surface heparin sulfate

University of Cape Town

proteoglycans (Saphire *et al.*, 2001), α4β7 intergrin (Arthos *et al.*, 2008; Cicala *et al.*, 2011), and dendritic cell specific intracellular adhesion molecule – 3 – grabbing non-intergrin (DC-SIGN) (Geijtenbeek *et al.*, 2000).

The entry process involves three important steps which are required in order for infection to occur, and these are CD4 binding, coreceptor binding, and membrane fusion. The first step, which is the binding of gp120 to the CD4 receptor, causes the rearrangement of the V1V2, and V3 regions, and exposes the conserved regions, which include the gp120 chemokine receptor binding site and the gp41 coiled coil domain (Kwong *et al.*, 1998; Zhou *et al.*, 2007). The interaction between gp120 and CD4 results in the formation of the bridging sheet, and the repositioning of the V3 region facilitates the binding to the coreceptor (Chen *et al.*, 2005; Kwong *et al.*, 1998). The CD4 receptor binds between both the inner and outer domain of gp120. This binding creates a highly protected and conserved 'cavity', which is also not glycosylated. A phenylalanine residue at position 43 (Phe-43) in CD4 has been found to be critical in binding to this cavity (Madani et al., 2004; Kwong et al., 2008). After CD4 binding, the conserved region of gp120 gets transformed from being in a rigid state to being in a flexible state, which allows for the interaction with the coreceptor (Kwong et al., 1998). The second step involved the subsequent binding to the coreceptors. The gp120-coreceptor interaction triggers the membrane fusion capacity of the virus by inducing conformational changes in the transmembrane protein, gp41, which exposes the fusion peptide and its penetration into the cell membrane resulting in the formation of the fusion pore (Brasseur *et al.*, 1988). The mapping of the gp120-coreceptor interaction suggests that for R5 viruses the N-terminus of the second extracellular loop (ECL2) of gp120 are important for coreceptor binding (Wu et al., 1997; Dragic et al., 1998), while the only the ECL2 is important for X4 viruses (Picard et al., 2007). After the CD4 and CCR5

University of Cape Town

binding steps, additional conformations occur which result in a shift by the viral Envelope from a non-fusogenic to a fusogenic state. The N- terminus of gp41 becomes exposed and gets inserted in the cell membrane through the fusion peptide. Structural changes within gp41 results in the formation of the six-helix bundle strucutre, which is essential for the fusion of the host and viral membranes. The change in free energy resulting from the six helix bundle formation provides the force needed for the formation of the fusion pore, and the viral genetic material enters the target cell through this process (Weiss et al., 2003; Poveda et al., 2005) (Doms and Moore, 2000; Pancera et al., 2010).

University of Cape Town

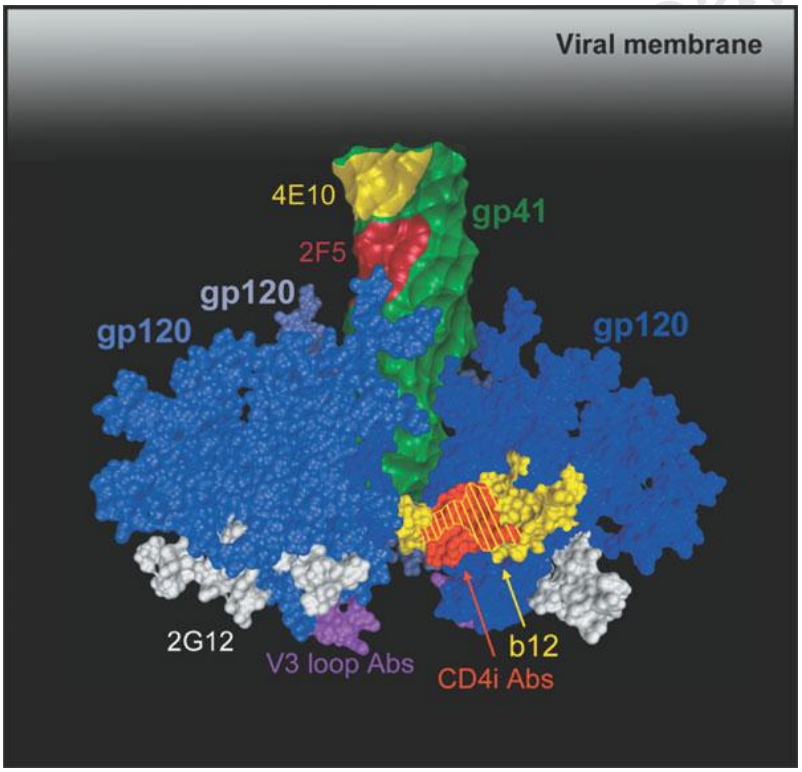


Figure 1.6. **Structure of the Envelope trimer showing the epitopes that are recognized by broadly neutralizing antibodies** (from Burton *et al.,* (2011).

As viral entry is dependent on Envelope binding to host cell surface receptors, the binding affinity of gp120 to CD4 and CCR5 as well as the capacity of gp41 to mediate membrane fusion influences how the virus will replicate in an environment, which is defined as viral replicative fitness. Natural mutations in the V3 loop region (sites 318 and 319) that decrease the binding affinity for receptor/coreceptor molecules (Maroszan *et al.*, 2005; Lobritz *et al.*, 2007), affect the sensitivity of HIV to entry inhibitors and the efficiency of host cell entry.

The specificity of binding to either CCR5 or CXCR4 is determined by the third variable (V3) region of gp120 (Fouchier *et al.*, 1992). Recent structural data has shed some light on how the interaction between gp120 with CD4 and CXCR4. The base of the V3 loop binds to the amino-terminal region of the coreceptor, while the tip of the V3 region binds to the extracellular loop (ECL2) of the coreceptor. A threshold number of CD4 molecules is required for successful membrane fusion however the number is not known (Wu *et al.*, 2010). Cryo-electron microscopy, together with antibodies and other reagents that block viral entry, has been used to study various conformational changes of the trimeric Envelope at different stages of the viral entry process (Harris *et al.*, 2011; Liu *et al.*, 2008; White *et al.*, 2010, 2011). It has been shown that the binding of antibody 17b to gp120, which mimics the binding mechanism of CD4 receptor, causes the activation of the envelope spike; however CD4 neutralizing antibodies VRC01, VRC02, and VRC03 block this activation, thus keeping the envelope in a closed, native state. Therefore, the conformation of Envelope in a 17b-bound sate has revealed an activated intermediate state during the entry process which could be useful for the design of novel immunogens (Tran *et al.*, 2012).

A number of studies have been done to determine regions or sites in the *env* gene that are critical in the various steps of host cell entry. These studies use entry inhibitor assays to indirectly

University of Cape Town

measure the affinity of the virus to CD4 and CCR5 receptors and also fusion capacity. A study by Reeves *et al.*, (2003) found that mutations that reduced CCR5 binding also resulted in increased sensitivity to CCR5 antagonist TAK779. Other regions in the Envelope protein such as the C4 region have also been found to be involved in the interaction between gp120 and the CCR5 coreceptor (Rizzuto & Sodroski, 2000). In order to identify viral determinants of membrane fusion, Derdeyn and colleagues (2000) identified a tripeptide sequence within HR1 (GIV at position 36 to 38) that influenced HIV sensitivity to the fusion inhibitor T-20, suggesting that this region might be an important interaction site for fusion. The study also demonstrated that the sensitivity of the virus to T-20 is modulated by the specificity in which gp120 interacts with the coreceptors. Mutations in the V3 region and the bridging sheet that reduce reduced the binding affinity of gp120 for CCR5 were found to increase the sensitivity of the virus to T-20 inhibition (Reeves *et al.*, 2002).

University of Cape Town

1.7.2 The role of DC-SIGN in cell-cell transfer

Dendritic cells are antigen presenting cells which are present in the mucosa and are one of the target cells that encounter the incoming virus during HIV transmission. The cells engulf the incoming antigen, migrate to the lymph nodes where they process and present the virus to T cells. Dendritic cells do not get productively infected by HIV, and this is partly due to their low CD4 and coreceptor expression levels, host restriction factors, and many other factors that block HIV at a postintergration level (Bakri *et al.*, 2001).

Dendiritic cells uses the C-type lectin DC-SIGN to attach to the gp120 region of the virus (McDonald *et al.*, 2003). The virus gets internalized by endocytosis (Geijtenbeek *et al.*, 2000) and migrates to the lymph nodes to form a virological synapse with the T cells (Felts *et al.*, 2010). The formation of these synapes, which is facilitated by attachment proteins like ICAM and LFA1 with gp120-CD4 interactions, facilitates the recruitment of CD4, CCR5, CXCR4, and $CD4^+$ T cells, resulting in productive infection in the T cells (Hübner *et al.*, 2009; McDonald *et al.*, 2003).

University of Cape Town

1.7.3 The role of intergrins during HIV infection

A group of investigators have identified the role of the homing receptor α4β7 during HIV infection. High levels of the α4β7 receptor, along with CD4 and CCR5, are expressed on the surface of mucosal $CD4^+$ T cells (Arthos *et al.*, 2008). When it is expressed, the α4β7 receptor is important in the migration of T cells from the gut (mainly the Peyer's patches and the messentric lymph nodes) to the lamina propia (von Adrian, UH; Mackay, 2000; Wagner *et al.*, 1996). However, the role of the α4β7 homing receptor in the vaginal mucosa is not yet fully understood. These gut-associated lymphoid tissues (GALT) play an important role during early stages of infection after sexual transmission. The high expression levels of CD4, CCR5, and α4β7 make them susceptible to HIV infection and may also favour the transmission of R5 viruses (Cicala *et al.*, 2011). The massive depletion of this cell population during the early stages of mucosal sexual transmission further highlights the role of these cells as one of the main target cells during transmission (McKinnon *et al.*, 2011).

These investigators hypothesized that HIV interacts with these cells through gp120 binding to the α4β7 receptor (Zeller *et al.,* 2001). The gp120 binding is mediated by a conserved LDV tripeptide in the V1V2 region of gp120 (Zeller *et al.,* 2001). This region has been previously found to be involved in transmission efficiency (Sagar *et al.,* 2006; Wu *et al.,* 2006), highlighting the possible role that gp120-α4β7 interactions could play in transmission. Binding of α4β7 to gp120 mimics the binding of MadCAM, VCAM, and fibronectin, which are natural ligands of α4β7. The α4β7 receptor is three times the size of the CD4 receptor, allowing easy viral capture. The interaction between α4β7 and gp120 also results in the activation of LFA-1, which is an adhesion-associated intergrin. The LFA-1 intergrin facilitates the formation of virological synapses and allows HIV to spread from one cell to another, and also influence *in vitro* host cell entry efficiency (Arthos *et al.,* 2009; Cicala *et al.,* 2009). As cell-to-cell spread of HIV is more efficient than cell free infection, this could be an essential method of virus production *in vivo* (Arthos *et al.,* 2008). The blockade of the intergrin α4β7 by monoclonal antibodies affects viral replication of HIV *in vitro*, further supporting the role of the receptor in facilitating entry (Cicala *et al.,* 2009). However, a recent study by Parrish *et al.,* (2012) found that transmitted viruses did not differ in their ability to bind CD4 and CCR5 compared to chronic viruses, and also found that anti- α4β7 antibodies were not effective at inhibiting infection. Hence the transmission bottleneck does not seem to result in the selection of viruses that utilize CD4, CCR5, and α4β7. In conclusion, the role of the intergrin α4β7 during HIV mucosal transmission is yet to be fully understood, and the effect of the blockage of gp120- α4β7 interaction on HIV replication is not yet known (Wilen *et al.,* 2012).

University of Cape Town

1.8 HIV transmission

1.8.1 Mucosal HIV transmission

Sexual transmission accounts for the majority of HIV infections, with women being the worst affected by the epidemic (UNAIDS, 2011). This thesis aims to understand viral factors that affect HIV transmission in a high risk population of women infected via the heterosexual transmission route. Therefore, a detailed understanding of early events during HIV transmission and the nature of the incoming virus will aid in interrupting HIV transmission by vaccination, microbicides, and pre- or post-exposure prophylaxis.

University of Cape Town

An intact vaginal epithelium consists of several thick layers of stratified squamous epithelial cells and the lamina propria, whereas the endocervix comprises of a single layer of columnar epithelial cells. These layers of cells serve as targets for HIV entry and provide a mucosal barrier to HIV infection. This barrier does not provide absolute protection and the virus can move across the intact epithelial barrier. (Pope & Haase, 2003). However, this barrier can be disrupted by sexually transmitted infections, microulcerations, and hormone-induced changes in the vaginal epithelium during the menstrual cycle (Hu *et al.*, 2000). This disruption can lead to inflammation, resulting in the influx of $CD4^+$ $CCR5^+$ T cells cells to the submucosa enabling HIV infection and then rapid dissemination to the lymhoid tissues.

Studies that have used the non human primate model have found that the infection is established in a small number of target cells that are infected by a small number of founder viral population. This was shown by the presence of SIV DNA within 3 to 4 days after viral exposure to the

mucosal tissue (Miller *et al.*, 2005; Zhang, 1999). This founder viral population was primarily identified in the endocervix and the transformation zone (which is the junction between ecto- and endocervix) (Haase, 2011; Sagar, 2010). This period before peak viremia is known as the eclipse phase (Haase, 2011). The small founder population thereafter expands locally as a result of recruitment of additional target cells to the site of infection (Li *et al.*, 2009a). The presence of virus results in an increase in MIP3-α/CCL20 expression by the endocervical epithelium, resulting in the recruitment of $CCR6^+$ plasmacytoid dendritic cells to the site. These dendritic cells in turn will secrete chemokines (which include MIP1α) resulting in the influx of T cells and macrophages. This creates an environment that is rich in $CD4^+$ T cells, and making it favourable for the founder population to expand (Haase, 2011). However, the $CD4^+$ T cells that are present in the cervical mucosa and submucosa show a 'resting' phenotype and low levels of CCR5 expression (Li *et al.*, 2005; Zhang, 1999). It is suspected that these cells are memory T cells that have reverted to a 'resting' state but have CCR5 levels that are able to support HIV/SIV replication. The expansion will initially move to the draining genital lymph nodes for further expansion, and ultimately lead to systemic infection in the blood, gut, spleen via the thoracic duct (Haase, 2011) (**Fig. 1.7**).

During the systemic stage of SIV and HIV infection, the innate immune response may or may not be able to control viral spread, and recruitment of more target cells could lead to further viral expansion. This has been shown in acute systemic SIV infection that an increase in viral load is associated with the expression of the chemokines and interferon genes (Abel *et al.*, 2005). The duration of time before systemic spread to establish a clinical infection can have implications for the effectiveness of vaccines. A vaccine may have a better chance of preventing viral replication if the initial infection involves a small number of cells resulting in low viral production.

University of Cape Town

Furthermore, it may be important to limit the influx of target cells to the site of infection (Haase, 2011). In SIV, the lipid glycerol monolaurate has been shown to be effective in preventing transmission by preventing signalling and the influx of target cells (Li *et al.*, 2009b). If a large number of viruses infect a large number of $CD4^+$ T cells and rapidly spread to the draining lymph nodes, then a vaccine will find it difficult to contain the infection even with a very rapid response.

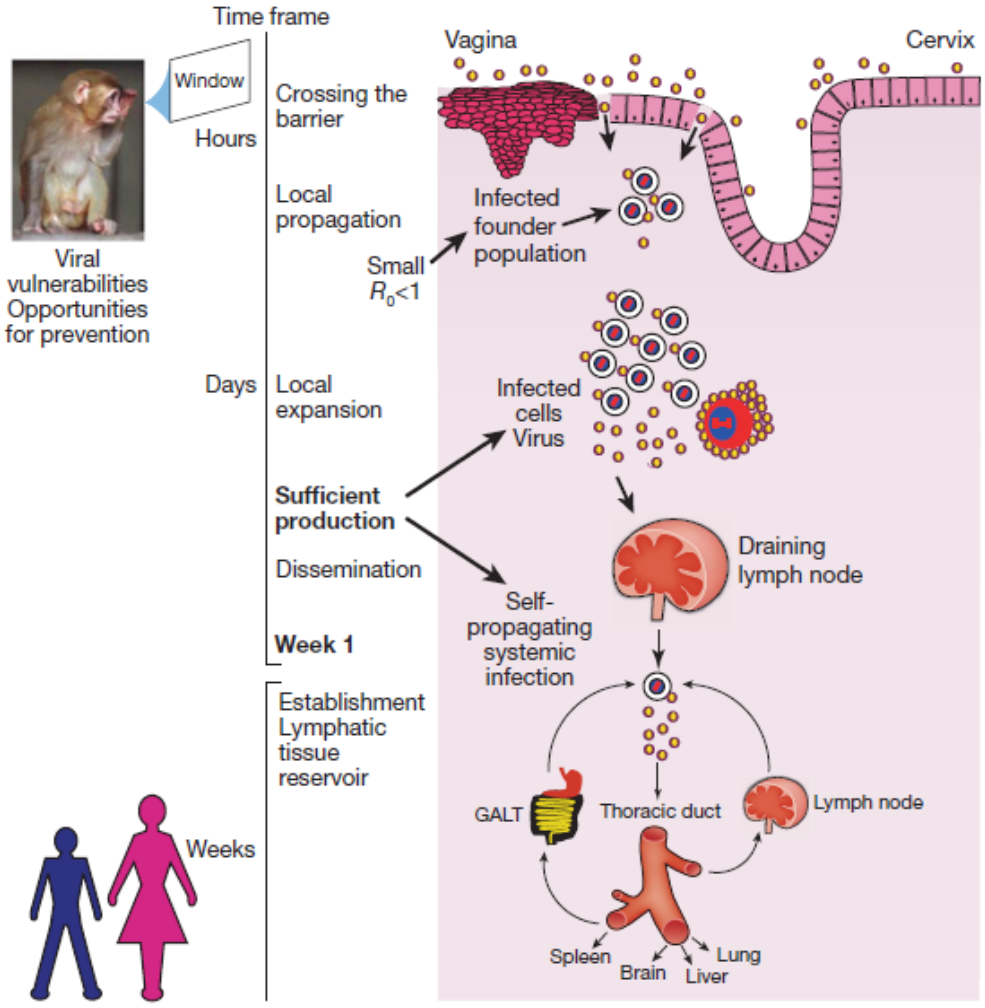


Figure 1.7. Illustration of the early events during HIV sexual transmission based on the rhesus macaque model. It is also shows phases of vulnerabilities which could be targeted by novel vaccines and microbicides (Haase *et al.*, 2010).

1.8.2 Isolation and enumeration of transmitted viruses

Identification of the transmitted variant is ideally performed in the context of transmission pairs. However, due to the difficulties in identifying transmission pairs, together with problems in defining the transmitted/founder virus population; there have been various methodologies developed to derive the transmitted virus/founder virus responsible for clinical infections.

Earlier studies that have investigated HIV transmission have used samples that were taken within the first six months of infection. These studies used viral sequences that were isolated from plasma or peripheral blood mononuclear cells (PBMCs) by bulk PCR, and subsequent cloning, sequencing, and phylogenetic analysis (Derdeyn *et al.*, 2004; Frost *et al.*, 2005b; Learn *et al.*, 2002; Long *et al.*, 2000; Ritola *et al.*, 2004; Rusert *et al.*, 2005; Sagar *et al.*, 2003). In some cases, the viral DNA was used to perform a Heteroduplex tracking assay (HTA), which involves the annealing of a short oligonucleotide probe to the amplified region of interest, and subsequent migration on a polyacrylamide gel. The migration pattern of the DNA fragment was used to determine the extent of genetic diversity in a participant's sample (Learn *et al.*, 2002; Long *et al.*, 2000). These methods have a number of limitations. The method of bulk PCR and cloning is compromised by the introduction of errors by Taq polymerase and errors caused by template switching (Li *et al.*, 2010; Palmer *et al.*, 2005; Salazar-Gonzalez *et al.*, 2008), resulting in extensive recombination (Salazar-Gonzalez *et al.*, 2008; Shriner *et al.*, 2004). HTA allows for a more qualitative analysis of genetic diversity as it does not provide sequence data for phylogenetic analysis (Palmer *et al.*, 2005; Salazar-Gonzalez *et al.*, 2008).

More recently, the single genome amplification (SGA) of viral RNA followed by direct sequencing of the uncloned amplicons was developed to more accurately enumerate the number

University of Cape Town

of viruses that have been transmitted, while also aim to address the limitations of previous approaches (Salazar-Gonzalez *et al.*, 2008). The method is based on dilution of the cDNA to an endpoint, where according on the Poisson distribution, a percentage of 30% or less of positive amplification enhances the probability of the products are derived from a single template. Sequences of these amplification products with no double peaks in the chromatograms were considered to be indicative of amplification from a single viral RNA template (Salazar-Gonzalez *et al.*, 2009). Using this approach on samples from acute infection, Keele *et al.*, (2008) developed a mathematical model of HIV sequence evolution which allowed for the identification of the transmitted/founder sequences based on an assumption that in the absence of selective pressure, the transmitted virus replicates exponentially and has a generation time of 2 days (Markowitz *et al.*, 2003), has a reproductive ratio of 6 (Stafford *et al.*, 2000), and an error rate of 2.16×10^{-5} (Mansky, 1995), and also undergoes constant mutations in all positions and lineages without any back mutations. The model showed that you could derive the transmitted/founder sequence at or near the time of transmission for sequences that fit this model of Poisson distribution of mutations and exhibits a star-like phylogeny (Keele *et al.*, 2008).

University of Cape Town

1.8.3 The population genetic bottleneck

It has been widely shown that in the majority of cases, that there is a severe genetic bottleneck associated with HIV transmission (Zhu et al., 1992; Abrahams *et al.*, 2009; Haaland *et al.*, 2009; Keele *et al.*, 2008; Salazar-Gonzalez *et al.*, 2009). Recent studies have shown that as much as 76% and 78% of sexual transmission events involve a single variant in both subtype B and C infections respectively, suggesting subtype differences do not seem to affect the frequency of the

transmission bottleneck (Abrahams *et al.*, 2009; Keele *et al.*, 2008). Transmission of multiple genetically distinct variants does occur in approximately 20% of individuals and has been found to be associated with faster disease progression compared to those infected with a single genetic variant (Sagar *et al.*, 2003). However, the association between infection with multiple variants and disease progression was not observed in other studies (Abrahams *et al.*, 2009; Woodman *et al.*, 2011).

There are various factors that may cause the bottleneck to be disrupted, which include genital tract infections and hormonal contraceptive use (Haaland *et al.*, 2009; Sagar *et al.*, 2003). Gender has also been shown to play a role in the degree of genetic diversity observed in individuals, with women being shown to be more susceptible to acquiring multiple genetic variants (Long *et al.*, 2000), however, this was not found in a study by Abrahams *et al.*, (2009). A high frequency of infection with multiple variants has been recently found in a population of men who have sex with men (Gottlieb *et al.*, 2004; Li *et al.*, 2010; Ritola *et al.*, 2004).

University of Cape Town

1.8.4 Characteristics of transmitted viruses

Transmitted viruses have a number of biological features that are important during transmission, some of which may impact on subsequent disease progression. The study reported in this thesis characterizes the phenotypic properties of the transmitted viruses and hypothesizes that certain properties within the Envelope protein of the transmitted viruses influences the disease outcome.

Despite the presence of R5 and X4 variants in the donor during chronic infection, variants that use the CCR5 receptor are almost exclusively acquired by the newly infected recipient (Berger *et*

al., 1999; Scarlatti *et al.*, 1997) although viruses that utilize CXCR4 or both CCR5 and CXCR4 have been identified in recently infected patients (Huang *et al.*, 2007). It has also been shown in donor-recipient transmission pairs that transmitted variants have Envelope proteins that are neutralization sensitive relative to the donor (Derdeyn *et al.*, 2004), with shorter variable loop length and less N-glycosylation than the chronically infected patients (Chohan *et al.*, 2005; Derdeyn *et al.*, 2004; Sagar *et al.*, 2006; Wu *et al.*, 2006). Similar results have been shown in Envelopes from subtype C transmission pairs (Haaland *et al.*, 2009), subtype A (Chohan *et al.*, 2005), and subtype A and D transmission pairs (Sagar *et al.*, 2006). The authors argue that the less compact Envelope glycoproteins may be more efficient at interacting with target cells. However, this finding was not observed in subtype B heterosexual and MSM infections, suggesting that subtype differences in the virus can affect the efficiency of transmission (Chohan *et al.*, 2005; Frost *et al.*, 2005a; Wilen *et al.*, 2012). This phenotype was also observed in SIV infected macaques in which changes in N-linked glycosylation along with increasing VIV2 length over time correlated with protection from neutralizing antibodies and loss of macrophage tropism (Rudensey *et al.*, 1995). The transmitted virus from known transmission pairs was shown to be more compact and closer to the ancestral virus. (Redd *et al.*, 2012).

There has been a considerable effort in trying to identify unique signatures in transmitted viral sequences in comparison with chronic viral sequence. Asmal and colleagues (2011) have recently found signatures near the CD4 and CCR5 binding sites, the gp41 cytoplasmic domain, and the signal peptide. They found a significantly high frequency of a histidine residue at position 12 of the leader sequence amongst transmitted viral sequences. This study showed that viruses that contain this residue are more efficient at transporting the Envelope glycoprotein to

University of Cape Town

the endoplasmic reticulum; and also have higher envelope expression and incorporation compared to those without the residue.

A number of recent studies have investigated whether viruses from individuals with acute infection have the ability to use certain cell types and have a higher affinity for CD4 and CCR5 receptors than chronic viruses. Early viruses have been shown to be more sensitive to CCR5 inhibitors than viruses at chronic infection, which suggests that transmitted viruses require high levels of CCR5 (Etemad *et al.*, 2009). The study also showed that transmitted viruses have slower fusion kinetics compared to those during chronic infection (Etemad *et al.*, 2009). Transmitted viruses also have the ability to replicate efficiently in $CD4^+$ T cells but poorly in monocyte-derived macrophages (Ochsenbauer *et al.*, 2012; Salazar-Gonzalez *et al.*, 2009). This finding suggests that $CD4^+$ T cells might be the main target cells and macrophages may not be critical during mucosal transmission as initially suspected.

University of Cape Town

1.9 HIV-1 viral replication capacity

1.9.1 Viral factors influencing viral replication capacity

HIV-1 infected individuals that have the ability to control viremia without the use of antiviral therapy are known as HIV controllers. These individuals maintain viremia at less than 2000 RNA copies/ml, while those that maintain viral loads below 50 copies/ml are termed elite suppressors. An understanding of the mechanism associated with this phenotype contributes to our understanding of HIV pathogenesis and could facilitate vaccine development. There have been a number of studies which have shown that disease outcome is linked between concordant

couples suggesting that properties of the virus may be inheritable (Alizon *et al.*, 2010; Hecht *et al.*, 2010; Hollingsworth *et al.*, 2010; Tang *et al.*, 2004; Yue *et al.*, 2013). A number of virological factors such as replicative rate, host cell tropism, linked to host factors which include genetic background, immune control, and target cell availability, have been associated with differential disease progression (Brockman *et al.*, 2007; Chopera *et al.*, 2008; Crawford *et al.*, 2007; Etemad *et al.*, 2009; Lassen *et al.*, 2009; Martinez-picado *et al.*, 2006; Nicastri *et al.*, 2003; Nijhuis *et al.*, 1999; Ochsenbauer *et al.*, 2012; Quiñones-Mateu *et al.*, 2000; Salazar-Gonzalez *et al.*, 2009; Schneidewind *et al.*, 2007, 2008; Troyer *et al.*, 2005).

University of Cape Town

1.9.2 Differences in replicative fitness between subtypes

Differences in pathogenicity and/or replicative fitness have been proposed to contribute to the uneven spread of HIV subtypes. A number of studies have compared *in vitro r*eplication between HIV Groups (Arie *et al.*, 2005) and subtypes (Abraha *et al.*, 2009; Rodriguez *et al.*, 2009). Using pairwise competition experiments in PBMCs from various donors, HIV group M isolates of any subtype have 100-fold more replication capacity than group O or HIV-2 strains (Arie *et al.*, 2005). Similar findings were also observed in a study by Abraha *et al.*, (2009) using an ex vivo transmission model. The study hypothesized that the lower replication capacity of group O and HIV-2 viruses may be one of the reasons for the limited geographical spreading of these viruses. Similar experiments were done comparing differences in replication fitness between HIV group M viruses. Subtype C is the most successful subtype globally and there have been several studies to investigate if there are properties of this subtype that would promote its dissemination. A study by (Rodriguez *et al.*, 2009) compared HIV-1 subtypes A and C viruses

in India, and found subtype C viruses have higher replication capacity than subtype A both in PBMCs and in an *ex vivo* cervical tissue derived organ culture. The authors propose that these findings may explain the uneven spread of subtype C over other subtypes in India. In contrast, a study by Abraha *et al.,* (2009) found that subtype C viruses have lower replicative fitness than subtypes B and D in an *ex vivo* transmission model using human explants from penile, rectal, and cervical tissues. The authors proposed that subtype C may have equal transmission fitness, but have lower pathogenic fitness than the other subtypes. However, caution must be exercised when extrapolating these laboratory results to what occurs *in vivo*. These *in vitro* replication models do not reflect the environment *in vivo*, and further some studies did not control for stage of infection from which the viruses were isolated from. As HIV rapidly evolves over time, these viruses would not reflect the properties of the transmitted/founder virus. Furthermore, the growth competition assays utilize low numbers of representative viruses, thus making subtype specific comparisons of viral fitness very difficult to interpret. Therefore, the role of replicative fitness in the pathogenicity and spread of HIV subtypes remains controversial.

Clinical studies have also been performed, which measures the association between being infected with a particular subtypes and the rate at which an individual will progress to disease (Kaleebu *et al.*, 2002; Kiwanuka *et al.*, 2008). A number of clinical studies that were done in Uganda found that individuals infected with subtype D, inter-subtype recombinants, and multiple subtypes progressed more rapidly to AIDS and death, compared to individuals infected with subtype A (Kaleebu *et al.*, 2002; Kiwanuka *et al.*, 2008).

University of Cape Town

1.9.3 The association between viral replication capacity and disease progression

Viral fitness describes an virus' ability to replicate in a particular environment and is partly influenced by the efficiency at which the virus is able to enter and infect host cells (Quiñones-Mateu *et al.*, 2000). Recent studies have found an association between viral replication capacity and disease progression, and this association has been found in viruses that are isolated during chronic infection (Lassen *et al.*, 2009; Miura *et al.*, 2009a; Quiñones-Mateu *et al.*, 2000). HIV-1 isolates from individuals that have progressed slowly to disease showed a significantly lower replicative fitness compared to patients with accelerated progression to AIDS (Quiñones-Mateu *et al.*, 2000). Miura and colleagues (2009) also demonstrated that elite controllers are infected with variants with lower replication capacity than those carried by rapid progressors. The escape mutations in Gag, particularly in the p24 capsid region, carry a fitness cost to the virus as these mutations may affect the conformation of the viral capsid (Chopera *et al.*, 2012; Martinez-picado *et al.*, 2006; Schneidewind *et al.*, 2008; Wright *et al.*, 2011). They have been associated with lower viral load and delayed disease progression in a number of cohorts (Brockman *et al.*, 2010; Chopera *et al.*, 2008; Crawford *et al.*, 2009; Prince *et al.*, 2012; Wright *et al.*, 2011). HIV controllers also harbour viruses that have low Envelope entry efficiency, suggesting that there are also determinants in Envelope that influence viral fitness and disease outcome (Lassen *et al.*, 2009).

University of Cape Town

1.9. 4 The effect of coreceptor switching on viral replication capacity

Individuals that are infected with certain subtypes such as subtype C (Morris *et al.*, 2001; Peeters *et al.*, 1999) rarely harbor viruses that use CXCR4. In subype B infections, the process of coreceptor switching occurs in 40 to 50% of individuals infected (Karlsson *et al.*, 1994; Koot *et al.*, 1993). A transition from a CCR5 utilizing (R5) phenotype to a CXCR4 (X4) phenotype is associated with increased replication capacity and progression to AIDS (Troyer *et al.*, 2005).

University of Cape Town

1.9. 5 Evolution of viral replication capacity over time

There have been conflicting findings regarding the evolution of HIV-1 replicative fitness over time. Arie *et al.*, (2005) found that historical HIV isolates (isolated between 1986 and 1989) had a higher replicative fitness than recent HIV isolates (isolated between 2002 and 2003), suggesting that HIV virulence has attenuated over time. However, a similar study in Amsterdam found an increase in HIV replicative fitness over time when early isolates (from 1986) were competed head–to–head with late viral isolates (from 1996-2003) (Gali *et al.*, 2007). A recent study by Gras *et al.*, (2010) compared differences in viral loads in ARV-naïve subjects that seroconverted at various time points, which include 1984 to 1995, 1996 to 2002, and 2003 to 2007. This study found that subjects that seroconverted between 1984 to 1995, and 1996 to 2005, had significantly lower viral loads and CD4 count at viral set point than those that seroconverted between 2003 and 2007. These findings may suggest an increase in viral replication capacity over time in the Netherlands. An increase in viral load and decrease in CD4 count was observed in a cohort in France (Potard *et al.*, 2009). However, a study that was done

in North America did not find any change in virulence (Herbeck *et al.*, 2008). It is very difficult to interpret or make comparison between these studies because of differences in laboratory techniques that were used, which may affect the assay readout. Therefore, the evolution of HIV replication capacity over time still remains unclear.

1.10 Study rationale

An understanding of early events that are involved during transmission, and the phenotype of the transmitted variant, is important for vaccine development which needs to block the incoming transmitted viruses. Recent studies have shown that there is evidence of a viral population bottleneck that occurs during transmission, resulting in the transmission of a single variant in approximately 80% of cases (Abrahams *et al.*, 2009; Haaland *et al.*, 2009; Keele *et al.*, 2008). These studies were done in subtype B and C cohorts and also in regions where only a single subtype is circulating. Due to previous findings that HIV subtypes can be a determinant of the rate of disease progression (Baeten *et al.*, 2007; Kaleebu *et al.*, 2002; Kiwanuka *et al.*, 2008; Kuritzkes, 2008), there is a need to understand the transmission characteristics in a region that has multiple subtypes that are co-circulating. Therefore, the aim of this study was to characterize the genotypic and phenotypic properties of transmitted viruses that are circulating in Mbeya Tanzania, which is region that has a number of HIV subtypes and recombinants.

A number of studies have shown that the *env* gene plays a major role in the competitive and adaptive ability of the virus (Ball *et al.*, 2003; Rangel *et al.*, 2003; Travers *et al.*, 2005) and that host cell entry efficiency largely determines the overall viral fitness of the virus (Lassen *et al.*,

University of Cape Town

2009; Marozsan *et al.*, 2005) and subsequent disease progression (Quiñones-Mateu *et al.*, 2000). Therefore, the study aims to determine if there are properties in the HIV-1 Envelope responsible for clinical infection that may impact subsequent disease progression.

This study forms part of the Mbeya Medical Research Programme, and was conducted using twenty-two acute infection plasma samples from a high risk population in the Mbeya region of Tanzania. These participants were followed up every three months for over 3 years as part of HIV Superinfection Study (HISIS) cohort (Hoelscher *et al.*, 1998; Riedner *et al.*, 2006).

The specific objectives of the project were as follows:

1. To characterize the virus population bottleneck in recently infected individuals from Mbeya, Tanzania.
2. To compare and characterize differences in Envelope entry efficiency of transmitted viruses circulating in the region.
3. To determine the association between host cell entry efficiency and disease progression.
4. To investigate the relationship between entry efficiency and replicative capacity of transmitted *env* chimeric viruses.

University of Cape Town

Chapter 2: Characterization of the HIV-1 transmission bottleneck in recently infected individuals from Mbeya, Tanzania.

University of Cape Town

ABSTRACT

The Mbeya region of Tanzania has a genetically complex HIV epidemic with multiple subtypes and recombinant forms circulating, together with a high frequency of dual infections with more than one subtype. This study aimed to determine whether this impacted the HIV-1 transmission bottleneck. A total of 122 *env* sequences from 14 participants were generated from recently infected women from Mbeya using the single genome amplification approach. These were combined with 88 sequences that were available from the laboratory yielding a total of 210 from 22 participants. The subtyping and multiplicity of infection was determined using phylogeny, together with the mathematical model of random evolution. Participants were infected with subtypes C (n = 9), A (n = 4), or D (n = 1); and recombinants AC (n = 4), CD (n = 2), AD (n = 1), or ACD (n = 1). In sixteen participants (73 %) clinical infection was the result of a single variant; whereas in five (23 %) participants there was evidence of multiple variant infection with more than one closely related quasispecies; and one (4 %) participant was found to be dually infected. Thus the frequency of single variant infections was similar to cohorts located in genetically restricted subtype B or C epidemics, suggesting that multiple circulating subtypes and unique recombinant forms do not have a significant impact on the transmission bottleneck.

University of Cape Town

2.1 INTRODUCTION

The extensive HIV-1 genetic diversity is one of the major obstacles in the development of an effective vaccine. In order for a vaccine to be effective, it will need to prevent the spread of the virus from the mucosal surfaces to the neighboring lymph nodes, and ultimately systemic spread of infection. A number of studies have shown that there is evidence of viral population bottleneck that occurs during transmission, and a single variant gets transmitted to establish clinical infection in approximately 80 % of cases (Abrahams *et al.*, 2009; Haaland *et al.*, 2009; Keele *et al.*, 2008). This finding presents a small 'window of opportunity' for vaccine development as the vaccine will need to be effective against viruses with low genetic diversity during acute infection.

