The Role of HIV-1 Transmitted/Founder Virus Characteristics in Driving Pathogenesis

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Dedication

This thesis is dedicated to the new family I acquired during my PhD…Omar and Ethan Williams!

And to my old family…dad, mom, Francis, Joyce and Kevin.

Declaration of originality

I, Gladys Njeri Macharia, declare that all work presented in this thesis is entirely of my own work and that the information derived from the published or unpublished work of others has been appropriately referenced.

Copyright Declaration

Abstract

HIV-1 transmission is associated with a severe bottleneck in which a limited number of variants from genetically diverse quasispecies establish infection. The IAVI protocol C cohort of discordant couples, female sex workers, other heterosexuals and men who have sex with men (MSM) present varying risks of HIV infection, diverse HIV-1 subtypes and present a unique opportunity to characterize transmitted/founder viruses (TFv) where disease outcome is known.

To identify the TFv, the HIV-1 repertoire of 38 MSM was sequenced close to transmission (median 21dpi) and assessment of multivariant infection, subtype and genetic polymorphisms done. Patient derived *gag* genes were cloned into a NL4.3 provirus to generate chimeric viruses which were characterised for replicative capacity (RC) and mechanism of spread between cells. Finally, an evaluation of how the virus characteristics that were predictors of disease progression modified the immune response at both acute and chronic HIV-1 infection was done.

There was higher incidence of multivariant infection compared with previously described heterosexual cohorts. TFv predictors of CD4 T-cell decline and set-point viral load included multivariant infection, subtype, drug resistance mutations and RC. A link was identified between these characteristics and both chronic immune activation and rapid $CD4^+$ T cell decline except in multivariant infection where perturbations were restored after control of viremia. The cell entry and CD4+ T cell depletion mechanisms by high RC TFv overlapped with those involved in cell-cell transmission but not cell free spread and involved increased expression of RNA that encodes proteins involved in apoptosis, autophagy and necrosis.

Strategies aimed at mitigating persistent immune activation could contribute toward improving HIV-1prognosis and research presented in this thesis suggests that this may involve strategies that sieve out high RC TFv and tighten the stringency of the transmission bottleneck. Furthermore, the sequences and chimeric viruses provide a useful resource in the field of immunogen design, for their utility in designing TFv peptide sets and for use in functional assays to probe effective immune responses against TFv.

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List of abbreviations and acronyms

ADP - Adenosine diphosphate
AIDS- Acquired immune deficiency syndrome
APOBEC - Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like

BAFF - B-cell activating factor
BCL2 - B-cell lymphoma 2
BSA - Bovine serum albumin

cART - Combination antiretroviral therapy
CCR5 - C-C chemokine receptor 5
CD - Cluster of differentiation
CDC - Centre for Diseases Control
cDNA - Complementary DNA
CMV - Cytomegalovirus
CRFs - Circulating recombinant forms
CTL - Cytotoxic T lymphocyte
CTLA 4 - Cytotoxic T lymphocyte associated protein 4
CXCL - C-X-C chemokine ligand
CXCR - C-X-C chemokine receptor

DC-SIGN - Dendritic cell specific intercellular adhesion molecule 3 grabbing non-integrin
DMEM - Dulbecco's modified eagle medium
DNA - Deoxynucleic acid
dNTP - Deoxyribonucleotide triphosphate
DRM - Drug resistance mutation
dsDNA - Double stranded DNA
DTR - Doutegravir

EBV- Epstein Bar virus
E. coli - Escherichia coli
EDI - Estimated date of infection
ESR1 - Estrogen receptor 1
ELISA - Enzyme linked immunosorbent assay
ERT - Etravirine
EFZ - Efavirenz
ELT - Elvitegravir

FCS - Foetal calf serum
FMO - Fluorescence minus one

GALT - Gut associated lymphoid tissue
GFP - Green fluorescent protein
GM-CSF - Granulocyte macrophage colony stimulating factor
GWAS - Genome-wide association study

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV - Human immunodeficiency virus
HLA - Human leucocyte antigen

ICAM-1 - Intercellular adhesion molecule 1
IDU - Intravenous drug user
IL - Interleukin
INDELs - Insertions or deletions
IMC - Infectious molecular clone
IFNα - Interferon alpha
KEMRI - Kenya Medical Research Institute
KIR - Killer cell immunoglobulin-like receptor
KLRG1 - Killer cell lectin-like receptor subfamily G member 1

LANL - Los Alamos National Library
LFA-1 - Lymphocyte function-associated antigen 1
LTR - Long terminal repeat

MIC A – MHC class 1 polypeptide-related sequence A
MHC - Major histocompatibility complex
MIP-1β - Macrophage inflammatory protein 1 beta
ML tree - Maximum likelihood tree
MOI - Multiplicity of infection
MSM - Men who have sex with men
MSD - Meso scale discovery

NAD - Nicotinamide adenine dinucleotide
NFLG - Near full-length genome
NIH - National Institute of Health
NJ tree - Neighbour joining tree
NK cell - Natural killer cell
NNRTI - Non-nucleoside reverse-transcriptase inhibitors
NVP - Nevirapine

PacBio - Pacific biosciences
PAMPS - Pathogen associated molecular patterns
PBMC - Peripheral blood mononuclear cells
PBS - Phosphate buffered saline
PCR - Polymerase chain reaction
PD-1 - Programmed death 1
PE - Phycoerythrin
PHA - Phytohemagglutinin
PI - Protease inhibitor
PTE - Potential T cell epitopes

RAL - Raltegravir
RC - Replicative capacity
RIP - Recombinant identification program
RNA - Ribonucleic acid

RPMI - Roswell Park Memorial Institute
RPV- Rilpivirine

SIV - Simian immunodeficiency virus
SMRT - Single molecule real time
SNP - Single nucleotide polymorphism
SPVL - Set point viral load
STD - Sexual transmitted disease
STI - Sexually transmitted infection

TARC – Thymus and activation regulated chemokine
TCID - Median tissue culture infective dose
T_{CM} - Central memory T cell
TDR - Transmitted drug resistance
T_{EM} - Effector memory T cell
T/F - Transmitted/founder
TLR - Toll-like receptor
TNFa - Tumour necrosis factor alpha
T-reg - Regulatory T cell
TRIM5 - Tripartite motif-containing protein 5
T_{TE} - Terminal effector T cell
T_{TM} - Transitional memory T cell

UK - United Kingdom
UNAIDS - United Nations program on HIV/AIDS
USA - United States of America

VL - Viral load
vRC - Viral replicative capacity
VEGF - Vascular endothelial growth factor

WHO - World Health Organization

ZEHRP - Zambia Emory HIV research project

Publications arising from this thesis

Conference presentations

Gladys Macharia, Ecco Staller, Ling Yue, Elina El-Badry, Dario Dilernia, Edward McGowan, Nesrina Imami, Eduard Sanders, Eric Hunter and Jill Gilmour. Infection With Multiple Transmitted/Founder (TF) HIV-1 Viruses impacts peak VL and HIV-1 pathogenesis. Research for Prevention 2018: AIDS Vaccine, Microbicide and ARV-based Prevention Science (HIV R4P). 21st - 26th October, Madrid, Spain.

Gladys Macharia, Ecco Staller, Ling Yue, Elina El-Badry, Dario Dilernia, Edward McGowan, Nesrina Imami, Eduard Sanders, Eric Hunter and Jill Gilmour. Replicative capacity of transmitted *gag* drives HIV-1 pathogenesis. Research for Prevention 2018: AIDS Vaccine, Microbicide and ARV-based Prevention Science (HIV R4P). 21st - 26th October, Madrid, Spain.

Gladys Macharia, Ling Yue, Dario Dilernia, Elina El-Badry, Edward McGowan, Nesrina Imami, Matt Price, Eduard Sanders, Jill Gilmour and Eric Hunter. Transmission of multiple HIV-1 founder viruses with high level of recombination in MSM in Kenya. Research for Prevention 2016: AIDS Vaccine, Microbicide and ARV-based Prevention Science (HIV R4P). 17th- 21st October, Chicago, USA.

Gladys Macharia, Ling Yue, Dario Dilernia, Ecco Staller, Daniel Wilkins, Elina El-Badry, Edward McGowan, Nesrina Imami, Eduard Sanders, Jill Gilmour and Eric Hunter. Transmission of multiple HIV-1 founder viruses with high level of recombination in Kenyan Men who have sex with men (MSM). HIV transmission meeting. 16th October 2016. Chicago, USA.

Elina El-Badry, **Gladys Macharia,** Daniel Claiborne, William Kilembe, Jill Gilmour, Susan Allen and Eric Hunter. Zambian women exhibit an exacerbated inflammatory response to early HIV infection compared to men. Keystone Symposia: HIV & Co-Infections. British Columbia, Canada. April 15th-19th 2018.

Manuscripts in preparation

Macharia *et al*., Multivariant HIV-1 infection is associated with lower CD4 T cell counts and modifies the immune response to HIV-1

Macharia *et al*., Replicative capacity conferred by *gag* drives HIV-1 subtype A pathogenesis

Chapter 1: Introduction

General introduction

1.1 Brief history of HIV and AIDS

In the period leading to 1981 groups of young previously healthy, homosexual men presented with unusual infections such as *Pneumocystis carinii* (now *Pneumocystis jirovecii*) pneumonia, mucosal candidiasis, Kaposi's sarcoma, and multiple viral infections (CDC 1981; Gottlieb et al. 1981; Siegal et al. 1981). These infections occurred after prolonged fevers of unknown origin and the patients were anergic, lymphopenic and lacked lymphocyte proliferative responses to soluble stimulants including the potent phytohemagglutinin (PHA). These patients were found to have low $CD4^+$ T cell counts and described as having acquired immunodeficiency syndrome (AIDS) (Siegal et al. 1981; Gerstoft et al. 1982). Subsequent documentation of cases of AIDS amongst individuals with haemophilia, blood transfusion recipients and heterosexual intravenous drug abusers and their sex partners suggested that an infectious agent was the cause of the immunologic defects associated with AIDS. Two years later in 1983, a retrovirus from the lentivirus family, human immunodeficiency virus type 1 (HIV-1), was identified as the etiologic agent of AIDS (Barre-Sinoussi et al. 1983; Gallo et al. 1983).