Recently, Keele and colleagues (2008) developed a mathematical model of HIV sequence evolution during acute infection that allows for the identification of the transmitted/founder sequences during acute infection. According to the model, the transmitted/founder sequences show a Poisson distribution and a star-like phylogeny which coalesce into a transmitted/founder genome at or near the time transmission occurs. This model accounts for a number of factors such as the rate of virus growth, reproductive ratio, virus generation time, and the reverse transcriptase error rate (Lee *et al.*, 2009). This model assumes early collection of sample and therefore limited sequence changes due to selection. Also, the consensus that is derived from the sequences that are samples before the onset of an immune response is the transmitted/founder virus that is responsible for establishing clinical infection. However, in cases where multiple viruses are transmitted, the viral sequences do not follow a Poisson distribution, and this implies that there is more than one virus responsible for establishing clinical infection (Keele *et al.*,

University of Cape Town

2008). These multiple transmission events vary between individuals and occur independently to each other, which may suggest that other factors such as STIs and hormonal contraceptives might play a role (Haaland *et al.*, 2009).

This approach allowed for the most recent common ancestor to be predicted, and was subsequently supported by both non-human primate and human studies. A study by Keele *et al.*, (2009) found that the viral *env* sequence in a newly infected Indian rhesus macaque was identical or differed by a few nucleotides to the virus in the inoculums. Another study by Haaland *et al.*, (2009) done on human transmission pairs also found the HIV *env* and full-length genomic sequences to be either identical of differ by a few amino acids in V1-V3 region in both donors and recipients.

The population genetic bottleneck has only been shown in regions where only a single subtype is prevalent. A number of studies have shown that HIV subtype can be a determinant of transmission efficiency and rate of disease progression (Baeten *et al.*, 2007; Kaleebu *et al.*, 2002; Kiwanuka *et al.*, 2008; Kuritzkes, 2008). Also, the genetic diversity at transmission has been previously associated the rate of disease progression (Sagar *et al.*, 2003). Therefore it is important to understand the impact of the population genetic bottleneck in a region where there are multiple subtypes that are circulating. Therefore, this study aimed to characterize the genetic bottleneck of transmitted variants infecting a Tanzanian cohort of high risk women. The region of Mbeya has multiple subtypes, which include subtype A, C, and D, a high number of unique recombinant viruses, as well as a high frequency (18%) of dual infection providing an opportunity to investigate if a genetically diverse HIV-1 epidemic impacts on the transmission bottleneck (Herbinger *et al.*, 2006; Hoelscher *et al.*, 2001).

University of Cape Town

2.2. MATERIALS AND METHODS

2.2. 1 Cohort description

Plasma was obtained from 22 participants from a high-risk population of female bar workers in Mbeya, Tanzania. Recently infected individuals were identified from an HIV-1 uninfected cohort who were screened every three months for the presence of HIV antibodies and/or RNA as part of a prospective HIV superinfection study (HISIS) (Ethics number: 168/2007) (Geldmacher *et al.*, 2007; Herbinger *et al.*, 2006; Hoelscher *et al.*, 2001; Riedner *et al.*, 2006) using the EnzygnostAntiHIV ½ Plus (Dade Behring, Liedrebach, Germany) and Determine HIV ½ (Abbott, Wiesbaden, Germany). Plasma RNA levels were measured using the Amplicor HIV Monitor Assay (Roche Diagnostics, Indianapolis, IN). The duration of HIV-1 infection was categorized into five stages based on evolving HIV-1 RNA or antibody profiles (Fiebig *et al.*, 2003). Individuals classified as being in Stage I/II were viral RNA positive, ELISA antibody negative; those in stage III were ELISA antibody positive but negative by Western blot; those in stage IV had an indeterminate Western blot; those in stage V were Western blot positive but without reactivity to the p31 integrase band; and those in stage VI were Western blot positive with a p31 band present. Western blots were performed using the GS HIV-1 Western Blot kit (Biorad, WA, USA) by Mrs R. Thebus at the University of Cape Town. The date of infection was estimated based on p24 antibody reactivity, western blot analysis of the seropositive samples and the time since the last seronegative using the algorithm in the study by Fiebig *et al.*, (2003) and Keele *et al.*, (2008). Therefore, Fiebig stages I/II was dated as 14 days post infection, Fiebig stage III/IV was dated as 30 days post infection, and Fiebig stage V or VI was dated as the

University of Cape Town

midpoint between the last seronegative and the first seropositive visit. This analysis was done by Mr. Gama Bandawe at the University of Cape Town.

2.2.2 Amplification and sequencing

2.2.2.1 RNA extraction and cDNA synthesis

RNA was extracted from the plasma samples using the MagNA Pure Compact RNA isolation kit (Roche, Germany). Complementary DNA was synthesized using the Superscript III™ RT-PCR System Kit (Invitrogen, GmbH, Karlsruhe, Germany) using the OFM19 primer (5' GCA CTC AAG GCA AGC TTT ATT GAG GCT TA 3') as described (Salazar-Gonzalez *et al.*, 2008).

2.2.2.2 PCR amplification using Single Genome Amplification (SGA) method

The resulting cDNA was amplified using Single Genome Amplification (SGA) approach as described (Abrahams *et al.*, 2009; Keele *et al.*, 2008). The full-length *env* sequences (2590 bp) were amplified from 22 participants with acute/early infection patient samples from Tanzania by using the Single Genome Amplification (SGA) approach (Salazar-Gonzalez *et al.*, 2008). This method involves diluting the cDNA in a two step nested PCR reaction so that initial amplification occurs from a single viral RNA template. According to the Poisson, if the percent of positive amplification product is 30% or less, it is likely to have been amplified from a single RNA template. The entire positive *env* amplification product is sequenced to determine whether there is a homogenous population in the reaction mix by detecting the presence of double peaks in the chromatograms. The absence of double peaks is indicative of sequence homogeneity. Amplification from a single template reduces the likelihood of artificial recombination occurring

University of Cape Town

in a test tube by bulk PCR. In addition, as each amplicon represents one amplifiable copy, it also ensures that sequences generated are representative of the variants present in the sample.

The amplification of the HIV-1 *env* by SGA was developed for HIV subtype C viral templates (Abrahams *et al.*, 2009). As Mbeya has multiple subtypes including subtypes A, C, and D, as well as recombinant forms, the initial phase of the study was to test whether the SGA primers designed for subtype C could amplify and sequence other subtypes. The primer sequences were submitted to PrimAlign (www.HIV.lanl.gov), to identify the homology of the primer sequences to subtype reference sequences in the database. The primer sequence was found to be complimentary subtype A, C, D, along with recombinant forms in the database with few nucleotide polymorphisms. These primers were found to reliably amplify the *env* region from all samples, which include subtype A, and a number of AC, CD, and AD recombinant viruses. Thus far, the patient samples had been successfully amplified using single genome amplification (SGA).

The cDNA was serially diluted and used for the first round PRC amplification. The first round PCR reaction mix for a 1X reaction include 10X Hi-Fi Buffer (Invitrogen, CA, US), $MgSO_4$ (Invitrogen, CA, US), DNTPs (10 mM each), 20 µM of both forward primer OFM19 (5' GCA CTCAAGGCAAGCTTTATTGAGGCTTA 3') and reverse primer VIF1 (5' GGG TTT ATT ACA GGG ACA GCA GAG 3'), Taq High Fidelity Platinum polymers (Invitrogen, CA, US), and distilled water was added up to a final volume of 20 µl. The samples were placed in a thermal cycle and the reaction was run under the following conditions: 94ºC for 2 minutes, 35 cycles of 94 ºC for 15 seconds, 55 ºC for 30 seconds, and 1 cycle of 68 ºC for 20 minutes.

University of Cape Town

The first round amplicon (1µl) was used for the second round (nested) PCR reaction. The PCR reaction mix for a 1X reaction included 10X Hi-Fi Buffer (Invitrogen, CA, US), $MgSO_4$ (Invitrogen, CA, US), dNTPs (10 mM each), 20 µM of both forward primer EnvA (5' (CACC)GGC TTA GGC ATC TCC TAT GGC AGG AAG AA 3') and reverse primer EnvN (5' CTG CCA ATC AGG GAA GTA GCC TTG TGT 3'), Taq High Fidelity Platinum polymerase (Invitrogen, CA, US), and distilled water was added up to a final volume of 20 µl. The samples were placed in a thermal cycle and the reaction was run under the following conditions: 94 ºC for 2 minutes, 45 cycles of 55 ºC for 30 seconds, 68 ºC for 30 seconds, and 1 cycle of 68 ºC for 20 minutes. The PCR product (3 µl) was loaded onto a 0.8 % agarose gel and migrated at 100V for 1 hour.

2.2.3 Intra-patient quasispecies diversity

Generation of phylogenetic trees and computation of pairwise DNA distances were done using MEGA version 4 (Tamura *et al.*, 2007) and differences were visualized using transition and transversion plots in the HIGHLIGHTER plots (www.hiv.lanl.gov). Sequences were analyzed for evidence of APOBEC3G-induced hypermutation using Hypermut 2.0 tool (www.hiv.lanl.gov).

2.2.4 Sequence analysis

Sequences of the full-length envelope gene were assembled and edited using the Sequence Assembly Pipeline (http://tools.caprisa.org). The sequences were aligned by the ClustalW application in the BioEdit Sequence Alignment Editor. Twenty-eight reference sequences representing all major subtypes and recombinants (www.hiv.lanl.gov) were included in the

University of Cape Town

phylogenetic trees. Phylogenetic trees constructed using the Neighbor-Joining method using MEGA 4 (Tamura *et al.*, 2007). The tree was constructed with 500 bootstrap replicates, with 1st, 2nd, 3rd codon positions as well as non-coding sites included, and the maximum composite likelihood nucleotide model was used. The REGA HIV Subtyping Tool Version 2.0 (http://www.bioafrica.net) was used for subtyping. Maxchi, Chimaera, and Bootscan analysis using RDP v3.34 (http://darwin.uvigo.es/rdp/rdp.html) (Martin *et al.*, 2005) was used to detect inter- and intrasubtype recombination events, and a bootstrap value equal to or greater than 70% was considered as significant. Recombination analysis was performed with a window size of 400 and step size 20.

2.2.5 Estimation of time to most recent common ancestor (tMRCA)

We used a Bayesian Markov Chain Monte Carlo method implemented in Bayesian Evolutionary Analysis Sampling Trees (BEAST) v1.4.7 (Drummond & Rambaut, 2007) which uses SGA-derived sequences to estimate the time to most recent common ancestor (tMRCA). This is based on the assumption that in the absence of selective pressure, the transmitted/founder virus replicates exponentially and has a generation time of 2 days (Markowitz *et al.*, 2003), has a reproductive ratio of 6 (Stafford *et al.*, 2000), and an error rate of 2.16 X 10^{-5} (Mansky, 1995), and also undergoes constant mutations in all positions and lineages without any back mutations.

2.2.6 Immune escape analysis

The identification of potential epitopes for analysis of cytotoxic T-lymphocyte (CTL) escape was done using Net MHCpan 2.0 server (www.cbs.dtu.dk) and Epitope Location Finder (www.hiv.lanl.gov).

University of Cape Town

2.3 RESULTS

2.3.1 Cohort description

Twenty-two participants were recruited within three months of their previous HIV negative sample (Herbinger *et al.*, 2006; Riedner *et al.*, 2006). This work was at the Mbeya Medical Research Clinic as part of the HIV superinfection study (HISIS) work in Mbeya, Tanzania (Geldmacher *et al.*, 2007; Herbinger *et al.*, 2006; Hoelscher *et al.*, 2001; Riedner *et al.*, 2006) (**Table 2.1**). Laboratory staging based on evolving viral RNA and antibody profiles was performed on 20 of the plasma samples (Fiebig *et al.*, 2003) by Mrs R Thebus at the University of Cape Town. Of these, 3 were in the acute/early stage of primary infection (Stage I-IV) and 17 individuals were in the later stage of primary infection (Stage V-VI). As expected the viral loads were higher for individuals with acute/early infection (median >750 001 copies/ml), compared to the 17 participants in later stages of primary infection (Stage V-VI) (median = 66 000 copies/ml) (**Table 2.1**). The estimated number of days post-infection ranges from 14-45 (median = 41.5 days).

University of Cape Town

Table 2.1 Data summary of 22 participant samples from a high risk population of female bar workers from Mbeya, Tanzania. The study participants are grouped based on whether they are infected with either single or multiple viruses.

PID	Lab stage**	No of days post infection	Viral Load (copies/ml)	Subtype	Seq (n)	Max DNA Distance (%)	Median GT (days)	Lower 95% CI	Upper 95% CI
89-F1	I/II	14	750 001	CD	10	0	0	0	**0**
216-F2	VI	14	1 840	A	7	0	0	0	**0**
49-F1	III	30	750 001	ACD	11	0.08	8.008	0.61	**22.476**
234-F1	V/VI	45	509 000	C	12	0.12	13.524	2.902	**32.046**
390-F1	V	45	750 001	C	11	0.23	23.89	9.902	**56.9**
401-F1	ND	45	51 500	CD	6	0.24	24.61	8.874	**73.046**
304-F2	V	45	750 001	C	11	0.23	31.625	17.486	**58.102**
54-F4	VI	41	28 200	D	9	0.27	45.006	27.83	**79.512**
605-F4	V/VI	41	434 000	AC	9	0.42	48.466	28.406	**83.512**
515-F4	VI	41	3 660	A	5	0.27	48.456	16.726	**126.648**
569-F1	V/VI	45	23 800	C	11	0.2	57.524	24.534	**114.418**
246-F3	VI	42	439 000	AC	18	0.27	62.442	36.166	**90.62**
***346-F4**	VI	41	26 700	C	8	0.23	78.238	28.164	**155.58**
541-F1	VI	ND	66 100	C	11	0.29	100.124	55.526	**162.136**
***398-F1**	ND	45	2 370	A	8	0.39	185.108	88.356	**354.172**
556-F3	V	44	43 300	C	11	1.45	365.69	188.84	**792.366**
142-F3	VI	44	366 000	C	9	1.56	469.844	289.914	**720.324**
98-F4	V	41	5 330	C	6	2.37	743.098	884.858	**1615.466**
21-F1	V/VI	42	21 200	C	11	0.97	1505.424	715.594	**2957.18**
532-F0	I/II	14	2 370	AD	10	1.18	1952.874	762.08	**4313.646**
410-F2	VI	ND	165 000	C/AC	11	4.61	2104.35	933.334	**4767.876**

* denotes participants that have diversified from single lineage based on shared mutations that are attributed to CTL and/or antibody pressure

** as according to Fiebig et al., 1993

MRCA – Most Recent Common Ancestor; GT – Generation Time; CI – Confidence Intervals; ND – not done

Participants that are infected with multiple variants are highlighted in grey.

University of Cape Town

2.3.2 HIV subytping and phylogenetic analysis

To investigate the extent of genetic diversity in a cohort of high risk women from Mbeya, the single genome amplification followed by sequencing was used. A total of 122 *env* sequences from 14 participants were generated from recently infected women from Mbeya using the single genome amplification approach. These were combined with 88 sequences that were generated from 8 participants (provided by G. Bandawe University of Cape Town) to make up a total of 210 from 22 participants. The *env* sequences from 20 of the 21 participants formed monophyletic clusters that showed that these participants were singly infected, while one participant (**410-F2**) had sequences that formed two distinct phylogenetic lineages, which suggests that the participant was dually infected with two distinct subtypes or RFs (**Fig. 2.1).**

The majority (14 out of 22 participants) of *env* sequences were identified as pure subtype with nine participants infected with subtype C, four with subtype A, and one with subtype D. Eight participants were found to be infected with intersubtype recombinant viruses , of which four infected with AC , two with CD, one with AD, and one with an ACD recombinant.

University of Cape Town

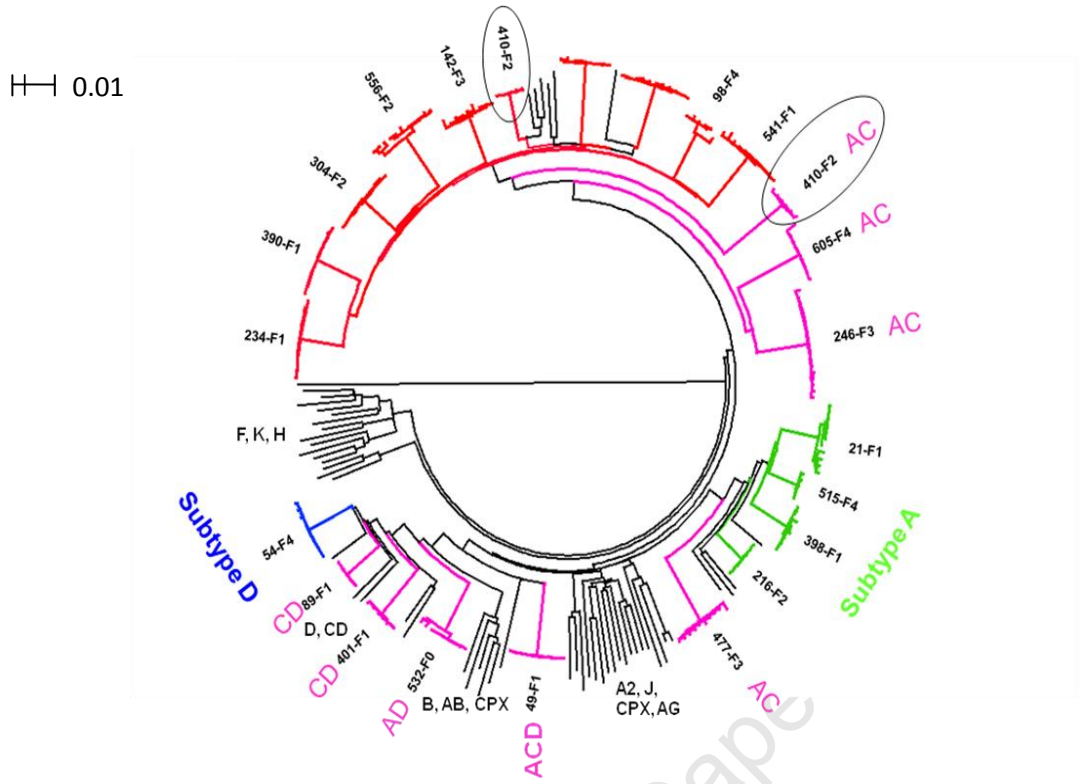


FIGURE 2.1 Neighbor-joining tree indicating the subtype distribution from 212 *env* sequences from 22 participants. The tree was constructed using the Dendroscope progam (http://ab.inf.uni-tuebingen.de/software/dendroscope/). The colours representing the various subtype in the participants' sequences, which is subtype C (red), D (blue), and AC, AD, CD, and ACD intersubtype recombinants (pink). The dually infected participant 410 who was infected with subtypes C and AC is circled. The tree was rooted using sequences from subtypes F, K, H, which represent the outgroups. The black branches represent the reference sequences.

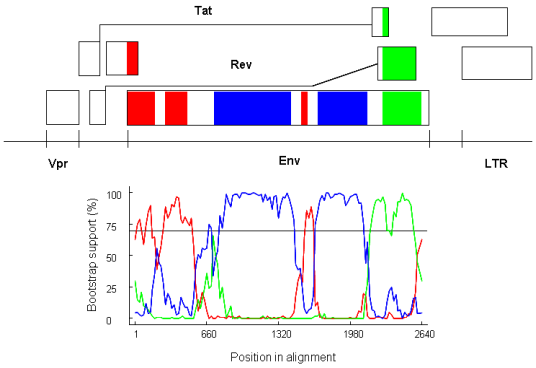
University of Cape

2.3.3 Recombination

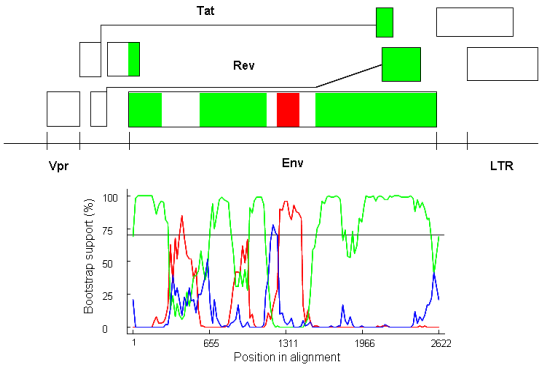
The recombination analysis identified eight participants were infected with intersubtype recombinant viruses. Two participants (**89-F1** and **401-F1**) were infected with CD recombinants, four participants (**246-F3**, **410-F2**, **477-F3**, and **605-F4**) were infected with AC recombinants, one participant (**54-F4**) with AD recombinant, and one participant (**49-F1**) was infected with an ACD recombinant **(Fig. 4)**. One of the participants**, 410-F2**, had a region in gp41 that was identified as subtype G by the REGA HIV Subtyping Tool, however, further analysis using BLAST (http://www.ncbi.nlm.nih.gov/blast) and RDP v3.34 identified the region as being subtype A. Therefore, we classified the virus as an AC recombinant (**Fig. 2.5**). All RF sequences from each participant were unique and did not share mosaic structures with viruses from other participants (**Fig. 2.2**) or any other recombinant forms from the East African region (data not shown). In addition, with the exception of participant 410-F2, all the viruses obtained from a given participant shared the same recombination mosaic structure with no variation in recombination breakpoints suggesting that these individuals were infected with the recombinant and that the recombinant virus was not a result of dual infection.

University of Cape Town

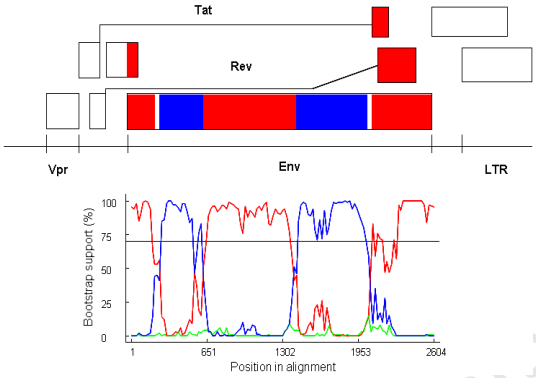
Participant 49-F1 (ACD recombinant)



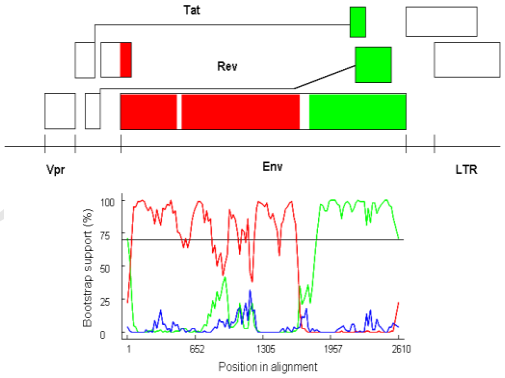
Participant 89-F2 (CD recombinant)



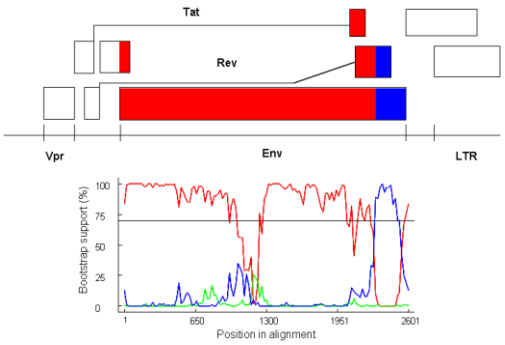
Participant 246-F4 (AC recombinant)



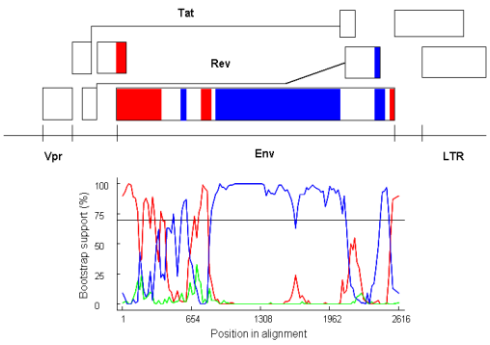
Participant 401-F1 (CD recombinant)



Participant 410-F2 (AC recombinant)

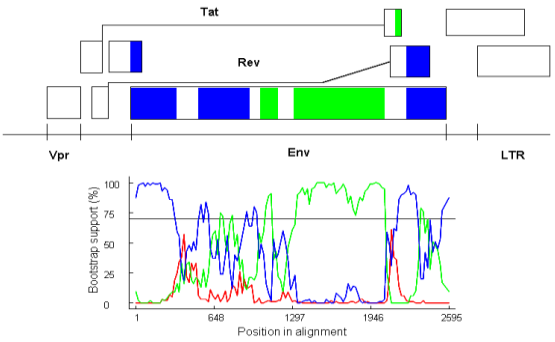


Participant 477-F3 (AC recombinant)



University of Cape Town

Participant 532-F0 (AD recombinant)



Participant 605-F4 (AC recombinant)

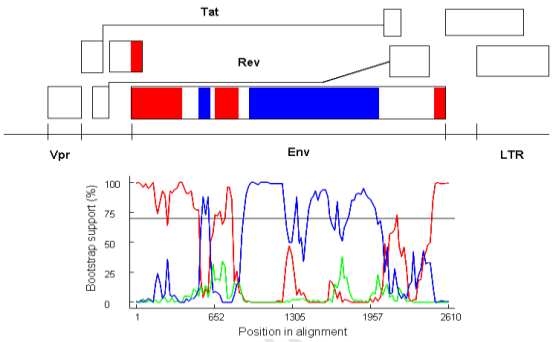


Figure 2.2 Recombination analysis of *env* sequences from eight participants. Participant **49-F1** was infected with an ACD recombinant, Participant **532-F0** was infected with an AD recombinant, Participants **89-F2** and **401-F1** were infected with CD recombinant viruses, and Participants **605-F4**, **477-F3**, **246-F4**, and **410-F2** were infected AC recombinant viruses. The recombination analysis was done using Bootscan within RDP 3.34. (Martin et al., 2005) (below) and REGA HIV-1 Subtyping Tool (above). The recombinants are shown to be derived from subtypes A (blue), C (red), and D (green).

University of Cape Town

2.3.4 Single and multiple variant transmissions

The study used a number of methods to define single and multiple variant transmission events amongst the participants. We defined single variant infection as sequences with low quasispecies diversity with a star-like phylogeny which would be expected for sequences from individuals infected with a single variant which undergoes neutral evolution prior to the onset of immune pressure (Keele *et al.*, 2008). In addition, for single variant infection the time of divergence from the most recent common ancestor (MRCA) should fall within 90 days since the last HIV negative blood sample. The MRCA analysis was done by Natasha Wood and Gama Bandawe at the University of Cape Town.

Based on this criterion, thirteen participants were found to be infected with a single virus. The viral populations in these participants had maximum pair-wise DNA distances ranging from 0 % to 0.42 % with concomitant lack of structure in the neighbor-joining phylogenetic trees.

A further three participants (**346-F4**, **398-F1** and **477-F3**) were regarded as being infected with a single variant as even though they showed some structure in the phylogenetic tree. These individuals were classified as infected with a single variant as these sequence changes could be accounted for by cytotoxic T-lymphocyte and/or antibody pressure. Participant 398-F1 who showed both CTL escape in a predicted epitope restricted by the participants HLA (A*6802), as well as antibody selection. Sequences from participant 346-F4 had an estimated tMRCA of 78 days, while participant 398-F1 had an estimated tMRCA of 185 days. The high estimated tMRCA is likely due to CTL escape driven diversification (**Fig. 2.6**). For participant 477-F3, we were only able to amplify sequences from the third follow-up visit, although there was evidence of low positive viral loads in the two visits in the previous 6 months (7 500 and 1 150 copies/ml

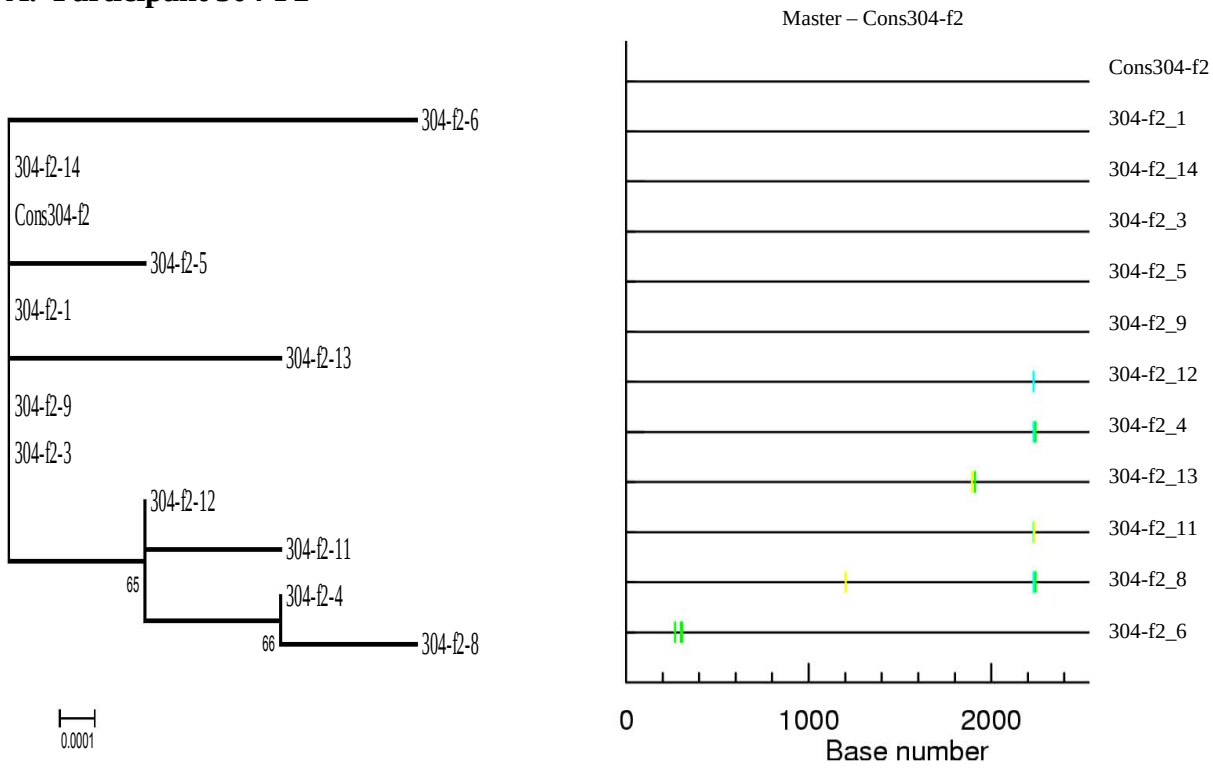
University of Cape Town

respectively). Sequences from this participant had a maximum DNA distance of 0.68 % and in line with estimated time of infection, the mean tMRCA of 324 days was estimated (**Table 2.1**). While it was not possible to resolve the infecting virus from this individual, she was classified as infected with a single variant as many changes of the sequences shared the same mutations which could be accounted for by both CTL and antibody selection (**Fig. 2.6**). Therefore, 16 out of 22 participants (73%) were classified as infected with viruses with a single variant (**Table 2.1**).

Participants were identified to be infected with multiple variants if there was high diversity together with monophyletic cluster with distinct structure in Neighbor joining trees (**Fig. 2.3**). Whereas, participants were classified as dual infected if infected with two distinct virus strains which could be separated on phylogenetic trees with epidemiologically unlinked viruses. Our expectation was that for multiple variants or dual infection transmission, the sequences in the recipient should coalesce at a time pre-dating the last seronegative visit (approximately 90 days prior to first seropositive visit) based on the estimated tMRCA. Five participants (23 %) were found to be infected with multiple variants and one with dual infection (4 %). In these participants, the maximum pair-wise DNA distances ranged from 0.97 % to 2.37 %. As a result of their extensive diversity, three participants (**21-F1**, **556-F3**, **and 142-F3**) showed evidence of intrapatient recombination (**Fig. 2.4**). In all five participants, the estimated number of days since the MRCA significantly exceeded the period for which the associated individual could realistically have been infected (MRCA range 365 to 1952 days) (**Table 2.1**).

University of Cape Town

A. Participant 304-F2



B. Participant 556-F2

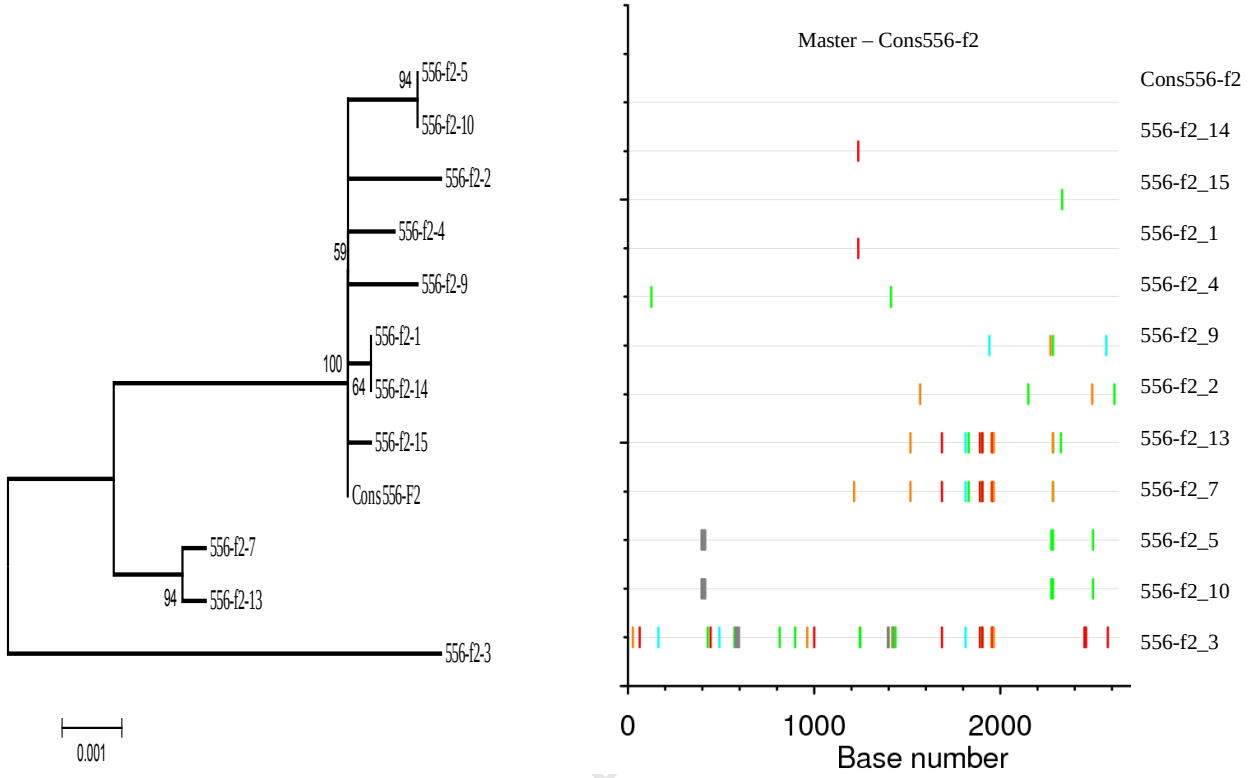
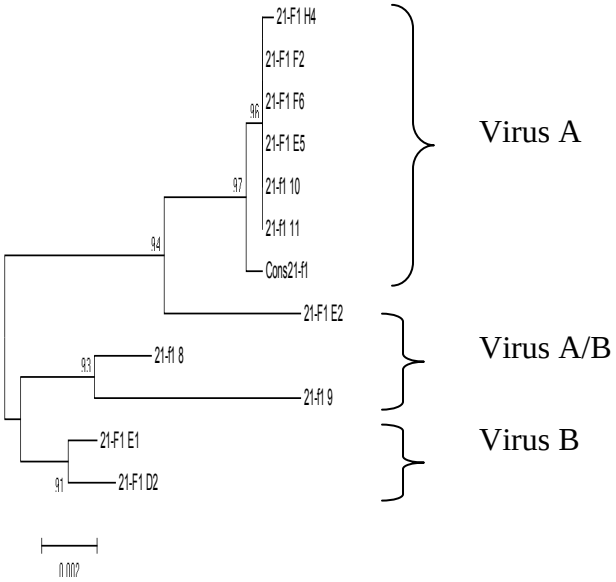


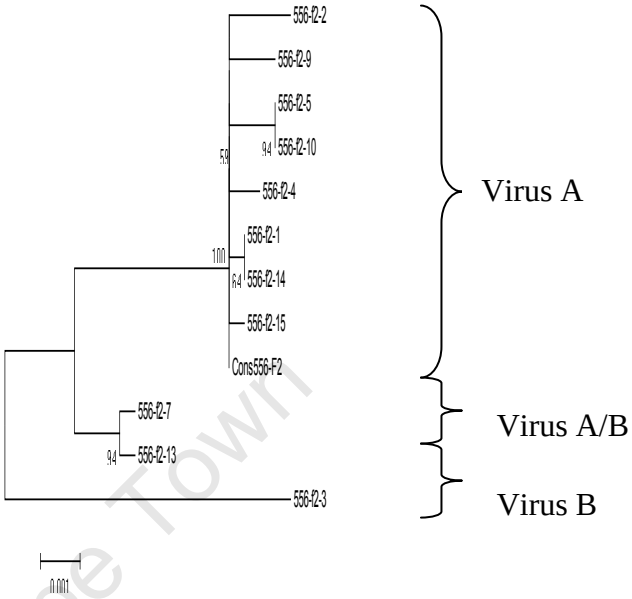
Figure 2.3 Neighbor-joining and Highlighter analyses that show the type of transmission event observed amongst the participants. Participant **304-F2** illustrates evidence of single variant transmission, and participant **556-F3** shows evidence of multiple variant transmission, and also the resulting recombination between viral lineages. The ticks in the HIGHLIGHTER plot represent the following: A: green, T: red, G: yellow, C: light blue, Gaps: gray.