Nearly four decades later, HIV-1 is a global pandemic, in which an estimated 36.7 million people were living with HIV in 2017, and another 1.8 million people becoming newly infected with HIV-1 annually (UNAIDS 2017). While advances in treatment methods have resulted in a decline of AIDS-related deaths by 48% since the peak in 2005, only 60% of people infected with HIV-1 knew their status and only 60% of those who knew their status had access to combination antiretroviral therapy (cART) as of June 2017. Thus, up to 1 million people still die from AIDS-related illnesses every year, the majority of whom are in lower and middle-income countries and often present for treatment too late when their immune system is damaged to the point of opportunistic infections (UNAIDS 2017). HIV infection is now considered a

life-long illness that remains a chronic disease manageable only by successfully suppressive cART but for which no cure or vaccines are available.

1.2 HIV subtypes

HIV is grouped into two main types, HIV-1 and 2, representing separate cross overs from non-human primates to humans (Peeters and Delaporte 1999; Gaschen et al. 2002). HIV-1 was discovered first and is more widespread, more virulent and therefore more widely studied than HIV-2 which is mostly restricted to West Africa and appears to be on the decline (Peeters, Piot, and van der Groen 1991; da Silva et al. 2008; Campbell-Yesufu and Gandhi 2011; Menendez-Arias and Alvarez 2014). HIV-1 and 2 differ by up to 55%. HIV-2 is believed to have been transmitted to humans around 1932, from a related simian immunodeficiency virus (SIV) in sooty mangabeys (SIVsm) while HIV-1 is believed to have been transmitted to humans on at least three separate occasions around 1908 from a SIV found in chimpanzees (SIVcpz) (Peeters, Piot, and van der Groen 1991; Korber et al. 2001).

Phylogenetically, HIV-1 is classified into groups M (major), O (outlier), N (non-M, non-O) and P (Putative) (Robertson et al. 2000; Sharp and Hahn 2011). Of these, only group M underwent pandemic spread and is the most widely studied circulating group. Mathematical models suggest a most recent common ancestor near 1910–1930 (Korber et al. 2000), followed by subclinical endemic spread of the virus in human populations in West Central Africa (Perrin, Kaiser, and Yerly 2003; Worobey et al. 2008). Within group M exist several clades/subtypes named A to K, along with their Circulating Recombinant Forms (CRFs). These can differ by up to 25%-35% in the *env* gene (Gaschen et al. 2002; Gilbert et al. 2007).

The global distribution of the different subtypes continues to evolve, with subtype C accounting for a majority of the infections worldwide (Hemelaar et al. 2006, 2011). Genetic variation within a subtype can be of the order of 15-20%, whereas variation between subtypes is approximately 25-35%, depending on the subtypes and genome regions studied (Korber et al. 2000). In addition to the main clades there are also 48 circulating recombinant forms (CRF), denoted by numbers (ascending in order of discovery) followed by letters of the parental clade. Recent advances in sequencing technology have enabled sequencing of full length HIV genomes, revealing the increasing role of inter-subtype recombinants in the HIV-1 pandemic (Hemelaar et al. 2011). Each HIV-1 Clade has a distinct geographical localization which appears to be relatively stable, although this is informed by studies employing partial genome sequencing, which may be inaccurate as it does not factor recombination events outside of the commonly sequenced genes. (Figure 1.1). Moreover, even within the same clade, clades isolated in different geographic locations form distinct phylogenetic clusters, further providing evidence for immune and genetic pressure on HIV-1‘s evolution. Clade C dominates sub-Saharan Africa and accounts for nearly half of all global infections (Miri et al. 2014; Mumtaz et al. 2011; Peeters, Jung, and Ayouba 2013).

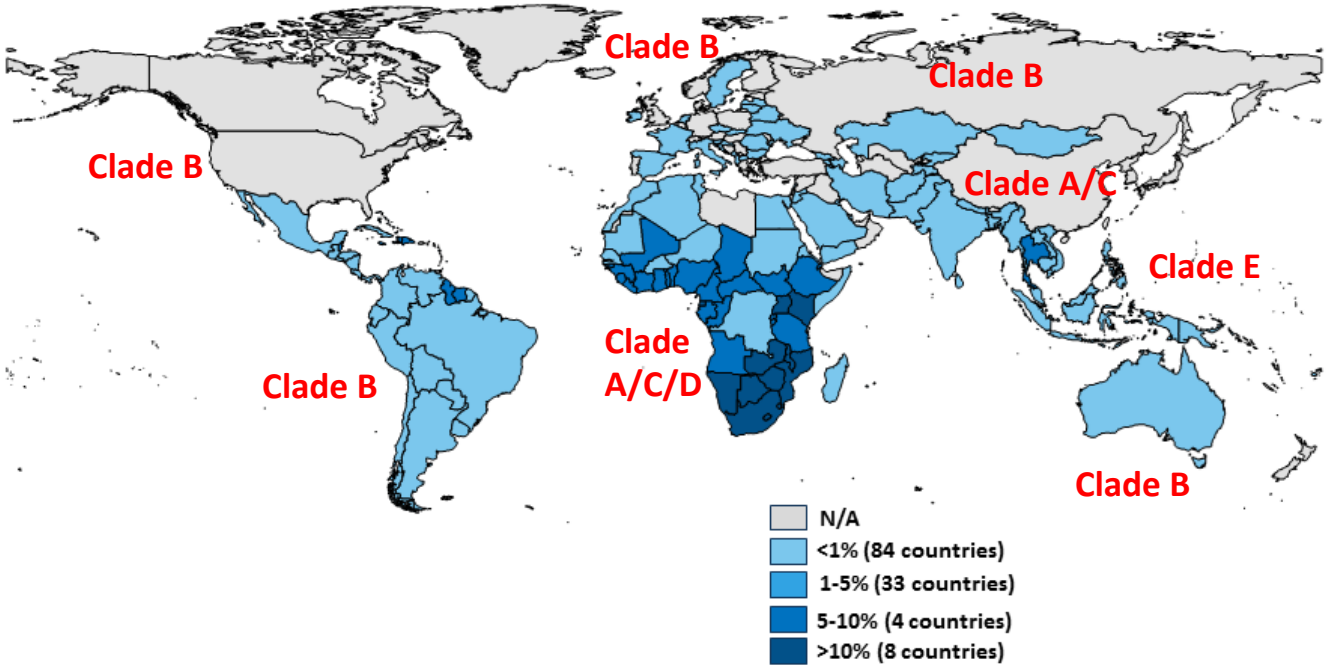


Figure 1.1: Prevalence and geographic distribution of HIV subtypes.

Map of the world showing the geographical distribution of the main circulating clades of HIV-1 virus with Africa harbouring the highest incidence of HIV-1. Whilst Clade B is the most common subtype in the Americas and Western Europe, Clade A, C and D dominate in Africa and account for majority of global infection. Modified from (KFF 2017)

1.3 Clinical stages and progression to AIDS

Despite the many routes by which HIV-1 can be transmitted from one individual to another, there is a sequential and reproducible appearance of viral and host markers of infection in blood (Busch et al. 1995). Fiebig and colleagues devised a laboratory staging system for acute and early infection based on this appearance of laboratory markers of new HIV-1 infection in peripheral circulation (Figure 1.2) (Fiebig et al. 2003). The initial eclipse period between when the first cell is infected but prior to the detection of viral RNA in plasma lasts around 7-21 days (Gaines et al. 1988; Lindback, Karlsson, et al. 2000; Schacker et al. 1996). During the eclipse

phase low levels of viral replication occur, primarily in the mucosa, submucosa and draining lymph nodes.

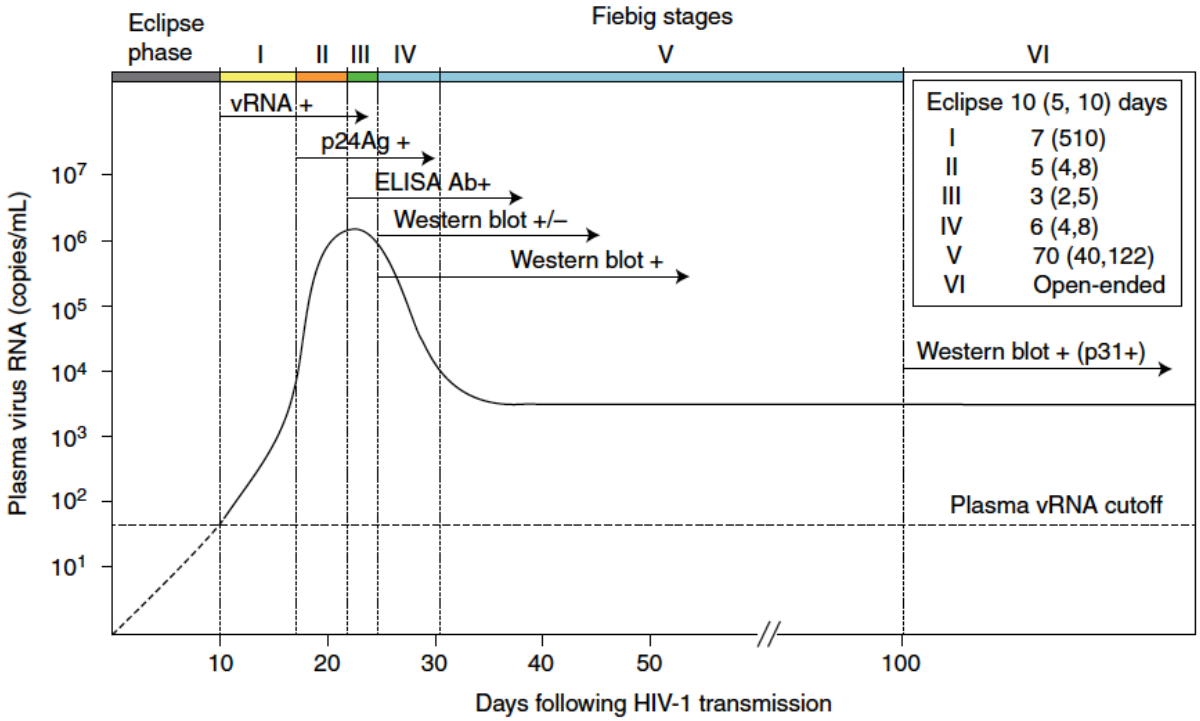


Figure 1.2: Staging of acute HIV-1 infection.

The weeks following HIV-1 infection can be divided into clinical stages that are defined by a stepwise detection of HIV-1 antigens and HIV-1-specific antibodies in laboratory assays (Fiebig et al. 2003; Oxenius et al. 2000).

HIV-1 is then transported into draining lymphoid tissue and ultimately gut-associated lymphoid tissue (GALT), where approximately 80% of the CD4 T cells in the GALT can be infected in the first three weeks of infection (Schacker 1996; Mehandru et al. 2004; Hladik et al. 2007; Brenchley and Douek 2008; Haase 2011). The precise mechanism by which HIV-1 is transported to the GALT remains unclear. Dendritic cells can capture and internalize HIV-1 through the interaction of DC-specific C-type lectin (DC-SIGN) and HIV-1 envelope

glycoprotein gp120, and then transmit HIV-1 to $CD4^+$ T cells (Geijtenbeek et al. 2000; Moris et al. 2006). The HIV-1 envelope can also directly interact with the gut homing receptor integrin $\alpha4\beta7$ on $CD4^+$ T cells and get trafficked to the gut (Arthos et al. 2008; Cicala et al. 2009; Perez et al. 2014). GALT remains a primary reservoir for HIV-1 throughout the course of HIV-1 infection, with latently infected cells maintaining low level viral replication in spite of cART (Chun et al. 2008)

Most patients experience a febrile illness coinciding with massive replication of the virus in mucosal $CD4^+$ T cells, the draining lymph nodes and the GALT, resulting in rapid damage to generative immune cell microenvironment (Gaines et al. 1988; Schacker et al. 1996). The virus then starts to spill into circulation and traces of viral RNA and eventually the HIV-1 protein p24 can be detected in peripheral blood. An exponential increase in plasma virus to a peak ensues, with eventual detection of HIV-1 specific antibodies, first by ELISA then by Western blot (Busch et al. 1995; Lindback, Thorstensson, et al. 2000; Fiebig et al. 2003). Diagnosing patients as soon as possible after infection is important for ensuring the prevention of forward transmission from the index patient and to ensure the safety of blood for transfusion and organs for transplant (Haase 2010; Cohen and Corbett 2011). Current treatment guidelines suggest that patients are started on treatment as soon as they are diagnosed, as preservation of immune cells and long term clinical benefit has been demonstrated in patients who are started on treatment during the acute stage of disease (Oxenius et al. 2000; Kok et al. 2015; Krebs and Ananworanich 2016; Schuetz et al. 2014; Haase 2010).

Following acute infection, the virus load is maintained at a more stable level (viral set point/set point viral load) albeit at different levels with some patients showing lower levels that others, by a balance of virus turnover and immune responses, typically declining 10-200-fold. This decline in viremia occurs in the absence of therapeutic intervention and is concomitant with the

emergence of HIV-1 specific $CD8^+$ T cell responses, and partial recovery of $CD4^+$ T cell counts (Goonetilleke et al. 2009; McMichael et al. 2010). In the absence of treatment, sustained hyperactivation and progressive immune dysfunction in chronic infection contributes to progressive decline in $CD4^+$ T cell count and in most patients, results in immune collapse and progression to AIDS, which is associated with a rise in plasma viremia and decline of CD4 lymphocyte numbers to below 200 cells/μl blood (Cohen et al. 2011). The combined effects of CD4 T cell loss and immune dysregulation leaves HIV-1 infected individuals susceptible to multiple co-infections such as pneumonia, hepatitis C virus and tuberculosis.

However, some rare individuals known as elite controllers are able to suppress virus load to below the limit of detection using standard methods and exhibit relative CD4 T cell preservation and delayed disease progression (Autran et al. 2011; Miura et al. 2008). On the other hand, long-term non progressors are able to maintain high levels of CD4 T cell counts in the absence of therapy (Imami et al. 2013; Westrop et al. 2017). These groups often overlap, but elite controller or long-term non progressor status can be lost in the race between viral evolution and the immune system (Brener et al. 2018; Chakrabarti 2004; Monaco, Ende, and Hunter 2017). Comparisons between these individuals and rapid progressors continue to reveal a role for both host genetics and viral characteristics in disease outcome.

1.4 The biology of HIV-1

1.4.1 The HIV-1 life cycle

HIV-1 is a lentivirus of the *Retroviridae* family. Structurally, the HIV-1 envelope encloses a capsid made up of the viral protein p24, containing two single strands of HIV RNA, each of which encodes a complete copy of the virus's 9 genes. The large polyproteins are then spliced

to the six structural proteins (Matrix, Capsid, Nucleocapsid, p6, gp120 and gp41), the three enzymes involved in its life cycle (Reverse transcriptase, Integrase and Protease) and 6 regulatory proteins (Tat, Rev, Nef, Vif, Vpr and Vpu) (Frankel and Young 1998).

HIV-1 enters target cells through the stepwise interaction of its envelope glycoproteins with CD4 and a co-receptor, usually either CCR5 or CXCR4 (Figure 1.3) (Cashin et al. 2013). Receptor binding induces a conformational change in Env that results in fusion pore formation and virus-cell membrane fusion. CCR5 (R5) tropic viruses are prevalent during the early stages of HIV-1 infection while CXCR4 (X4) tropic viruses emerge during chronic infection (Eckert and Kim 2001; Gorry and Ancuta 2011).

Fusion with the host cell membrane delivers the capsid which shuttles across the cytoplasm and delivers virion's contents into the nucleus, with reverse transcription of HIV-1 RNA to single stranded DNA using a reverse transcriptase enzyme that the virion carries occurring within the capsid. The newly synthesized viral dsDNA is then translocated across the nuclear pore as part of the pre-integration complex to the nucleus, where the HIV-1 enzyme integrase catalyses the integration of viral DNA into the host chromosomal DNA (Whittaker, Kann, and Helenius 2000). The choice of integration site is important in the establishment of a viral reservoir, which ultimately prevents clearance of the virus (Chun et al. 1997; Schroder et al. 2002; MacNeil et al. 2006). Figure 1.4 summarizes the timing of each of the steps in the HIV-1 life cycle.

Viral transcripts from the integrated viral genome are expressed when the HIV-1 Tat binds to the 5' long terminal repeat (LTR) and initiates transcription and elongation (Freed 2001). The viral transcripts are then transported to the cytoplasm where the majority are translated and capsid assembly occurs, a process regulated by the Rev protein. The envelope proteins are not included in the assembly but are instead transported to the cell surface for incorporation into the outer membrane of the viral particle. As the viral particle buds, it takes a fragment of the

cell membrane with the envelope proteins and undergoes maturation (Freed 2001; Morita and Sundquist 2004; Mohammadi et al. 2013).