University of

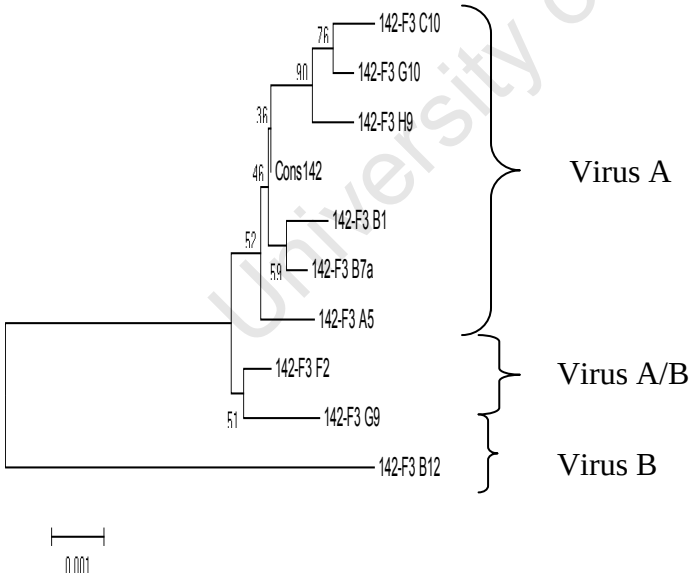
A. Participant 21-F1



B. Participant 556-F2



C. Participant 142-F3



University of Cape Town

Figure 2.4. Neighbor-joining tree showing evidence of intrasubype recombinant observed in three participants (21-F1, 142-F3, and 556-F2). Virus A and B represented the parental viruses and virus A/B represent the recombinant virus.

2.3.5 Dual infection

The presence of various subtypes in this region has also resulted in dual infections, with a frequency as much as 18% in this region (Herbinger *et al.*, 2006). By means of phylogeny analysis, the study aims to identify any evidence of dual infection amongst the participants. Participants were classified as dual infected if infected with two distinct virus strains which could be separated on phylogenetic trees with epidemiologically unlinked viruses. One of the participants 410-F2 was found to by dually infected (**Fig. 2.1**). Subtyping analysis of the participant sequences showed one subtype C population and the other being an AC recombinant. The gp120 regions of the two viral populations showed homogeneity with a maximum DNA distance 0.16 %, with a single recombination breakpoint in gp41. Phylogenetic analysis based on gp41 sequences showed two separate lineages clustering with high bootstrap support (**Fig. 2.1**). The sequences in the gp41 region differed by as much as 4.6 %. The number of sequences that were generated from this participant allowed for the identification of only one parental sequence, which is the subtype C virus. The other sequence, which is an AC recombinant, appears is likely to be a progeny virus that is derived from the subtype A and C parental viruses. In the absence of the other parent, it remains unclear as to whether this individual was originally infected with both subtype A and C viruses, with the generation of the AC recombinant occurring post infection; or whether co-transmission of the C and AC recombinant virus occurred from a dually infected donor.

University of Cape Town

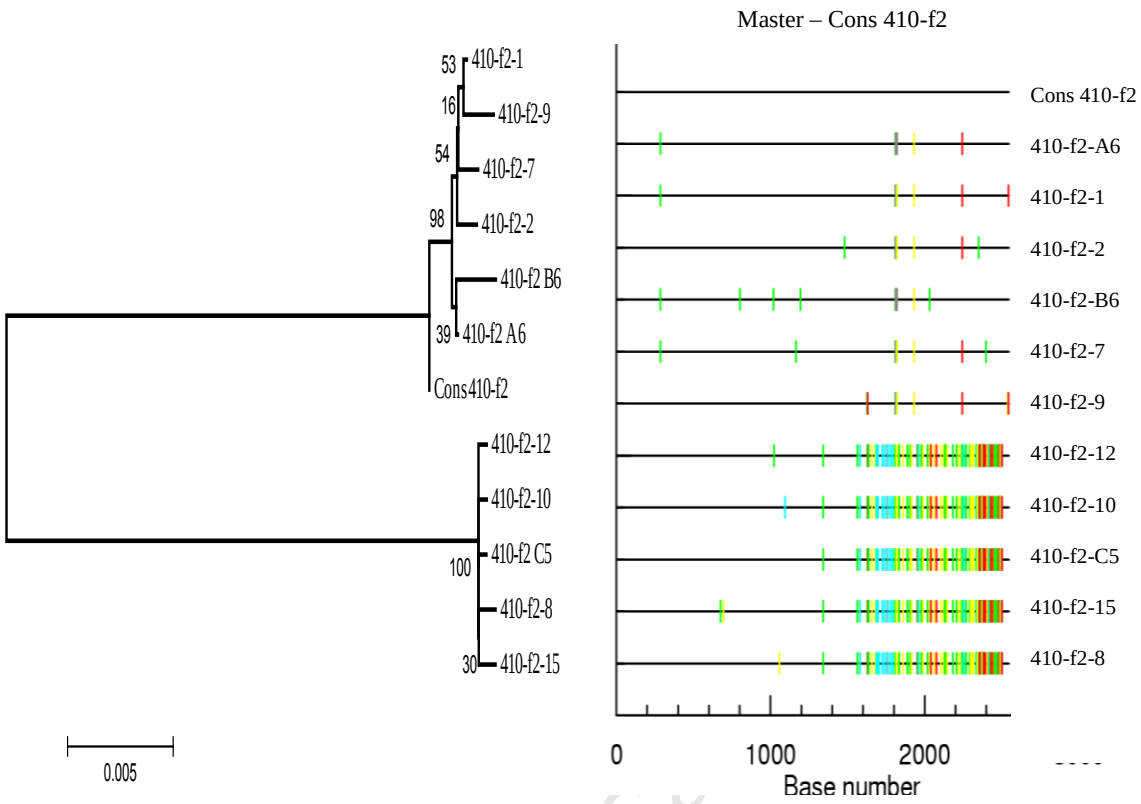
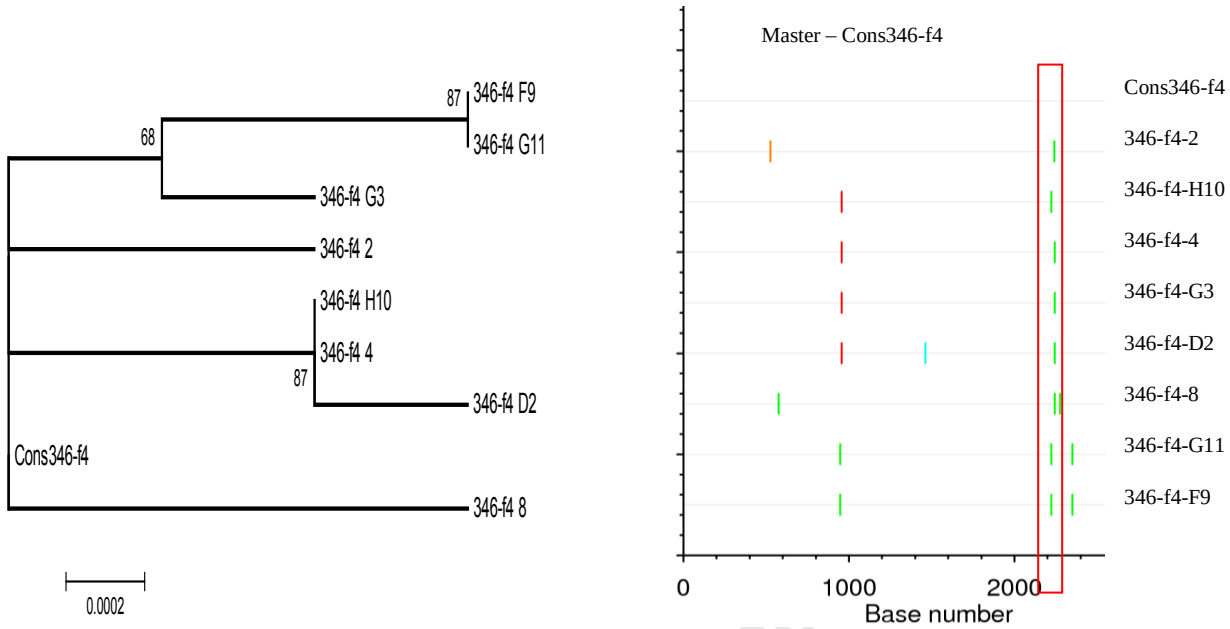


Figure 2.5. **Neighbor-joining and Highlighter analyses from the dually infected participant participant 410-F2**. This participant was infected with subtype C and with an AC recombinant. The ticks in the HIGHLIGHTER plot represent the following: A: green, T: red, G: yellow, C: light blue, Gaps: gray.

University of Ca

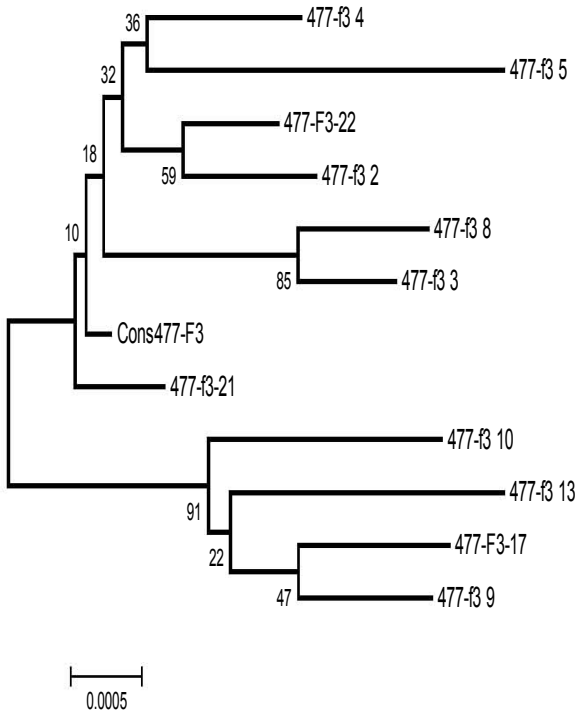
A. Participant 346-F4

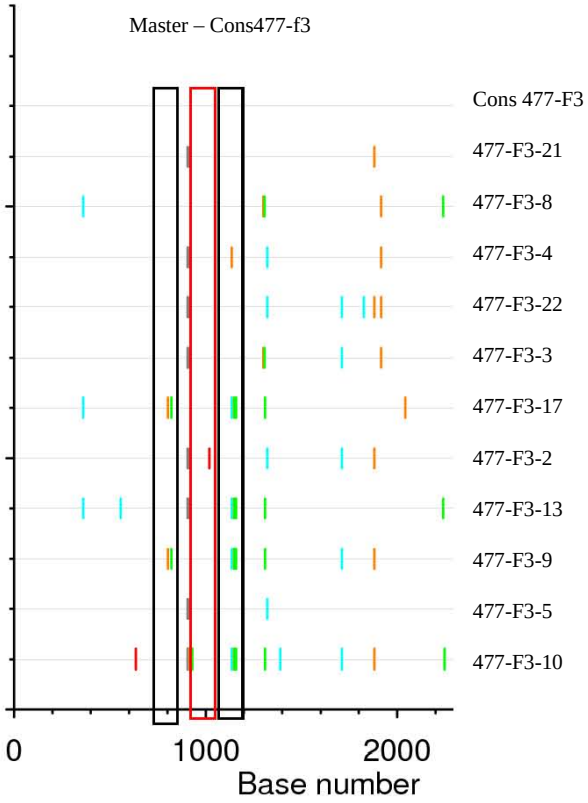


Participant sequences	HLA genotype	CTL selection
	HLA -A*202	RLVNGFLALVWDDL (HXB2 747-760)
346-f4-2		S..............
346-f4-4		....
346-f4-8		H..........N..
346-f4_D2		....
346-f4_F9		....
346-f4_G3		....
346-f4_G11		....
346-f4_H10		....

University of Cape

B. Participant 477-F3

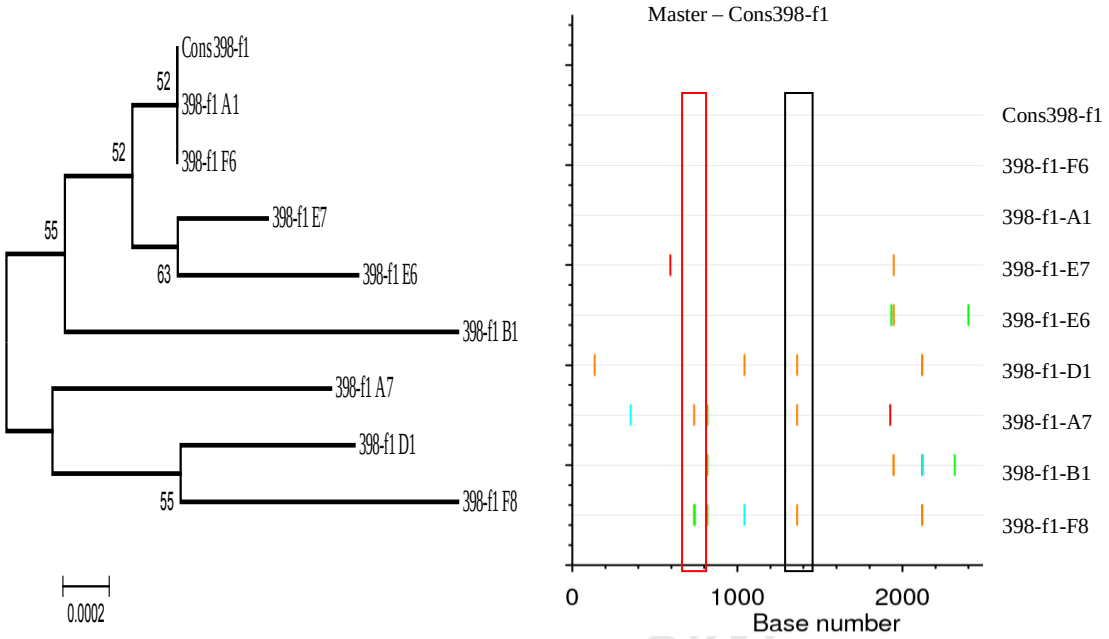




Participant sequences	HLA genotype	CTL selection	Antibody selection
		KVARQLSKY	CNNKTFNGT (HXB2 228-236)
477-f3-21	HLA A*2902	(HXB2 342-350)	.....
477-f3_8		.....	.....
477-f3_4		.....	.....
477-F3-22		.....	.....
477-f3_3		.....	.....
477-F3-17		.....	....A....
477-f3_2		.....	.....
477-f3_13		...GK.R..	.....
477-f3_9		.....	....A....
477-f3_10		...GK.R..	.....
477-f3_5		...GK.R..	.....
		...GK.R..	
		.....	NSMEWLNDT (HXB2 397-405)
477-f3-21			.....
477-f3_8			.....
477-f3_4			..T.....
477-F3-22			..T.....
477-f3_3			.....
477-F3-17			.....
477-f3_2			..T.....
477-f3_13			.....
477-f3_9			.....
477-f3_10			.....
477-f3_5			..T.....

University of Cape Town

C. Participant 398-F1



University of Ca

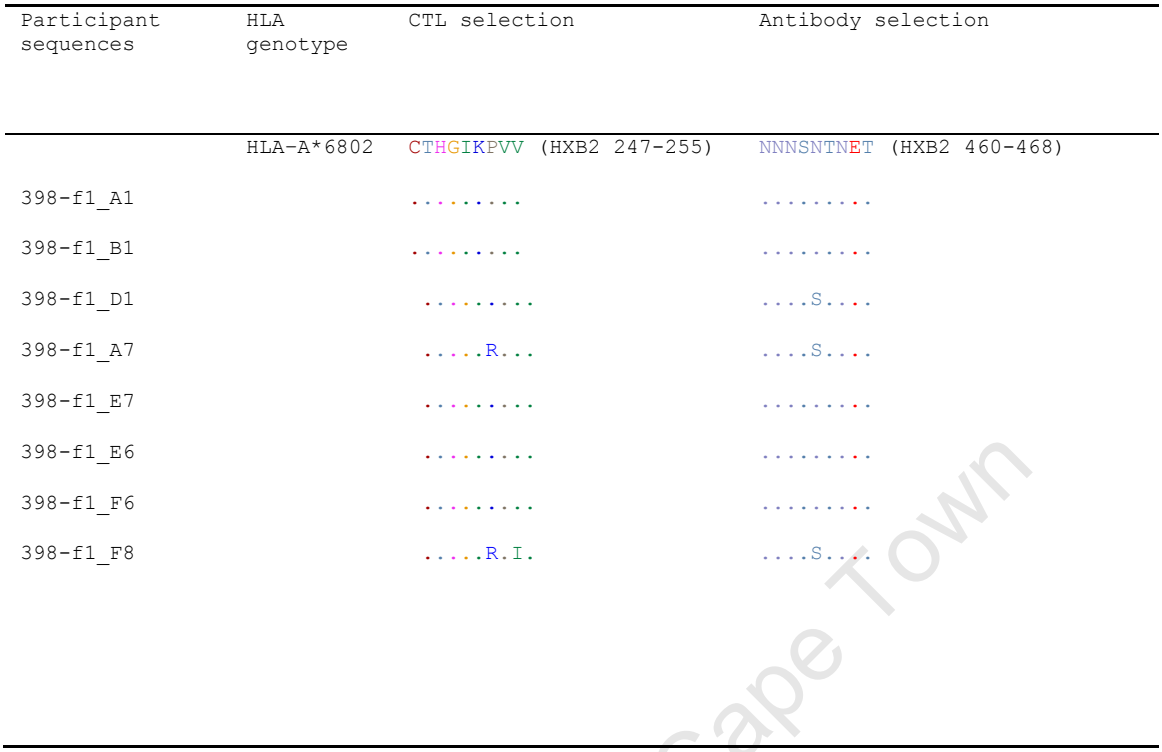


Figure 2.6. Putative CTL pressure (red blocks) in participants 346-F4 (A), 477-F3 (B) and 398-F1 (C) shown by Neighbor Joining phylogenetic trees and HIGHLIGHTER analyses. Epitope Location Finder (www.hiv.lanl.gov) and Net MHCpan 2.0 server (www.cbs.dtu.dk) were used to identify known or predicted HLA class I associations are shown along with the known or predicted HLA restriction for each participant. Putative antibody pressure (mutations in N-linked glycosylation sites) is shown in black blocks. Putative CTL epitopes. colours in the HIGHLIGHTER plot for participants 477 and 398 represent the following: A: green, T: red, G: yellow, C: light blue, Gaps: gray.

University of Cape Town

2.4 DISCUSSION

This study aimed to determine the frequency of the population transmitted/founder bottleneck in the cohort of high risk women in Mbeya region of Tanzania. This cohort has been previously known to be infected with a number of subtypes A, C, D; along with a number of intersubtype recombinants. The population bottleneck has been investigated in other cohorts (Abrahams *et al.*, 2009; Keele *et al.*, 2008), but only in region where there is a single subtypes that is circulating, mainly in subtype C and B cohorts. The study founds in the HISIS cohort a 73 % frequency of single variant transmission event, compared to a frequency of 76 % (Keele *et al.*, 2008) and 78 % (Abrahams *et al.*, 2009) that has been shown in other studies. Therefore, these findings illustrate that subtype differences does not seem to have an impact on the transmission bottleneck. It must be noted that the studies by (Abrahams *et al.*, 2009; Keele *et al.*, 2008) used 20 or more sequences to investigate the transmission bottleneck. Based on the power analysis in a study by (Keele *et al.*, 2008), this would allow for a 95 % probability to detect sequences that are present at a frequency of more than 15 %. Our study only used 10 sequences per participant which only allowed for an overall probability of 80%, and therefore this should be taken into consideration.

We found a high frequency of subtype C infections in the participants, both as the infecting subtype in the majority of participants and also certain regions of it being present in recombinant viruses. Analysis of full-length *env* genomes identified subtypes A, C, and D as the main

University of Cape Town

subtypes that circulate in the region with a high percentage of intersubtype recombinants. This subtyping profile is similar to a previous study which used a multi-region hybridization (MHA) assay to identify the subtypes (Herbinger *et al.*, 2006). There was a relative dominance of infection with subtype C, which was found in 45 % of the individuals. The recombination analysis also found that all the recombinant viruses have regions that are derived from subtype C. Also, similar to the findings by (Hoelscher *et al.*, 2001), the recombinant viruses were shown to be unique and more sampling would be needed to determine if they are spreading in the region.

This study identified only one participant with dual infection out of the 22 participants (4 %) that were screened. This frequency is much lower compared to the frequency of 18% that has been previously found in this cohort (Herbinger *et al.*, 2006), which used the MHA method to screen for dual infections. Despite not being able to detect certain strains due to occasional primer or probe mismatch, the MHA was more sensitive at identifying the dual infections than the SGA approach which we used an average of 10 sequences per participant and also used only one region to detect dual infection, as opposed to the MHA which uses multiple genes in HIV genome. However, the increased prevalence of dual infection in the study by (Herbinger *et al.*, 2006) could be due to the fact that these authors were detecting both co-infection and superinfection, whereas in our study during acute infection we were likely to only detect co-infection.

University of Cape Town

In conclusion, similar approaches to other studies, this study found that the population transmission bottleneck remains constant in heterosexual transmissions irrespective of the geographical region or the complexity of the circulating viruses. These findings have implications on vaccine design as vaccines that aim block transmitted viruses can be effective not only in regions where there is a single subtypes, but also in regions where there are multiple subtypes.

University of Cape Town

Chapter 3: Characterization of Envelope function of transmitted/founder viruses circulating in Mbeya and its impact on disease progression.

University of Cape Town

ABSTRACT

A better understanding of the biological characteristics of transmitted viruses will provide important insights into HIV pathogenesis and aid in vaccine development. The aim of this study was to determine the relationship between viral entry efficiency and disease progression and to characterize the Envelope function of transmitted/founder (T/F) viruses representative of different subtypes and recombinant forms. HIV-1 Envelope pseudoviruses, representative of subtypes C (n = 6), D (n = 1) and recombinants CD (n = 2) and AC (n = 1), that matched the consensus of single genome derived sequences from early infection were generated. These molecular clones, assumed to represent the founder virus responsible for clinical infection, had a >100 fold range in entry efficiencies. An association was found between entry efficiency and viral load at 3 months (p = 0.0022) and 12 months postinfection (p = 0.0347) suggesting that viruses carrying Envelope with enhanced entry function at transmission may influence subsequent disease progression. Using the HEK 293 Affinofile cells to measure entry efficiency, the relative Envelope entry efficiency of the T/F viruses was shown to be dependent on both high CD4 and CCR5 levels (CD4 levels = 146 397 molecules/cell; CCR5 levels = 428 032 molecules/cell) as only 30 % of the viruses were able to mediate entry of cells with low levels of CD4 (CD4 levels = 3 041 molecules/cell) , and absolute entry efficiency levels dropped 4 to 5-fold when CCR5 levels were lowered to 2 566 molecules/cell. The ability of the pseudoviruses to enter cells with low CD4 and CCR5 levels was significantly associated with the IC_{50} values for soluble CD4 (p = 0.0438) and CCR5 antagonist, TAK779 (p = 0.0138), suggesting that receptor affinity contributed to these differences in entry efficiency. Furthermore, some viruses with high entry efficiency (3/5) had better incorporation of gp120 into pseudovirions compared to

University of Cape Town

those with low entry efficiency, suggesting that surface Envelope expression may also influence entry efficiency. This data suggests that viruses with low entry efficiency, and low affinity for CD4 and CCR5 host receptors are still transmitted. However, viruses with high entry may have a replication advantage subsequent to transmission, leading to increased viral loads.

University of Cape Town

3.1 INTRODUCTION

In most cases, HIV transmission is characterized by a genetic bottleneck whereby one or few closely related variants establish productive infection (Abrahams *et al.*, 2009; Haaland *et al.*, 2009; Keele *et al.*, 2008; Nofemela *et al.*, 2011). T/F variants have been shown to share common genotypic and phenotypic characteristics such as R5 tropism, fewer potential N-glycosylation sites (PNGs) and more compact Envelopes (Chohan *et al.*, 2005; Derdeyn *et al.*, 2004; Zhang, 1999). It has also been suggested that availability of target cells in the genital tract could play a role in the preferential transmission of certain variants (Hu *et al.*, 2000; Miller *et al.*, 2005; Ochsenbauer *et al.*, 2012; Salazar-Gonzalez *et al.*, 2009; Zhang, 1999). Together these findings suggest that viral and host factors might act in concert to select specific variants for transmission. The elucidation of the biological characteristics of transmitted variants that impact HIV-1 transmission fitness and subsequent pathogenicity is important for the design of vaccines effective against these incoming viruses.

Previous studies have shown that biological properties such as viral replicative fitness are determining factors in viral control with variants with low replicative fitness associated with long-term non-progression and elite control during chronic (Miura *et al.*, 2009b; Quiñones-Mateu *et al.*, 2000) and acute infection (Miura *et al.*, 2010). We and others have also shown that infection with transmitted variants carrying known attenuating mutations in Gag was associated with lower viral load set point and higher CD4 counts (Chopera *et al.*, 2008; Goepfert *et al.*, 2008). A recent study of mother-to-child transmission demonstrated that viruses transmitted from mothers with chronic infection to their infants had V1-V5 Envelope regions that conferred higher replication fitness (Kong *et al.*, 2008). This corroborates earlier evidence that the viral

University of Cape Town

Envelope plays a major role in the competitive and adaptive ability of the virus (Rangel *et al.*, 2003; Travers *et al.*, 2005) and that host cell entry efficiency is a determinant of overall viral fitness (Marozsan *et al.*, 2005) and thus disease progression (Lassen *et al.*, 2009). While there is limited information on elucidating the phenotypic properties of transmitted viruses in subtype B and C cohorts (Ochsenbauer et al., 2010; Salazar-Gonzalez et al., 2009; Lassen et al., 2009; Ping et al., 2013), the identification of the properties of T/F viruses in a genetically diverse setting has not been fully explored. This study aimed to characterize T/F viruses representative of different HIV-1 subtypes circulating in Mbeya, Tanzania. These Envelopes, cloned from single variant transmission events, were characterized in order to elucidate potential biological properties associated with selection mechanisms at work during HIV transmission.

University of Cape Town

3.2 METHODS

3.2.1 Cohort description

The HIV Superinfection Study (HISIS) high risk cohort from Mbeya, Tanzania recruited participants within three months of their previous HIV negative sample (Herbinger *et al.*, 2006; Riedner *et al.*, 2006). Analysis of 212 *env* sequences from 22 participants (n = 10 sequences per participant) generated using single genome amplification (SGA) indicated that sixteen participants were infected with a single infectious unit at transmission (**see Chapter 2**) (Nofemela *et al.*, 2011). Neighbor-joining trees and HIGHLIGHTER plots (http://www.hiv.lanl.gov) of the SGA sequences were used to identify the sequence of the T/F virus, and the amplicons identical to the consensus sequence were cloned. The clones were generated as part of the Vaccine Immune Monitoring Consortium, Collaboration for AIDS Vaccine Discovery (VIMC – CAVD) (http://www. cavd.org.za) which involves the generation of functional clones from different subtypes and diverse geographical regions to assess vaccine-elicited neutralizing antibodies and to study broadly neutralizing antibodies.

3.2.2 PCR amplification and cloning of the *env* gene.

The PCR amplification of the HIV-1 *env* gene was done using the SGA approach previously described (Abrahams *et al.*, 2009; Keele *et al.*, 2006). The second round PCR reaction was repeated using the high fidelity Phusion Hot Start DNA Polymerase (Finnzymes), together with, 0.2 mM dNTPs (Roche), 4 uM of Env 1A-Rx (5' CAC CGG CTT AGG CAT CTC CTA TAG CAG GAA GAA 3') and EnvN (5’ CTG CCA ATC AGG GAA AGT AGC CTT GT 3') in a final

University of Cape Town

Volume of 50 µl. The cycling conditions for this reaction were as follows: denaturing at 94 ºC for 5 minutes, 45 cycles of 94 ºC for 30 seconds; 55 ºC for 30 seconds; 72 ºC for 4 minutes, and final extension step at 72 ºC for 10 minutes. The amplicons were gel purified and cloned into the mammalian expression vectors pcDNA3.1D/V5-His-TOPO (Invitrogen) or pTarget (Promega, US) according to the manufacturer's instructions. Functional Envelope clones were selected using a 96-well plate format, and the Envelope clones with a RLU that is 2.5 times above background were considered to be functional.

3.2.3 Pseudovirion entry efficiency assays

Pseudoviruses were generated by co-transfection of HEK 293T cells with 2.5 µg *env* and 5 µg of either the subtype B HIV-1 backbone pSG3Δ*env* (gift from L. Morris, NICD) or the subtype C backbone pBR264F-MluΔ*env* (gift from B. Hahn, UAB) using PolyFect Transfection Reagent (QIAGEN). After 48 hours, the viral supernatant (2 ml) was harvested and clarified through a 0.45 µm filter and stored at -80° C. Pseudovirus titer was normalized using p24 ELISA (Vironostika HIV-1 Antigen microelisa system, Biomerieux, FRA) according to the manufacturer's instructions. TZM-bl cells (1 X 10^4 cells) were infected in triplicate with 50 ng/ml p24 of pseudovirus for 48 hours before medium was removed and cells were lysed with Bright-Glo buffer (Promega, US). Luciferase activity [relative light units (RLU)] was measured using a Glomax 96 microplate luminometer (Promega, US). The functional subtype C clone, Du151a was used as a positive control (van Harmelen *et al.*, 2001) and included in all experiments so that clonal Envelope entry efficiency could be measured relative to DU151a.

University of Cape Town

Pseudovirion stock generated by the transfection of the pSG3Δ*env* backbone was used as a negative control, and the RLU reading of cells only was considered as background signal. A RLU reading of 2.5 times above background was considered a positive infection. Relative infectivity in TZM-bl cells was measured as the % infection relative to Du151a).

3.2.4 Coreceptor phenotype

Pseudoviruses were generated by co-transfecting Envelope clones with the luciferase encoding pNL4-lucR$^-$E$^-$ backbone (NIH Aids Reagent Programme). U87 cell lines that express CD4 and either CCR5 or CXCR4 coreceptor were infected with 50 ng/ml p24 of virus and luciferase activity was measured after 48 hours. Viruses that were able to infect the U87- CD4 - CCR5 cells were classified as R5 tropic, and viruses that infected U87 – CD4- CXCR4 were classified as X4-tropic. The Envelope clones QHO (Li *et al.*, 2005), RPI (Cilliers *et al.*, 2005), and Du179 (Van Harmelen *et al.*, 2001) were obtained from Penny Moore (NICD), and were used as positive controls for R5, X4, and dualtropic R5X4 variants, respectively. A pseudovirion stock generated with the transfection of the pNL4-lucR$^-$E$^-$ backbone was used as a negative control, and the RLU reading of wells containing cells only was considered as background signal.

3.2.5 Pseudovirus entry inhibition assay

TZM-bl cells (1 X 10^4 cells per well) were infected in triplicate with pseudovirus (50 ng p24) in the presence of increasing concentration of TAK779 (0.04 - 400 nM) and T-20 (0.3 μg/ml – 25 μg/ml) (NIH Aids Reagent Programme), and luciferase activity was measured after 48 hours, and the 50 % inhibitory concentration (IC_{50}) was determined using the GraphPad Prism software 5

University of Cape Town

(CA, USA). Soluble CD4 IC_{50} was measured as part of the VIMC-CAVD (http://www.cavd.org) study and included in this analysis.

3.2.6 Cell-cell fusion assay

HEK 293T cells (4×10^5) were seeded in 6 well plates overnight, and co- transfected with 3.75 µg of each individual *env* plasmid and 3.75 µg of pSVtat72 (NIH AIDS Reagent Programme) for 48 hours using the PolyFect Transfection Reagent (QIAGEN, US) according to the manufacturer's instructions. The cell medium was removed and the cells were lifted using 0.04 % EDTA. The 293T cells (1×10^4 cells per well) were added to TZM-bl cells (1×10^4 cells per well) and allowed to incubate at 37 ^oC for 24 hours. The media (100 µl) was removed from the cells, and 100 µl of Bright-Glo buffer (Promega, US) was added. The plate was incubated for 2 minutes at room temperature with mild shaking. After cell lysis, 150 µl was transferred to a Co-Star black plate, and the plate was read immediately using a Glomax 96 microplate luminometer (Promega, US). The percentage fusion efficiency was measured relative to the reference clone, Du151a.

3.2.6 Pseudovirion infection of Affinofile cells.

Pseudoviruses were generated by co-transfecting HEK 293T cells with 5 µg of *env* plasmid and 10 µg of the luciferase encoding pNL4-lucR^-E^- backbone (NIH Aids Reagent Programme). Pseudovirus-containing cell culture medium was collected after 48 hours, and filtered (0.45 µM) before p24 levels were measured (Vironostika HIV-1 Antigen microelisa system, Biomerieux, FRA). The Affinofile cell lines, a generous donation from Benhur Lee (UCLA), were induced and infected as previously described by Lassen *et al.*, (2009) and colleagues. Briefly, the 293

University of Cape Town

Affinofile cells (1 X 10^4 cells per well) were seeded in 24-well plates, and induced with a concentration range of minocyline (0 to 5ng/ml) and ponasterone A (0 to 4 μM) to induce CD4 and CCR5 expression, respectively. The cells were collected after 24 hours and washed with PBS before staining. The cells were stained with PE anti-human CD4 and PE anti-human CD195 (CCR5) (BioLegend, US) for 30 minutes at room temperature. The absolute number of CD4 and CCR5 was quantified using the Quantibrite beads (BD Biosciences, US) according to the manufacturer's instructions. The data acquisition and flow cytometry analysis was done using Cellquest and Flowjo software, respectively.

Affinofile cells (1 X 10^4 cells per well) were seeded in a 96-well plate for 48 hours, and were induced with minocyline (2.5 – 5 ng/ml) and ponasterone A (2 - 4 μM) for 24 hours. The cells were then infected with 100 ng/ml p24 pseudovirus and infection was determined after 48 hrs using luminescence. Entry efficiency in 293Affinofile cells was determined based on luminescence. CCR5 dependence (% infection relative to high CD4/high CCR5) was determined using the following equation: RLU (high CD4/low CCR5)/RLU (high CD4/high CCR5) X 100 (Ping *et al.*, 2012).

University of Cape Town

3.2.7 Pseudovirus Envelope Western Blotting

Pseudoviral stocks were generated by co-transfecting HEK 293T cells (2 X 10^6 cells) with 5 μg of cloned Envelope and 10 μg of the subtype B HIV-1 backbone pSG3Δ*env*. After 48hrs, the clarified culture medium was centrifuged at 26 000 rpm for 2 hrs through a 20 % glycerol cushion at 4° C in a RW55 rotor (Beckman Coulter, GER) to harvest pseudovirus. The viral pellet was resuspended in PBS for p24 determination and viral quantitation using the Vironostika HIV-1 Antigen microelisa system (Biomerieux, FRA) before viral lysis in SDS Laemlli loading

buffer [40 MM Tri-HCL (pH 6.8), 10 % glycerol, 10 % β mercaptethanol, and 1 % SDS]. The equivalent of 1.5ug/ml (for gp120) and 50ng/ml (for 24) p24 pseudoviral lysate was resolved using 8 % SDS PAGE before transfer to nitrocellulose membrane. The primary antibodies (sheep anti-gp120 and rabbit ARP 432) raised against gp120 and p24, respectively were obtained from the NIH AIDS Reagent Programme. Primary antibodies for gp120 and p24 were detected with horseradish peroxidase-conjugated goat-anti-sheep IgG (Sigma, US) and alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma, US) secondary antibodies respectively. The gp120 Western blot was detected using the LumiGlo chemiluminescent substrate (KPL) and exposed to X-ray film and the p24 Western Blot was visualised with NCIP/BCIP tablets (Roche, GER).

3.2.8 Statistical analysis

The association of entry efficiency with viral load and fusion capacity was calculated using the Spearman correlation test. These figures were constructed using GaphPad Prism 5 software (CA, USA). Classification of the Envelope clones into high and low entry efficiency groups were tested using Mann-Whitney U test.

University of Cape Town

3. 3 RESULTS

3.3.1 Cloning of the transmitted/founder (T/F) virus envelope

The *env* sequences from recently infected participants from the Mbeya region of Tanzania has been previously described (**see Chapter 2**) (Nofemela *et al.*, 2011) and for this study we selected ten participants identified as infected with a single variant belonging to either subtype C (n = 6), D (n = 1) or recombinant CD (n = 2) or AD (n = 1). In order to characterize the fitness properties of Envelope of the virus that caused clinical infection, we generated functional Envelope clones whose sequences were identical to the consensus of SGA-derived sequences. These molecular clones were assumed to represent the transmitted/founder (T/F) virus responsible for clinical infection. The T/F Envelope clones characterized in this study were generated from samples collected at the first seroconversion visit. These individuals were estimated to be infected for between 14 – 45 days (median = 42.5 days post-infection), and the plasma viral loads at the time of sampling ranged from 2 378 to > 750 000 copies/ml (**Table 3.1).**

University of Cape Town

Table 3.1 Description of transmitted/founder Envelope clones from a high risk population of female bar workers from Mbeya, Tanzania

Clone ID	Time post-infection (Days)	Subtype	Fiebig staging	Viral Load (Time of sampling) (copies/ml)	Cell Tropism	Soluble CD4 IC_{50} (µg/ml)	TAK779 IC_{50} (nM)	T20 IC_{50} (µg/ml)
390_T/F_TZ	45	C	V	>750 001	R5	2.50	21.28	1.86
89_T/F_TZ	14	CD	I/II	>750 001	R5	2.20	9.84	1.16
216_T/F_TZ	14	C	VI	>750 001	R5	>50	125.50	2.41
605_T/F_TZ	41	AC	V/VI	434 000	R5	7.4	20.32	0.75
401_T/F_TZ	45	CD	ND	51 500	R5	10.0	60.77	1.35
234_T/F_TZ	45	C	V/VI	509 000	R5	5.40	101.33	1.43
569_T/F_TZ	45	C	V/VI	23 800	R5	14.40	42.94	3.30
346_T/F_TZ	41	C	VI	26 700	R5	1.56	33.63	5.46
54_T/F_TZ	42	D	VI	28 200	R5	>50	>400	>25
398_T/F_TZ	45	C	ND	2 378	R5	16.4	8.18	>25

>50: Resistant to 50 µg/ml sCD4; Resistant to 400nM TAK779; 25µg/ml T20.