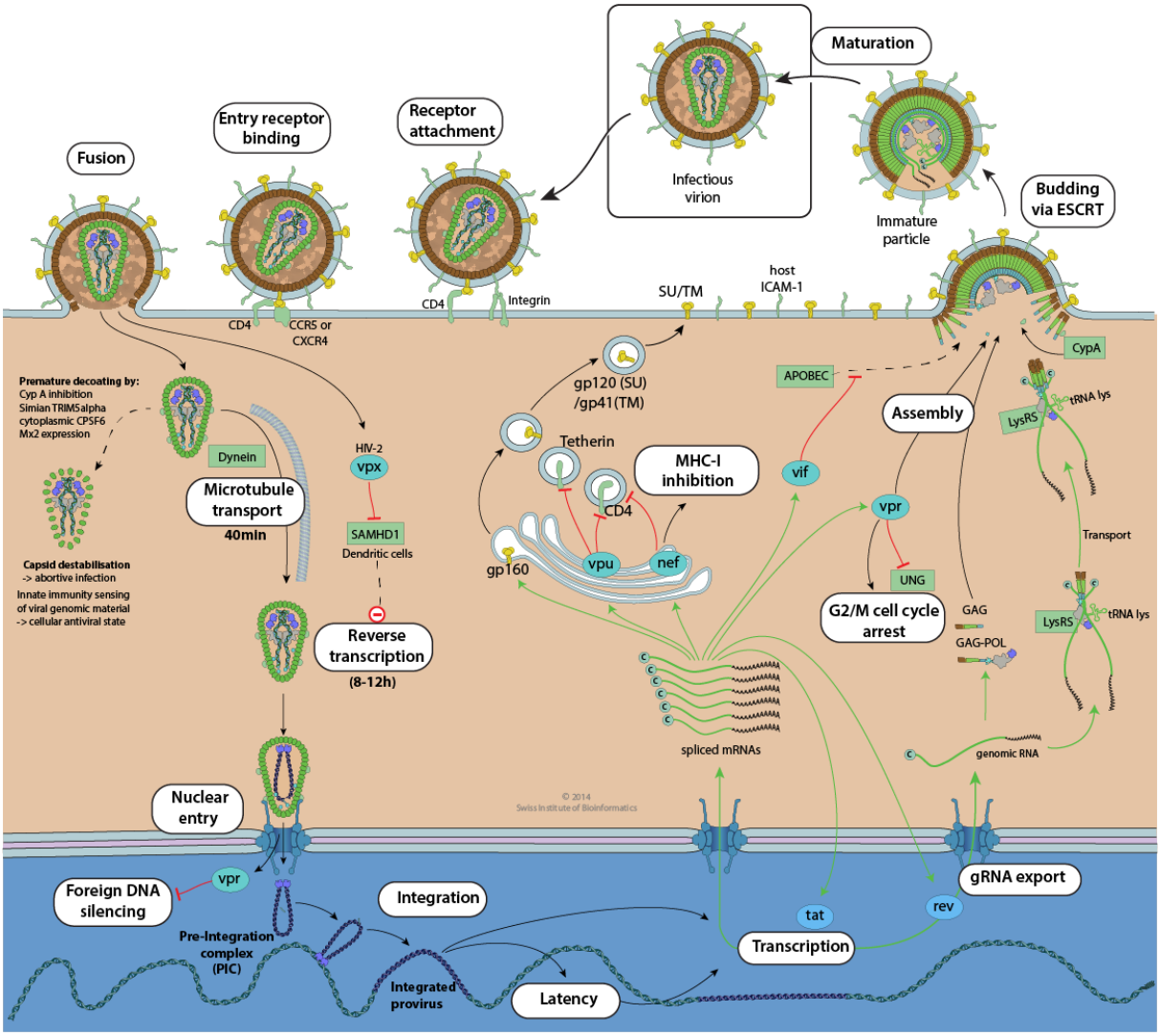


Figure 1.3: Life cycle of HIV-1.

The *env* (envelope) gene encodes the proteins of the outer envelope of the virus, the *gag* (group-specific antigen) gene encode the components of the inner capsid protein, whereas the *pol* (polymerase) gene codes for the enzymes that are used in viral replication (Ambrose and Aiken 2014).

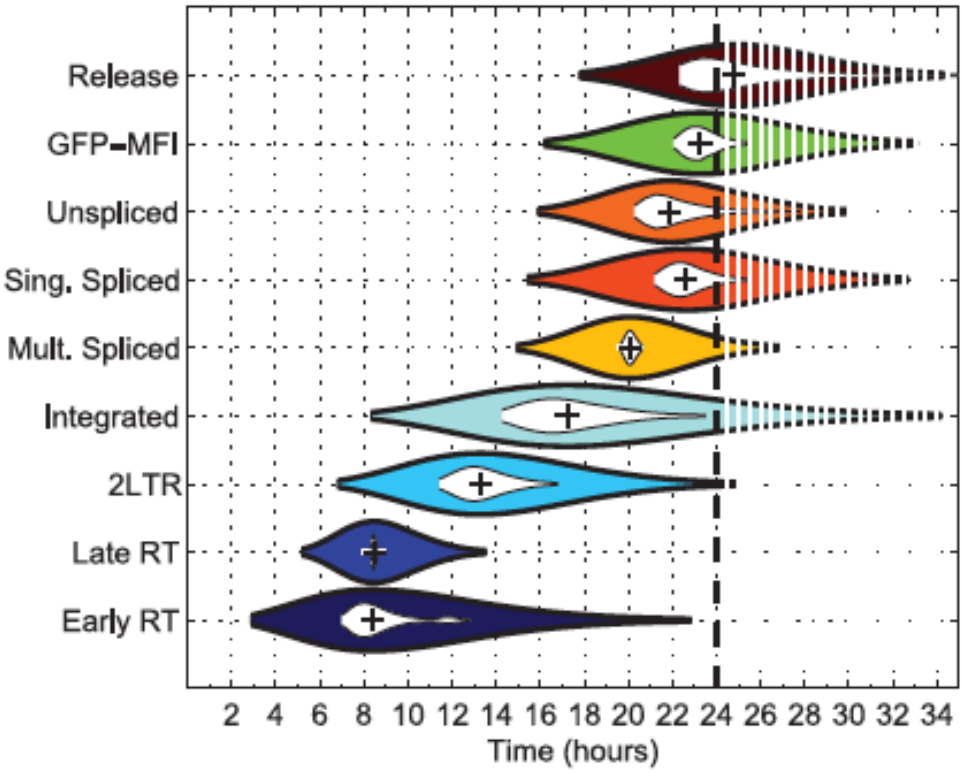


Figure 1.4: Activity profile of the HIV-1 life cycle.

Each violin spans the 98% quantile of the viral step with width proportional to activity level at each given point in time. The plus symbol denotes the peak of the activity and the inner white violin its 95% bootstrap confidence interval. In the shaded area, expected values have been extrapolated beyond the last observed time point, which is the dashed line at 24 hours. (Mohammadi et al. 2013).

Several factors have been shown to contribute to HIV-1's evasion from immunity including the destruction of virus-specific T helper cells, the emergence of immune escape, and the expression of an envelope complex that structurally minimizes antibody access to conserved epitopes. Various genes of HIV-1 have also been implicated in immune evasion including *nef*'s

ability to prevent presentation of viral antigens by the major histocompatibility complex (MHC) and *vif*'s counteraction of APOBEC3G and APOBEC3H activity.

Antiretroviral drugs target different steps in the HIV-1 life cycle, most commonly by blocking fusion, preventing reverse transcription, inhibiting integration of HIV-1 genomes into host genomic DNA or by blocking the viral protease enzyme that is necessary for virion maturation.

1.4.2 HIV-1 transmission

HIV-1 is transmitted by contact across mucosal surfaces for instance through sexual intercourse, by percutaneous inoculation as happens though intravenous drug use and needle stick injuries, or from mother to child through breastmilk. Sexual intercourse accounts for the majority of HIV infections. Because mucosal exposures in humans are inaccessible to direct analysis, our understanding of the transmission event comes from studies of HIV-1 epidemiology, viral and host genetics, risk factor analyses, animal models, human explant tissues, and *in vitro* studies of virus-target cell interactions.

The efficiency of HIV-1 transmission varies by the route of exposure, with multiple sexual exposures being required for most of the heterosexual transmissions (Powers et al. 2008; Attia et al. 2009; Dosekun and Fox 2010). The probability of infection for each encounter with the virus is estimated to be as low as 1 infection per 200-1000 contacts for heterosexual transmission based on studies in discordant couples (Hladik and McElrath 2008) but much higher during acute HIV-1 infection when viral load is higher, and the viral characteristics closest to consensus sequence. However, the likelihood of the transmission event can be increased by conditions that compromise mucosal integrity, increase the number of susceptible cells present at the mucosa or increase the inoculation dose. These include the presence of

genital inflammation, sexually transmitted infections, high donor viral load, transmission routes and male circumcision (Attia et al. 2009; Auvert et al. 2005; Boeras et al. 2011; Dosekun and Fox 2010; Galvin and Cohen 2004; Kahn, Marseille, and Auvert 2006; Keele and Estes 2011; Li et al. 2010; Powers et al. 2008; Quinn 2007; Wamai et al. 2015). Penile-anal transmission carries the highest risk of mucosal transmission with infectivity exceeding 1 successful transmission per 3 contacts (Attia et al. 2009; Dosekun and Fox 2010). Heterosexual transmission is responsible for nearly 70% of all new infections worldwide, with the remainder being attributable to mother-child transmission, men who have sex with men (MSM) and intravenous drug use (IDU) (Li et al. 2010; Dosekun and Fox 2010).

The mechanism by which HIV-1 crosses the genital mucosal milieu is poorly characterised owing to a lack of perfect mucosal transmission model, but $CD4^+$ T cells, dendritic cells, macrophages and Langerhans cells are HIV-1's earliest targets (Miller et al. 2005). HIV-1 virions traverse the mucosal epithelium through transcytosis, endocytosis, or by penetrating through gaps in compromised epithelium to make initial cell contact with underlying mucosal Langerhans cells (Moris et al. 2006; Miller et al. 2005). Infection of activated and memory $CD4^+$ T cells is more efficient than that of quiescent cells (Spira et al. 1996).

1.4.3 HIV-1 transmission bottleneck and selection bias

Several human and animal studies describe a population bottleneck in which a limited number of HIV-1 variants from genetically diverse quasi-species in the transmitting partner establish productive infection in the newly infected partner (Wolfs et al. 1992; Wolinsky et al. 1992; Zhu et al. 1996) (Figure 1.5).

Subsequent studies continue to explore the genetic and biologic characteristics of what is now termed the "founder virus", as well as host factors that can modify the stringency of this transmission bottleneck (Derdeyn et al. 2004; Frange et al. 2013; Gottlieb et al. 2004; Keele et

al. 2008; Powers et al. 2008; Ritola et al. 2004; Sagar, Kirkegaard, et al. 2004; Sagar, Lavreys, et al. 2004; Salazar-Gonzalez et al. 2009; Zhu et al. 1996). These studies found out that approximately 80% of heterosexual patients involve productive infection by a single viral genome (Abrahams et al. 2009; Haaland et al. 2009; Keele and Estes 2011) and approximately 60%, 40% and 68% of MSM, IDUs and mother to child transmissions respectively are productively infected by single genomes. The range in transmitted/founder genomes observed has been 1–16 genomes (Bar et al. 2010; Masharsky et al. 2010). The observed differences in the multiplicity of viral infection associated with different routes of transmission roughly correlate to the relative risk of clinical infection (Dosekun and Fox 2010).