> 750 001 shows the limit of detection for the Amplicor HIV Monitor assay

* Participant identification: clone number_transmitted/founder virus_country of origin

Envelope clones with low entry efficiency are highlighted in grey

University of Cape Town

3.3.2 Entry efficiency differs between T/F Envelope pseudoviruses

Pseudovirion entry efficiency was compared to a reference clone, Du151a measured in parallel to determine a relative fitness score and to control for inter-experimental variation (Gottlieb *et al.*, 2004; Nofemela *et al.*, 2011). Pseudovirion entry efficiency was compared using both subtype B, (pSG3.1Δ*env)* and subtype C, (pBR264F-MluΔ*env)* HIV-1 backbones to determine whether subtype differences would impact Envelope function. The relative entry efficiency of the pseudoviruses generated using subtype B and the subtype C backbone vectors varied 164 - fold and 52 - fold respectively (data not shown). Furthermore, there was a direct correlation between the relative entry efficiency of the pseudoviruses generated using subtype B and subtype C backbone vectors ($p = 0.0047$). Lastly, subtype C Envelopes did not have enhanced function when coupled with a subtype C backbone. Together these results suggest that the subtype of the HIV-1 backbone did not impact the relative entry efficiency of the Envelope clones suggesting that subtype specific protein-protein interactions did not influence the assay. This heterogeneity in entry efficiency between Envelopes irrespective of the backbone used, suggests that in the ten samples that were analyzed, high Envelope entry efficiency is not a prerequisite for HIV transmission (**Fig. 3.1**).

University of Cape Town

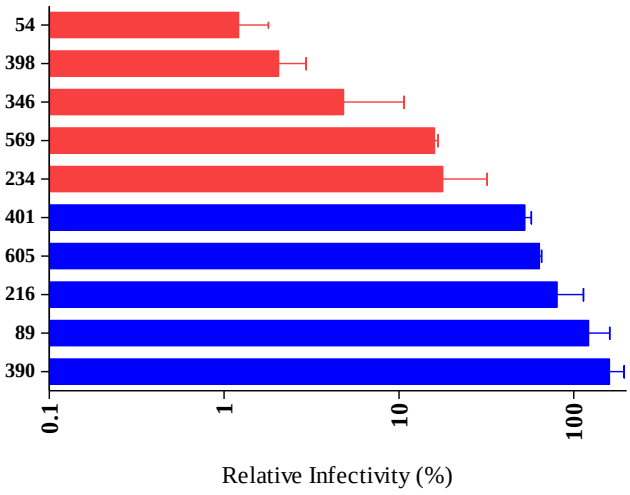


Figure 3.1 Envelope clones of transmitted/founder viruses have a wide range of entry efficiency. The entry efficiency of 10 transmitted/founder *env* variants, relative to Du151a (positive control) was determined using pseudovirions generated with pSG3.1Δ*env*. The red and blue bars represent those clones with low and high TZM-bl entry efficiency. Infection was performed in triplicate for each of the variants and this is a combined result of two pseudovirus preparations with the error bars indicating standard deviation.

University of Cape Town

3.3.3 T/F Entry efficiency is associated with disease progression

In order to investigate the relationship between Envelope entry efficiency of T/F variants and viral loads subsequent to transmission in a cohort of women infected with multiple circulating subtypes and recombinants, the association between entry efficiency and viral loads at 3 and 12 months post infection was determined. The Envelope from T/F variants infecting individuals with high viral load at 3 and 12 months post infection had higher entry efficiency compared to those variants infecting individuals with low viral loads ($p = 0.0022$ and $p = 0.0347$, respectively). The same relationship was observed with pseudoviruses generated using the subtype C backbone at both 3 and 12 months post infection ($p = 0.0392$ and 0.0234, respectively)

(data not shown), suggesting that entry efficiency of T/F variants could be a predictor of disease outcome (**Fig. 3.2**). However, in noted that the viral measurement of >750 001 for three samples is somewhat truncated and represented the limit of detection for the assay. While it would be ideal to dilute the plasma samples to determine the absolute viral load levels, this is however unlikely to affect the correlation that was observed . Also, these findings support other previous studies that have shown a similar correlation between viral replicative fitness and viral load (Quinones-Mateu *et al.*, 2000; Prince *et al.*, 2013).

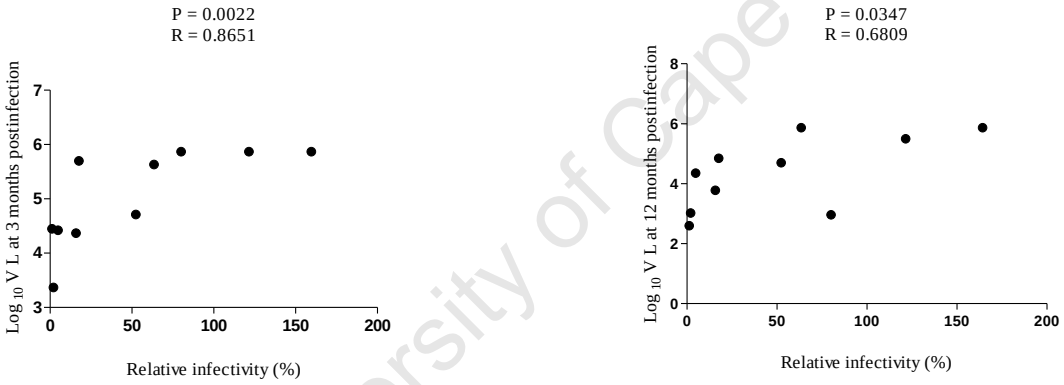


Figure 3.2 The Envelope entry efficiency of T/F variants is associated with viral load. Pseudovirions were generated using the Subtype B backbone pSG3Δ*env* and entry efficiency was measured using TZM-bl cells relative to Du151 (% infection). The association between relative infectivity and viral load at A) 3 and B) 12 months post-infection was analysed using a Spearman correlation test.

3.3.4 Coreceptor tropism and inhibition kinetics are not associated with entry efficiency of TZM-bl cells

CCR5-utilising viruses are preferentially transmitted (Berger *et al.*, 1999) while the development of CXCR4 tropism has been linked to increased disease progression (Koot *et al.*, 1993; Sucupira

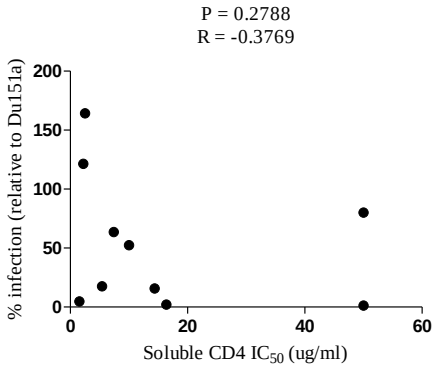
University of Cape Town

et al., 2012). Furthermore, previous studies have demonstrated the role of coreceptor usage in HIV-1 replicative fitness (Arie *et al.*, 2005; Quiñones-Mateu *et al.*, 2000). In order to determine whether tropism played a role in the entry efficiency of the T/F variants, U87 cells expressing the CD4 receptor as well as either CCR5 or CXCR4 coreceptors were infected with pseudovirus. All the Envelope clones were R5 tropic as only U87 - CD4 – CCR5 cells were infected and not those expressing CXCR4 (data not shown), suggesting that viral tropism did not contribute to the observed differences in entry efficiency of the variants (**Table 3. 1**).

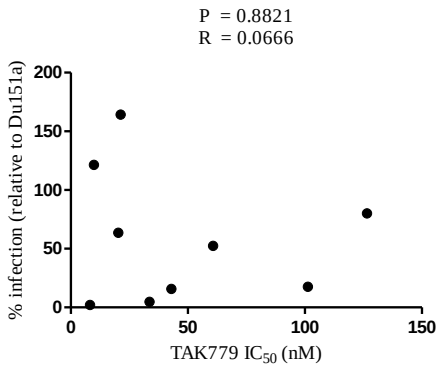
In order to examine whether differences in entry efficiency of the T/F variants were due to changes in binding affinity to host receptors (Reeves *et al.*, 2002), we determined the IC_{50} of TAK779 (CCR5 antagonist) and T-20 (fusion inhibitor). The sensitivity of the viruses to soluble CD4 was also determined. However, this was not used as a measure of binding affinity as a number of studies have shown that soluble CD4 can either enhance (Allan *et al.*, 1990; Allan *et al.*, 1991; Clapham *et al.*, 1992) or inhibit HIV infection (Deen et al., 1988; Fisher et al., 1988; Hussey *et al.*, 1988; Smith *et al.*, 1987; Traunecker *et al.*, 1988). There was variation in sensitivity of the Envelope clones to sCD4 (IC_{50} median = 6.4 μg/ml; range = 1.6 – 16.4 μg/ml), TAK779 (IC_{50} median = 33.63 nM; range = 8.18 – 126.50 μM), and T-20 (IC_{50} median = 1.64 μg/ml; range = 1.15 – 5.45 μg/ml). One pseudovirus (clone 398) was resistant to T-20, and one (clone **54**) was resistant to both TAK779 and T-20 and these clones were excluded from the statistical analysis. There was no significant correlation between entry efficiency of TZM-bl cells and the IC_{50} of sCD4 ($p = 0.2788$), TAK779 ($p = 0.8821$) and T20 ($p = 0.2162$) (**Fig. 3.3**).

University of Cape Town

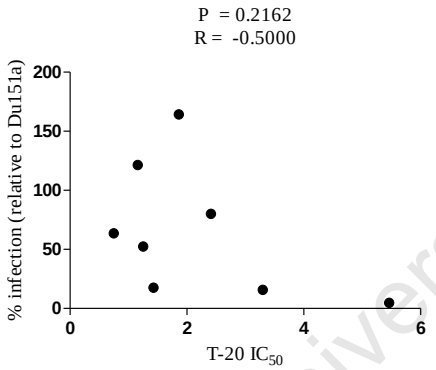
A



B



C



University of Cape Town

Figure 3.3 Association of Envelope entry efficiency with sensitivity to entry inhibitors IC_{50} of entry inhibitors: sCD4 (A), TAK779 (B) and T-20 (C) was determined for each Envelope clone and its association with the corresponding entry efficiency of each transmitted variants was determined using a Spearman correlation test.

3.3.5 Cell surface CD4 is critical for successful host cell entry of transmitted viruses

CD4 and CCR5 density is an important determinant of HIV infectivity (Heredia *et al.,* 2007; Kabat *et al.,* 1994; Lee *et al.,* 1999; Parker *et al.,* 2012; Platt *et al.,* 1998; Reynes *et al.,* 2000; Walter *et al.,* 2005) and while the number of CD4 molecules on the surface of peripheral T cells remains constant (Poncelet *et al.,* 1991), the levels of CCR5 receptors differs between individuals and is thought to play a more critical role (Lee *et al.,* 1999; Reynes *et al.,* 2000). It is possible that high expression levels of CCR5 by TZM-bl cells may mask subtle differences in the ability of T/F Envelope clones to mediate entry and membrane fusion as high CCR5 density has been shown to hasten membrane fusion kinetics (Heredia *et al.,* 2007; Reeves *et al.,* 2002) and infectivity of cells with high CCR5 were shown to be independent of CD4 levels (Platt *et al.,* 1998). Therefore, by changing the level of receptors at the cell surface we aimed to determine the ability of T/F viruses to infect cells with low receptor levels and also to distinguish those envelope clones with high receptor affinity from those with low affinity for CD4 and /or CCR5.

HEK 293 Affinofile cells were induced to express different levels of surface CD4 and CCR5 to generate cells with: 1) high CD4 (146 397 molecules/cell), low CCR5 (2 566 molecules/cell); 2) low CD4 (2 397 molecules/cell), high CCR5 (428 032 molecules/cell; and 3) high CD4 and high CCR5 levels (146 397 and 428 032 molecules/cell respectively).

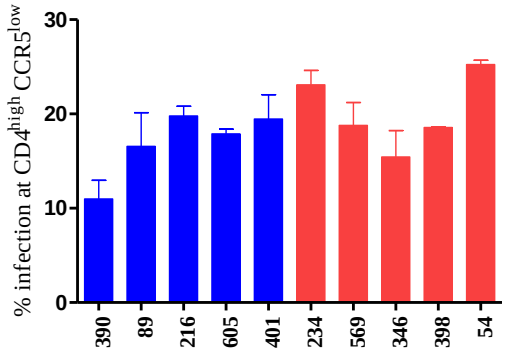
All the T/F Envelope pseudovirions were able to infect cells with high CD4 and high CCR5 levels and the Envelope clones with low entry efficiency in TZM-bl cells also had low entry efficiency in Affinofile cells. All of the Envelope pseudovirions were able to infect cells when CCR5 levels were lowered to 2 566 molecules/cell although infection dropped by 4 to 5-fold (% infection relative to $CD4^{high}/CCR5^{high}$ range = 10.9% - 25%; median = 18.6%), suggesting a

University of Cape Town

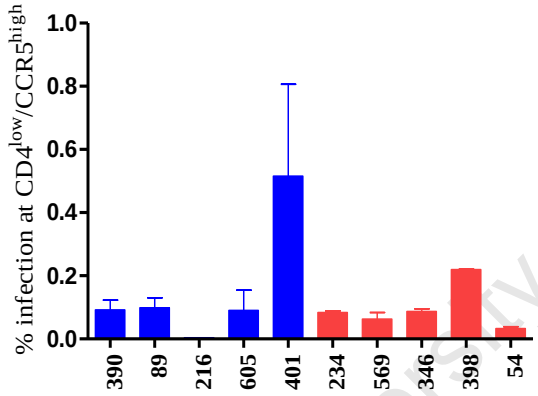
dependency on high levels of CD4 for entry (**Fig 3.4 A**). This supports previous findings of Platt *et al.*, (1998) which showed that trace amounts of CCR5 could support HIV infection if CD4 numbers were high. However, when CD4 receptor levels were lowered (3 401 molecules/cell) and CCR5 numbers were high (428 032 CCR5 molecules/cell), the virus mediated entry very poorly with nearly 100 - fold decrease compared to cells with high CD4 and high CCR5 levels (% infection relative to $CD4^{high}/CCR5^{high}$ range = 0.003 – 0.5%; median = 0.08%) (**Fig. 3.4B**). This suggests that the Envelope pseudovirions in this study require a minimum level of CD4 receptors in order to infect target cells irrespective of CCR5 levels.

University of Cape Town

A



B



University of Cape Town

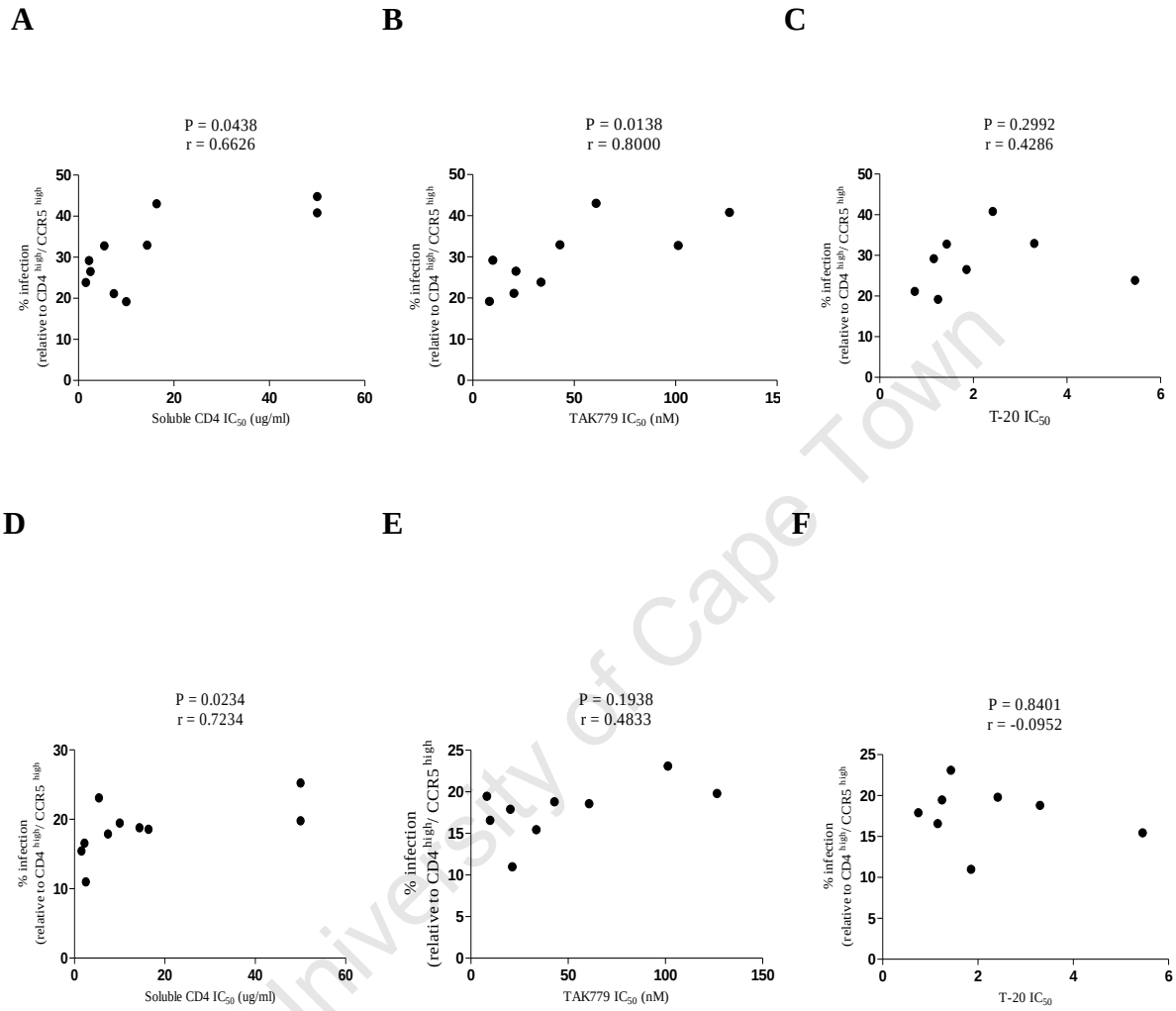
Figure 3. 4 Pseudovirion entry efficiency in dual inducible HEK293 Affinofile cells at (A) High CD4 and Low CCR5 levels; (B) Low CD4 and High CCR5 levels. Entry efficiency is calculated as % infection relative to $CD4^{high}/CCR5^{high}$. The grey bars indicate the pseudoviruses with low entry efficiency and the black bars are the pseudoviruses with high entry efficiency as previously described. The level of entry efficiency was measured in RLU. This figure is a result of two assays using two pseudovirus preparations, and the error bars represents the standard deviation between the two assays.

3.3.6 CD4 and CCR5 binding affinity a determinant of entry under limiting receptor/coreceptor levels

As only three out of five Envelope clones with high entry efficiency were able to infect cells with low CD4 levels, and there was a relative 4 - 5 fold drop in entry efficiency for all clones when CCR5 levels were lowered, we wanted to determine whether binding affinity to either CD4 or CCR5 played a role in this dependency on high CCR5 receptor numbers. We therefore tested the relationship between the IC_{50} values obtained using soluble CD4, TAK779 and T-20 and CCR5 dependence (which is calculated as % infection relative to high CD4/ high CCR5 levels) (Ping *et al.,* 2012). The lower the percentage value, the higher the dependency on CCR5 levels and the greater the difficulty in entering cells with low CCR5 density. In cells with high CD4 levels and low CCR5 levels there was a positive correlation between CCR5 dependence and the IC_{50} measurements of soluble CD4 and TAK779 ($p = 0.0438$; $p = 0.0138$, respectively) (**Fig. 3.6 A, B**), suggesting that the ability to enter cells with low CCR5 levels was determined by binding affinity to CD4 and CCR5. The correlation between soluble CD4 IC_{50} measurements and coreceptor dependence became more significant when the CD4 levels were lowered to 69 988 molecule/cell ($p = 0.0234$) whereas significance was lost with TAK779 affinity ($p = 0.1938$) (**Fig. 3.6 D, E**). The ease with which pseudovirions are able to enter cells with low CCR5 levels is thus directly related to Envelope binding affinity for CD4 and CCR5 and influenced by the relative abundance of the receptor and coreceptor on the host cell. Overall, this also suggests that binding affinity to CD4 and CCR5 is a determining factor in entry efficiency, as T/F clones, classified as having high efficiency in TZM-bl cells, infected Affinofile cells with limiting receptor levels better than the low TZM-bl entry efficiency group (**Fig. 3.5**). Finally, there was no positive correlation between T-20 IC_{50} measurements and CCR5 dependence at either CD4

University of Cape Town

levels tested (p = 0.2992, p = 0.8401, respectively) (**Fig. 3.6 C, F**) suggesting that membrane fusion kinetics was not a determinant of entry efficiency under limiting host receptor conditions.



University of Cape Town

Figure 3.6 Infection of cells with low CCR5 levels is associated with binding affinity for CD4 and CCR5. Pseudovirion entry was determined of Affinofile cells induced to express different levels of CD4: (A, B and C) 146 397 molecules/cell and (D, E and F) 69 988 molecule/cell with constant low levels of CCR5 (2 566 molecules/cell). Spearman correlation was carried out to determine the relationship between CCR5 dependence and soluble CD4, TAK779 and T-20 IC50's determined using TZM-bl cells.

3.3.7 Fusion capacity is not associated with entry efficiency

As both TZM-bl and Affinofile entry under both high and low host receptor levels was not associated with sensitivity to T - 20, a cell-cell fusion assay was performed to determine whether this step contributed to differences in entry efficiency. HEK 293T cells, co-expressing Envelope and Tat, were added to TZM-bl cells which stably express the LTR - luciferase reporter gene. Luciferase activity was measured after 24 hours as fusion between the two cells would enable the transfer of Tat to TZM-bl cells resulting in luciferase expression. Although there was no statistically significant association between fusion and entry efficiency ($p = 0.8916$) (**Fig. 3.7B**), Envelope clones classified with high entry efficiency had relatively high fusion capacity (71 – 138 % relative to Du151a), and three out of the five clones with low entry efficiency had lower fusion capacity (43 – 53 % relative to Du151a) (**Fig. 3.7A**). However, two of the clones (**54** and **569**) with low entry efficiency had high fusion capacity (relative fusogenicity = 240 % and 140 % respectively) suggesting, at least with these clones, that fusion was not the major determinant accounting for differences in entry efficiency.

University of Cape Town

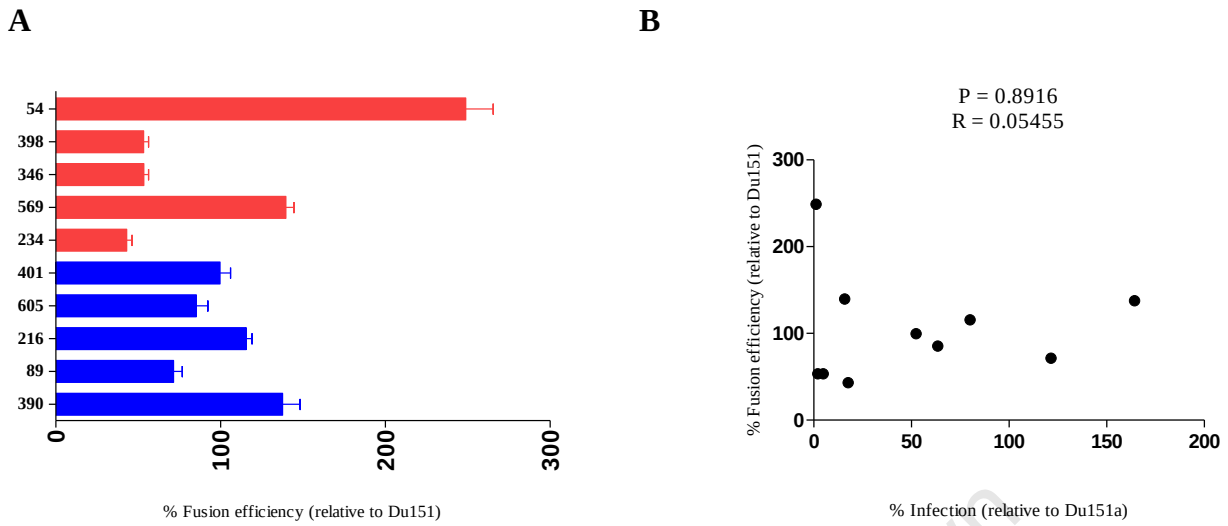


Figure 3.7 Cell-cell fusion capacity is not always associated with TZM-bl entry efficiency.(A) Envelope fusion capacity of the transmitted variants was determined by using a cell-cell fusion assay and (B) the association between entry efficiency and fusion capacity was determined using a Spearman Correlation test. The blue bars indicate the viruses with high entry efficiency and the red bars are the viruses with low entry efficiency.

University of Cape Town

3.3.8 Envelope incorporation into pseudovirion particles affects entry efficiency

The number of functional Envelope trimers incorporated into the viral particle (Moore *et al.*, 2006), as well as correct processing of the immature protein (gp160) into gp120 and gp41, impacts HIV-1 infection (Bachrach *et al.*, 2005; Blay *et al.*, 2007; Provine *et al.*, 2009). SDS page was used to characterize Env processing and to determine the amount of protein that is required to ensure the visibility of the protein on the blotting membrane. This is done by means of a semi quantitative method which involves the serial dilution of the pseudovirion samples to determine the p24 concentration that provides a linear range of detection. In order to determine whether entry efficiency was linked to Envelope incorporation and processing, pseudovirion stocks were concentrated using ultracentrifugation and normalized for p24 concentration before

SDS PAGE and Western blotting against gp120 and p24. Equivalent pseudoviral particles, as determined by p24 concentration were loaded per lane. The Western blot showed that the Envelope pseudoviruses with the highest levels of entry efficiency (**89**, **216**, **390**) had high levels of both gp160 and gp120 incorporation. The Envelope pseudoviruses with the lowest entry efficiency (**54**, **398**, and **569**) had a single, band which was difficult to identify as either gp160 or gp120 due to the heterogeneity in molecular weight of the Envelope clones, however the low intensity of the band suggested poor incorporation compared to those with high entry efficiency. However, pseudoviruses **234** and **346** had good gp120 incorporation despite having low entry efficiency, and pseudovirus **605** showed low or very little processing despite having high entry efficiency (**Figure 3.8**). Overall, these differences suggest heterogeneity in the level of gp120 incorporation into the virus which may contribute to high entry efficiency of some T/F variants.

of Cape Town

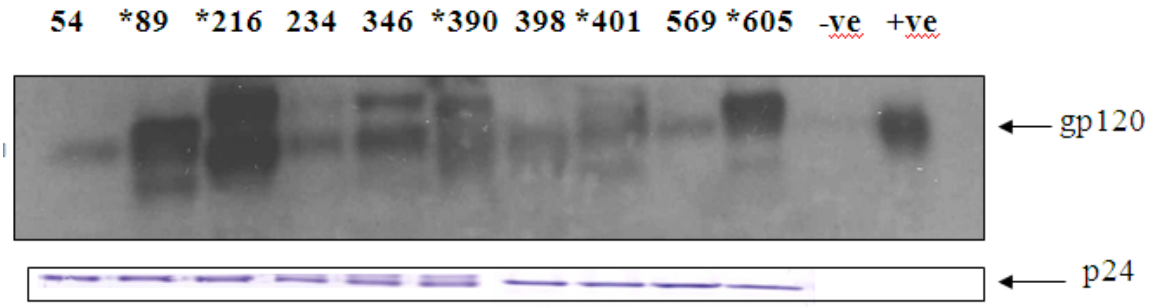


Figure 3.8 Gp120 pseudovirion incorporation varies between Envelope clones. Pseudovirions were generated in HEK 293T cells, and culture supernatants were layered over 20% glycerol and ultracentrifuged at 26 000 RPM for 2 hours. The pelleted viruses were lysed and 1.5 ug/ml and 50ng/ml of p24 was loaded per lane for gp120 and p24 respectively. The Envelope clones highlighted with an asterisk are those with high entry efficiency in TZM-bl cells Negative control (-ve) comprises viral particles without *env* and the positive control is HIV-1 gp120 CM cat #2968 (AIDS Reagent Programme).

3.4 DISCUSSION

The genetic bottleneck during HIV heterosexual transmission selects for variants that almost exclusively use CCR5 receptors and carry more compact Envelope with fewer potential N-glycosylation sites (Abrahams *et al.*, 2009; Haaland *et al.*, 2009; Keele *et al.*, 2008; Zhang, 1999), suggesting that mucosal factors select Envelope variants enriched with specific functional attributes. Although a few studies have shown a link between Envelope function and HIV replicative fitness (Kong *et al.*, 2008; Rangel *et al.*, 2003) and replicative fitness and disease progression (Miura *et al.*, 2010; Quiñones-Mateu *et al.*, 2000), only one study on subtype B infected elite suppressors demonstrated a link between Envelope function and disease progression (Lassen *et al.*, 2009). The study by Lassen *et al.*, (2009) showed that elite suppressors had significantly lower Env entry efficiency than chronic progressors. In this study we report on a study of high-risk women from Tanzania, recruited soon after infection, and show that the entry efficiency of multiple subtype and recombinant T/F Envelope pseudoviruses was positively correlated with viral loads at 3 months and 12 months post-infection. These findings support the findings by Lassen *et al.*, (2009), which show that low viral loads observed in elite suppressors was associated with low Env entry efficiency. Furthermore, we find that the T/F Envelopes clones exhibited large differences in entry efficiency that was not associated with a specific subtype, and that this together with the heterogeneity in dependency on CCR5 and CD4 suggests entry efficiency is not an absolute requirement for transmission.

University of Cape Town

Due to the relationship between Envelope function in TZM-bl cells and viral loads, we wanted to identify the underlying biological factors(s) driving Envelope entry efficiency and thus potentially, replication fitness. As HIV replication fitness may be influenced by the level of CD4 and CCR5 at the surface of T cells (Alexander *et al.*, 2010), we investigated the effect of limiting host receptor levels on entry efficiency using Affinofile cells induced to express different levels of CD4 and CCR5. There was a positive correlation of the relative entry efficiency of the Envelope clones between TZM-bl cell and Affinofile cells at high levels of CD4 and CCR5, however the absolute level of entry efficiency decreased by 4 -5 fold when the CCR5 levels were lowered and 100 - fold when the CD4 levels were decreased. Only three viruses, from the high TZM-bl entry efficiency group, were able to infect cells with low CD4 levels, despite high CCR5 levels. This suggests that these viruses are dependent on a specific number of CD4 molecules per cell and thus unlikely to be macrophage tropic similar to recent studies on subtype B and C viruses (Ochsenbauer *et al.*, 2012; Salazar-Gonzalez *et al.*, 2009). The dependency on CCR5 density correlated significantly with sensitivity to CD4 and CCR5 inhibitors suggesting that pseudoviruses with high affinity for CCR5 were able to infect cells with low CCR5 density. Furthermore, the large variation of dependence on high CCR5 levels between the clones, suggests that the extensive variability of host CCR5 density could influence selection during transmission.

As previous studies have shown that high levels of surface Envelope expression result in increased infectivity (Bachrach *et al.*, 2005; Provine *et al.*, 2009), we investigated whether the entry efficiency of the T/F Envelope clones is due to enhanced Envelope incorporation. This study found that incorporation/processing of Envelope into virions may impact entry efficiency as some T/F viruses with the highest entry efficiency had better gp120 incorporation compared to

University of Cape Town

other viruses. However, the relationship between Envelope incorporation/cleavage/processing and entry efficiency was not consistent for all the clones, suggesting that other factors may be playing a role in the entry efficiency of the T/F viruses. A recent study by Parrish et al (2013) found that T/F are more infectious than viruses at chronic infection, and this was partly due to the presence of Envelope particles on the surface, which suggests that Envelope incorporation/processing may play a role in entry efficiency. In terms of cell-cell fusion assay, despite some viruses with high entry efficiency having high cell-cell fusion capacity, there was no correlation between entry efficiency and cell-cell fusion. This discrepancy could be due to the two assays measuring different parameters, with the pseudovirus assay being dependent on Envelope incorporation into viral particles, and the cell-cell fusion assay reliant on Envelope expression on the surface of cells. Clone **54**, which had low entry efficiency and the highest cell-cell fusion capacity, illustrates this phenomenon. Clone **54** was resistant to sCD4, TAK779 and T20 and is therefore likely to have high affinity for CCR5. Reeves *et al.*, (2002) demonstrated that increased coreceptor affinity resulted in faster fusion kinetics which may explain the high fusion capacity of clone 54. However, its incorporation into viral particles was very poor which would lower its ability to mediate entry of TZM-bl cells.

Finally, this study investigated the entry efficiency of T/F Envelope clones from 10 participants, six infected with subtype C, three infected with recombinant forms (CD, AC and AD) and one with a subtype D variant. Phenotypic coreceptor tropism results indicated that all were R5 tropic even though subtype D variants are more likely to utilize CXCR4 (Huang *et al.*, 2007). There was no apparent association between entry efficiency, inhibitor sensitivity, receptor dependence, fusion capacity or Envelope incorporation and HIV subtype or recombinant form. However, given the small sample size and subtype related genotypic differences of transmitted variants

University of Cape Town

(Chohan *et al.*, 2005; Derdeyn *et al.*, 2004; Frost *et al.*, 2005a), further studies on non-subtype B and subtype C T/F variants might provide important insights into the mechanism of HIV transmission.

In conclusion, this study has shown that Envelopes of T/F variants infecting participants with high viral loads had higher entry efficiency compared to those with low viral loads. This association was determined at viral set point (12 months), which suggests that entry efficiency of transmitted viruses could influence disease outcome. The viruses were dependent on high levels of CD4 for entry, with many of them unable to infect cells with low CD4 density despite high levels of CCR5. Therefore, T/F variants from multiple subtypes and recombinant forms are unlikely to be macrophage tropic, corroborating earlier subtype B and subtype C studies. The phenotypic properties of the viruses were also highly heterogeneous, similar to a previous study comparing acute infection subtype B Envelope clones from progressors to those of elite suppressors, suggesting that, irrespective of subtype, the genetic bottleneck does not select for viruses with equal Envelope fitness.

University of Cape Town

Chapter 4: Generation of replication competent viruses carrying transmitted/ founder Envelope clones and measuring their replicative fitness in lymphocytes and macrophages.

University of Cape Town

University of Cape Town

ABSTRACT

As studies have shown discrepancies between pseudoviruses and infectious molecular clones, we were interested in characterizing *env* expressed in chimeric infectious molecular clones (IMCs). The Envelope clones were shuttled into a NL4-3 backbone using a yeast gap repair method to generate recombinant viruses, and their replication was measured in TZM-bl, PBMCs and monocyte-derived macrophages. In TZM-bl cells, the majority of recombinant viruses (80%) showed similar entry efficiency profiles to those in the pseudovirion system. The replication capacity of the recombinant viruses in PBMCs varied between donors, however the recombinant viruses replicated much more efficiently in T lymphocytes than in monocyte-derived macrophages. Although the pseudovirion assays showed that the higher replication kinetics *in vitro* was associated with high viral loads *in vivo*, due to host to host variation we could not confirm this finding in PBMCs. The preferential replication in T-cells is in agreement with our results which showed the inability of pseudovirions to infect Affinofile cells induced to express low levels of CD4 receptor, and together these results support published studies that T/F viruses, irrespective of subtype, preferentially replicate in T-cells and not macrophages.

University of Cape Town

4.1 INTRODUCTION

A number of investigators have used the pseudovirus based assay to measure the Envelope phenotypic properties of viruses. While these assays are highly reproducible and are high throughput, they use cell lines and can only measure a single cycle of infection. Therefore, in order to circumvent this, it is important to use a system that measures replication fitness over multiple rounds of infection, and one that resembles what is likely to be taking place *in vivo*. One approach involves the construction of chimeric infectious molecular clones containing the *env* gene of interest in the context of a full HIV-1 proviral genome backbone (Etemad *et al.*, 2009; Provine *et al.*, 2009), and measuring replication in peripheral blood mononuclear cells (PBMCs) (Arie *et al.*, 2005; Ochsenbauer *et al.*, 2012; Quiñones-Mateu *et al.*, 2000; Salazar-Gonzalez *et al.*, 2009). Our previous findings using the pseudovirus (PSV) system has shown certain properties in Envelope are important for enhanced entry efficiency during acute infection, and could possibly predict disease outcome (**see See Chapter 2**). Therefore, in order to validate these findings under biologically relevant conditions, this study aimed to determine the efficiency at which these viruses replicated in different target cells including TZM-bl, PBMCs and monocyte-derived macrophages.