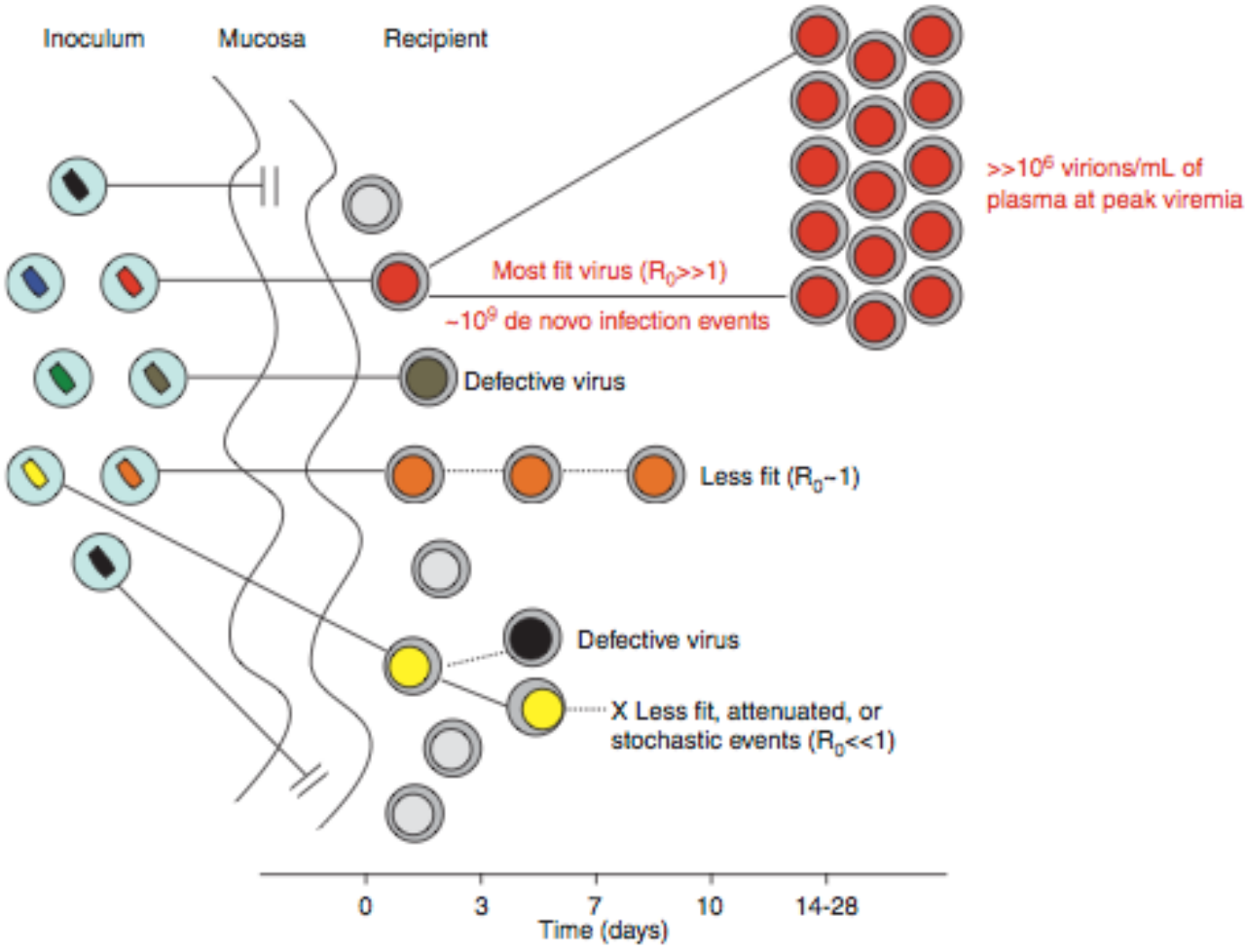


Figure 1.5: HIV-1 transmission model.

HIV-1 virions that breach the mucosa may have different fates. R0 is the reproductive ratio. R0>1 leads to productive clinical infection, whereas R0<1 results in an extinguished infection (Shaw and Hunter 2012).

Breakthrough viruses may have unique properties that confer a higher capacity to transmit. Approaches using single genome amplification of endpoint diluted plasma or peripheral blood mononuclear cells (PBMC) followed by direct sequencing of uncloned DNA amplicons combined with comparisons between virus sequences at acute infection with those at chronic infection or those from a donor patient reveal that the founder virus is rarely the dominant variant in the plasma or genital tract of the transmitting partner (Boeras et al. 2011; Frange et al. 2013; Salazar-Gonzalez et al. 2008). This suggests that the transmission bottleneck involves selection of biological and genetic traits that make the founder virus more transmissible.

Founder virus envelopes tend to be CCR5 tropic, sensitive to neutralisation by autologous antibodies and encode shorter and less glycosylated V1-V4 regions (Ashokkumar et al. 2018; Derdeyn et al. 2004; Keele et al. 2008; Oberle et al. 2016; Ritola et al. 2004; Salazar-Gonzalez et al. 2008), suggesting that transmission is related to the loss of *env* adaptations required for neutralisation in the donor patient, but unimportant in the newly infected seronegative partner. Surprisingly, these characteristics are not observed in subtype B infection. By comparing founder virus sequences to those in chronic patients in epidemiologically linked transmission pairs using mathematical modelling, Carlson et al observed increased transmission of amino acids within *gag, pol* and *nef* that favour fitness and that were closest to consensus (Carlson et al. 2016; Carlson et al. 2014; Deymier et al. 2015). Subtype A, B and C founder viruses have also been shown to replicate more efficiently in primary human $CD4^+$ T cells but much less in monocyte derived macrophages (Ochsenbauer et al. 2012; Salazar-Gonzalez et al. 2009).

Interestingly, a substantial proportion of subtype D transmitted/founder viruses replicated efficiently in both $CD4^+$ T cells and macrophages (Baalwa et al. 2013). Studies of full length infectious molecular clones (IMC) of founder viruses in comparison to viruses from chronic infection have demonstrated increased particle infectivity and enhanced resistance to IFNα in some cohorts but not others (Deymier et al. 2015; Fenton-May et al. 2013; Parrish et al. 2013; Parrish et al. 2012; Iyer et al. 2017).

1.4.4 HIV-1 spread between cells

While both cell-free and cell-associated HIV-1 are present in the mucosa, cell free spread is the most widely studied. *In vitro*, cell-to-cell transfer of HIV-1 between T cells leads to a massive and very efficient infection that may be up to 1000-fold more efficient than infection carried out by cell-free viral particles (Martin and Sattentau 2009).

Productive cell-to-cell infection requires interaction between the viral envelope glycoproteins on the surface of the infected cell and HIV-1 receptors on the surfaces of target cells, leading to the formation of virological synapses. The synapse is initiated by the CD4 molecule on an uninfected cell binding to gp120 on the surface of an infected cell, leading to the recruitment of Gag polyprotein precursor to the intercellular interface, which then triggers the recruitment of CCR5 or CXCR4 and eventually LFA-1 and ICAM-1 for stabilisation of the synapse (Rodriguez-Plata et al. 2013; Puigdomenech et al. 2008; Jolly, Mitar, and Sattentau 2007a, 2007b). At the cell-cell contact sites, the interaction between Env and the receptors on the target cell mediates the creation of fusion pores between the two plasma membranes. HIV-1 then goes through these pores without extracellular budding as fully assembled infectious enveloped virions. The presence of the co-receptor is dispensable for the formation of the virological synapse but is essential for efficient infection, actin cytoskeleton remodelling and microtubule polarization toward the virological synapse (Jolly and Sattentau 2004; Jolly et al. 2004).

Less studied nanotubules can mediate and facilitate the transfer of cytoplasmic and plasma membrane molecules. The frequency of nanotubules is not affected by HIV-1, but studies have reported that HIV-1 can surf along the surface of nanotubules on T cells and macrophages and gain entry to an uninfected cell (Hashimoto et al. 2016; Eugenin, Gaskill, and Berman 2009; Sowinski et al. 2008). Gp120 and the CD4 receptor independent macrophage phagocytosis of infected $CD4^+$ T cells has also been described to lead to their infection (Waki and Freed 2010). The engulfment of infected dying T cells is demonstrably higher compared with uninfected healthy T cells, indicating that the cell death promoted T-cell engulfment by macrophages (Kadiu and Gendelman 2011; Groot, Welsch, and Sattentau 2008).

Cell-to-cell fusion between infected $CD4^+$ T cells, or between infected and uninfected $CD4^+$ T cells resulting in giant cells 5-100 times bigger than individual cells has also been described (Freel et al. 2003). Cell-to-cell fusion that occurs through actin cytoskeleton rearrangements, is dependent on LFA-1/ICAM-1 interaction and is mediated through interaction between envelope glycoproteins expressed at the cell surface of infected cells and CD4 expressed on the target cells (Barbeau et al. 1998).

Direct cell-to-cell transmission avoids several physical and immunologic barriers and is therefore a more rapid and efficient mode of spread (Figure 1.6) (Chen et al. 2007; Carr et al. 1999; Dimitrov et al. 1993; Sourisseau et al. 2007). The high efficiency of cell-to-cell infection is thought to be a mechanism for HIV-1 to escape antiretroviral therapy and neutralizing antibodies, but is also due to a high-multiplicity of infection at the site of the cell–cell contact that allows for the integration, and accelerated viral gene expression of multiple proviruses in the target cell (Boulle et al. 2016; Duncan et al. 2014; Durham et al. 2012; Gupta et al. 1989; Sourisseau et al. 2007; Zhong, Agosto, Ilinskaya, et al. 2013). Galloway et al demonstrated that the mode of cell spread is important in determining the mechanism of $CD4^+$ T cell death, with cell-to-cell transmission leading to massive depletion of uninfected bystander cells that were

refractory and non-permissive to HIV-1 infection within lymph nodes. Cell-free HIV-1 virions even when added in large quantities failed to activate bystander pyroptotic cell death (Galloway et al. 2015; Law et al. 2016).