In order to fulfill the objective of the study, replication competent recombinant viruses carrying Envelope clones from transmitted/founder (T/F) viruses (**see Chapter 2**) were generated using the NL4-3 backbone and the yeast gap repair system. This process occurs by means of homologous recombination in yeast cells. Homologous recombination is a natural mechanism that is used by yeasts to repair its chromosomal DNA. It uses regions of homology between the damaged DNA and the chromosome in order to repair the gap. This method offers several

University of Cape Town

advantages over the bacterial cloning in that it is very efficient, precise, and also uses negative selection markers that ensure the insertion of the *env* gene in the correct orientation (Baudin *et al.*, 1993; Lorenz *et al.*, 1995; Sikorski & Hieter, 1989). Also, this method does not require restriction enzyme digestions, gel purifications, *in vitro* ligations, and screening of the inserts in order to produce an infectious full-length proviral DNA vectors (Dudley *et al.*, 2009). The replication capacity of the recombinant viruses was subsequently evaluated in PBMCs and macrophages.

University of Cape Town

4.2 MATERIALS AND METHODS

4.2.1 Amplification of the HIV-1 *env* gene by Single Genome Amplification

Refer to Chapter 2.2.2.2

4.2.2 DNA gel isolation and purification

The gel purification was done using the QIAquick Gel Extraction Kit (QIAGEN, US) according to the manufacturer's instructions.

4.2.3 Plasmids

The subtype B backbone CMV –PBS-LTR-NL4-3Δgp160 URA) was used for the yeast gap repair method. The 5' LTR has been replaced with a CMV promoter in order to prevent recombination of the entire HIV genome and thus expression of the HIV genes is under the control of the CMV promoter. The *env* gene is replaced by the (URA3) gene, which encodes the orotidine – 5' phosphate decarboxylase protein which is involved in the biosynthesis of uracil. The plasmid also contains the beta – isopropylmalate dehydrogenase gene for the biosynthesis of leucine (LEU), an autonomously replicating sequence (ARSH4), and the yeast centromere sequence (CEN6), which all enables the recombinant plasmid to be cultured in yeast cells. The CMV NL4-3 Gag4 was used as a helper plasmid. A subtype B *env*-expressing plasmid derived from NL4-3 (CMV NL4-3 GT) was used as a positive control in the TZM-bl infections. These plasmids were provided by Dr. Manish Sagar, Brigham and Women's Hospital, Harvard Medical School, US. The subtype B virus NL4-3 was used as a positive control for PBMC infection, and the macrophage tropic virus Yu-2 was used as a positive control of macrophage infection.

University of Cape Town

4.2.4 Yeast gap-repair homologous recombination

The yeast competent cells were initially thawed at room temperature. The sonicated salmon sperm DNA (Agilec Technologies, US) was heated at 95 °C and put on ice for at least 2 minutes. The vials of 100 µl of yeast cells (*Saccharomyces cerevisiae* Hanson (MYA-906), MAT α ade6 can1 his3 leu2 trpl *URA3*) were centrifuged at 13 000 rpm for 30 seconds, and the supernatant was discarded. The amplified DNA (1 µg/ul) and vector (CMV –PBS-LTR-NL4-3Δgp160 URA) (200 ng/µl) were added to the cells, and distilled water was added to a final volume of 74 µl. The yeast cells were gently resuspended, and 10 µl of 10 mg/ml sonicated salmon sperm DNA, 36 µl of 1M Lithium Acetate (**see Appendix D**), and 240 µl of 50% sterile polyethylene glycol (PEG) (Sigma-Aldrich, US) (**see Appendix D**) was added to the cells and mixed by pipetting gently several times. The tubes were then initially incubated at 30 °C for 30 minutes, with subsequent heat shocking at 42 °C for 15 minutes. The tubes were then centrifuged at 13 000 rpm for 30 seconds. The supernatant was discarded, and the pelleted cells were resuspended in 100 µl of 1 X TE buffer. The cells were then plated on (C – Leu/5- FOA) selective plates (**see Appendix D**) and incubated at 30 °C for 3 - 4 days. The mixture of yeast cells with only the vector was used as a negative control. The construction of recombinant NL4-3 viruses using the yeast-gap repair method was done in Dr Manish Sagar's Laboratory at Brigham and Women's Hospital, Harvard Medical School, Cambridge, MA, USA.

University of Cape Town

4.2.5 Yeast DNA plasmid rescue and transformation

Two colonies were picked from each plate and grown in 2 ml of C – Leu culture media (**see Appendix D**) at 30 °C with shaking overnight. The overnight cultures were used to extract plasmid DNA from the yeast cells.

The overnight culture was transferred to a sterile 2 ml tube and the culture was centrifuged at 13 000 rpm for 5 minutes, after which the supernatant was discarded. The yeast pellets were resuspended in 200 µl of Yeast Lysis Buffer (**see Appendix D**). The acid washed baked beads (Sigma-Aldrich, US) (200 µl) were added together with 200 µl of phenol/chloroform (Sigma-Aldrich, US). The mixture was vortexed for 2 minutes, and then centrifuged at 13 000 rpm for a further 2 minutes. The top (aqueous) layer was removed and transferred to a new sterile 1.5 ml tube. The volume of the fluid was determined and 1/10 volume of 3 M Sodium Acetate (**see Appendix C**) was added to the fluid, after which two volumes of 100 % ethanol was then added. The mixture was placed on ice for 5 minutes and then centrifuged at 13 000 rpm for 10 minutes. The supernatant was discarded and 100 µl of 70 % ethanol was added to the pellet. The tubes were again centrifuged at 13 000 rpm for 5 minutes. The supernatant was discarded, and the pellet was air dried for 5 minutes and resuspension in 100 µl of distilled water.

University of Cape Town

4.2.6 Transformation of yeast plasmids into competent cells

The plasmid (2 µl) was added to a 50 µl vial of TOP 10® electrocompetent cells and transferred to electroporation curvettes (Fisher Scientific, US), and were then electroporated at 1.80 V using the Gene Pulser II electroporator (Bio-Rad, US). The SOC medium (Invitrogen, US) (250 µl)

was added to the cells, and incubated at 30 °C with shaking for 1 hour. The cells were then plated onto LB plates (**see Appendix D**) and stored at 30 °C overnight.

One colony was picked from each plate and cultured overnight at 30 °C in 5 ml of LB broth (**see Appendix D**) containing 100 µg/ml ampicillin.

4. 2.7 Plasmid DNA extraction

The overnight culture (4 ml) was used to extract plasmid DNA from the bacterial cells using the QIAprep Spin Miniprep Kit (QIAGEN, US), and this was done according to the manufacturer's instructions.

4. 2.8 Screening of recombinant plasmids by PCR amplification and sequencing

The recombinant plasmids were screened for the presence of *env* by PCR using Env-IF and Env-IR. The plasmids were also screened by DNA sequencing using primers Env- OF (5' GGC TTA GGC ATC TCC TAT GGC AGG AAG AA 3') and Env- OR (5' TAG CCC TTC CAG TCC CCC CCT TTT CTT TTA 3').

University of Cape Town

4. 2.9 Viral production by DNA transfection

The replication competent viruses were generated by co-transfection of HEK 293T cells with 1 µg recombinant NL4-3 containing the transmitted *env* and 1 µg of the helper plasmid CMV NL4-3 Gag4 using Fugene 6 Transfection Reagent (Roche, GER). As a positive control, 1µg of NL4-3 CMV GT (recombinant NL4-3 carrying the *env* gene) was transfected with the CMV

NL4-3 Gag4 helper plasmid. After 48 hours, the viral supernatant (2 ml) was harvested and clarified through a 0.45 µm filter and stored at - 80 °C.

4. 2.10 Viral RLU titration

DMEM medium (100 µl) was added to all the wells of 96 well costar plates. The viral samples (100 µl) were added in triplicate onto the plates, and up to four serial dilutions were made for each virus. TZM-bl cells (100 µl) (1 X 10^4 cells per well of media) was added to the plates and incubated at 37 °C, 5 % CO_2 for 48 hours.

After 48 hours, the media was removed from the plates and the cells were washed with 1 X PBS. The PBS was removed and 20 µl of the Tropix® Lysis Solution (Applied Biosystems, US) was added to each well and left at room temperature for 10 minutes. A 1:100 dilution of the Tropix® Galacton-Plus substrate solution was made using the Tropix® Galacto Reaction Buffer diluent and 70 µl was added to the plates. The plates were left on the shaker at room temperature for 1 hour. The Tropix® Accelerator II (100 µl) (Applied Biosystems, US) was added and then plates were read using Fluostar Optima (BMG Labtech, US) luminometer.

University of Cape Town

4. 2.11 Measuring entry efficiency of recombinant NL-4 viruses

The entry efficiency was measured using the method used in the pseudovirion assay (**See Chapter 2**). The recombinant NL4-3 viruses were generated by transfecting with NL4-3 CMV GT, which was used as a positive control (provided by the Sagar Laboratory, US).

4.2.12 Replication of recombinant viruses in PBMCs and monocyte-derived macrophages

4.2.12.1 PBMC isolation from blood

The lymphocytes and macrophages were isolated from blood obtained from donors at the Massachusetts General Hospital using Ficoll-gradient centrifugation method (**see Appendix C**). The lymphocytes were counted and stimulated with complete RPMI containing PHA and IL-2 for 24 - 48 hours, and subsequently maintained using RPMI containing fetal bovine serum, penicillin-streptomycin, L-glutamine, and HEPES (**see Appendix D**).

University of Cape Town

4.2.12.2 Viral expansion in activated lymphocytes

The recombinant viruses were expanded in lymphocytes in order to generate high titers. The activated lymphocytes (2 X 10^6 cells) were transferred to a sterile 15 ml tube, and centrifuged at 2500 rpm for 10 minutes. The supernatant was removed and the cells were resuspended with 500 µl of 293T cell supernatant and 50 µl of 1:100 diluted DEAE dextran and mixed by vortexing. The mixture of cells and virus were incubated for 2 hours at 37 °C, 5 % CO_2 for 2 hours.

The cells were centrifuged at 2500 rpm for 10 minutes. The supernatant was discarded and the cells were washed with 1X PBS. The washing step was repeated up to four times. The infected cells were then resuspended in 1 ml of RPMI with IL-2 and transferred to T-25 tissue culture

flasks. The flasks were placed vertically and incubated at 37 °C, 5 % CO_2 and the virus was collected on Day 4 and 7.

During every virus collection, the cells were centrifuged at 2500 rpm for 10 minutes. The supernatant was removed and aliquoted in cryovials and frozen at - 80 °C. The cells were then resuspended in fresh RPMI containing IL-2 and transferred to the flasks and incubated at 37 °C, 5 % CO_2 for further culturing.

4.2.12.3 Viral titration using B-gal staining method

The TZM-bl cells (2 X 10^6 cells per plate) were seeded in 48 well plates overnight. A 1:10 dilution of each virus was initially done, followed by a further three 1:10 serial dilutions, and the volume was made up to 200 µl per dilution. The media was removed from the TZM-bl cell plates, and 50 µl was added in triplicate for each dilution. The TZM-bl cells were placed in the 37 °C incubator for 2 hours, and 500 µl of DMEM was added to the cells. The plates were incubated at 37 °C, 5 % CO_2 for 48 hours.

After 48 hours, the media was removed and the cells were washed with 1X PBS. The fixing solution (**see Appendix D**) was added to the TZM-bl cells for 5 minutes. The fixing solution was removed and 200 µl of the staining solution (**see Appendix D**) was added to the cells. The plates were covered in aluminum foil and placed at 37 °C for 2 hours. The number of infectious viral particles was determined by directly counting β-galactosidase positive "blue" foci in the plates.

University of Cape Town

4. 2.12.4 Infection of activated lymphocytes by recombinant viruses

Activated lymphocytes and macrophages (10 X 10^6 cells/ ml) were transferred to a sterile 15 ml tube, and centrifuged at 2500 rpm for 10 minutes. The supernatant was removed and the cells were resuspended with 500 IP of 500 µl total volume of virus and 50 µl of DEAE dextran and mixed by vortexing. The mixture of cells and virus were incubated for 2 hours at 37 °C, 5 % CO_2 for 2 hours. The cells were thereafter centrifuged at 2500 rpm for 10 minutes. The supernatant was discarded and washed with 1X PBS. The washing step was repeated up to four times. The infected cells were resuspended in 1 ml of RPMI containing IL-2 and were transferred to T-25 tissue culture flasks. The flasks were placed vertically and incubated at 37 °C, 5 % CO_2 and the virus was collected on Days 4, 7, 10, and 14 days post infection.

During every virus collection, 500 µl of the cells were removed and placed in a 2 ml tube, and centrifuged at 2500 rpm for 10 minutes. The supernatant was removed and aliquoted in cryovials and frozen at -80 °C. Fresh RPMI with IL-2 (500 µl) was added to the plates and incubated at 37 °C, 5 % CO_2 for further culturing.

4.2.12.5 Infection of viruses in activated macrophages

The activated macrophages (1 X 10^6 cells/ml) were seeded in a 24 well plate after 7 days of activation. The medium was removed and the virus (5000 IP in 500 µl) was added to the cells, and incubated at 37 °C overnight. The media was removed from the cells and washed with 1X PBS four times, and replaced with fresh macrophage media (**see Appendix D**) for further culturing. The virus was collected at Days 4, 7, 10, and 14.

University of Cape Town

During every virus collection, 500 µl of the cells were removed and placed in a 2 ml tube, and centrifuged at 2500 rpm for 10 minutes. The supernatant was removed and aliquoted in cryovials and frozen at - 80 °C. Fresh macrophage media (500 µl) was added to the plates and incubated at 37 °C, 5 % CO_2 for further culturing.

4.2.13.6 Measuring replication using an in-house p24 ELISA

Flat bottomed 96 well plates (Nunc) were first coated with 100 µl of coating antibody (4 µl of HIV-Ig (50 mg/ml) per 10.5 ml of PBS and then add 100 µl per well). HIV-Ig is from NIH Catalog # 3957, and incubated at 37 °C overnight. The plates were washed by adding 300 µl of 1 X PBS (**see Appendix D**) to each well. The washing step was done up to six times. The plates were then blocked by adding 200 µl of blocking solution (**see Appendix D**), and incubated at 37 °C for 2 hours. The plates were washed using p24 Washing Solution (**see Appendix D**) using Labsystem Wellwash 4 Mk2 plate washer (Fisher Scientific, US), after which the 100 µl of the standards and the test samples were added to the plates. The p24 protein standard (Perkin Elmer) was used as the standard, with a starting concentration of 16 ng/ml that underwent two-fold serial dilutions to 0.25 ng/ml. The test samples were added, both undiluted and at a 1:10 dilution for each sample to a final volume of 300 µl. The sample diluent (**see Appendix D**) was used to make up the dilution of the standards and the test samples. The plates were incubated at 37 °C for 2 hours. The plates were washed again six times with washing solution. A 1:10 dilution of the primary antibody (mouse anti-p24 hybridoma 183 Clone H12-5C) (100 µl) was added to the plates, and were incubated at 37 °C for 1 hour. The plates were washed again with washing solution as outlined above. A 1:70 000 dilution of the secondary antibody (goat anti-mouse HRP; Sigma-Aldrich) was made, and 100 µl of secondary antibody was added to the plates. The

University of Cape Town

plates were then incubated at 37 °C for 1 hour. The plates were washed again six times with washing solution, and 100 µl of detection solution (**see Appendix D**) was added and before incubation in the dark for 30 minutes. The reaction was stopped by adding 50 µl of 2 M/4 N H_2SO_4, and the plates were read immediately at a wavelength of 490 nm and 490/650 nm using the Vmax Kinetic Microplate Reader (Molecular Devices, CA, US).

The p24 levels were also measured using the Alliance® HIV-1 p24 ELISA Kit (PerkinElmer, Waltham MA, US) according to the manufacturer's instructions. This kit was used to measure p24 levels from culture supernatants isolated during macrophage infection.

University of Cape Town

4.3. RESULTS

To directly compare the results obtained in the pseudovirus assay to virus replication using the IMCs, we used the transmitted/founder Envelope clones (**see Chapter 3**) to generate 10 *env* chimeric NL4-3 viruses representative of subtype C (n = 6), subtype D (n = 1), and AC (n = 1), CD (n = 2) recombinants **(Table 4.1)**. The *env* gene was amplified from the plasmid before being shuttled into a subtype B backbone CMV –PBS-LTR-NL4-3Δgp160 URA using the yeast gap repair recombinant method. The backbone contains the URA3 gene in place of the *env* gene, and contains 40 nucleotide sequences in both the 5' and 3' region that are homologous to the HIV-1 *env* target gene and a 'gap' after enzyme digestion with *PacI*. This allows for the replacement of URA3 gene with the target *env* gene. After the transformation of the yeast cells, the recombinant plasmid was selected on plates containing CMM – Leucine + 5 – flouro – 1, 2, 3, 6 – tetrahydro – 2, 6 – dioxo – 4 – pyrimidine carboxylic acid (FOA) (which is toxic to the yeast cells expressing URA3), thus allowing the growth of the yeast cells that contain the recombinant plasmid. In order to confirm the presence of the correct insert in the correct orientation, the plasmid was sequenced using the Env - IF and Env – IR primers, which are located on either side of the *env* gene. The plasmid was extracted from the yeast and shuttled into *E.coli* to produce many copies of the plasmid. The plasmids were thereafter transfected into 293T cells to generate the recombinant NL4-3 viruses. The titers and levels of entry efficiency were done using the TZM-bl cells.

University of Cape Town

4.3.1 Infection of the recombinant viruses in TZM-bl cells

In order to determine whether the recombinant viruses displayed similar infectivity in TZM-bl cells compared to pseudovirus restricted to a single round of infection, TZM-bl cells were infected with the equivalent of 50 ng/ml p24 replication competent viruses, and infection was measured after 48 hours using luminescence. Of the five viruses classified as having high entry efficiency using the pseudovirion assay, four also had high infectivity in the IMC assay, with the fifth recombinant virus carrying clone 390 (**recNL4-3_390**) having low infectivity. Similarly of five viruses classified as low entry efficiency using the pseudovirion assay, four has low infectivity in the IMC assay except for one virus (**recNL4-3_234**), with had high infectivity in the recombinant NL4-3 backbone system **(Fig. 4.1)**.

University of Cape Town

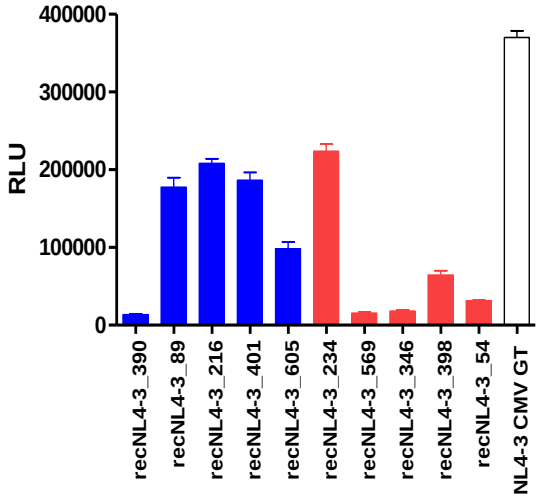


Figure 4.1 Comparing the infectivity of the recombinant viruses in TZM-bl cells normalized using p24. The plasmid NL4-3 CMV GT was used as a positive control. The clones that were previously characterized in the pseudovirus system as having high entry efficiency are colored in blue, while those having low entry efficiency are colored in red. This is a representative figure of two biological repeats. The infection was measured after 48 hours using luminescence. A RLU reading of 2.5 times above background was considered a positive infection and the RLU reading of wells containing cells only was considered as background signal. The NL4-3 virus was used as a positive control for the assay. The NL4-3 CMV GT virus was used as a positive control for the assay.

University of Cape Town

4.3.2 Viral expansion in activated PBMCs

Recombinant viruses were first used to infect activated PBMCs to expand their viral titers. The viruses were collected after four and seven days of infection and added to TZM-bl cells before β-galactosidase staining method was used to monitor the number of infected cells. Seven of the ten Envelope clones mediated infection after 4 days of viral expansion whereas by day 7, all of the T/F recombinant viruses infected TZM-bl cells. Therefore, viruses were expanded for 7 days in activated PBMCs prior to the replication assays **(Table 4.1)**

Table 4.1 Viral titers of the recombinant viruses after viral expansion. The number of infectious particles was determined using the β-galactosidase staining method after infecting TZM-bl cells for 48 hours. The recombinant viruses carrying Envelope clones with high entry efficiency in the pseudovirion assay are highlighted in grey.

Recombinant virus ID	Subtype	Day 4 virus titers (IP per µl)	Day 7 virus titers (IP per µl)
recNL4-3_390	**C**	**NI**	99
recNL4-3_89	CD	101.0	8 366.0
recNL4-3_216	C	74.6	2 080
recNL4-3_401	CD	67	2 200
recNL4-3_605	AC	126.3	22 000
recNL4-3_234	C	7.8	162.3
recNL4-3_569	**C**	**NI**	53
recNL4-3_346	C	11.7	96
recNL4-3_398	C	13	30
recNL4-3_54	**D**	**NI**	1.1

*NI – no infection observed

*****IP – infectious particles

Nomenclature: recombinant NL4-3_clone number

University of Cape Town

4.3.3 Replication of recombinant viruses in PBMCs

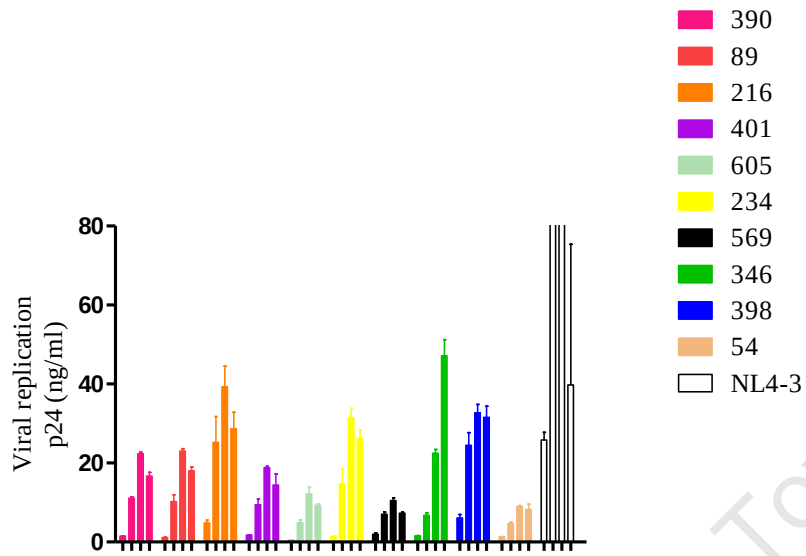
Equivalent viral titers (500 IP in 500 µl total volume) were used to infect PBMCs. Viruses were collected on days 4, 7, 10, and 14 after infection. The recombinant viruses from the 10 participants were assayed using two different PBMC donors.

Of the ten IMCs, only four have similar relative replicative capacity in both donors (IMCs expressing Envelope clones **398**, **216**, **401** and **54**). The virus **recNL4-3_346** showed the highest

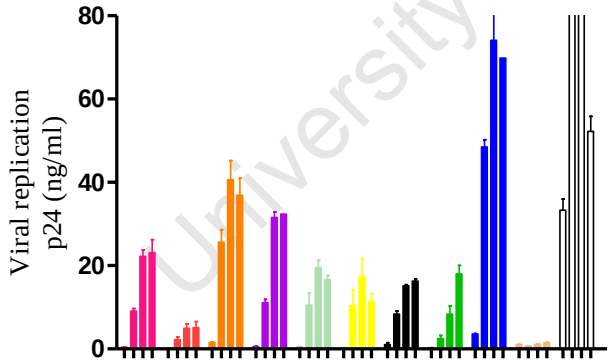
level of viral production, with peak infection at 47.1 ng/ml p24 at 14 days post infection in one PBMC donor, while **recNL4-3_398** showed the highest levels of virus production in another donor (with peak infection at 74.1 ng/ml p24 at day 10 post infection). Also, the recombinant virus **recNL4-3_54** replicated very poorly in one donor, and the replication improved by 4 to 5 fold in the second donor. This variability of infectivity can be attributed to differences in donor PBMCs which is well known to affect viral replication kinetics (Spira & Ho, 1995).

University of Cape Town

A



B

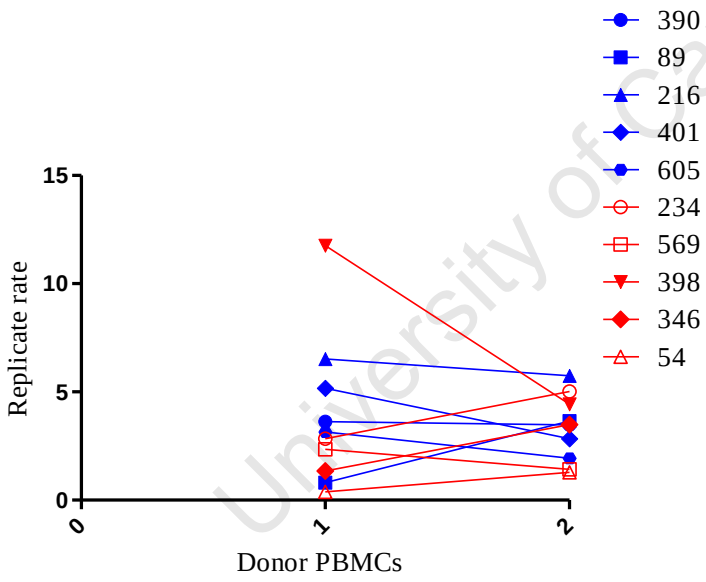


Days post-infection

University of Cape Town

Figure 4.2 Replicative capacity of recombinant viruses in PBMCs from two different donors. Each bar represents the time the viruses were collected postinfection: days 4, 7, 10, and 14. Viruses 390, 89, 216, 401, and 605 represent viruses with high entry efficiency in the pseudotype virus system, and viruses 234, 569, 346, 398, and 54 represents those with low entry efficiency. Viral replication was measured using p24 ELISA. The plasmid backbone pNL4-3 was used as a positive control.

In order to further compare the relative replication fitness of the recombinant viruses between the two donors, the replicative rate was determined based on the slope of the curve from day 4 to 10 for all the recombinant viruses and this was compared between the assays in both donors. The rationale for using these time points was based on the observation that the majority of the viruses showed exponential growth up to day 10 post infection before plateauing or decreasing at day 14 post-infection. The majority of the recombinant viruses had different replicative rates between the two donors, and there was no similar clustering in terms of replication rate between viruses that were previously characterized as having either high or low entry efficiency from both donors (**Fig. 4.3**).



University of Cape Town

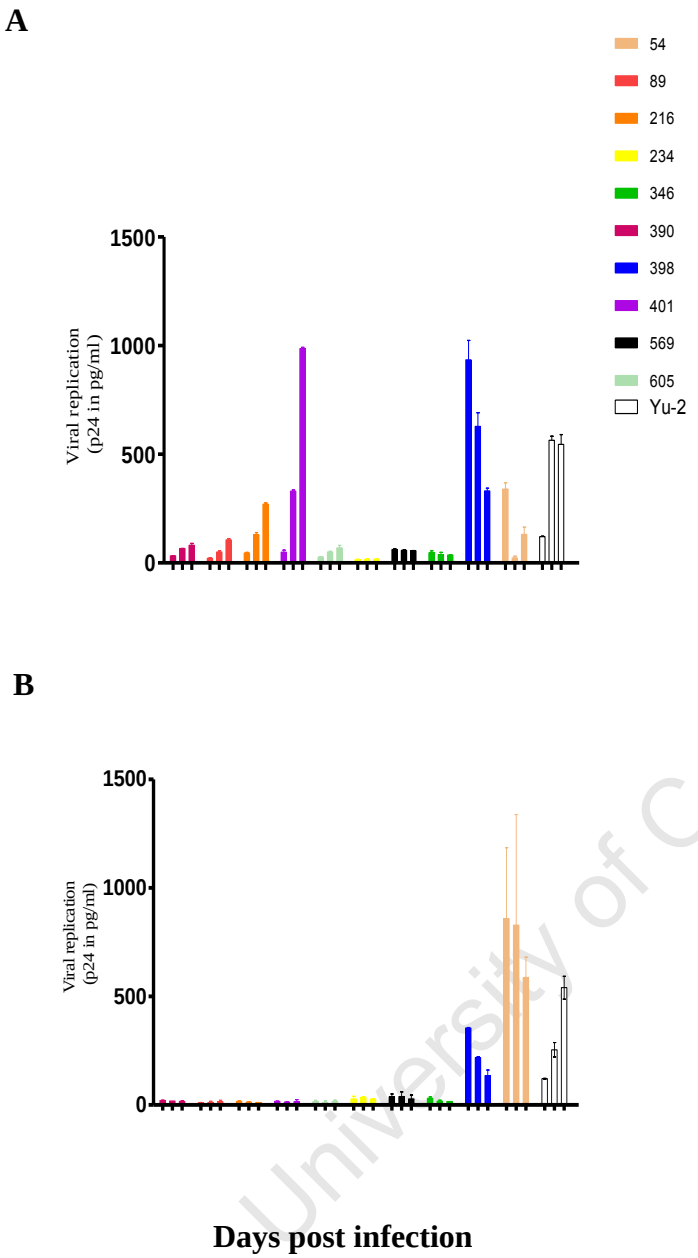
Figure 4.3 Comparing differences in replication rate of the T/F viruses in two different PBMC donors. The line between the points indicates the change in replication rate between the donors. The replicate rate was measured as the slope of the replication curve between days 4 and 10 of infection. The clones that were previously characterized in the pseudovirus system as having high entry efficiency are colored in blue, while those having low entry efficiency are colored in red.

Overall, the relative order of replication fitness in either donor did not mimic the entry efficiency of TZM-bl cells using the pseudovirion assay (**Fig. 4.2**).

4.3.4 Replication of recombinant viruses in monocyte-derived macrophages

Similarly, equivalent amounts of viruses (5000 IP per µl in 500 µl total volume) were used to infect monocyte-derived macrophages and viral titer was determined on days 4, 7, and 10. In the case of **recNL4-3_54** (which had the lowest number of infectious particles), the viruses was concentrated by ultracentrifugation before infection in macrophages. The macrophage tropic virus Yu-2 was used as a positive control. In comparison to PBMC infection, the levels of replication were very low in macrophages, with virus production only reaching 800 pg/ml. There was also extreme variation in the replicative capacity of the viruses in macrophages from the different donors, and the level of viral production in the macrophages fluctuated at various days post infection for viruses **recNL4-3_54** and **recNLA4-3_398**. The recombinant virus **recNL4-3_398** was the only virus that showed consistently high replication capacity in both donors. Viruses that replicated well in one of the PBMC donors (**recNL4-3_54**, **recNL4-3_216**, **recNL4-3_401**) did not replicate with the same efficiency in another donor, except for virus **recNL4-3_54** which had enhanced replication capacity when infected in another donor. All of these viruses (**recNL4-3_54**, **recNL4-3_216**, **recNL4-3_398 recNL4-3_401**) showed higher levels of replication in macrophages, but this varied between donors (**Fig. 4.4**). Overall, the level of viral replication was much lower in monocyte-derived macrophages compared to what was observed in PBMCs.

University of Cape Town



University of Cape Town

Figure 4.4 Replicative capacity of recombinant viruses in monocyte-derived macrophages from two different PBMC donors. Viruses **390**, **89**, **216**, **401**, and **605** represent viruses with high entry efficiency in the pseudotype virus system, and viruses **234**, **569**, **346**, **398**, and **54** represents those with low entry efficiency. Each bar represents the time post-infection when the viruses were collected: days 4, 7, and 10. Viral replication was measured using p24 ELISA. The macrophage tropic virus Yu-2 was used as a positive control.

4.4 DISCUSSION

Studies have shown that virions produced in the pseudovirus system do not always resemble the virus produced as an IMC (Ochsenbauer & Kappes, 2009), with one study demonstrating that differences in Envelope incorporation and cleavage of the envelope resulted in differences in infectivity and sensitivity to entry inhibitors (Provine *et al.*, 2009). This is partly because the 293T cell lines, which are used to produce pseudoviruses, produce high levels of unprocessed gp160 (Herrera *et al.*, 2005). Also, certain stages of Envelope processing and biosynthesis such as the addition of N- glycans to the *env* at a post-translational level (Willey *et al.*, 1996) and the incorporation of host proteins in the progeny virus (Bastiani *et al.*, 1997; Fortin *et al.*, 1997; Goonetilleke *et al.*, 2009a; Hioe *et al.*, 2001; Rizzuto & Sodroski, 1997) can affect viral infectivity and sensitivity to neutralization between viruses (Sawyer *et al.*, 1994; Bastiani *et al.*, 1997; Willey *et al.*, 1996; Zhang *et al.*, 1997). Other factors that affect the consistency in results between the PBMC and pseudovirion assays is the use of reagents like DEAE dextran or polybrene, the mode of host cell entry of the viruses (plasma membrane fusion compared to endocytosis) (Miyauchi *et al.*, 2010), as well as differences in CCR5 expression (Polonis *et al.*, 2008).

This study was done to compare the infectivity in TZM-bl cell lines of infectious chimeric viruses expressing the same T/F Envelopes as evaluated in the pseudovirion single cell replication assay, and to investigate replication of chimeric *env* IMCs in more biologically relevant conditions, including PBMCs and monocyte-derived macrophages. In order to measure replication capacity, the SGA-generated Envelope clones were shuttled into the NL4-3 backbone to generate recombinant viruses using the yeast gap repair method. For the majority (80%) of

University of Cape Town

the viruses, the infectivity (measured by luminescence in TZM-bl cells after 48 hours) followed the same trend as that was observed in the pseudovirus system: that is, viruses with higher infectivity generally were classified has having high pseudovirion *env* entry efficiency. This result suggests that entry efficiency using the pseudovirion assay, at least in the most part, was a good surrogate marker of infectivity. However, this observation was not upheld when PBMCs were used as target cells due to variation in PBMCs between donors with only 40% concordance in relative replication capacity between donors. This is not surprising as viral infection can vary by as much as 40 - fold between healthy donors (Spira & Ho, 1995), and this variability between donors has also been shown to affect the neutralizing activity of certain monoclonal antibodies (Binley *et al.*, 2004; Polonis *et al.*, 2008). This is mainly due to variation in expression levels of CD4 and CCR5 that exists between donors (Polonis *et al.*, 2008).

There were high levels of replication in $CD4^+$ T lymphocytes, and low levels of replication observed in monocyte-derived macrophages, supporting our findings using the PSV approach, as well as results from recent studies (Alexander *et al.*, 2010; Isaacman-Beck *et al.*, 2009; Salazar-Gonzalez *et al.*, 2009). The low levels of replication of transmitted viruses in macrophages suggest that these cells are not the critical target cells during HIV transmission (Walter *et al.*, 2005). Interestingly, one virus in this study the recombinant **recNL4-3 virus 54**, replicated more efficiently in macrophages than in PBMCs, which might suggest that this virus may be macrophage tropic. This virus had a subtype D envelope, was collected ~42 days post infection and, although it was found to use CCR5 and not CXCR4 as a co-receptor, it was resistant to both CD4 and CCR5 antagonists, and also to the fusion inhibitor (**Chapter 3**).

University of Cape Town

In this study, we would need to expand our evaluation to many more donors, use pooled PBMCs from different healthy donors, or use cell lines that more closely mimic natural infection. Evaluation of viral replication in PBMCs is important because $CD4^+$ T cells are the main targets for HIV infection, however the inter-assay variation between donors is a major limitation of this approach (Polonis *et al.*, 2008). Furthermore, the PBMC assays are labor intensive, expensive, and are not sufficient for high throughput analysis (Gauduin *et al.*, 1996; Souza *et al.*, 1997). An alternative approach is the use of suspension cells that have similar expression levels to $CD4^+$ T lymphocytes, because of their robust nature and high levels of reproducibility. This approach has been used in a number of studies to measure viral replication (Alexander *et al.*, 2010; Miura *et al.*, 2009b, 2010). Other methods that have been proposed include the incorporation of host cell proteins such as ICAMs, MHC class I and II molecules in 293T cells, which are proteins that the virus particles incorporate prior to budding from the primary cells (Cantin *et al.*, 2005), and the development of hybrid assays that use pseudoviruses to infect PBMCs and primary isolates to infect TZM-bl cells (Polonis *et al.*, 2008).

In conclusion, we find that these infectious chimeric viruses carrying Envelope from the T/F virus preferentially replicate in T cells and not macrophages, confirming observations using the Affinofiles / pseudovirus approach. Therefore, T/F viruses utilize the CCR5 coreceotor and require high levels of CD4 of entry. This was confirmed in our PBMC replication assay, which showed that T/F viruses replicate efficiently in T cells and not macrophages irrespective of the infecting subtype or recombinant form. These findings support recently published studies that have investigated the phenotypic properties of transmitted viruses (King *et al.*, 2013; Parrish *et al.*, 2013; Ping *et al.*, 2013). Using the recombinant NL4-3 virus system, we find that infectivity of TZM-bl largely tracked entry efficiency, supporting our hypothesis that virus entry is an

University of Cape Town

important virological determinant influencing viral load in infected individuals. However we were not able to validate our observation in PBMCs, and further work is required to determine if this is due to limitations of thePBMCassay.

Chapter 5: Summary and Conclusion

High HIV-1 diversity remains one of the major obstacles in the design of an effective vaccine. Vaccines need to target the incoming transmitted virus and prevent it from establishing infection. Most studies to date have focused on understanding the properties of subtype B and C T/F viruses, and there is limited information on the characteristics of viruses in cohorts from East Africa, which may differ in circulating subtypes, prevalence of STIs, and risk behaviours. In this thesis, we describe the diversity of circulating viruses in the Mbeya region of Tanzania, and characterize the genotypic and phenotypic properties of the T/F viral populations. Furthermore, we investigate the effect of host cell entry efficiency on disease progression. This study utilized samples from the HIV Superinfection Study (HISIS) Cohort, NIMR - Mbeya Medical Research Center, Tanzania.

It is known that the genital mucosa acts as a formidable barrier which usually allows only a single virus to be transmitted from the donor to the recipient. In subtype B and C cohorts in USA and southern/central Africa, studies have shown that this occurs in approximately 70 to 90% of cases (Abrahams et al., 2009; Haaland et al., 2009; Keele et al., 2008). In the HISIS cohort, women were infected with subtype A, C, D, or a number of unique intersubtype recombinants. Seventy three percent (16/22) were infected with a single virus based on *env* sequencing (Nofemela et al., 2011), which is very similar to the 76 % and 78 % that has been shown in subtype B and C cohorts respectively (Abrahams et al., 2009; Keele et al., 2008). These results provide further evidence of the genetic transmission bottleneck, irrespective of cohort and genetic subtype of the circulating viruses.

University of Cape Town

There has been a great deal of interest in the field to understand why certain viruses are selected over others to cross the mucosal barrier. It is well known that these viruses predominantly utilize the CCR5 receptor for entry (Berger *et al.*, 1999; Scarlatti *et al.*, 1997; Zhu *et al.*, 1993), and some studies in donor-recipient transmission pairs have shown that T/F variants have Envelope proteins that are neutralization sensitive relative to the donor (Derdeyn *et al.*, 2004). These viruses were shown to have shorter variable loop lengths and less N-glycosylation than the chronically infected individuals (Chohan *et al.*, 2005; Derdeyn *et al.*, 2004; Sagar *et al.*, 2006; Wu *et al.*, 2006). A number of unique signatures in transmitted viral sequences were also recently observed near the CD4 and CCR5 binding sites, the gp41 cytoplasmic domain, and the signal peptide when compared with the chronic viral sequences (Asmal *et al.*, 2011). Here we aimed to characterize the Envelope of transmitted viruses and to determine whether they share a common biological property. We found major differences in entry efficiency between the transmitted Envelope pseudoviruses, suggesting that the mucosal barrier in the host does not select for viruses with the same ability to enter cells. We found that high CD4 density on the target cells was critical for the entry of the transmitted viruses, with 70 % of viruses unable to infect cells with low levels of CD4, even at the highest levels of CCR5. This supports recently published data that suggests that the transmitted viruses are not macrophage - tropic (Alexander *et al.*, 2010; Ochsenbauer *et al.*, 2012; Salazar-Gonzalez *et al.*, 2009; King et al., 2013; Parrish et al., 2013; Ping et al., 2013). As the pseudovirus system may differ from viral infection *in vivo* in terms of *env* processing and incorporation, thus we validated these results using chimeric IMCs expressing the T/F *env* gene. We showed that viruses had a vast range of replicative fitness, and replicated efficiently in $CD4^+$ T cells and very poorly in macrophages. The preferential replication in $CD4^+$ T cells and not in macrophages occurs irrespective of the infecting subtype

University of Cape Town

or recombinant form. Therefore, despite being present at the site of infection, macrophages may not play a critical role during transmission.

Our study found that entry efficiency of T/F viruses was associated with viral load at 3 and 12 months post infection, and that this entry efficiency was related to CD4 and CCR5 binding affinity and, partly to Envelope incorporation and cell-cell fusion capacity. Thus, viruses that have Envelope with enhanced function may influence disease progression. The role of viral replicative fitness in disease progression has been supported by a number of other studies (Ball *et al.*, 2003; Miura *et al.*, 2010; Quiñones-Mateu *et al.*, 2000; Rangel *et al.*, 2003; Travers *et al.*, 2005); however, in addition to our study, there has only been one other subtype B study in elite suppressors have showed a link between Envelope function and disease progression (Lassen *et al.*, 2009).

In conclusion, the presence of multiple subtypes circulating in a region does not seem to affect the transmission population bottleneck. The characterization of the T/F viruses that established infection shows that host cell entry efficiency during transmission is dependent on both host and viral factors: host factors included cell surface CD4 and CCR5 receptor levels; while viral factors include CD4 and CCR5 affinity, and to some extent, Envelope processing or incorporation and cell-cell fusion capacity. Our study showed that CD4 and CCR5 affinity of T/F viruses at transmission can play an important role in enhanced host cell entry, which may affect viral loads and subsequent disease progression. Overall, this study has provided valuable insight into the viral and host factors that contribute to effective transmission by T/F viruses, and how these viral properties can influence the disease outcome of the infected individuals. In an intact epithelial barrier of a healthy individual, the macrophage and dendritic cells were initially

University of Cape Town

believed be the major target cells during transmission, whereby they capture and transfer the virus to the $CD4^+$ T cells, This would subsequently lead to the recruitment of more $CD4^+$ T cells to the site of infection, resulting in the propagation and dissemination of the virus to the neighbouring lymph nodes. Our study together with recent publications, show that macrophages are not an important target cell during HIV acquisition. The preferential replication of the transmitted viruses in the $CD4^+$ T cells suggests that these cells are the primary target cells during HIV transmission. Therefore, HIV crosses the mucosal barrier and establishes a small founder population. This population infects the target cells, which are the resting $CD4^+$ T cells, resulting in a local expansion and subsequent migration to the genital lymph nodes where more viruses will be produced.

University of Cape Town

APPENDICES

Appendix A: List of sequencing primers

Primer Name	HXB2 Location	Primer sequence
Env - AF	6202 - 6223	5' - GAA AGA GCA GAA GAC AGT GGC - 3'
Env - ER	6886 – 6900	5' - TTA GAA TCG CAT AAC CAG – 3'
Env - DF	7211 – 7226	5' - AGC ACA TTG TAA CAT TAG T – 3'
Env - AR	7783 – 7799	5' - TGC TGC TCC CAA GAA CCC AA – 3'
Env - BF	6829 – 6849	5' - TAA CAC AAG CCT TTG CCA AAG GT – 3'
Env – BR	6838 – 6856	5' - AAT TTC CTA AGG TCC CCT CTG A – 3'
Env – MF	7644 – 7661	5' – GCA GGA ATG AGG GAC AAT TGG – 3'
Env – NF	8104 – 8117	5' - TGA CCT GGA TGC AGT GG – 3'
Env – NR	8342 – 8342	5' – GGT GAG TAT CCC TGC CTA ACT CTA – 3'
Env - KR	8830 – 8843	5' - CTT ATA GCA GGC CAT CC – 3'
Env – 1A	5953 – 5982	5' – CAC CGG CTT AGG CAT CTC CTA TGG CAG GAA GAA – 3'
Env-1M	9068 – 9093	5' – TAG CCC TTC CAG TCC CCC CTT TTC TTT TA – 3'
Env – E15	7101 - 7121	5' – GTA GAA ATT AAT TGT ACA AGA CCC – 3'
Env -TUG	7862 - 7879	5' – GTC TGG TAT AGT GCA ACA GCA – 3'
Env - ZAR	7629 - 7652	5' – GTC CCT CAT ATC TCC TCC TCC AGG TCT – 3'
Env – JL74	8797 – 8817	5' – CTG TTC TAC CCA CTT GCC ACC CAT – 3'
Env – JL71	8797 – 8814	5' – TTT TGA CCA CTT GCC ACC CAT CAT – 3'
Env – JL89	7006 – 7025	5' – TCC TTC TGC TAG ACT GCC ATT TA – 3'
Env – Z1F	6330 – 6353	5' – TGG GTC ACA GTC TAT TAT GGG GTA CCT – 3'
Env – TUJ	8346 – 8362	5' – GGT GAG TAT CCC TGC CTA AC – 3'
Env – TUH	7914 – 7937	5' – GCC CCA GAC TGT GAG TTG CAA CAG ATG – 3'
Env–JL109	7669 – 7689	5' – GTG AAT TAT ATA AAA TAT AAA GTA G 3'
Env – E260	8523 – 8544	5' – TTC AGC TAC CAC CGA TTG AGA GAC T – 3'

University of Cape Town

Appendix B: HIV-1 *env* sequences

Genbank accession numbers

The Genbank accession numbers for the 210 *env* sequences are HQ659584 – HQ659642, HQ697934 – HQ697993, HQ697994 – HQ698053, and HQ688994 – HQ689024.

Appendix C: Isolation of PBMC using Ficoll-gradient centrifugation from blood

University of Cape Town

1. Sterilize a pair of scissors and cut open your buffy bag.
2. Empty the contents of the bag in one 50 ml centrifuge tube. There is no need to do wash for the remnants with PBS.
3. Make the volume up to the smallest volume dividable by 15 ml using HANKES E.g. if you have 40 ml of blood, make up to 60 ml (= 2 x 15 ml).
4. Dispense the blood in aliquots of 15 ml in 50 ml centrifuge tubes.
5. Make the volume up to 30 ml per tube using HANKES. Invert to mix.
6. Prepare new centrifuge tubes with Ficoll, one per tube of blood. Ficoll is light sensitive!
7. Use 2 parts of Ficoll for 3 parts of blood. Thus, for 30 ml of diluted blood use 20 ml of Ficoll per tube.

   Carefully add the 30 ml of blood in one tube of Ficoll. Try to incline the tube so that you have a bigger surface of Ficoll and layer the blood gently. Starting at an angle, gradually bring the tube back to a vertical position as layering progresses.

8. Centrifuge the tubes at 1500 rpm at room temperature for 30 min. Please note the following:

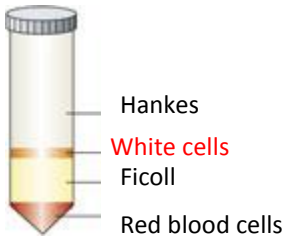
   - Balance the centrifuge precisely as any vibrations will disturb the separation.

   -Use slow acceleration.

   - Disable the centrifuge brake.

(this will add about 15 min for deceleration).

9. Prepare a new set of 50 ml blue-top tubes.
10. After centrifugation, remove the tubes slowly to avoid disturbing the separation. The contents of the tube should look as in the diagram below.



11. Aspirate off most of the HANKES layer, until it is about 1-2 cm above the white cells layer.
12. Carefully select the white cell layer by pipetting it up in a circular movement.
    It is ok if you take some HANKES and Ficoll.
13. Transfer the selected fraction into a new 50 ml tube.
14. Wash cells by topping up the volume to 50 ml with HANKES and centrifuging for 10 min at 1500 rpm (you can put the centrifuge brake back on).
15. Decant supernatant.
16. Repeat wash (resuspend the cell pellet in HANKES and repeat step 14).
17. Decant supernatant.
18. Pool all the cells from all the 6 tubes in 1 tube with 40 ml of R-20 medium.
19. Sample the cell suspension and make two serial 1:10 dilutions of it in trypan-blue dye.
20. Count the cells and if you need to activate them, bring them to a final concentration of 2 x 10^6/ ml in RPMI complete and add PHA and IL-2:
    a. PHA add 1µl per 1 ml
    b. IL-2 add 1µl per 5 ml,

and incubate them at 37 °C for 24 h.

21. Alternatively, proceed to monocyte / lymphocyte separation.

University of Cape Town

Monocyte Isolation

1. Make a percoll (Amersham Biosciences) gradient. Percoll should be at room temperature.
2. Make "Percoll Solution": take 18 ml Percoll (product) and add 2 ml 1.5M NaCl.
   Make "Denser Solution": 12 ml Percoll Solution and add 8 ml 3.8 % Citrate in PBS.

   Make "Less Dense Solution": 8 ml Percoll Solution and add 8 ml 3.8 %Citrate in PBS.

   Make up Resuspension Medium (which consists of the following):

   - 12.6 ml RPMI (not complete)

   - 1.2 ml human serum

   - 1.2ml 3.8 % Citrate in PBS.

3. Gently layer 16 ml of Less Dense Solution on top of 20 ml of Denser Solution.
4. Resuspend the washed PBMC in Resuspension Medium (use all volume).
5. Gently layer PBMC on Less Dense/Denser layered solution
6. Spin @ 400 RCF X (i.e. ~1.5 rpm) for 30 minutes with slow acceleration and slow brake.
7. Take off layer between less and dense gradient, which contains monocytes. The cells at the bottom are lymphocytes.
8. Wash both the monocytes and the lymphocytes with HANKES.

To differentiate monocytes to macrophages (adherent cells):

Put monocytes in Macrophage media and grow for 7 days. Change media on Day 4.

Seed the macrophages directly in the plate that you will use for your experiment.

University of Cape Town

Macrophage media	Total Volume 50 ml
Macrophage SFM Medium (Gibco)	41.5 ml
10% Human Serum (heat-inactivated 55°C, 1h)	5 ml
5% FBS (ready heat-inactivated)	2.5 ml
1% Glutamine	500 µl
1% Pen-Strep	500 µl

University of Cape Town

Appendix D: Buffers and solutions

- Pseudovirion Western Blotting

10X SDS running buffer

30 g Trizma (250mM)

114 g glycine (2M)

50 ml 20% SDS

Make up to 1 liter with ddH_2O

10X TBS

24.2 g Tris (200mM)

80 g NaCl

Add 800 ml ddH_2O. pH to 7.6 with HCl. Make up to 1 liter with ddH_2O

4X SDS loading buffer

2.5 ml 1M Tris pH 6.8

1 ml 20% SDS

2 ml β-mercaptoethanol

4 ml glycerol

0.5 ml Bromophenol blue

Store at 4ºC

Block/Wash Buffer

1 liter 1X TBS

40 g low fat milk powder

5 ml Tween-20

Store at 4 ºC (Short term)

Transfer Buffer

3.03 g Trizma base

14.4 g Glycine

Add 1 liter ddH_2O and store at room temperature

Ponceau S stock solution

2 g Ponceau S

30 g trichloroacetic acid

30 g 5-sulfosalicyclic acid

Add ddH_2O to 100 ml

For working stock, dilute 1:10. Reusable

University of Cape Town

10% SDS separating gel (for 2 gels)

5 ml 30 % acrylamide mix (37:5:1)

5.6 ml 1M Tris pH 8.8

75 µl 20% SDS

4.36 ml H_20

40 µl 20% ammonium persulphate

20 µl TEMED

5% SDS stacking gel (for 2 gels)

0.85 ml 30 % acrylamide mix (37:5:1)

0.625 ml 1M Tris pH 6.8

25 µl 20% SDS

3.5 ml H_20

10 µl 20% ammonium persulphate

2.5 µl TEMED

- Yeast gap repair

Luria- Betani (LB) Broth

Add to 1 liter of ddH_2O the following:

10 g tryptone

10 g NaCl

5 g Yeast Extract

Adjust the pH with NaOH pellets

If antibiotic media is required, add after autoclaving.

University of Cape Town

Yeast Amino Acid Dropout Media (YEPD)

Add 6.7 g Yeast Nitrogen Base, 6.7 g CSM-LEU to 500 ml ddH_20.

Mix and autoclave.

Add 20 g dextrose to 500 ml ddH20. Mix the solutions to make up to 1 liter.

YEPD Broth

Add to 1 liter ddH_20 the following:

10 g Yeast Extract

20 g Peptone

20 g Dextrose

Mix and autoclave.

LB Amp Plates

Add 40 g LB Agar mix to 1 liter of ddH_20.

Adjust pH to 7 with NaOH pellets

Mix and autoclave

Cool to 55 °C in waterbath.

Add 1 ml of $Amp^{100.}$ Mix and pour into plates.

Add the plates to dry at room temperature before storing at 4 °C.

University of Cape Town

Yeast Amino Acid Dropout plates + FOA

1. To 500 ml ddH_20 add the following:

20 g Agar

6.7 g Yeast Nitrogen Base with Ammonium sulfate

6.7 g CSM-LEU (Complete Supplement Mixture minus Leu)

Mix and then autoclave.

2. To 350 ml ddH20 add the following:

20 g dextrose

1 g 5-FOA

Mix and check the pH of the solution.

Allow the plates to cool at room temperature in the dark due to the possible inactivation of 5 - FOA by light.

- Yeast plasmid rescue

10 X TE Buffer

10 ml 1M Tris-HCl

2 ml 0.5M EDTA pH 8.0

Add 988 ml ddH20. Store at room temperature.

1 M Lithium Acetate

Add 10.2 g Lithium Acetate to 100ml ddH_20

50 % PEG

Add 25 g to 30 ml ddH20 while stirring. Add 20 ml ddH20, autoclave, and store at room temperature.

University of Cape Town

Yeast Lysis Buffer

0.1 M Tris-HCl (pH 8.0)
50 mM EDTA

1 % SDS

3 M Sodium Acetate

20.4 g sodium acetate

6 ml acetic acid

Add to 50 ml ddH_20

- Replication assays in PBMCs and macrophages

Complete DMEM

Remove 70 ml DMEM from the bottle, and add the following:

50 ml FBS

10 ml Pen Strep

5 ml L-glutamine

5 ml HEPES buffer

Complete RPMI

Remove 70 ml RPMI from the bottle, and add the following:

50 ml FBS

10 ml Pen Strep

5 ml L-glutamine

5 ml HEPES buffer

University of Cape Town

- PCR and agarose gel electrophoresis

50 X TAE

To 600 ml ddH_20 add the following:

242 g Tris Base

57.1 ml glacial acetic acid

100 ml 0.5 EDTA (pH 8.0)

Adjust the volume to 1 liter using ddH_20

0.8% agarose

Weigh 0.4 g agarose and add to 100ml Schoot bottle. Add 50 ml 1X TAE. Boil in microwave and leave it to cool.

- Viral titration assays

Fixing solution

To 1 liter of ddH_20 add the following:

10 ml formaldehyde (1 %)

2 ml glutaraldehyde (0.2 %)

Staining solution (for one plate)

Add the following to a 50 ml conical tube:

9.5 ml 1 X PBS

200 ul 0.2 M Potassium ferrocyanide (light sensitive)

200 µl 0.2 M Potassium ferricyanide (light sensitive)

University of Cape Town

20 µl 2 M $MgCl_2$

0.2 M Potassium ferrocyanide 100 ml

Formula weight = 422.41 g/mol

Add 8.45 g potassium ferrocyanide to 100 ml ddH_20

0.2 M Potassium ferricyanide 100 ml

Formula weight = 329.26 g/mol

Add 6.59 g to 100 ml ddH_20

- In-house p24 ELISA

Coating solution

5 % Newborn Calf Serum (NCS): Dilute 1 ml of NCS in 19 ml PBS.

Washing solution

To a 1 liter bottle, add 1 liter 1 X PBS and 2 ml Tween-20

Sample Diluent

To 500 ml of sterile PBS, add 50 ml NCS and 2.5 ml of Triton X-100.

Store at -20 °

Detection Solution

For one plate, add 5.25 ml of TMB peroxidase substrate and 5.25 ml of TMB peroxidase solution.

Stop solution

In a 500 ml bottle, add 53.3 ml of neat H_2SO_4 to 446.7 ml ddH_20.

University of Cape Town

References

Abel, K., Rocke, D. M., Chohan, B., Miller, C. J. & Fritts, L. (2005). Temporal and Anatomic Relationship between Virus Replication and Cytokine Gene Expression after Vaginal Simian Immunodeficiency Virus Infection Temporal and Anatomic Relationship between Virus Replication and Cytokine Gene Expression after Vaginal Simian Immunodeficiency Virus Infection. *Journal of Virology* **79**, 12164-72

Abraha, A., Nankya, I. L., Gibson, R., Demers, K., Tebit, D. M., Johnston, E., Katzenstein, D., Siddiqui, A., Herrera, C. & other authors. **(2009).** CCR5- and CXCR4-tropic subtype C human immunodeficiency virus type 1 isolates have a lower level of pathogenic fitness than other dominant group M subtypes: implications for the epidemic. *Journal of Virology* **83**, 5592–605.

Abrahams, M.-R., Anderson, J. a, Giorgi, E. E., Seoighe, C., Mlisana, K., Ping, L.-H., Athreya, G. S., Treurnicht, F. K., Keele, B. F. & other authors. **(2009).** Quantitating the multiplicity of infection with human immunodeficiency virus type 1 subtype C reveals a non-poisson distribution of transmitted variants. *Journal of Virology* **83**, 3556–67.

Allan, J.S. (1991). Receptor- mediated activation of immunodeficiency viruses in viral fusion. *Science* **252**: 1322 - 1323.

Allan, J.S., Strauss, J., Duck, D.W. (1990). Enhancement of SIV infection with soluble receptor molecules. *Science* **247**: 1084 - 1088.

Von Adrian, UH; Mackay, C. (2000). T-cell function and migration: Two sides of the same coin. *New England Journal of Medicine* **343**, 1020–34.

Alexander, M., Lynch, R., Mulenga, J., Allen, S., Derdeyn, C. a & Hunter, E. (2010). Donor and recipient envs from heterosexual human immunodeficiency virus subtype C transmission pairs require high receptor levels for entry. *Journal of Virology* **84**, 4100–4.

Alizon, S., Von Wyl, V., Stadler, T., Kouyos, R. D., Yerly, S., Hirschel, B., Böni, J., Shah, C., Klimkait, T. & other authors. **(2010).** Phylogenetic approach reveals that virus genotype largely determines HIV set-point viral load. *PLoS pathogens* **6**, e1001123.

Arie, K. K., Abraha, A., Quin, M. E., Kestens, L., Vanham, G. & Arts, E. J. (2005). The Replicative Fitness of Primary Human Immunodeficiency Virus Type 1 (HIV-1) Group M , HIV-1 Group O , and HIV-2 Isolates. *Journal of Virology* **79**, 8979–8990.

Arroyo, M. a, Hoelscher, M., Sateren, W., Samky, E., Maboko, L., Hoffmann, O., Kijak, G., Robb, M., Birx, D. L. & McCutchan, F. E. (2005). HIV-1 diversity and prevalence differ between urban and rural areas in the Mbeya region of Tanzania. *AIDS* **19**, 1517–24.

University of Cape Town

Arthos, J; Cicala, C; Martinelli, E; Macleod, K; Van Ryk, D; Wei, D; Xiao, Z; Veenstra, TD; Conrad, TP; Lempicki, RA; McLaughlin, S; Pascussio, M; Gopaul, R; McNally, J; Cruz, CC; Censoplano, N; Chung, E; Reitano, KN; Kottilil, S; Goode, DJ; Fauci, S. (2008). HIV-1 envelope protein binds to and signals through intergrin alphabeta7, the gut mucosal homing receptor for peripheral T cells. *Nature Immunology* **9**, 301–9.

Asmal, M., Hellmann, I., Liu, W., Keele, B. F., Perelson, A. S., Gnanakaran, S., Daniels, M., Haynes, B. F., Korber, B. T. & other authors. **(2011).** A Signature in HIV-1 Envelope Leader Peptide Associated with Transition from Acute to Chronic Infection Impacts Envelope Processing and Infectivity. *PLOS One* **6**, e23678

Bachrach, E., Dreja, H., Lin, Y.-L., Mettling, C., Pinet, V., Corbeau, P. & Piechaczyk, M. (2005). Effects of virion surface gp120 density on infection by HIV-1 and viral production by infected cells. *Virology* **332**, 418–29.

Baeten, J. M., Chohan, B., Lavreys, L., Chohan, V., McClelland, R. S., Certain, L., Mandaliya, K., Jaoko, W. & Overbaugh, J. (2007). HIV-1 subtype D infection is associated with faster disease progression than subtype A in spite of similar plasma HIV-1 loads. *The Journal of infectious diseases* **195**, 1177–80.

Bakri, Y., Schiffer, C., Zennou, V., Charneau, P., Kahn, E., Benjouad, a, Gluckman, J. C. & Canque, B. (2001). The maturation of dendritic cells results in postintegration inhibition of HIV-1 replication. *Journal of immunology* **166**, 3780–8.

Ball, S. C., Abraha, A., Collins, K. R., Andre, J., Baird, H., Quiñones-mateu, M. E., Murray, M., Richard, N., Zimmerman, P. A. & other authors. **(2003).** Comparing the Ex Vivo Fitness of CCR5-Tropic Human Immunodeficiency Virus Type 1 Isolates of Subtypes B and C Comparing the Ex Vivo Fitness of CCR5-Tropic Human Immunodeficiency Virus Type 1 Isolates of Subtypes B and C. *Journal of Virology* **77**, 1021-38.

Bastiani, L., Laal, S., Kim, M. & Zolla-Pazner, S. (1997). Host cell-dependent alterations in envelope components of human immunodeficiency virus type 1 virions. *Journal of Virology* **71**, 3444–50.

Basu, D., Kraft, C. S., Murphy, M. K., Campbell, P. J., Yu, T., Hraber, P. T., Irene, C., Pinter, A., Chomba, E. & other authors. **(2012).** HIV-1 subtype C superinfected individuals mount low autologous neutralizing antibody responses prior to intrasubtype superinfection. *Retrovirology* **9**, 76

Baudin, a, Ozier-Kalogeropoulos, O., Denouel, a, Lacroute, F. & Cullin, C. (1993). A simple and efficient method for direct gene deletion in Saccharomyces cerevisiae. *Nucleic acids research* **21**, 3329–30.

University of Cape Town

Berger, E. a, Murphy, P. M. & Farber, J. M. (1999). Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annual review of immunology* **17**, 657–700.

Bernstein, H. B., Tucker, S. P., Hunter, E., Schutzbach, J. S. & Compans, R. W. (1994). envelope glycoprotein is modified by O-linked Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Is Modified by 0-linked Oligosaccharides. *Journal of Virology* **68**, 463-68.

Binley, J. (2009). Specificities of broadly neutralizing anti-HIV-1 sera. *Current opinion in HIV and AIDS* **4**, 364–72.

Binley, J. M., Wrin, T., Korber, B., Zwick, M. B., Wang, M., Chappey, C., Stiegler, G., Zolla-pazner, S., Katinger, H. & other authors. **(2004).** Comprehensive Cross-Clade Neutralization Analysis of a Panel of Anti-Human Immunodeficiency Virus Type 1 Monoclonal Antibodies. *Journal of Virology* 78, 13232-52.

Blay, W. M., Kasprzyk, T., Misher, L., Richardson, B. a & Haigwood, N. L. (2007). Mutations in envelope gp120 can impact proteolytic processing of the gp160 precursor and thereby affect neutralization sensitivity of human immunodeficiency virus type 1 pseudoviruses. *Journal of Virology* **81**, 13037–49.

Blish, C. A., Dogan, O. C., Jaoko, W., Mcclelland, R. S., Mandaliya, K., Odem-davis, K. S., Richardsonb, B. A. & Overbaugh, J. (2012). Research Letters 643–651.

Borrow, P; Lewicki, H; Wei, X; Howitz, MS; Peffer, N; Meyers, H; Nelson, JA; Gairin, JE; Hahn, BH; Oldstone, MB; Shaw, G. (1997). Antiviral pressure exerted by HIV-1-specific cytotoxic T lymphocytes (CTLs) during primary infection demonstrated by rapid selection of CTL escape virus. *Nature medicine* **3**, 205–11.

Brasseur, R; Cornet, B; Burny, A; Vandenbranden, M; Ruysschaert, J. (1988). Mode of inserion into a lipid membrane of the N-terminal HIV gp41 peptide segment. *AIDS Research and Human Retroviruses* **4**, 83–90.

Brockman, M. a, Schneidewind, A., Lahaie, M., Schmidt, A., Miura, T., Desouza, I., Ryvkin, F., Derdeyn, C. a, Allen, S. & other authors. **(2007).** Escape and compensation from early HLA-B57-mediated cytotoxic T-lymphocyte pressure on human immunodeficiency virus type 1 Gag alter capsid interactions with cyclophilin A. *Journal of Virology* **81**, 12608–18.

Brockman, M. a, Brumme, Z. L., Brumme, C. J., Miura, T., Sela, J., Rosato, P. C., Kadie, C. M., Carlson, J. M., Markle, T. J. & other authors. **(2010).** Early selection in Gag by protective HLA alleles contributes to reduced HIV-1 replication capacity that may be largely compensated for in chronic infection. *Journal of Virology* **84**, 11937–49.

University of Cape Town

Brown, B. K., Wieczorek, L., Sanders-Buell, E., Rosa Borges, A., Robb, M. L., Birx, D. L., Michael, N. L., McCutchan, F. E. & Polonis, V. R. (2008). Cross-clade neutralization patterns among HIV-1 strains from the six major clades of the pandemic evaluated and compared in two different models. *Virology* **375**, 529–38.

Bunnik, E. M., Pisas, L., Van Nuenen, A. C. & Schuitemaker, H. (2008). Autologous neutralizing humoral immunity and evolution of the viral envelope in the course of subtype B human immunodeficiency virus type 1 infection. *Journal of Virology* **82**, 7932–41.

Bures, R., Morris, L., Williamson, C., Deers, M., Fiscus, S. A., Abdool-karim, S., Montefiori, D. C. & Ramjee, G. (2002). Regional Clustering of Shared Neutralization Determinants on Primary Isolates of Clade C Human Immunodeficiency Virus Type 1 from South Africa Regional Clustering of Shared Neutralization Determinants on Primary Isolates of Clade C Human Immunodeficiency . *Journal of Virology* **76**, 2233-44.

Burton, DR; Desrosiers, RC; Doms, RW; Koff, WC; Kwong, PD; Moore, JP; Nabel, GJ; Sodroski, J; Wilson, IA; Wyatt, R. (2004). HIV vaccine design and the neutralizing antibody problem. *Nature Immunology* **5**, 233–236.

Cantin, R., Méthot, S. & Tremblay, M. J. (2005). Plunder and Stowaways: Incorporation of Cellular Proteins by Enveloped Viruses. *Journal of Virology* **79, 6577-87**.

Cao, J., Sullivan, N., Desjardin, E., Parolin, C., Robinson, J., Wyatt, R., Cao, J. I. E., Sullivan, N., Desjardin, E. & other authors. **(1997).** Replication and neutralization of human immunodeficiency virus type 1 lacking the V1 and V2 variable loops of the gp120 envelope. *Journal of Virology* **71**, 9808-12.

Carrington, M. (1999). HLA and HIV-1: Heterozygote Advantage and B*35-Cw*04 Disadvantage. *Science* **283**, 1748–1752.

Carrington, M. & O'Brien, S. J. (2003). The influence of HLA genotype on AIDS. *Annual review of medicine* **54**, 535–51.

Chan, D. C., Fass, D., Berger, J. M. & Kim, P. S. (1997). Core structure of gp41 from the HIV envelope glycoprotein. *Cell* **89**, 263–73.

Checkley, M. A., Luttge, B. G. & Freed, E. O. (2011). HIV-1 envelope glycoprotein biosynthesis, trafficking, and incorporation. *Journal of molecular biology* **410**, 582–608. Elsevier B.V.

Chen, B., Vogan, E. M., Gong, H., Skehel, J. J., Wiley, D. C. & Harrison, S. C. (2005). Determining the structure of an unliganded and fully glycosylated SIV gp120 envelope glycoprotein. *Structure* **13**, 197–211.

University of Cape Town

Chohan, B., Lang, D., Sagar, M., Korber, B., Lavreys, L., Richardson, B. & Overbaugh, J. (2005). Selection for Human Immunodeficiency Virus Type 1 Envelope Glycosylation Variants with Shorter V1-V2 Loop Sequences Occurs during Transmission of Certain Genetic Subtypes and May Impact Viral RNA Levels. *Journal of Virology* **79**, 6528–6531.

Chopera, D. R., Woodman, Z., Mlisana, K., Mlotshwa, M., Martin, D. P., Seoighe, C., Treurnicht, F., De Rosa, D. A., Hide, W. & other authors. **(2008).** Transmission of HIV-1 CTL escape variants provides HLA-mismatched recipients with a survival advantage. *PLoS pathogens* **4**, e1000033.

Chopera, D. R., Cotton, L. a, Zawaira, A., Mann, J. K., Ngandu, N. K., Ntale, R., Carlson, J. M., Mlisana, K., Woodman, Z. & other authors. **(2012).** Intersubtype differences in the effect of a rare p24 gag mutation on HIV-1 replicative fitness. *Journal of Virology* **86**, 13423–33.

Cicala, C., Martinelli, E., McNally, J. P., Goode, D. J., Gopaul, R., Hiatt, J., Jelicic, K., Kottilil, S., Macleod, K. & other authors. **(2009).** The integrin alpha4beta7 forms a complex with cell-surface CD4 and defines a T-cell subset that is highly susceptible to infection by HIV-1. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 20877–82.

Cicala, C., Arthos, J. & Fauci, A. S. (2011). HIV-1 envelope, integrins and co-receptor use in mucosal transmission of HIV. *Journal of translational medicine* **9 Suppl 1**, S2. BioMed Central Ltd.

Cilliers, T., Moore, P., Coetzer, M. & Morris, L. (2005). In vitro generation of HIV type 1 subtype C isolates resistant to enfuvirtide. *AIDS research and human retroviruses* **21**, 776–83.

Clapham, P.R., McKnight, A., Weiss, R.A. (1992). Human Immunodeficiency virus type 2 infec tion and fusion of CD4 negative human cell lines: induction and enhancement by soluble CD4. *Journal of Virology* **66**: 3531 - 3537.

Cohen, M.S., Shaw, G.M., McMichael, A.J., & Haynes, B.F. (2011). Acute infection. *New England Journal of Medicine* **364**, 1943-54.

Connor, R. I., Sheridan, K. E., Ceradini, D., Choe, S. & Landau, N. R. (1997). Change in coreceptor use correlates with disease progression in HIV-1--infected individuals. *The Journal of experimental medicine* **185**, 621–8.

Corbet, S., Müller-trutwin, M. C., Versmisse, P., Delarue, S., Ayouba, A., Lewis, J., Martin, P., Brun-vezinet, F., Barre-sinoussi, F. & Mauclere, P. (2000). env Sequences of Simian Immunodeficiency Viruses from Chimpanzees in Cameroon Are Strongly Related to Those of Human Immunodeficiency Virus Group N from the Same Geographic Area env

University of Cape Town

Sequences of Simian Immunodeficiency Viruses from Chimpanzees in Cameroon. *Journal of Virology* **74**, 529-34.

Cormier, E. G. & Dragic, T. (2002). The Crown and Stem of the V3 Loop Play Distinct Roles in Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Interactions with the CCR5 Coreceptor. *Journal of Virology* **76**, 8953–8957.

Cormier, E. G., Tran, D. N. H., Yukhayeva, L., Olson, W. C. & Irol, J. V. **(2001).** Mapping the Determinants of the CCR5 Amino-Terminal Sulfopeptide Interaction with Soluble Human Immunodeficiency Virus Type 1 gp120-CD4 Complexes. *Journal of Virology* **75**, 5541–5549.

Cortez, V., Odem-Davis, K., McClelland, R. S., Jaoko, W. & Overbaugh, J. (2012). HIV-1 superinfection in women broadens and strengthens the neutralizing antibody response. *PLoS pathogens* **8**, e1002611.

Crawford, H., Prado, J. G., Leslie, A., Hué, S., Honeyborne, I., Reddy, S., Van der Stok, M., Mncube, Z., Brander, C. & other authors. **(2007).** Compensatory mutation partially restores fitness and delays reversion of escape mutation within the immunodominant HLA-B*5703-restricted Gag epitope in chronic human immunodeficiency virus type 1 infection. *Journal of Virology* **81**, 8346–51.

Crawford, H., Lumm, W., Leslie, A., Schaefer, M., Boeras, D., Prado, J. G., Tang, J., Farmer, P., Ndung'u, T. & other authors. **(2009).** Evolution of HLA-B*5703 HIV-1 escape mutations in HLA-B*5703-positive individuals and their transmission recipients. *The Journal of experimental medicine* **206**, 909–21.

Curlin, M. E., Zioni, R., Hawes, S. E., Liu, Y., Deng, W., Gottlieb, G. S., Zhu, T. & Mullins, J. I. (2010). HIV-1 envelope subregion length variation during disease progression. *PLoS pathogens* **6**, e1001228.

Deen, K., McDougal, T.S., Inacker, R., Folena-Wasserman, G., Arthos, J., Rosenberg, J., Maddon, P., Axel, R., Sweet, R. (1988). A soluble form of CD4 (T4) ptotein inhibits AIDS virus infection. *Nature* **331**: 82-84.

Derdeyn, C. A., Decker, J. M., Sfakianos, J. N., Wu, X., Brien, W. A. O., Ratner, L., John, C., Shaw, G. M. & Hunter, E. (2000). Sensitivity of Human Immunodeficiency Virus Type 1 to the Fusion Inhibitor T-20 Is Modulated by Coreceptor Specificity Defined by the V3 Loop of gp120. *Journal of Virology* **74,** 2358-67

Derdeyn, C. A., Decker, J. M., Bibollet-ruche, F., Mokili, J. L., Muldoon, M., Denham, S. A., Heil, M. L., Kasolo, F., Musonda, R. & other authors. **(2004).** Envelope-Constrained After Heterosexual Transmission. *Science* **303**, 2019–2022.

University of Cape Town

Doms, R. W. & Moore, J. P. (2000). HIV-1 membrane fusion: targets of opportunity. *The Journal of cell biology* **151**, F9–14.

Doria-Rose, N. a, Klein, R. M., Manion, M. M., O'Dell, S., Phogat, A., Chakrabarti, B., Hallahan, C. W., Migueles, S. a, Wrammert, J. & other authors. **(2009).** Frequency and phenotype of human immunodeficiency virus envelope-specific B cells from patients with broadly cross-neutralizing antibodies. *Journal of Virology* **83**, 188–99.

Douagi, I., Forsell, M. N. E., Sundling, C., O'Dell, S., Feng, Y., Dosenovic, P., Li, Y., Seder, R., Loré, K. & other authors. **(2010).** Influence of novel CD4 binding-defective HIV-1 envelope glycoprotein immunogens on neutralizing antibody and T-cell responses in nonhuman primates. *Journal of Virology* **84**, 1683–95.