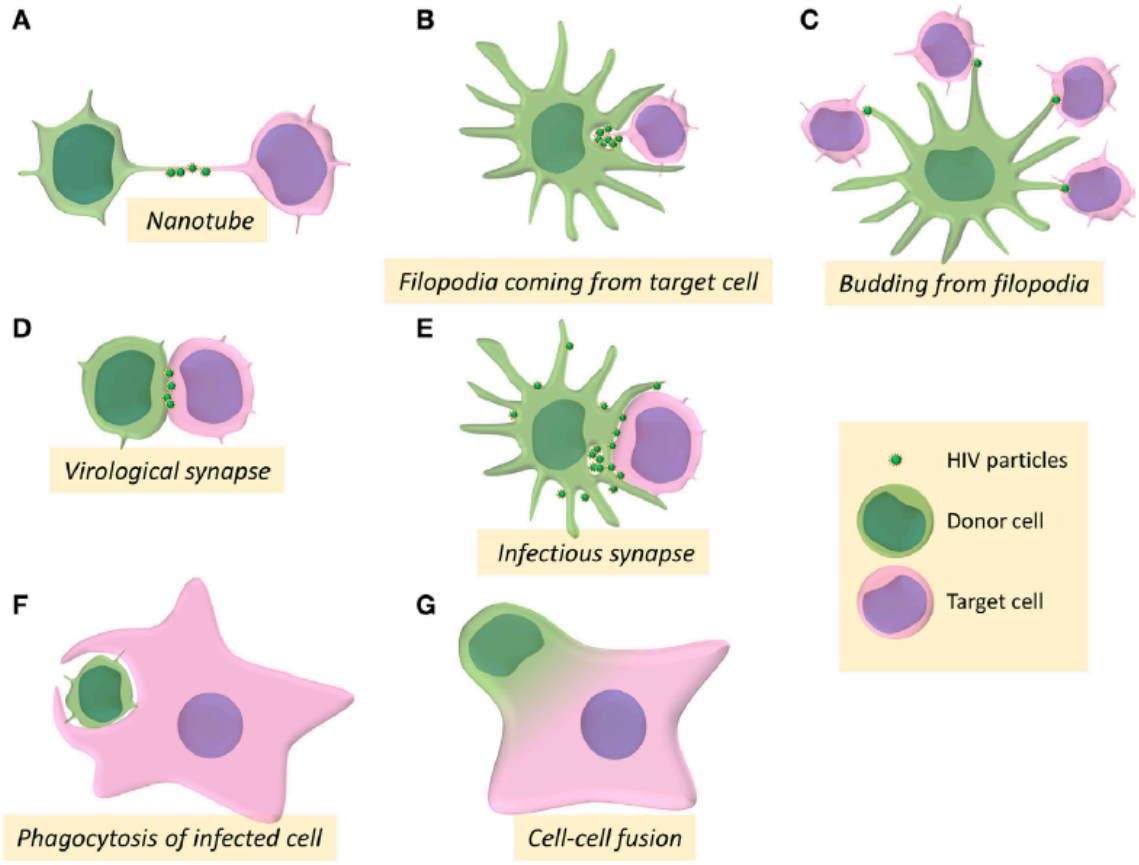


Figure 1.6: Modes of HIV-1 spread between cells.

Schemes represent the different pathways for HIV-1 cell to cell transfer between donor cells in green and target cells in pink. HIV's ability to take advantage of normal cellular processes allows it to improve its spread through a multiplicity of intercellular structures and membrane protrusions, like tunneling nanotubes, filopodia, or lamellipodia-like structures. Other features of immune cells, like the immunological synapse or the phagocytosis of infected cells are hijacked by HIV-1 and used as gateways to infect target cells. Finally, HIV-1 can induce fusion between infected donor cells and target cells, and to form infected syncytia with high capacity of viral production and improved capacities of motility or survival. Taken from (Bracq et al. 2018).

1.5 Role of transmitted/founder virus characteristics in HIV-1 pathogenesis

The host and viral factors determining sterilizing or relative resistance to HIV-1 transmission and early dissemination are still unclear. The most likely viral candidates are features that modulate the entry process, replicative capacity of the virus, immune evasion, and those that influence its capacity to induce immune activation.

1.5.1 Virus fitness

Some studies have reported that one of the features that distinguishes elite controllers is that they are infected by viruses of low replicative capacity (Song et al. 2012; Yue et al. 2015). Prediction of replicative capacity from viral sequences based on statistical models trained on *in vitro* measurements also demonstrated a direct correlation with set point viral load (Kouyos et al. 2011; Nicastri et al. 2003). Escape within *gag* and *pol* encoded epitopes can significantly reduce viral replicative capacity and has been associated with low viral loads (Barbour et al. 2004; Prince et al. 2012). Prince et al inserted the *gag* gene from the transmitted virus in 149 subtype C newly infected epidemiologically linked transmissions pairs into a proviral vector and determined the replicative capacity of the gag chimeras *in vitro* (Claiborne, Prince, and Hunter 2014; Prince et al. 2012). They showed that certain mutations in the transmitted *gag* conferred an increased replicative capacity while some reduced the virus' replicative capacity independent of HLA type. In addition, they looked at how the replicative capacity of the transmitted *gag* influenced the clinical markers of disease progression. Individuals infected with attenuated *gag* sequences were delayed in their progression to $CD4^+$ T cell counts <300 cells/μl of blood compared to high replicating viruses indicating a long-term clinical benefit associated with the transmission of less fit viruses. There was no difference in the viral load, suggesting that replicative capacity affected $CD4^+$ T cell decline in a manner that may be

independent of viral load, and that this played a role in defining pathogenesis (Claiborne et al. 2015). They also observed increased activation of CD8 T cells and higher levels of pro inflammatory cytokines, positioning the ability to resolve chronic immune activation as a link between viral fitness and disease progression.

Transmitted preadaptation to the newly infected host has also been associated with faster $CD4^+$ T cell decline in several studies (Carlson et al. 2016; Monaco et al. 2016). However, these studies have mainly been done on subtype C variants and not on other subtypes that vary in disease progression.

1.5.2 HIV-1 subtype

There is evidence for subtype differences in HIV pathogenesis. After adjustment for age, sex, and human leukocyte antigen (HLA) alleles subtype C-infected participants progress faster than subtype A to all three endpoints: $CD4^+$ T cell decline, viral load and duration to onset of AIDS (Amornkul et al. 2013; Baeten et al. 2007; Easterbrook et al. 2010; Kaleebu et al. 2002; Kaleebu et al. 2001; Kiwanuka et al. 2008; Vasan et al. 2006). Subtype D-infected participants reach high viral load more rapidly and progress nearly twice as fast to AIDS compared to subtype A (Amornkul et al. 2013; Baeten et al. 2007). In a multicenter study by Sanders and colleagues, individuals with subtype A were more likely than individuals with subtypes C or D to report acute retroviral syndrome symptoms, with each individual symptom other than rash being more prevalent in subtype A than in subtype C or D (Sanders et al. 2017).

1.5.3 Diversity of infecting virus population

Studies of the HIV-1 breakthrough infections in the Step and RV144 HIV-1 vaccine efficacy trials showed that although recently infected individuals with multiple phylogenetically linked HIV-1 founder variants represent a minority of HIV-1 infections, more diverse HIV-1

populations in early infection were associated with significantly higher viral load 1 year after HIV-1 diagnosis (Janes et al. 2015). In both trials, HIV-1 infections were established by a single viral variant in most individuals, but with heterogeneity of founder viruses being identified as a viral determinant of $CD4^+$ T cell decline.

1.6 Role of protective host genetics

1.6.1 CCR5 polymorphisms

Before the era of genome-wide association studies (GWAS), single nucleotide polymorphism (SNP) variants in numerous candidate genes had been reported as important determinants of HIV-1 susceptibility and disease progression. The first to be described was CCR5-Δ32, a 32-base pair deletion that truncates C-C chemokine receptor 5 (CCR5), the HIV-1 entry coreceptor on lymphoid cells (Huang et al. 1996; Michael, Louie, and Sheppard 1997). Epidemiological studies showed that individuals homozygous for CCR5-Δ32 had a 100-fold reduction in HIV-1 infection incidence (Huang et al. 1996). Individuals that were heterozygous for CCR5-Δ32, although susceptible to infection, consistently had a delayed onset of AIDS by two to four years (Huang et al. 1996; Quillent et al. 1998; Laurichesse et al. 2010). Furthermore, AIDS patients on CART suppress viral load faster and live longer when they are heterozygous for the CCR5-Δ32 mutation. An outstanding example of CCR5-Δ32 clinical translation involves treatment of the Berlin patient who received a stem cell transplant from an HLA-matched donor who was homozygous for CCR5-Δ32, resulting in viral control and elimination with no rebound after treatment interruption (Hutter et al. 2009; Allers et al. 2011).

1.6.2 HLA polymorphisms

HLA allelic resistance and susceptibility to AIDS progression is well accepted, irrespective of differences in ethnicity, virus clade, and risk group. Most of the HLA polymorphism is on residues around the peptide-binding groove, and therefore defines the specificity of the peptides that can be loaded onto the pocket. Both GWAS and epidemiological data point towards a bigger association of HLA-B than HLA-A and HLA-C with HIV viral set point (Tang et al. 1999; Peterson et al. 2013; O'Brien, Gao, and Carrington 2001; Kiepiela et al. 2004). Faster disease progression is seen also in patients with HLA class I homozygosity. HLA-B*5701, HLA-B*5801, HLA-B*27 and HLA-B*51 are associated with good control of HIV-1 while HLA-B*3502 and HLA-B*3503 are associated with rapid progression to AIDS (Prentice et al. 2013; Ngumbela et al. 2008; Migueles et al. 2003; Matthews et al. 2012; Matthews et al. 2011; Kloverpris et al. 2012; Carrington et al. 1999; Altfeld et al. 2003). HLA-B*5701/5801 are capable of binding the TW10 epitope while HLA-B*27 binds the KK10 epitope to which a cytotoxic T lymphocyte (CTL) response in acute infection is associated with low viral load, and escape mutations on this epitope associated with a fitness cost to the virus. T cell receptors for the HIV-1 KK10 epitope from patients with differential immunologic control are functionally indistinguishable, with differences in immunodominance patterns accounting for the differences is disease outcome (Joglekar et al. 2018).

It is not clear why HLA-B correlates more with various outcomes than the other types. It has been suggested that NK cell Killer cell Immunoglobulin-like Receptors (KIRs) that bind HLA-B may contribute to the observed effect, further supported by the observation that the HLA class I alleles most commonly associated with viral control bear the Bw4 motif, a ligand for KIRs (Altfeld and Goulder 2007). However, the association of specific MHC class I alleles with control of viremia is independent of KIRs, and the interaction is most likely synergistic. A role for HLA-C has also been suggested on the basis of the observation that Nef mediated

downregulation of HLA selectively applies to HLA-A and HLA-B but not HLA-C, but HLA-C can still be downregulated by Vpu. (Zipeto and Beretta 2012; Kulpa and Collins 2011; Cohen et al. 1999; Boelen et al. 2018). Recent work by Boelen et all demonstrated that in the context of HCV, HTLV-1 and HIV-1, inhibitory KIRs enhance HLA class I associations and that this can be explained by an increase in $CD8^+$ T cell survival in the presence of functional inhibitory KIRs (Boelen et al. 2018). They proposed that inhibitory KIRs could increase $CD8^+$ T cell survival through direct effects on the lifespan of T cells or indirectly such as through NK cell activity.

At least one HLA genotype, HLAB*5701, seems to predispose carriers to a severe immunological disorder (fever, gastrointestinal and respiratory distress) when they are treated with the nucleoside reverse transcriptase HIV inhibitor abacavir (Mallal et al. 2002; Manglani et al. 2018).

SNP variants within loci that mediate HIV-1 restriction *in vitro* and against other viruses (TRIM5, APOBEC, TSG101, CUL5) have shown modest effects on AIDS progression in association studies (Merindol and Berthoux 2015; Singh et al. 2018).

1.7 Mechanisms of $CD4^+$ T cell decline

The progressive loss of $CD4^+$ T cells in untreated HIV-1 infection is at the root of AIDS, allowing opportunistic infections and malignancies to thrive. Loss of $CD4^+$ T cells can occur from reduced production, redistribution to other anatomical sites, or through cellular destruction by direct cytotoxicity of infected cells, programmed cell death in infected cells, or in uninfected bystander cells triggered by soluble or membrane-bound viral or host immune

factors. However, the relative contribution of each of these mechanisms in clinical HIV-1 infection remains poorly understood.

Early studies indicated decreased T-cell production in the thymus in HIV-1 infection due to a combination of direct cytopathicity of HIV-1-infected thymocytes and apoptosis of uninfected immature thymocyte precursors (Chrobak et al. 2010; Pedroza-Martins et al. 2006; Brunner et al. 2011; Zou et al. 2012). HIV-1 also infects and induces apoptosis of multipotent hematopoietic progenitor cells, thereby potentially decreasing progenitor cell input into the thymus (Cummins and Badley 2010; Donnenberg et al. 1995; Ye, Kirschner, and Kourtis 2004).

Redistribution of $CD4^+$ T cells away from peripheral circulation with subsequent sequestration in secondary lymphoid organs has been shown to occur. Resting $CD4^+$ T cells upregulate CD62L when exposed to HIV-1 therefore potentially home to lymph nodes (Wang, Robb, and Cloyd 1997; Hengel et al. 1999; Wang et al. 1999). Increased splenic sequestration has also been shown in cells that upregulate CCR6 (Ye, Kirschner, and Kourtis 2004). Moreover, increased homing to the gut mediated by $\alpha 4\beta 6$ expression also leads to accumulation and eventual destruction of CD4 cells in the GALT (Wang et al. 1999).

Most studies have focused on the death of productively infected cells circulating in peripheral blood. Productively infected $CD4^+$ T cells expressing MHC bound HIV-1 antigens are targeted and killed by CTL, or through direct cytopathic effects of the virus. However, apoptosis of circulating $CD4^+$ T cells does not always correlate with viral load suggesting either not all $CD4^+$ T cell apoptosis is triggered by active viral replication, or that circulating T cells though the easiest to characterize, are not necessarily the most physiologically relevant compartment to gauge $CD4^+$ T cell loss. Both of these possibilities are now supported by evidence.

Bystander killing occurs, resulting in around 80% of CD4 T cells in the GALT being depleted in the first three weeks of HIV-1 infection. This has been shown in resting $CD4^+$ T cells that

take in HIV-1 but are not productively infected due to their quiescent state. Such resting cells represent the main target encountered by HIV-1, and die by an alternative mechanism of cell death, pyroptosis (Cummins and Badley 2010; Church 2014; Cummins and Badley 2014; Doitsh et al. 2014; Monroe et al. 2014). As opposed to apoptosis, which is immunologically silent, pyroptosis is highly inflammatory and is mediated by a distinct set of caspases and intracellular signalling molecules. $CD4^+$ T cells release their cytoplasmic contents including inflammatory cytokines and fragments of Pathogen Associated Molecular Patterns (PAMPs) to the extracellular milieu (Doitsh et al. 2014; Monroe et al. 2014; Doitsh and Greene 2016; Liu and Lieberman 2017). $CD4^+$ T-cell death by pyroptosis *in vivo* would therefore be accompanied by increased inflammation, generalized immune activation and possibly exhaustion that is characteristic of fast disease progression. Pyroptosis has been well demonstrated in controlled *in vitro* systems, but not *in vivo* or in any patient cohort. It is unclear whether some viruses induce pyroptosis better than others, but recent data shows that pyroptosis occurs in quiescent cells that are infected through virological synapse-induced cell-to-cell transmission as opposed to cell free transmission (Galloway et al. 2015; Munoz-Arias et al. 2015). However, a systematic characterization of the ability of founder viruses from patients who differ in disease progression to induce pyroptosis has yet to be performed; and the ensuing consequence of pyroptosis to other immune cells is unclear.

HIV-1 proteins including gp160, Tat, Nef, Vpr, Vpu and protease have also been shown to induce apoptosis (Kolesnitchenko et al. 1995; Akari et al. 2001; Chen et al. 2002; Giacca 2005; Lum, Schnepple, and Badley 2005; Cummins and Badley 2010).

1.8 Immune responses to HIV-1 infection

The nature of the immune response in the period before the establishment of a viral set point is one of the major determinants of whether the virus remains localized or disseminated into a systemic infection (Figure 1.7) (Borrow 2011; McMichael et al. 2010). Furthermore, the observation that a majority of heterosexual transmissions are established by a single founder virus suggests that there exists a robust innate barrier at the mucosa. These barriers include both physical properties such as epithelial integrity, mucus and low pH, as well as secreted anti-HIV-1 factors. Human cells express a number of restriction factors that are able to block or reduce HIV-1 transmission including APOBEC3G/F, TRIM5a and tetherin. However, HIV-1 has evolved mechanisms to counteract these factors (Alter, Teigen, et al. 2007; Collins and Baltimore 1999; Jost and Altfeld 2012).

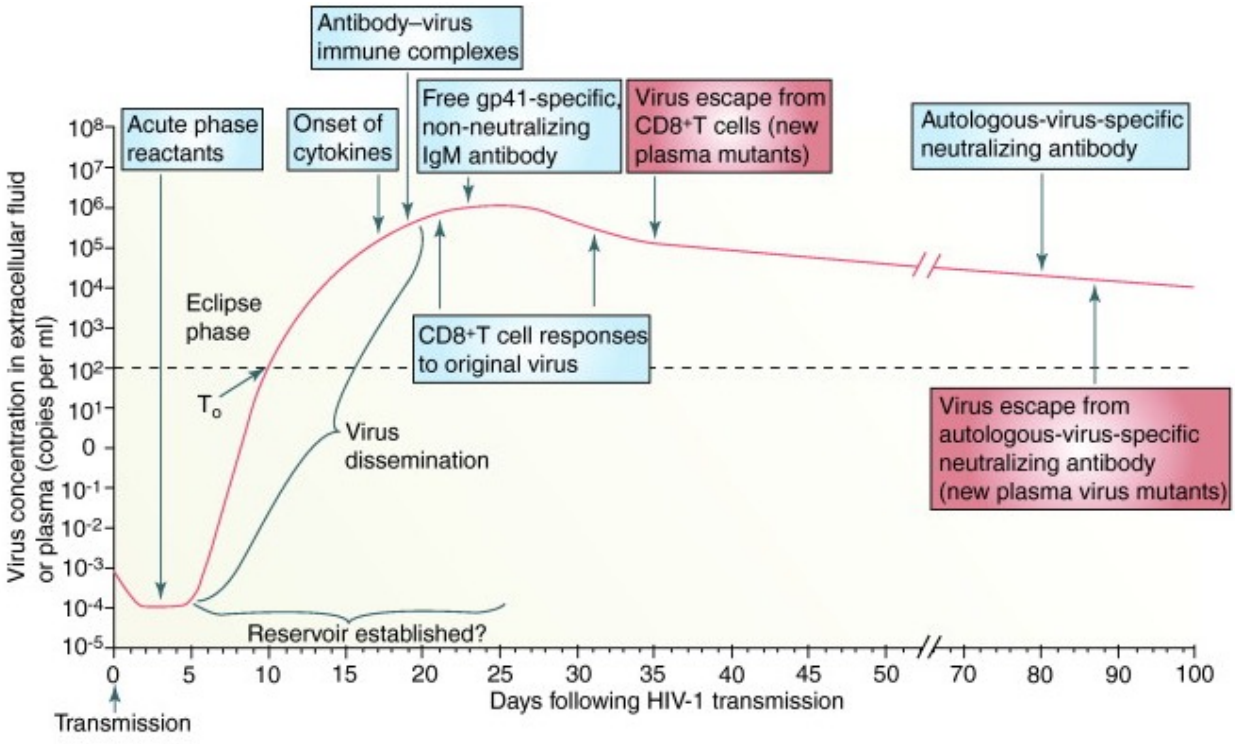


Figure 1.7: Earliest immune responses after HIV-1 transmission.