Dragic T, Trkola A, Lin SW, Nagashima KA, Kajumo FA, Maxwell E, Lin SW, Ying W, Smith SO, Sakmar T, & Moore JP. (1998). Amino-terminal substitutions in the CCR5 coreceptor impair gp120 binding and HIV type 1 entry. *Journal of Virology* **72**, 279-85.

Dreja, H., O'Sullivan, E., Pade, C., Greene, K. M., Gao, H., Aubin, K., Hand, J., Isaksen, A., D'Souza, C. & other authors. **(2010).** Neutralization activity in a geographically diverse East London cohort of human immunodeficiency virus type 1-infected patients: clade C infection results in a stronger and broader humoral immune response than clade B infection. *The Journal of General Virology* **91**, 2794–803.

Drummond, A. J. & Rambaut, A. (2007). BEAST: Bayesian evolutionary analysis by sampling trees. *BMC evolutionary biology* **7**, 214.

Dudley, D. M., Gao, Y., Nelson, K. N., Henry, K. R., Nankya, I., Gibson, R. M. & Arts, E. J. (2009). A novel yeast-based recombination method to clone and propagate diverse HIV-1 isolates. *BioTechniques* **46**, 458–67.

Egan, M. A., Carruth, L. M., Rowell, J. F. & Yu, X. (1996). Human immunodeficiency virus type 1 envelope protein endocytosis mediated by a highly conserved intrinsic internalization signal in the cytoplasmic domain of gp41 is suppressed in the presence of the Pr55gag precursor protein . *Journal of Virology* **70**, 6547-56.

Etemad, B., Fellows, A., Kwambana, B., Kamat, A., Feng, Y., Lee, S. & Sagar, M. (2009). Human immunodeficiency virus type 1 V1-to-V5 envelope variants from the chronic phase of infection use CCR5 and fuse more efficiently than those from early after infection. *Journal of Virology* **83**, 9694–708.

Euler, Z., Van Gils, M. J., Bunnik, E. M., Phung, P., Schweighardt, B., Wrin, T. & Schuitemaker, H. (2010). Cross-reactive neutralizing humoral immunity does not protect from HIV type 1 disease progression. *The Journal of infectious diseases* **201**, 1045–53.

University of Cape Town

Fellay, J., Shianna, K. V, Ge, D., Colombo, S., Ledergerber, B., Weale, M., Zhang, K., Gumbs, C., Castagna, A. & other authors. **(2007).** A whole-genome association study of major determinants for host control of HIV-1. *Science* **317**, 944–7.

Felts, R. L., Narayan, K., Estes, J. D., Shi, D., Trubey, C. M., Fu, J. & Hartnell, L. M. (2010). 3D visualization of HIV transfer at the virological synapse between dendritic cells and T cells. *Proceedins of the National Academy of Sciences of the United States of America* **107**, 13336-41.

Fenouillet, E; Gluckman, JC; Jones, I. (1994). Functions of HIV envelope glycans. *Trends in Biochemical Sciences* **19**, 65–70.

Fiebig, E. W., Wright, D. J., Rawal, B. D., Garrett, P. E., Schumacher, R. T., Peddada, L., Heldebrant, C., Smith, R., Conrad, A. & other authors. **(2003).** Dynamics of HIV viremia and antibody seroconversion in plasma donors: implications for diagnosis and staging of primary HIV infection. *AIDS* **17**, 1871–9.

Fisher, R., Berfoms, J., Meier, W., Johnson, V., Cosopoulos, D., Liu, T., Tizard, R., Walder, B., Hirsch, M., Schooley, & authors. (1988). HIV infection is blocked by in vitro by recombinant soluble CD4. *Nature* **331**: 76-78.

Fontella, R., Soares, M. a & Schrago, C. G. (2008). On the origin of HIV-1 subtype C in South America. *AIDS* **22**, 2001–11.

Fortin, JF; Cantin, R; Lamontagne, G; Tremblay, M. (1997). Host-derived ICAM glycoproteins incorporated on human immunodeficiency virus type 1 are biologically active and enhance viral infectivity. *Journal of Virology* **71**, 3588–96.

Fouchier, R. a, Groenink, M., Kootstra, N. a, Tersmette, M., Huisman, H. G., Miedema, F. & Schuitemaker, H. (1992). Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. *Journal of Virology* **66**, 3183–7.

Freed, E. O. & Martin, M. a. **(1995).** The role of human immunodeficiency virus type 1 envelope glycoproteins in virus infection. *The Journal of biological chemistry* **270**, 23883–6.

Freed, E. O., Myers, D. J. & Risser, R. (1989). Mutational analysis of the cleavage sequence of the human immunodeficiency virus type 1 envelope glycoprotein precursor gp160. *Journal of Virology* **63**, 4670–5.

Freed, E. O., Myers, D. J. & Risser, R. (1990). Characterization of the fusion domain of the human immunodeficiency virus type 1 envelope glycoprotein gp41. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 4650–4.

University of Cape Town

Freed, E. O., Myers, D. J. & Risser, R. (1991). Identification of the principal neutralizing determinant of human immunodeficiency virus type 1 as a fusion domain. *Journal of Virology* **65**, 190–4.

Frost, S.D.W., Wrin, T., Smith, D.M., Pond, S.L.K., Liu, Y., Paxinor, E., Chappey, C., Galovich, J., Beauchalne, J., Petropoulos, C.J., Little, S.J., & Richman, D.D. (2005a). Neutralizing antibody response drive the evolution of human immunodeficiency virus type 1 envelope during recent HIVinfection. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 18514-19.

Frost, S. D. W., Liu, Y., Pond, S. L. K., Chappey, C., Wrin, T., Petropoulos, C. J., Little, S. J. & Richman, D. D. (2005b). Characterization of Human Immunodeficiency Virus Type 1 (HIV-1) Envelope Variation and Neutralizing Antibody Responses during Transmission of HIV-1 Subtype B. *Journal of Virology* **79,** 6523-27.

Gali, Y., Berkhout, B., Vanham, G., Bakker, M., Back, N. K. T. & Ariën, K. K. (2007). Survey of the temporal changes in HIV-1 replicative fitness in the Amsterdam Cohort. *Virology* **364**, 140–6.

Gao, F., Bailes, E., Robertson, D. L., Chen, Y., Rodenburg, C. M., Michael, S. F., Cummins, L. B., Arthur, L. O., Peeters, M. & other authors. **(1999).** Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. *Nature* **397**, 436–41.

Gao, X., Nelson, G. W., Karacki, P., Martin, M. P., Phair, J., Kaslow, R., Goedert, J. J., Buchbinder, S., Hoots, K. & other authors. **(2001).** Effect of a single amino acid change in MHC class I molecules on the rate of progression to AIDS. *The New England journal of medicine* **344**, 1668–75.

Gauduin, M.C., Allaway, G.P., Maddon, P.J., Barbas, C.F. 3rd , Burton, D.R., & Koup, R.A. (1996). Effective ex vivo neutralization of human immunodeficiency virus type 1 in plasma by recombinant immunoglobulin molecules. *Journal of Virology* **70**, 2586-92.

Geijtenbeek, T. B. ., Torensma, R., Van Vliet, S. J., Van Duijnhoven, G. C. ., Adema, G. J., Van Kooyk, Y. & Figdor, C. G. (2000). Identification of DC-SIGN, a Novel Dendritic Cell–Specific ICAM-3 Receptor that Supports Primary Immune Responses. *Cell* **100**, 575–585.

Geldmacher, C., Currier, J. R., Herrmann, E., Haule, A., Kuta, E., McCutchan, F., Njovu, L., Geis, S., Hoffmann, O. & other authors. **(2007).** CD8 T-cell recognition of multiple epitopes within specific Gag regions is associated with maintenance of a low steady-state viremia in human immunodeficiency virus type 1-seropositive patients. *Journal of Virology* **81**, 2440–8.

University of Cape Town

Gilbert, M. T. P., Rambaut, A., Wlasiuk, G., Spira, T. J., Pitchenik, A. E. & Worobey, M. (2007). The emergence of HIV/AIDS in the Americas and beyond. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 18566–70.

Gnanakaran, S., Lang, D., Daniels, M., Derdeyn, C. A., Korber, B. & Bhattacharya, T. (2007). Clade-Specific Differences between Human Immunodeficiency Virus Type 1 Clades B and C: Diversity and Correlations in C3-V4 Regions of gp120. *Journal of Virology* **81**, 4886-91

Goepfert, P. a, Lumm, W., Farmer, P., Matthews, P., Prendergast, A., Carlson, J. M., Derdeyn, C. a, Tang, J., Kaslow, R. a & other authors. **(2008).** Transmission of HIV-1 Gag immune escape mutations is associated with reduced viral load in linked recipients. *The Journal of experimental medicine* **205**, 1009–17.

Goonetilleke, N., Liu, M. K. P., Salazar-Gonzalez, J. F., Ferrari, G., Giorgi, E., Ganusov, V. V, Keele, B. F., Learn, G. H., Turnbull, E. L. & other authors. **(2009a).** The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *The Journal of experimental medicine* **206**, 1253–72.

Goonetilleke, N., Liu, M. K. P., Salazar-Gonzalez, J. F., Ferrari, G., Giorgi, E., Ganusov, V. V, Keele, B. F., Learn, G. H., Turnbull, E. L. & other authors. **(2009b).** The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *The Journal of experimental medicine* **206**, 1253–72.

Gottlieb, G. S., Nickle, D. C., Jensen, M. a, Wong, K. G., Grobler, J., Li, F., Liu, S.-L., Rademeyer, C., Learn, G. H. & other authors. **(2004).** Dual HIV-1 infection associated with rapid disease progression. *Lancet* **363**, 619–22.

Gras, L., Jurriaans, S., Bakker, M., Van Sighem, A., Bezemer, D., Fraser, C., Lange, J., Prins, J. M., Berkhout, B. & De Wolf, F. (2009). Viral load levels measured at set-point have risen over the last decade of the HIV epidemic in the Netherlands. *PloS one* **4**, e7365.

Gray, C. M., Mlotshwa, M., Riou, C., Mathebula, T., De Assis Rosa, D., Mashishi, T., Seoighe, C., Ngandu, N., Van Loggerenberg, F. & other authors. **(2009a).** Human immunodeficiency virus-specific gamma interferon enzyme-linked immunospot assay responses targeting specific regions of the proteome during primary subtype C infection are poor predictors of the course of viremia and set point. *Journal of Virology* **83**, 470–8.

Gray, E. S., Moore, P. L., Choge, I. a, Decker, J. M., Bibollet-Ruche, F., Li, H., Leseka, N., Treurnicht, F., Mlisana, K. & other authors. **(2007).** Neutralizing antibody responses in acute human immunodeficiency virus type 1 subtype C infection. *Journal of Virology* **81**, 6187–96.

Gray, E. S., Madiga, M. C., Moore, P. L., Mlisana, K., Abdool Karim, S. S., Binley, J. M., Shaw, G. M., Mascola, J. R. & Morris, L. (2009b). Broad neutralization of human

University of Cape Town

immunodeficiency virus type 1 mediated by plasma antibodies against the gp41 membrane proximal external region. *Journal of Virology* **83**, 11265–74.

Gray, E. S., Madiga, M. C., Hermanus, T., Moore, P. L., Wibmer, C. K., Tumba, N. L., Werner, L., Mlisana, K., Sibeko, S. & other authors. **(2011a).** The neutralization breadth of HIV-1 develops incrementally over four years and is associated with CD4+ T cell decline and high viral load during acute infection. *Journal of Virology* **85**, 4828–40.

Gray, E. S., Madiga, M. C., Hermanus, T., Moore, P. L., Wibmer, C. K., Tumba, N. L., Werner, L., Mlisana, K., Sibeko, S. & other authors. **(2011b).** The neutralization breadth of HIV-1 develops incrementally over four years and is associated with CD4+ T cell decline and high viral load during acute infection. *Journal of Virology* **85**, 4828–40.

Grobler, J., Gray, C. M., Rademeyer, C., Seoighe, C., Ramjee, G., Karim, S. A., Morris, L. & Williamson, C. (2004). Incidence of HIV-1 dual infection and its association with increased viral load set point in a cohort of HIV-1 subtype C-infected female sex workers. *The Journal of infectious diseases* **190**, 1355–9.

Haaland, R. E., Hawkins, P. a, Salazar-Gonzalez, J., Johnson, A., Tichacek, A., Karita, E., Manigart, O., Mulenga, J., Keele, B. F. & other authors. **(2009).** Inflammatory genital infections mitigate a severe genetic bottleneck in heterosexual transmission of subtype A and C HIV-1. *PLoS pathogens* **5**, e1000274.

Haase, A. T. (2010). Targeting early infection to prevent HIV-1 mucosal transmission. *Nature* **464**, 217–23.

Haase, A. T. (2011). Early events in sexual transmission of HIV and SIV and opportunities for interventions. *Annual review of medicine* **62**, 127–39.

Van Harmelen, J., Williamson, C., Kim, B., Morris, L., Carr, J., Karim, S. S. & McCutchan, F. (2001). Characterization of full-length HIV type 1 subtype C sequences from South Africa. *AIDS research and human retroviruses* **17**, 1527–31.

Harris, A., Borgnia, M. J., Shi, D., Bartesaghi, A., He, H., Pejchal, R. & Kenneth, Y. (2011). native HIV-1 envelope glycoproteins display the same closed and open quaternary molecular architectures. *Proceeds of the National Academy of Sciences OF THE United States of America* **108**, 11440-45.

Harris, RS; Liddament, M. (2004). Retroviral restriction by APOBEC proteins. *Nature Reviews Immunology* **4**, 868–77.

Hecht, F. M., Hartogensis, W., Bragg, L., Bacchetti, P., Atchison, R., Grant, R., Barbour, J. & Deeks, S. G. (2010). HIV RNA level in early infection is predicted by viral load in the transmission source. *AIDS* **24**, 941–5.

University of Cape Town

Helseth, E., Olshevsky, U., Furman, C. & Sodroski, J. (1991). Human immunodeficiency virus type 1 gp120 envelope glycoprotein regions important for association with the gp41 transmembrane glycoprotein. *Journal of Virology* **65**, 2119–23.

Herbeck, J. T., Gottlieb, G. S., Li, X., Hu, Z., Detels, R., Phair, J., Rinaldo, C., Jacobson, L. P., Margolick, J. B. & Mullins, J. I. (2008). Lack of evidence for changing virulence of HIV-1 in North America. *PloS one* **3**, e1525.

Herbinger, K.-H., Gerhardt, M., Piyasirisilp, S., Mloka, D., Arroyo, M. a, Hoffmann, O., Maboko, L., Birx, D. L., Mmbando, D. & other authors. **(2006).** Frequency of HIV type 1 dual infection and HIV diversity: analysis of low- and high-risk populations in Mbeya Region, Tanzania. *AIDS research and human retroviruses* **22**, 599–606.

Heredia, A., Gilliam, B., DeVico, A., Le, N., Bamba, D., Flinko, R., Lewis, G., Gallo, R. C. & Redfield, R. R. (2007). CCR5 density levels on primary CD4 T cells impact the replication and Enfuvirtide susceptibility of R5 HIV-1. *AIDS* **21**, 1317–22.

Herrera, C., Spenlehauer, C., Fung, M. S., Burton, D. R., Beddows, S. & Moore, J. P. (2003). Nonneutralizing Antibodies to the CD4-Binding Site on the gp120 Subunit of Human Immunodeficiency Virus Type 1 Do Not Interfere with the Activity of a Neutralizing Antibody against the Same Site. *Journal of Virology* **77**, 1084-91.

Herrera, C., Klasse, P. J., Michael, E., Kake, S., Barnes, K., Kibler, C. W., Campbell-Gardener, L., Si, Z., Sodroski, J. & other authors. **(2005).** The impact of envelope glycoprotein cleavage on the antigenicity, infectivity, and neutralization sensitivity of Env-pseudotyped human immunodeficiency virus type 1 particles. *Virology* **338**, 154–72.

Hioe, C. E., Jr, P. C. C., Lu, C., Timothy, A., Wang, X., Bandres, J. & Tuen, M. (2001). LFA-1 Expression on Target Cells Promotes Human Immunodeficiency Virus Type 1 Infection and Transmission LFA-1 Expression on Target Cells Promotes Human Immunodeficiency Virus Type 1 Infection and Transmission. *Journal of Virology* **75**, 1077-82.

HIV Sequence Database. (2012). HIV and SIV Nomenclature. http://www.hiv.lanl.gov.

Hoelscher, M., Hanker, S., Barin, F., Cheingsong-Popov, R., Dietrich, U., Jordan-Harder, B., Olaleye, D., Nägele, E., Markuzzi, a & other authors. **(1998).** HIV type 1 V3 serotyping of Tanzanian samples: probable reasons for mismatching with genetic subtyping. *AIDS research and human retroviruses* **14**, 139–49.

Hoelscher, M., Kim, B., Maboko, L., Mhalu, F., Von Sonnenburg, F., Birx, D. L. & McCutchan, F. E. (2001). High proportion of unrelated HIV-1 intersubtype recombinants in the Mbeya region of southwest Tanzania. *AIDS* **15**, 1461–70.

University of Cape Town

Hollingsworth, T.D., Laeyendecker, O., Shirrett, G., Donnelly, C.A., Serwadda, D., Wawer, M.J., Kiwanuka, N., Nalugoda, F., Collinson-Streng, A., Ssempijja, V., Hanage, W.P., Quinn, T.C., Gray, R.H., & Fraser, C. (2010). HIV transmission couples have similar viral load set-point in Rakai, Uganda. *PLOS Pathogens* **6**, e1000876.

Hu, J., Gardner, M. B. & Miller, C. J. (2000). Simian Immunodeficiency Virus Rapidly Penetrates the Cervicovaginal Mucosa after Intravaginal Inoculation and Infects Intraepithelial Dendritic Cells Simian Immunodeficiency Virus Rapidly Penetrates the Cervicovaginal Mucosa after Intravaginal Inoculation. *Journal of Virology* **74**, 6087-95.

Huang, W., Eshleman, S. H., Toma, J., Fransen, S., Stawiski, E., Paxinos, E. E., Whitcomb, J. M., Young, A. M., Donnell, D. & other authors. **(2007).** Coreceptor tropism in human immunodeficiency virus type 1 subtype D: high prevalence of CXCR4 tropism and heterogeneous composition of viral populations. *Journal of Virology* **81**, 7885–93.

Hübner, W., Mcnerney, G. P., Chen, P., Dale, B. M., Gordon, R. E., Chuang, F. Y. S., Li, X., Asmuth, D. M., Huser, T. & Chen, B. K. (2009). *Virological Synapses* **323**, 1743–1747.

Hussey, R., Richardson, N., Kowalski, M., Brown, N., Change, H., Siliciano, R., Dorfman, F., Walker, B., Sodroski, J., Reinherz, E. (1988). A soluble CD4 protein selectively inihibits HIV replication and synctium formation. *Nature* **331**: 78 - 81.

Isaacman-Beck, J., Hermann, E. a., Yi, Y., Ratcliffe, S. J., Mulenga, J., Allen, S., Hunter, E., Derdeyn, C. a. & Collman, R. G. (2009). Heterosexual Transmission of Human Immunodeficiency Virus Type 1 Subtype C: Macrophage Tropism, Alternative Coreceptor Use, and the Molecular Anatomy of CCR5 Utilization. *Journal of Virology* **83**, 8208–8220.

Itescu, S; Mathu-Wagh, U; Skovron, ML; Brancato, LJ; Marmor, M; Zeleniuch-Jacquotte, A; Winchster, R. (1992). HLA-B35 is associated with accelerated progression to AIDS. *Journal of acquired immune deficiency syndromes* **5**, 37–45.

Janeway, CA; Travers, P. (1994). *Immunobiology: the immune system in health and disease*. Current Biology/Garland.

Kabat, D., Kozak, S. L., Wehrly, K. & Chesebro, B. (1994). Differences in CD4 dependence for infectivity of laboratory-adapted and primary patient isolates of human immunodeficiency virus type 1. *Journal of Virology* **68**, 2570–7.

Kaleebu, P., French, N., Mahe, C., Yirrell, D., Watera, C., Lyagoba, F., Nakiyingi, J., Rutebemberwa, A., Morgan, D. & other authors. **(2002).** Effect of human immunodeficiency virus (HIV) type 1 envelope subtypes A and D on disease progression in a large cohort of HIV-1-positive persons in Uganda. *The Journal of infectious diseases* **185**, 1244–50.

University of Cape Town

Kalia, V., Sarkar, S., Gupta, P., Montelaro, C. & Montelaro, R. C. (2003). Rational Site-Directed Mutations of the LLP-1 and LLP-2 Lentivirus Lytic Peptide Domains in the Intracytoplasmic Tail of Human Immunodeficiency Virus Type 1 gp41 Indicate Common Functions in Cell-Cell Fusion but Distinct Roles in Virion Envelope Incorporation. *Journal of Virology* **77**, 3634-46.

Karlsson, A., Parsmyr, K., Sandstrom, E., Fenyo, E. V. A. M. & Albert, J. A. N. (1994). MT-2 cell tropism as prognostic marker for disease progression in human immunodeficiency virus type 1 infection . MT-2 Cell Tropism as Prognostic Marker for Disease Progression in Human Immunodeficiency Virus Type 1 Infection. *Journal of Clinical Microbiology* **32,** 364-70.

Kaslow, R.A., Carrington, M., Apple, R., Park, L., Munoz, A., Saah, A.J., Goedert, J.J., Winkler, C., O'Brien, S.J., Rinaldo, C., Detels, R., Blattner, W., Phair, J., Erlich, & H., Mann, D.L. (1996). Influence of combinations of human major histocompatibility complex genes on the course of HIV-1 infection. *Nature Medicine* **2**, 405-11.

Keele, B. F., Van Heuverswyn, F., Li, Y., Bailes, E., Takehisa, J., Santiago, M. L., Bibollet-Ruche, F., Chen, Y., Wain, L. V & other authors. **(2006).** Chimpanzee reservoirs of pandemic and nonpandemic HIV-1. *Science* **313**, 523–6.

Keele, B. F., Giorgi, E. E., Salazar-Gonzalez, J. F., Decker, J. M., Pham, K. T., Salazar, M. G., Sun, C., Grayson, T., Wang, S. & other authors. **(2008).** Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 7552–7.

Kiepiela, P., Leslie, A. J., Honeyborne, I., Ramduth, D., Thobakgale, C., Chetty, S., Rathnavalu, P., Moore, C., Pfafferott, K. J. & other authors. **(2004).** Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. *Nature* **432**, 769–75.

Kiepiela, P., Ngumbela, K., Thobakgale, C., Ramduth, D., Honeyborne, I., Moodley, E., Reddy, S., De Pierres, C., Mncube, Z. & other authors. **(2007).** CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. *Nature medicine* **13**, 46–53.

Kiwanuka, N., Laeyendecker, O., Robb, M., Kigozi, G., Arroyo, M., McCutchan, F., Eller, L. A., Eller, M., Makumbi, F. & other authors. **(2008).** Effect of human immunodeficiency virus Type 1 (HIV-1) subtype on disease progression in persons from Rakai, Uganda, with incident HIV-1 infection. *The Journal of infectious diseases* **197**, 707–13.

King, DF., Siddiqui, AA., Buffa, V., Fischetti, L., Gao, Y., Stieh, D., McKay, PF., Rogers, P., Ochsenbauer, C., Kappes, JC. & other authors. (2013). Mucosal tissue tropism and

University of Cape Town

dissemination of HIV-1 subtype B acute envelope - expressing chmeric virus. *Journal of Virology* **87**, 890-97.

Kondo, N., Miyauchi, K., Meng, F., Iwamoto, A. & Matsuda, Z. (2010). Conformational changes of the HIV-1 envelope protein during membrane fusion are inhibited by the replacement of its membrane-spanning domain. *The Journal of biological chemistry* **285**, 14681–8.

Kong, X., West, J. T., Zhang, H., Shea, D. M., M'soka, T. J. & Wood, C. (2008). The human immunodeficiency virus type 1 envelope confers higher rates of replicative fitness to perinatally transmitted viruses than to nontransmitted viruses. *Journal of Virology* **82**, 11609–18.

Koot, M., Keet, I. P., Vos, a H., De Goede, R. E., Roos, M. T., Coutinho, R. a, Miedema, F., Schellekens, P. T. & Tersmette, M. (1993). Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4+ cell depletion and progression to AIDS. *Annals of internal medicine* **118**, 681–8.

Kuritzkes, D. R. (2008). HIV-1 subtype as a determinant of disease progression. *The Journal of infectious diseases* **197**, 638–9.

Kwong, P. D., Wyatt, R., Robinson, J., Sweet, R. W., Sodroski, J. & Hendrickson, W. A. (1998). Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. *Nature* **393,** 648-59.

Kwong, P; Wyatt, R; Majeed, S; Robnson, J; Sweet, RW; Sodroski, J; Hendrickson, W. (2000). Structure of HIV-1 gp120 envelope glycoproteins from laboratory-adapted and primary isolates. *Structure* **8**, 1329–39.

Land, A. M., Ball, T. B., Luo, M., Pilon, R., Sandstrom, P., Embree, J. E., Wachihi, C., Kimani, J. & Plummer, F. a. (2008). Human immunodeficiency virus (HIV) type 1 proviral hypermutation correlates with CD4 count in HIV-infected women from Kenya. *Journal of Virology* **82**, 8172–82.

Lassen, K. G., Lobritz, M. a, Bailey, J. R., Johnston, S., Nguyen, S., Lee, B., Chou, T., Siliciano, R. F., Markowitz, M. & Arts, E. J. (2009). Elite suppressor-derived HIV-1 envelope glycoproteins exhibit reduced entry efficiency and kinetics. *PLoS pathogens* **5**, e1000377.

Learn, G. H., Muthui, D., Brodie, S. J., Zhu, T., Diem, K., Mullins, J. I. & Corey, L. (2002). Virus Population Homogenization following Acute Human Immunodeficiency Virus Type 1 Infection Virus Population Homogenization following Acute Human Immunodeficiency Virus Type 1 Infection. *Journal of Virology* **76**, 11953-59.

University of Cape Town

Lee, B., Sharron, M., Montaner, L. J., Weissman, D. & Doms, R. W. (1999). Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 5215–20.

Lee, H. Y., Giorgi, E. E., Keele, B. F., Gaschen, B., Athreya, G. S., Salazar-Gonzalez, J. F., Pham, K. T., Goepfert, P. a, Kilby, J. M. & other authors. **(2009).** Modeling sequence evolution in acute HIV-1 infection. *Journal of theoretical biology* **261**, 341–60.

Lee, S. F., Wang, C. T., Liang, J. Y., Hong, S. L., Huang, C. C. & Chen, S. S. (2000). Multimerization potential of the cytoplasmic domain of the human immunodeficiency virus type 1 transmembrane glycoprotein gp41. *The Journal of biological chemistry* **275**, 15809–19.

Lee, S.-F., Ko, C.-Y., Wang, C.-T. & Chen, S. S.-L. (2002). Effect of point mutations in the N terminus of the lentivirus lytic peptide-1 sequence of human immunodeficiency virus type 1 transmembrane protein gp41 on Env stability. *The Journal of biological chemistry* **277**, 15363–75.

Le Gall, S., Stamenga, P., & Walker, B.P (2007). Portable flanking sequences modulate CTL epitope processing. *Journal of Clinical Investigation* **117**, 3563-75.

Leonard, C. K., Spellman, M. W., Riddle, L., Harris, R. J., Thomas, J. N. & Gregory, T. J. (1990). 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. *The Journal of biological chemistry* **265**, 10373–82.

Li, H., Bar, K. J., Wang, S., Decker, J. M., Chen, Y., Sun, C., Salazar-Gonzalez, J. F., Salazar, M. G., Learn, G. H. & other authors. **(2010a).** High Multiplicity Infection by HIV-1 in Men Who Have Sex with Men. *PLoS pathogens* **6**, e1000890.

Li, M., Salazar-Gonzalez, J. F., Derdeyn, C. a, Morris, L., Williamson, C., Robinson, J. E., Decker, J. M., Li, Y., Salazar, M. G. & other authors. **(2006).** Genetic and neutralization properties of subtype C human immunodeficiency virus type 1 molecular env clones from acute and early heterosexually acquired infections in Southern Africa. *Journal of Virology* **80**, 11776–90.

Li, Q., Duan, L., Estes, J. D. & Ma, Z. (2005). Peak SIV replication in resting memory CD4 + T cells depletes gut lamina propria CD4 + T cells. *Nature* **434,** 1148-52.

Li, Q., Skinner, P. J., Ha, S., Duan, L., Mattila, T. L., Hage, A., White, C., Barber, D. L., Mara, L. O. & other authors. **(2009a).** Visualizing Antigen-Specific and Infected Cells in Situ Predicts Outcomes in Early Viral Infection. *Science* **323**, 1726–1729.

University of Cape Town

Li, Q., Estes, J. D., Schlievert, P. M., Duan, L., Brosnahan, A. J., Southern, P. J., Reilly, C. S., Peterson, M. L., Schultz-Darken, N. & other authors. **(2009b).** Glycerol monolaurate prevents mucosal SIV transmission. *Nature* **458**, 1034–8.

Lichterfeld, M., Yu, X. G., Cohen, D., Addo, M. M., Malenfant, J., Perkins, B., Pae, E., Johnston, M. N., Strick, D. & other authors. **(2004).** HIV-1 Nef is preferentially recognized by CD8 T cells in primary HIV-1 infection despite a relatively high degree of genetic diversity. *Aids* **18**, 1383–1392.

Liu, J., Bartesaghi, A., Borgnia, M. J., Sapiro, G. & Subramaniam, S. (2008). Molecular architecture of native HIV-1 gp120 trimers. *Nature* **455**, 109–13.

Liu, R., Paxton, W. a, Choe, S., Ceradini, D., Martin, S. R., Horuk, R., MacDonald, M. E., Stuhlmann, H., Koup, R. a & Landau, N. R. (1996). Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86**, 367–77.

Lobritz, M. a, Marozsan, A. J., Troyer, R. M. & Arts, E. J. (2007). Natural variation in the V3 crown of human immunodeficiency virus type 1 affects replicative fitness and entry inhibitor sensitivity. *Journal of Virology* **81**, 8258–69.

Long, E. M., Martin, H. L., Kreiss, J. K., Rainwater, S. M., Lavreys, L., Jackson, D. J., Rakwar, J., Mandaliya, K. & Overbaugh, J. (2000). Gender differences in HIV-1 diversity at time of infection. *Nature medicine* **6**, 71–5.

Lorenz, M. C., Muir, R. S., Lim, E., McElver, J., Weber, S. C. & Heitman, J. (1995). Gene disruption with PCR products in Saccharomyces cerevisiae. *Gene* **158**, 113–117.

Madani, N., Perdogroto, A., Srinivasan, K., Cox, J.M., Chruma, J.J., LaLonde, J., Head, M., Smith, A.B. 3rd, and J.G. Sodroski. (2004). Localized changes in the gp120 envelope glycoprotein confer resistance to HIV entry inhibitors BMS-806 and #155. *Journal of Virology* **78**, 3742-52.

Mansky, L.M. & Temin, H. M. (1995). Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse Lower In Vivo Mutation Rate of Human Immunodeficiency Virus Type 1 than That Predicted from the Fidelity of Purified Reverse Transcriptase. *Journal of Virology* **69**, 5087-94.

Mao, Y., Wang, L., Gu, C., Herschhorn, A., Xiang, S.-H., Haim, H., Yang, X. & Sodroski, J. (2012). Subunit organization of the membrane-bound HIV-1 envelope glycoprotein trimer. *Nature structural & molecular biology* **19**, 893–9.

Markowitz, M., Louie, M., Hurley, A., Mascio, M. Di, Perelson, A. S., Ho, D. D. & Sun, E. (2003). A Novel Antiviral Intervention Results in More Accurate Assessment of Human

University of Cape Town

Immunodeficiency Virus Type 1 Replication Dynamics and T-Cell Decay In Vivo. *Journal of Virology* **77**, 5037–5039.

Marozsan, A. J., Moore, D. M., Lobritz, M. A., Fraundorf, E., Abraha, A., Reeves, J. D. & Arts, E. J. (2005). Differences in the Fitness of Two Diverse Wild-Type Human Immunodeficiency Virus Type 1 Isolates Are Related to the Efficiency of Cell Binding and Entry. *Journal of Virology* **79**, 7121–7134.

Martin, D. P., Williamson, C. & Posada, D. (2005). RDP2: recombination detection and analysis from sequence alignments. *Bioinformatics* **21**, 260–2.

Martinez-picado, J., Prado, J. G., Fry, E. E., Pfafferott, K., Leslie, A., Chetty, S., Honeyborne, I., Crawford, H., Pillay, T. & other authors. **(2006).** Fitness Cost of Escape Mutations in p24 Gag in Association with Control of Human Immunodeficiency Virus Type 1 Fitness Cost of Escape Mutations in p24 Gag in Association with Control of Human Immunodeficiency Virus Type 1. *Journal of Virology* **80**, 3617-23.

Masharsky, A. E., Dukhovlinova, E. N., Verevochkin, S. V, Toussova, O. V, Skochilov, R. V, Anderson, J. a, Hoffman, I., Cohen, M. S., Swanstrom, R. & Kozlov, A. P. (2010). A substantial transmission bottleneck among newly and recently HIV-1-infected injection drug users in St Petersburg, Russia. *The Journal of infectious diseases* **201**, 1697–702.

Mayr, L. M., Powell, R. L., Ngai, J. N., Takang, W. a, Nádas, A. & Nyambi, P. N. (2012). Superinfection by discordant subtypes of HIV-1 does not enhance the neutralizing antibody response against autologous virus. *PloS one* **7**, e38989.

McCune, JM; Rabin, LB; Feinberg, MB; Lieberman, M; Kosek, JC; Resek, JC; Reyes, G. W. (1988). Endoproteolytic cleavage of gp120 is required for the activation of human immunodeficiency virus. *Cell* **53**, 55–67.

McCutchan, F. E., Hegerich, P. a, Brennan, T. P., Phanuphak, P., Singharaj, P., Jugsudee, a, Berman, P. W., Gray, a M., Fowler, a K. & Burke, D. S. (1992). Genetic variants of HIV-1 in Thailand. *AIDS research and human retroviruses* **8**, 1887–95.

McDonald, D., Wu, L., Bohks, S. M., KewalRamani, V. N., Unutmaz, D. & Hope, T. J. (2003). Recruitment of HIV and its receptors to dendritic cell-T cell junctions. *Science* **300**, 1295–7.

McKinnon, L. R., Nyanga, B., Chege, D., Izulla, P., Kimani, M., Huibner, S., Gelmon, L., Block, K. E., Cicala, C. & other authors. **(2011).** Characterization of a human cervical CD4+ T cell subset coexpressing multiple markers of HIV susceptibility. *Journal of immunology* **187**, 6032–42.

McMichael, A. (1998). T cell responses and viral escape. *Cell* **93**, 673–6.

University of Cape Town

Migueles, S. a, Sabbaghian, M. S., Shupert, W. L., Bettinotti, M. P., Marincola, F. M., Martino, L., Hallahan, C. W., Selig, S. M., Schwartz, D. & other authors. **(2000).** HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 2709–14.

Miller, C. J., Li, Q., Abel, K., Kim, E., Ma, Z., Wietgrefe, S., Franco-scheuch, L. La, Compton, L., Duan, L. & other authors. **(2005a).** Propagation and Dissemination of Infection after Vaginal Transmission of Simian Immunodeficiency Virus. *Journal of Virology* **79**, 9217–9227.

Miura, T., Brockman, M. a, Schneidewind, A., Lobritz, M., Pereyra, F., Rathod, A., Block, B. L., Brumme, Z. L., Brumme, C. J. & other authors. **(2009a).** HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte recognition. *Journal of Virology* **83**, 2743–55.

Miura, T., Brockman, M. a, Brumme, Z. L., Brumme, C. J., Pereyra, F., Trocha, A., Block, B. L., Schneidewind, A., Allen, T. M. & other authors. **(2009b).** HLA-associated alterations in replication capacity of chimeric NL4-3 viruses carrying gag-protease from elite controllers of human immunodeficiency virus type 1. *Journal of Virology* **83**, 140–9.

Miura, T., Brumme, Z. L., Brockman, M. a, Rosato, P., Sela, J., Brumme, C. J., Pereyra, F., Kaufmann, D. E., Trocha, A. & other authors. **(2010).** Impaired replication capacity of acute/early viruses in persons who become HIV controllers. *Journal of Virology* **84**, 7581–91.

Miyauchi, K., Kim, Y., Latinovic, O., Morozov, V. & Melikyan, G. B. (2010). NIH Public Access **137**, 433–444.

Mlotshwa, M., Riou, C., Chopera, D., De Assis Rosa, D., Ntale, R., Treunicht, F., Woodman, Z., Werner, L., Van Loggerenberg, F. & other authors. **(2010).** Fluidity of HIV-1-specific T-cell responses during acute and early subtype C HIV-1 infection and associations with early disease progression. *Journal of Virology* **84**, 12018–29.

Modrow, S., Hahn, B. H., Shaw, G. M., Gallo, R. C., Wong-staal, F. & Wolf, H. (1987). Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions. *Journal of Virology* **61**, 570-78.

Montavon, C; Toure-Kane, C; Liegeois, F; Mpoudi, E; Bourgeois, A; Vergne, L; Perret, JL; Boumah, A; Saman, E; Mboup, S; Delaporte, E; Peeters, M. (2000). Most env and gag subtype A HIV-1 viruses circulating in West and Central Africa are similar to the prototype AG recombinant virus IBNG. *Journal of acquired immune deficiency syndromes* **23**, 363–74.

University of Cape Town

Moore, J. P., Cao, Y., Ho, D. D. & Koup, R. A. (1994). Development of the anti-gp120 antibody response during seroconversion to human immunodeficiency virus type 1 . Development of the Anti-gpl20 Antibody Response during Seroconversion to Human Immunodeficiency Virus Type 1. *Journal of Virology* **68,** 5142-55.

Moore, P. L., Crooks, E. T., Porter, L., Zhu, P., Cayanan, C. S., Grise, H., Corcoran, P., Zwick, M. B., Franti, M. & other authors. **(2006).** Nature of Nonfunctional Envelope Proteins on the Surface of Human Immunodeficiency Virus Type 1. *Journal of Virology* **80**, 2515–2528.

Moore, P. L., Gray, E. S., Choge, I. a, Ranchobe, N., Mlisana, K., Abdool Karim, S. S., Williamson, C. & Morris, L. (2008). The c3-v4 region is a major target of autologous neutralizing antibodies in human immunodeficiency virus type 1 subtype C infection. *Journal of Virology* **82**, 1860–9.

Moore, P. L., Ranchobe, N., Lambson, B. E., Gray, E. S., Cave, E., Abrahams, M.-R., Bandawe, G., Mlisana, K., Abdool Karim, S. S. & other authors. **(2009).** Limited neutralizing antibody specificities drive neutralization escape in early HIV-1 subtype C infection. *PLoS pathogens* **5**, e1000598.

Morris, L., Cilliers, T., Bredell, H., Phoswa, M. & Martin, D. J. (2001). CCR5 is the major coreceptor used by HIV-1 subtype C isolates from patients with active tuberculosis. *AIDS research and human retroviruses* **17**, 697–701.

Muñoz-Barroso, I., Salzwedel, K., Hunter, E. & Blumenthal, R. (1999). Role of the membrane-proximal domain in the initial stages of human immunodeficiency virus type 1 envelope glycoprotein-mediated membrane fusion. *Journal of Virology* **73**, 6089–92.

Nicastri, E., Sarmati, L., d'Ettorre, G., Palmisano, L., Parisi, S. G., Uccella, I., Rianda, A., Concia, E., Vullo, V. & other authors. **(2003).** Replication capacity, biological phenotype, and drug resistance of HIV strains isolated from patients failing antiretroviral therapy. *Journal of medical virology* **69**, 1–6.

Nijhuis, M., Schuurman, R., De Jong, D., Erickson, J., Gustchina, E., Albert, J., Schipper, P., Gulnik, S. & Boucher, C. a. **(1999).** Increased fitness of drug resistant HIV-1 protease as a result of acquisition of compensatory mutations during suboptimal therapy. *AIDS* **13**, 2349–59.

Nofemela, A., Bandawe, G., Thebus, R., Marais, J., Wood, N., Hoffmann, O., Maboko, L., Hoelscher, M., Woodman, Z. & Williamson, C. (2011). Defining the human immunodeficiency virus type 1 transmission genetic bottleneck in a region with multiple circulating subtypes and recombinant forms. *Virology* **415**, 107–13.

University of Cape Town

Nyombi, B. M., Kristiansen, K. I., Bjune, G., Müller, F. & Holm-Hansen, C. (2008). Diversity of human immunodeficiency virus type 1 subtypes in Kagera and Kilimanjaro regions, Tanzania. *AIDS research and human retroviruses* **24**, 761–9.

Ochsenbauer, C. & Kappes, J. C. (2009). New virologic reagents for neutralizing antibody assays. *Current opinion in HIV and AIDS* **4**, 418–25.

Ochsenbauer, C., Edmonds, T. G., Ding, H., Keele, B. F., Decker, J., Salazar, M. G., Salazar-Gonzalez, J. F., Shattock, R., Haynes, B. F. & other authors. **(2012).** Generation of transmitted/founder HIV-1 infectious molecular clones and characterization of their replication capacity in CD4 T lymphocytes and monocyte-derived macrophages. *Journal of Virology* **86**, 2715–28.

Olshevsky, U., Helseth, E., Furman, C., Li, J., Haseltine, W. & Sodroski, J. (1990). Identification of individual human immunodeficiency virus type 1 gp120 amino acids important for CD4 receptor binding. *Journal of Virology* **64**, 5701–7.

Overbaugh, J. & Rudensey, L. M. (1992). Alterations in potential sites for glycosylation predominate during evolution of the simian immunodeficiency virus envelope gene in macaques. *Journal of Virology* **66**, 5937–48.

Overbaugh, J., Rudensey, L. M., Papenhausen, M. D., Benveniste, R. E. & Morton, W. R. (1991). Variation in simian immunodeficiency virus env is confined to V1 and V4 during progression to simian AIDS. *Journal of Virology* **65**, 7025–31.

Pace, C., Keller, J., Nolan, D., James, I., Gaudieri, S., Moore, C. & Mallal, S. (2006). Population level analysis of human immunodeficiency virus type 1 hypermutation and its relationship with APOBEC3G and vif genetic variation. *Journal of Virology* **80**, 9259–69.

Palmer, S., Kearney, M., Maldarelli, F., Elias, K., Bixby, C. J., Bazmi, H., Rock, D., Jr, R. T. D., Dewar, R. L. & other authors. **(2005).** Multiple , Linked Human Immunodeficiency Virus Type 1 Drug Resistance Mutations in Treatment-Experienced Patients Are Missed by Standard Genotype Analysis. *Journal of Clinical Microbiology* **43**, 406-13.

Pancera, M., Majeed, S., Ban, Y.-E. A., Chen, L., Huang, C., Kong, L., Kwon, Y. Do, Stuckey, J., Zhou, T. & other authors. **(2010).** Structure of HIV-1 gp120 with gp41-interactive region reveals layered envelope architecture and basis of conformational mobility. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 1166–71.

Parker, Z. F., Iyer, S. S., Wilen, C. B., Parrish, N. F., Chikere, K. C., Lee, F.-H., Didigu, C. a, Berro, R., Klasse, P. J. & other authors. **(2012).** Transmitted/Founder and Chronic HIV-1 Envelope Proteins are Distinguished by Differential Utilization of CCR5. *Journal of Virology* **87**, 2401-11.

University of Cape Town

Parrish, N. F., Wilen, C. B., Banks, L. B., Iyer, S. S., Pfaff, J. M., Salazar-Gonzalez, J. F., Salazar, M. G., Decker, J. M., Parrish, E. H. & other authors. **(2012).** Transmitted/founder and chronic subtype C HIV-1 use CD4 and CCR5 receptors with equal efficiency and are not inhibited by blocking the integrin α4β7. *PLoS pathogens* **8**, e1002686.

Parrish, N.F., Gao, F., Li, H., Giorgi, E.E., Barbian, H.J., Parrish, E.H., Zajic, L., Iyer, S.S., Decker, J.M., Kumar, & other authors. (2013). Phenotypic features of transmitted founder HIV-1. *Procedings of the National Academy of Sciences of the United States of America* **110**, 6626- 33.

Peeters, M., Vincent, R., Perret, J. L., Lasky, M., Patrel, D., Liegeois, F., Courgnaud, V., Seng, R., Matton, T. & other authors. **(1999).** 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. *Journal of acquired immune deficiency syndromes and human retrovirology: official publication of the International Retrovirology Association* **20**, 115–21.

Pejchal, R., Doores, K. J., Walker, L. M., Khayat, R., Huang, P.-S., Wang, S.-K., Stanfield, R. L., Julien, J.-P., Ramos, A. & other authors. **(2011).** A potent and broad neutralizing antibody recognizes and penetrates the HIV glycan shield. *Science (New York, NY)* **334**, 1097–103.

Piantadosi, A., Humes, D., Chohan, B., McClelland, R. S. & Overbaugh, J. (2009a). Analysis of the percentage of human immunodeficiency virus type 1 sequences that are hypermutated and markers of disease progression in a longitudinal cohort, including one individual with a partially defective Vif. *Journal of Virology* **83**, 7805–14.

Piantadosi, A., Panteleeff, D., Blish, C. a, Baeten, J. M., Jaoko, W., McClelland, R. S. & Overbaugh, J. (2009b). Breadth of neutralizing antibody response to human immunodeficiency virus type 1 is affected by factors early in infection but does not influence disease progression. *Journal of Virology* **83**, 10269–74.

Picard, L., Wilkinson, D., McKnight, A., Gray, P.W., Hoxie, J.A., Clapham, P.R., and Weiss, R.A. (1997). Role of the amino-terminal extracellular domain of CXCR4 in HIV type 1 entry. *Virology* 231. 105-11.

Piller, S. C., Dubay, J. W., Derdeyn, C. A. & Hunter, E. (2000). Mutational Analysis of Conserved Domains within the Cytoplasmic Tail of gp41 from Human Immunodeficiency Virus Type 1: Effects on Glycoprotein Incorporation and Infectivity. *Journal of Virology* **74**, 11717-23.

Ping, JS; Anderson, J; Kincer, L; Morris, L; Hahn, BH; Williamson, C; Swanstrom, C. (2012). Heterosexual transmission selects for virus that requires high levels of CD4 to enter

University of Cape Town

cells and is more homogenous in its sensitivity to CCR5 antagonists. In *19th Conference on Retroviruses and Opportunistic Infections, Seattle*.

Ping, L.H., Joseph, S.B., Anderson, J.A., Abrahams, MR., Salazar-Gonzalez, J.F., Kincer, L.P., Treunicht, F.K., Arney, L., Ojeda, S., Zhang, M. & other authors. (2013). Comparison of Viral Env proteins from acute and chronic infection with subype C Human Immunodeficiency Virus type 1 identifies differences in glycosylation and CCR5 utilization and suggest new strategy for immunogen design. *Journal of Virology* **87**, 7218-33.

Platt, E. J., Wehrly, K., Kuhmann, S. E., Chesebro, B. & Kabat, D. (1998). Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. *Journal of Virology* **72**, 2855–64.

Pollakis, G., Abebe, A., Kliphuis, A., Chalaby, M. I. M., Bakker, M., Mengistu, Y., Brouwer, M., Goudsmit, J., Schuitemaker, H. & Paxton, W. A. (2004). Phenotypic and Genotypic Comparisons of CCR5- and CXCR4-Tropic Human Immunodeficiency Virus Type 1 Biological Clones Isolated from Subtype C-Infected Individuals. *Journal of Virology* **78**, 2841–2852.

Polonis, V. R., Brown, B. K., Rosa Borges, A., Zolla-Pazner, S., Dimitrov, D. S., Zhang, M.-Y., Barnett, S. W., Ruprecht, R. M., Scarlatti, G. & other authors. **(2008).** Recent advances in the characterization of HIV-1 neutralization assays for standardized evaluation of the antibody response to infection and vaccination. *Virology* **375**, 315–20.

Poncelet, P; Poinas; Corbeau, P; Devaux, C; Tubaina, N; Muloko, N; Tamalet, C; Chermann, JC; Kourilsky, F; Sampol, J. (1991). Surface density remains constant on lymphocytes of HIV-infected patients in the progression disease. *Immunologic Research* **142**, 291–8.

Pope, M. & Haase, A. T. (2003). Transmission, acute HIV-1 infection and the quest for strategies to prevent infection. *Nature medicine* **9**, 847–52.

Potard, V., Weiss, L., Lamontagne, F., Rouveix, E., Beck-Wirth, G., Drogoul-Vey, M.-P., Souala, M. F. & Costagliola, D. (2009). Trends in post-infection CD4 cell counts and plasma HIV-1 RNA levels in HIV-1-infected patients in France between 1997 and 2005. *Journal of acquired immune deficiency syndromes (1999)* **52**, 422–6.

Prince, J. L., Claiborne, D. T., Carlson, J. M., Schaefer, M., Yu, T., Lahki, S., Prentice, H. a, Yue, L., Vishwanathan, S. a & other authors. **(2012).** Role of transmitted Gag CTL polymorphisms in defining replicative capacity and early HIV-1 pathogenesis. *PLoS pathogens* **8**, e1003041.

Provine, N. M., Puryear, W. B., Wu, X., Overbaugh, J. & Haigwood, N. L. (2009). The infectious molecular clone and pseudotyped virus models of human immunodeficiency

University of Cape Town

virus type 1 exhibit significant differences in virion composition with only moderate differences in infectivity and inhibition sensitivity. *Journal of Virology* **83**, 9002–7.

Quiñones-Mateu, M. E., Ball, S. C., Marozsan, a J., Torre, V. S., Albright, J. L., Vanham, G., Van Der Groen, G., Colebunders, R. L. & Arts, E. J. (2000). A dual infection/competition assay shows a correlation between ex vivo human immunodeficiency virus type 1 fitness and disease progression. *Journal of Virology* **74**, 9222–33.

Rambaut, A; Posada, D; Crandall, KA; Holmes, C. (2004). The causes and consequences of HIV evolution. *Nature Reviews Genetics* **5**, 52–61.

Rangel, H. R., Weber, J., Chakraborty, B., Gutierrez, A., Marotta, M. L., Mirza, M., Kiser, P., Martinez, M. A., Este, J. A. & Quin, M. E. (2003a). Role of the Human Immunodeficiency Virus Type 1 Envelope Gene in Viral Fitness. *Journal of Virology* **77**, 9069–9073.

Redd, A. D., Laeyendecker, O., Kong, X., Kiwanuka, N., Lutalo, T., Huang, W., Gray, R. H., Wawer, M. J., Serwadda, D. & Eshleman, S. H. (2012). Efficiency of CCR5 Coreceptor Utilization by the HIV Quasispecies Increases over Time , *AIDS Research and Human Retroviruses* **28,** 289-94.

Reeves, J. D., Gallo, S. a, Ahmad, N., Miamidian, J. L., Harvey, P. E., Sharron, M., Pohlmann, S., Sfakianos, J. N., Derdeyn, C. a & other authors. **(2002).** Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 16249–54.

Reynes, J., Portales, P., Segondy, M., Baillat, V., André, P., Réant, B., Avinens, O., Couderc, G., Benkirane, M. & other authors. **(2000).** CD4+ T cell surface CCR5 density as a determining factor of virus load in persons infected with human immunodeficiency virus type 1. *The Journal of infectious diseases* **181**, 927–32.

Richman, D. D., Wrin, T., Little, S. J. & Petropoulos, C. J. (2003). Rapid evolution of the neutralizing antibody response to HIV type 1 infection. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 4144–9.

Riedner, G., Hoffmann, O., Rusizoka, M., Mmbando, D., Maboko, L., Grosskurth, H., Todd, J., Hayes, R. & Hoelscher, M. (2006). Decline in sexually transmitted infection prevalence and HIV incidence in female barworkers attending prevention and care services in Mbeya Region, Tanzania. *AIDS* **20**, 609–15.

Ritola, K., Pilcher, C. D., Fiscus, S. A., Hoffman, N. G., Nelson, J. A. E., Kitrinos, K. M., Hicks, C. B., Eron, J. J. & Swanstrom, R. (2004). Multiple V1 / V2 env Variants Are

University of Cape Town

Frequently Present during Primary Infection with Human Immunodeficiency Virus Type 1 **78**, 11208–11218.

Rizzuto, C. & Sodroski, J. (2000). Fine definition of a conserved CCR5-binding region on the human immunodeficiency virus type 1 glycoprotein 120. *AIDS research and human retroviruses* **16**, 741–9.

Rizzuto, C. D. & Sodroski, J. G. (1997). Contribution of virion ICAM-1 to human immunodeficiency virus infectivity and sensitivity to neutralization . Contribution of Virion ICAM-1 to Human Immunodeficiency Virus Infectivity and Sensitivity to Neutralization. *Journal of Virology* **71**, 4847-51.

Robertson, DL.; Anderson, JP.; Bradac, JA.; Carr, JK.; Foley, B.; Funkhouser, RK.; Gao, F.; Hahn, BH.; Kalish, ML.; Kuiken, C.; Learn, GH; GH;McCutchan Leitner, L.; Osmanov, S.; Peeters, M.; Pieniazek, D.; Salminen, L; Sharp, P.M.; Wolinsky, Korber, B. (2000). HIV-1 Nomenclature Proposal. *Science* **288**, 55.

Rodriguez, M. a, Ding, M., Ratner, D., Chen, Y., Tripathy, S. P., Kulkarni, S. S., Chatterjee, R., Tarwater, P. M. & Gupta, P. (2009). High replication fitness and transmission efficiency of HIV-1 subtype C from India: Implications for subtype C predominance. *Virology* **385**, 416–24.

Rong, R., Li, B., Lynch, R. M., Haaland, R. E., Murphy, M. K., Allen, S. A., Pinter, A., Shaw, G. M., Hunter, E. & other authors. **(2009).** Escape from Autologous Neutralizing Antibodies in Acute / Early Subtype C HIV-1 Infection Requires Multiple Pathways **5**.

Rowell, J. F., Stanhope, P. E. & Siliciano, R. F. (1995). Endocytosis of Endogenously Synthesized HIV-1 Envelope protein. The Journal of Immunology 155, 473-88.

Rudensey, LM; Kimata, JT; Benveniste, RE; Overbaugh, J. (1995). Progression to AIDS in macaques is associated with changes in the replication, tropism, and cytopathic properties of the simian immunodeficiency virus variant population. *Virology* **207**, 528–42.

Rusert, P., Kuster, H., Joos, B., Gujer, C., Leemann, C., Stiegler, G., Katinger, H., William, C., Weber, R. & other authors. **(2005).** Virus Isolates during Acute and Chronic Human Immunodeficiency Virus Type 1 Infection Show Distinct Patterns of Sensitivity to Entry Inhibitors. *Journal of Virology* **79**, 8454-69.

Sagar, M. (2010). HIV-1 transmission biology: selection and characteristics of infecting viruses. *The Journal of infectious diseases* **202**, S289–96.

Sagar, M., Lavreys, L., Baeten, J. M., Richardson, B. A., Mandaliya, K., Chohan, B. H., Kreiss, J. K. & Overbaugh, J. (2003). Infection with Multiple Human Immunodeficiency Virus Type 1 Variants Is Associated with Faster Disease Progression. *Journal of Virology* 77, 12921–12926.

University of Cape Town

Sagar, M., Wu, X., Lee, S. & Overbaugh, J. (2006). 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. *Journal of Virology* **80**, 9586–98.

Salazar-Gonzalez, J. F., Bailes, E., Pham, K. T., Salazar, M. G., Guffey, M. B., Keele, B. F., Derdeyn, C. a, Farmer, P., Hunter, E. & other authors. **(2008).** Deciphering human immunodeficiency virus type 1 transmission and early envelope diversification by single-genome amplification and sequencing. *Journal of Virology* **82**, 3952–70.

Salazar-Gonzalez, J. F., Salazar, M. G., Keele, B. F., Learn, G. H., Giorgi, E. E., Li, H., Decker, J. M., Wang, S., Baalwa, J. & other authors. **(2009).** Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. *The Journal of experimental medicine* **206**, 1273–89.

Santiago, M. L., Rodenburg, C. M., Kamenya, S., Bibollet-Ruche, F., Gao, F., Bailes, E., Meleth, S., Soong, S.-J., Kilby, J. M. & other authors. **(2002).** SIVcpz in wild chimpanzees. *Science* **295**, 465.

Saphire, A. C. S., Bobardt, M. D., Zhang, Z. H. E. & David, G. (2001). Syndecans Serve as Attachment Receptors for Human Immunodeficiency Virus Type 1 on Macrophages. *Journal of Virology* **75**, 9187–9200.

Sather, D. N., Armann, J., Ching, L. K., Mavrantoni, A., Sellhorn, G., Caldwell, Z., Yu, X., Wood, B., Self, S. & other authors. **(2009).** Factors associated with the development of cross-reactive neutralizing antibodies during human immunodeficiency virus type 1 infection. *Journal of Virology* **83**, 757–69.

Sawyer, L.S., Wrin, M.T., Crawford-Miksza, L., Potts, B., Wu, Y., Webber, P.A., Alfonso, R.D., & Hanson (1994). Neutralization sensitivity of human immunodeficiency virus type 1 is determined in part by the cell in which the virus is propagated. *Journal of Virology* **68**, 1342-49.

Scarlatti, G; Tresoldi, E; Bjorndal, A; Freriksson, R; Colognesi, C; Deng, HK; Malnati, MS; Plebani, A; Siccardi, AG; Littman, RD; Fenyo, EM; Lusso, P. (1997). In vivo evolution of HIV co-receptor usage and sensitivity to chemokine-mediated suppression. *Nature medicine* **3**, 1259–65.

Schneidewind, A., Brockman, M. a, Yang, R., Adam, R. I., Li, B., Le Gall, S., Rinaldo, C. R., Craggs, S. L., Allgaier, R. L. & other authors. **(2007).** Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication. *Journal of Virology* **81**, 12382–93.

University of Cape Town

Schneidewind, A., Brockman, M. a, Sidney, J., Wang, Y. E., Chen, H., Suscovich, T. J., Li, B., Adam, R. I., Allgaier, R. L. & other authors. **(2008).** Structural and functional constraints limit options for cytotoxic T-lymphocyte escape in the immunodominant HLA-B27-restricted epitope in human immunodeficiency virus type 1 capsid. *Journal of Virology* **82**, 5594–605.

Schuitemaker, H., Koot, M., Kootstra, N. A., Dercksen, M. W., Steenwijk, R. P. Van, Lange, J. M., Schattenkerk, J. K. & Tersmette, M. (1992). 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 Populations. *Journal of Virology* **66**, 1354-60.

Shang, L. & Hunter, E. (2010). Residues in the membrane-spanning domain core modulate conformation and fusogenicity of the HIV-1 envelope glycoprotein. *Virology* **404**, 158–67.

Shang, L., Yue, L. & Hunter, E. (2008). Role of the membrane-spanning domain of human immunodeficiency virus type 1 envelope glycoprotein in cell-cell fusion and virus infection. *Journal of Virology* **82**, 5417–28.

Sheehy, A. M., Gaddis, N. C., Choi, J. D. & Malim, M. H. (2002). Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* **418**, 646-50.

Shioda, T; Levy, JA; Cheng-Mayer, C. (1991). Macrophage and T cell-line tropisms of HIV-1 are determined by specific regions of the enveloppe gp120 gene. *Nature* **349**, 167–9.

Shriner, D., Rodrigo, A. G., Nickle, D. C. & Mullins, J. I. (2004). Pervasive genomic recombination of HIV-1 in vivo. *Genetics* **167**, 1573–83.

Sikorski, R. S. & Hieter, P. (1989). A System of Shuttle Vectors and Yeast Host Strains Designed for Efficient Manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19-27.

Simek, M. D., Rida, W., Priddy, F. H., Pung, P., Carrow, E., Laufer, D. S., Lehrman, J. K., Boaz, M., Tarragona-Fiol, T. & other authors. **(2009).** Human immunodeficiency virus type 1 elite neutralizers: individuals with broad and potent neutralizing activity identified by using a high-throughput neutralization assay together with an analytical selection algorithm. *Journal of Virology* **83**, 7337–48.

Smith, D., Byrn, R., Marstens, S., Grgory, T., Groopman, J., Capan, D. (1987). Blocking of HIV infectivity by a soluble secreted form of the CD4 antigen. *Science* **238**: 1704 - 1707.

Smith, D. M., Richman, D. D. & Little, S. J. (2005). HIV Superinfection. *The Journal of Infectious Diseases* **192,** 438-44.

University of Cape Town

Smith, D. M., Strain, M. C., Frost, S. D. W., Pillai, S. K., Wong, J. K., Wrin, T., Liu, Y., Petropolous, C. J., Daar, E. S. & other authors. **(2006).** Lack of neutralizing antibody response to HIV-1 predisposes to superinfection. *Virology* **355**, 1–5.

Souza, M. P. D., Livnat, D. & Bradac, J. A. Sandra H . Bridges, the AIDS Clinical Trials Group, Antibody Selection Working Group and Collaborating Investigators (1997). Evaluation of Monoclonal Antibodies to Human Immunodeficiency Virus Type 1 Primary Isolates by Neutralization Assays: Performance Criteria for Selecting Candidate Antibodies for Clinical Trials,. *The Journal of Infectious Diseases* **175**, 1056–1062.

Spira, A. I. & Ho, D. D. (1995). Effect of different donor cells on human immunodeficiency virus type 1 replication and selection in vitro . Effect of Different Donor Cells on Human Immunodeficiency Virus Type 1 Replication and Selection In Vitro. *Journal of Virology* **69,** 422-29.

Stafford, M. a, Corey, L., Cao, Y., Daar, E. S., Ho, D. D. & Perelson, a S. (2000). Modeling plasma virus concentration during primary HIV infection. *Journal of theoretical biology* **203**, 285–301.

Sucupira, M. C. A., Sanabani, S., Cortes, R. M., Giret, M. T. M., Tomiyama, H., Sauer, M. M., Sabino, E. C., Janini, L. M., Kallas, E. G. & Diaz, R. S. (2012). Faster HIV-1 disease progression among Brazilian individuals recently infected with CXCR4-utilizing strains. *PloS one* **7**, e30292.

Takehisa, J., Zekeng, L., Ido, E., Yamaguchi-Kabata, Y., Mboudjeka, I., Harada, Y., Miura, T., Kaptu, L. & Hayami, M. (1999). Human immunodeficiency virus type 1 intergroup (M/O) recombination in cameroon. *Journal of Virology* **73**, 6810–20.

Tamura, K., Dudley, J., Nei, M. & Kumar, S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular biology and evolution* **24**, 1596–9.

Tang, H., Robinson, J. E., Gnanakaran, S., Li, M., Rosenberg, E. S., Perez, L. G., Haynes, B. F., Liao, H.-X., Labranche, C. C. & other authors. **(2011).** epitopes immediately below the base of the V3 loop of gp120 as targets for the initial autologous neutralizing antibody response in two HIV-1 subtype B-infected individuals. *Journal of Virology* **85**, 9286–99.

Tang, J., Tang, S., Lobashevsky, E., Zulu, I., Aldrovandi, G., Allen, S. & Kaslow, R. a. **(2004).** HLA allele sharing and HIV type 1 viremia in seroconverting Zambians with known transmitting partners. *AIDS research and human retroviruses* **20**, 19–25.

Teeraputon, S., Louisirirojchanakul, S. & Auewarakul, P. (2005). N-linked glycosylation in C2 region of HIV-1 envelope reduces sensitivity to neutralizing antibodies. *Viral immunology* **18**, 343–53.

University of Cape Town

Tomaras, G. D., Yates, N. L., Liu, P., Qin, L., Fouda, G. G., Chavez, L. L., Decamp, A. C., Parks, R. J., Ashley, V. C. & other authors. **(2008).** Initial B-cell responses to transmitted human immunodeficiency virus type 1: virion-binding immunoglobulin M (IgM) and IgG antibodies followed by plasma anti-gp41 antibodies with ineffective control of initial viremia. *Journal of Virology* **82**, 12449–63.

Tomiyama, H., Akari, H., Adachi, A. & Takiguchi, M. (2002). Different Effects of Nef-Mediated HLA Class I Down-Regulation on Human Immunodeficiency Virus Type 1-Specific CD8 + T-Cell Cytolytic Activity and Cytokine Production. *Journal of Virology* **76**, 7535-43.

Tran, E. E. H., Borgnia, M. J., Kuybeda, O., Schauder, D. M., Bartesaghi, A., Frank, G. a, Sapiro, G., Milne, J. L. S. & Subramaniam, S. (2012). Structural mechanism of trimeric HIV-1 envelope glycoprotein activation. *PLoS pathogens* **8**, e1002797.

Traunecker, A., Luke, W., Karjalainen, K., (1988). Soluble CD4 molecules neutralizes human immunodeficiency virus type 1. *Nature* **331**: 84-86

Travers, S. A. A., Connell, M. J. O., Grace, P., Mcinerney, J. O. & Mccormack, G. P. (2005). Evidence for Heterogeneous Selective Pressures in the Evolution of the env Gene in Different Human Immunodeficiency Virus Type 1 Subtypes. *Journal of Virology* **79**, 1836-41.

Troyer, R. M., Collins, K. R., Abraha, A., Moore, D. M., Krizan, R. W., Toossi, Z., Colebunders, R. L., Jensen, M. A., Mullins, J. I. & other authors. **(2005).** Changes in Human Immunodeficiency Virus Type 1 Fitness and Genetic Diversity during Disease Progression. *Journal of Virology* 79, 9006-18.

Ulenga, N. K., Sarr, A. D., Hamel, D., Sankale, J.-L., Mboup, S. & Kanki, P. J. (2008). The level of APOBEC3G (hA3G)-related G-to-A mutations does not correlate with viral load in HIV type 1-infected individuals. *AIDS research and human retroviruses* **24**, 1285–90.

UNAIDS World AIDS Day Report. (2012). http://www.unaids.org

Wagner, N; Lohler, J; Kunkel, EJ; Ley, K; Leung, E; Krissansen, G; Rajewsky, K; Muller, W. (1996). Critical role of beta7 intergrins in formation of the gut-associated lymhoid tissue. *Nature* **382**, 366–70.

Walker, B. & McMichael, A. (2012). The T-cell response to HIV. *Cold Spring Harbor perspectives in medicine* **2,** doi:pii: a007054.

Walter, B. L., Wehrly, K., Swanstrom, R., Platt, E., Kabat, D. & Chesebro, B. (2005). Role of Low CD4 Levels in the Influence of Human Immunodeficiency Virus Type 1 Envelope V1 and V2 Regions on Entry and Spread in Macrophages **79**, 4828–4837.

University of Cape Town

Wang, J., Sen, J., Rong, L. & Caffrey, M. (2008). Role of the HIV gp120 conserved domain 1 in processing and viral entry. *The Journal of biological chemistry* **283**, 32644–9.

Wei, X., Decker, J. M., Wang, S., Hui, H., Kappes, J. C., Wu, X., Salazar-Gonzalez, J. F., Salazar, M. G., Kilby, J. M. & other authors. **(2003).** Antibody neutralization and escape by HIV-1. *Nature* **422**, 307–12.

Weinfurter, J. T., May, G. E., Soma, T., Hessell, A. J., León, E. J., Macnair, C. E., Piaskowski, S. M., Weisgrau, K., Furlott, J. & other authors. **(2011).** Macaque long-term nonprogressors resist superinfection with multiple CD8+ T cell escape variants of simian immunodeficiency virus. *Journal of Virology* **85**, 530–41.

Weiss, C. (2003). Mediator of fusion and target for inhibition. *AIDS Review* 5, 214-21.

Weissenhorn, W, Dessen, A, Harrison, SC, Skehel, J. (1997). Atomic structure of the ectodomain from HIV gp41. *Nature* **387**, 426–30.

White, T. a, Bartesaghi, A., Borgnia, M. J., Meyerson, J. R., De la Cruz, M. J. V, Bess, J. W., Nandwani, R., Hoxie, J. a, Lifson, J. D. & other authors. **(2010).** Molecular architectures of trimeric SIV and HIV-1 envelope glycoproteins on intact viruses: strain-dependent variation in quaternary structure. *PLoS pathogens* **6**, e1001249.

White, T. a, Bartesaghi, A., Borgnia, M. J., De la Cruz, M. J. V, Nandwani, R., Hoxie, J. a, Bess, J. W., Lifson, J. D., Milne, J. L. S. & Subramaniam, S. (2011). Three-dimensional structures of soluble CD4-bound states of trimeric simian immunodeficiency virus envelope glycoproteins determined by using cryo-electron tomography. *Journal of Virology* **85**, 12114–23.

Wilen, C. B., Tilton, J. C. & Doms, R. W. (2012). HIV: cell binding and entry. *Cold Spring Harbor perspectives in medicine* **2**. doi:pii: a006866. 10.1101/cshperspect.a006866.

Willey, R. L., Shibata, R., Freed, E. O., Cho, M. W. & Martin, M. a. **(1996).** Differential glycosylation, virion incorporation, and sensitivity to neutralizing antibodies of human immunodeficiency virus type 1 envelope produced from infected primary T-lymphocyte and macrophage cultures. *Journal of Virology* **70**, 6431–6.

Wood, N., Bhattacharya, T., Keele, B. F., Giorgi, E., Liu, M., Gaschen, B., Daniels, M., Ferrari, G., Haynes, B. F. & other authors. **(2009).** HIV evolution in early infection: selection pressures, patterns of insertion and deletion, and the impact of APOBEC. *PLoS pathogens* **5**, e1000414.

Woodman, Z. & Williamson, C. (2012). HIV Molecular Epidemiology: transmission and adaption to human population. *Current Opinion in HIV and AIDS* **4**, 247–252.

University of Cape Town

Woodman, Z., Mlisana, K., Treurnicht, F., Abrahams, M., Thebus, R., Karim, S. A., Williamson, C., Acute, C. & Study, I. (2011). Short Communication Decreased Incidence of Dual Infections in South African Subtype C-Infected Women Compared to a Cohort Ten Years Earlier. *AIDS Research and Human Retrviruses* **27,** 1167-72.

Wooley, D. P., Smith, R. A., Czajak, S., Desrosiers, R. C., Wooley, D. P., Smith, R. A. & Czajak, S. (1997). Direct demonstration of retroviral recombination in a rhesus monkey . *Journal of Virology* **71,** 9650-53.

Wright, J. K., Novitsky, V., Brockman, M. A., Brumme, Z. L., Brumme, C. J., Jonathan, M., Heckerman, D., Wang, B., Losina, E. & other authors. **(2011).** Influence of Gag-Protease-Mediated Replication Capacity on Disease Progression in Individuals Recently Infected with HIV-1 Subtype C. *Journal of Virology* **86**, 3996-4006.

Wu, L., LaRosa, G., Kassam, N., Gordon, C.J., Heath, H., Ruffling, N., Chen, H., Humblias, J., Samson, M., Parmentire, M. (1997). Intercation of chemokine coreceptors CCR5 with its ligands: multiple domains for HIV-1 gp120 binding and a single domain for chemokine binding. *Journal of Experimental Medicine* **186**, 1373-81.

Wu, S., Löving, R., Lindqvist, B., Hebert, H., Koeck, P. J. B., Sjöberg, M. & Garoff, H. (2010). Single-particle cryoelectron microscopy analysis reveals the HIV-1 spike as a tripod structure. *Proceedings of the National Academy of Sciences of the Unites States of America* **107**, 18844-49.

Wu, X., Parast, A. B., Richardson, B. A., John-stewart, G., Mbori-ngacha, D., Rainwater, S. M. J., Overbaugh, J. & Nduati, R. (2006). Neutralization Escape Variants of Human Immunodeficiency Virus Type 1 Are Transmitted from Mother to Infant Neutralization Escape Variants of Human Immunodeficiency Virus Type 1 Are Transmitted from Mother to Infant. *Journal of Virology* **80**, 835-44

Wyatt, R., Sullivan, N., Thali, M., Repke, H., Ho, D., Robinson, J., Posner, M. & Sodroski, J. (1993). Functional and immunologic characterization of human immunodeficiency virus type 1 envelope glycoproteins containing deletions of the major variable regions. *Journal of Virology* **67**, 4557–65.

Wyatt, R., Moore, J., Accola, M., Desjardin, E., Robinson, J., Wyatt, R., Moore, J., Accola, M., Desjardin, E. & other authors. **(1995).** Involvement of the V1 / V2 variable loop structure in the exposure of human immunodeficiency virus type 1 gp120 epitopes induced by receptor binding. *Journal of Virology* **69**, 5723-33.

Wyatt, R. (1998). The HIV-1 Envelope Glycoproteins: Fusogens, Antigens, and Immunogens. *Science* **280**, 1884–1888.

University of Cape Town

Yu, X., Yu, Y., Liu, B., Luo, K., Kong, W., Mao, P. & Yu, X.-F. (2003). Induction of APOBEC3G ubiquitination and degradation by an HIV-1 Vif-Cul5-SCF complex. *Science* **302**, 1056–60.

Yue, L., Prentice, H. a, Farmer, P., Song, W., He, D., Lakhi, S., Goepfert, P., Gilmour, J., Allen, S. & other authors. **(2013).** Cumulative impact of host and viral factors on HIV-1 viral-load control during early infection. *Journal of Virology* **87**, 708–15.

Zeller, Y., Mechtersheimer, S. & Altevogt, P. (2001). Critical Amino Acid Residues of the a4 Subunit for a4b7 Integrin Function. *Journal of Cellular Biochemistry* **319**, 304–319.

Zhang, Y. J., Fredriksson, R., McKeating, J. a & Fenyö, E. M. (1997). Passage of HIV-1 molecular clones into different cell lines confers differential sensitivity to neutralization. *Virology* **238**, 254–64.

Zhang, Z. (1999). Sexual Transmission and Propagation of SIV and HIV in Resting and Activated CD4+ T Cells. *Science* **286**, 1353–1357.

Zhou, T., Xu, L., Dey, B., Hessell, A. J., Van Ryk, D., Xiang, S.-H., Yang, X., Zhang, M.-Y., Zwick, M. B. & other authors. **(2007).** Structural definition of a conserved neutralization epitope on HIV-1 gp120. *Nature* **445**, 732–7.

Zhu, T., Wang, N., Carr, A., Wolinsky, S., Ho, D. D., Zhu, T., Wang, N., Carr, A. & Wolinsky, S. (1995). Evidence for coinfection by multiple strains of human immunodeficiency virus type 1 subtype B in an acute seroconvertor. *Journal of Virology* **69,** 1324-27.

Zhu, T; Mo, H; Wang, N; Nam, DS; Cao, Y; Koup, RA; Ho, D. (1993). Genotypic and phenotypic characterization of HIV-1 patients in primary infection. *Science* **261**, 1179–81.

Zhuang, J., Jetzt, A. E., Sun, G., Yu, H., Klarmann, G., Ron, Y., Preston, B. D. & Dougherty, J. P. (2002). Human Immunodeficiency Virus Type 1 Recombination: Rate , Fidelity , and Putative Hot Spots. *Journal of Virology* **76**, 11273–11282.

University of Cape Town