The first systemically detectable immune responses to HIV-1 infection are the increases in levels of acute-phase proteins in the plasma and plasma cytokine levels, followed by antibody–virus immune complexes, HIV-1-specific $CD8^+$ T cells, and finally detection of the first free gp41-specific but non-neutralizing IgM antibodies (McMichael et al. 2010).

1.8.1 Acute cytokine storm

One of the earliest innate responses observed just before the peak of viremia is a wave of pro-inflammatory cytokines produced by activated innate cells, infected T cells and eventually HIV-1 specific T cells (Figure 1.8). There is transient expression of IL-15, type 1 interferons and CXCL10, while IL-18, IFN-γ, TNF, IL-10 and IL-18 levels are sustained for a longer time. Some of these cytokines such as the type 1 interferons up-regulate the transcription of interferon-stimulated genes, many of which are antiviral. In an attempt to kill the infected cells and support an adaptive response, the early innate response potentially also recruits additional susceptible T cells to the site of infection.

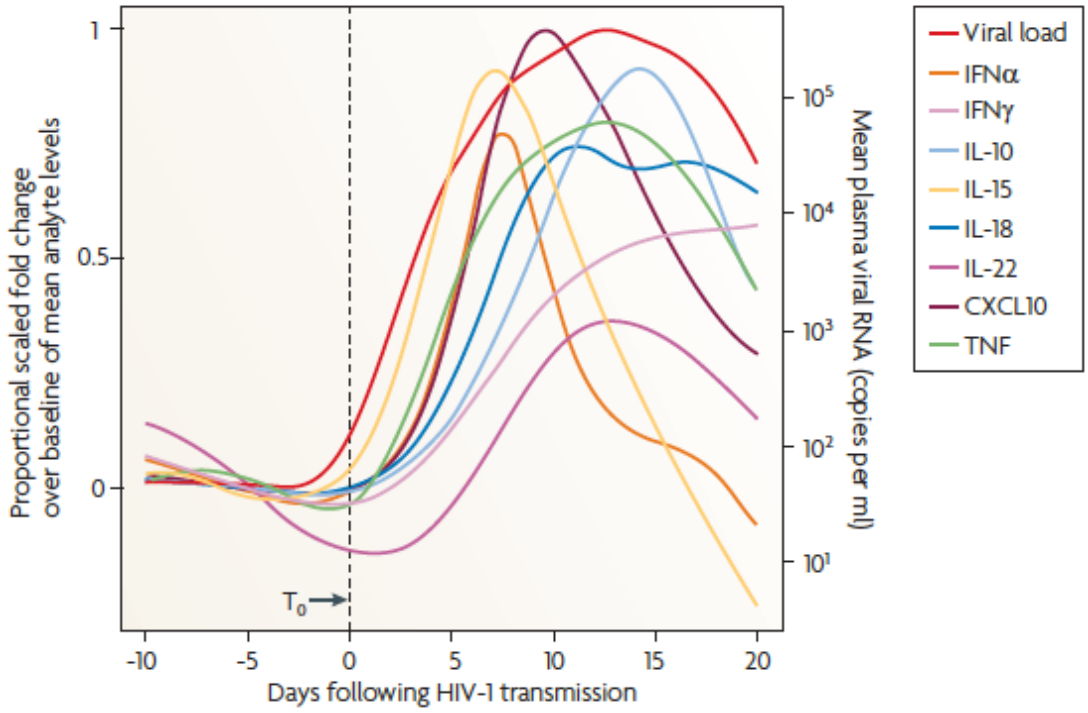


Figure 1.8: The acute cytokine storm in HIV-1 infection.

Comprising of acute-phase proteins, cytokines and chemokines in the plasma during acute HIV-1 infection. There are two initial waves of cytokines: interleukin-15 and interferon-α, followed by tumor necrosis factor, interleukin-18, interleukin-10 and CXCL10 (McMichael et al. 2010).

1.8.2 NK cells

One of the few subsets of innate cells that can be studied in frozen PBMC are NK cells. Once activated, NK cells have both antiviral and immunomodulatory functions through the secretion of cytokines and antiviral proteins. NK cell numbers expand rapidly during acute HIV-1 infection, which has been postulated to be driven by the acute cytokine storm (Alter and Altfeld 2009; Alter et al. 2004; Altfeld and Goulder 2007; Lodoen and Lanier 2006; Peppa et al. 2018). However, to compensate for this early burst of cytotoxicity, HIV-1 has devised strategies of down regulating NK cell activation, indicating that these cells are able to place pressure on the

virus therefore suggesting a role for NK cells in early control of viremia. The HIV-1 proteins Nef and Vpu are known to target two families of NK cell receptors; KIRs whose ligand is MHC class I, and NKG2D, which binds to stress induced proteins (Apps et al. 2016; Cerboni et al. 2007; Cohen et al. 1999; Galaski et al. 2016; Matusali et al. 2012). Firstly, they accelerate endocytosis of the dominant T cell receptor ligands HLA-A and HLA-B to facilitate escape from recognition by $CD8^+$ T cells while retaining expression of HLA-C, the dominant ligand for the inhibitory NK cell receptors. Secondly, Nef also down regulates the expression of stress-induced ligands of the NK cell activatory receptor NKG2D such as MIC A on HIV-1 infected cells. It is thought that one way that HIV avoids both CTLs and NK cells is by not downregulating HLA-C and HLA-E which are ligands to NK cell inhibitory receptors (iNKRs) while down modulating HLA-A and -B. However, NK cell expression of specific iNKRs is variegated and as such only some, but not all, NK cells are affected. The proportion of NK cells that express HLA-E specific receptors (*i.e.*, NKG2A/CD94) is significantly decreased in viraemic subjects compared to NK cells from aviraemic individuals (Davis et al. 2016).

The role for particular KIR/HLA combinations in HIV-1 control has been well established (Alter, Martin, et al. 2007; Alter, Teigen, et al. 2007). NK cells expressing the activatory KIR3DS1 and KIR3DS1 receptor expand robustly during acute HIV-1 infection and de-granulate more potently in response to HIV-1 infected T cells expressing the HLA-Bw-80I allele and are able to control viral replication (Alter et al. 2009; Martin et al. 2007). Besides, elevated KIR3DS1 transcripts have been found in HIV-1 exposed seronegative individuals suggesting that KIR3DS1 may be involved in their protection (Ravet et al. 2007). Furthermore, distinct peptide footprints have been described to emerge preferentially in HLABw-80I expressing patients whose NK cells express this KIR suggesting that NK cells are part of the immune pressure that drives HIV-1 evolution (Alter et al. 2011). In addition to KIR3DS1, co-

expression of HLA-Bw-80I with the KIR3DL1 has also been associated with slower progression to AIDS (Alter et al. 2009).

Crystal structures of KIR/MHC class I complexes reveal that KIRs have direct contact with the α1 and α2 subunits of MHC class I, and the carboxy terminal of the bound peptide (Boyington et al. 2000; Fan, Long, and Wiley 2001). Changes in amino acid residues within the MHC presented peptide therefore have the potential to alter the binding affinity of the MHC/Peptide complex to a KIR. Decreased binding to an inhibitory KIR such as KIR3DL1 may trigger 'missing self' NK activation while increased binding to an activatory KIR such as KIR3DS1 may activate NK cell cytotoxicity.

1.8.3 The $CD8^+$ T cell response

The first T cell response to HIV-1 is detected as viremia approaches its peak and then starts to decline (Goonetilleke et al. 2009; Koup and Ho 1994; Koup et al. 1994; McMichael et al. 2010). This inverse relationship between viral load and the appearance of $CD8^+$ T cell responses at acute infection is the first indication that $CD8^+$ T cells play a role in controlling viremia. Further evidence is provided by *ex vivo* and *in vitro* viral inhibition assays (Mutua et al. 2016), the association of specific HLA class I molecules with particular disease outcomes (Leslie et al. 2006; Wang et al. 2009), the observation of escape mutations in key $CD8^+$ T cell epitopes (Bernardin et al. 2005; Carlson and Brumme 2008; Borrow et al. 1997; Goulder et al. 1997), and $CD8^+$ T cell depletion studies in primates (Bosinger et al. 2013; Pandrea et al. 2011; Jin et al. 1999; Matano et al. 1998; Schmitz et al. 1999) .

During acute HIV-1 infection, HIV-1 specific $CD8^+$ T cell clones expand and respond to HIV-1 with multiple effector functions such as the production of IL-2, TNF-α, IFN-γ, MIP-1β and Granzyme/Perforin mediated cytotoxicity (Burgers et al. 2009; Imami et al. 2001; Streeck, Brumme, et al. 2008; Streeck, van Bockel, and Kelleher 2008). There is evidence from multiple

studies that the ability of $CD8^+$ T cells to mediate two or more of these effector functions (polyfunctionality) at acute infection is associated with the ensuing viral load set point. The polyfunctional profile of the $CD8^+$ T cell response to different epitopes within the same HIV-1 infected patient differs but tends to decrease from acute to chronic infection in untreated patients as HIV-1 specific $CD8^+$ T cells ultimately display signs of exhaustion (Bernardin et al. 2005; Imami et al. 2013; Rehr et al. 2008; Streeck, van Bockel, and Kelleher 2008; Winstone et al. 2009) .

However, the first $CD8^+$ T cell responses are narrowly directed against a limited number of epitopes following a predictable ordered immunodominance pattern and despite an increase in the breadth and magnitude of the CTL response in the chronic stage of infection, no increased control of viral replication is observed (Turnbull et al. 2009). This suggests that the first $CD8^+$ T cell responses are unique in their ability to efficiently suppress viral replication, whereas $CD8^+$ T cell responses generated later in infection are increasingly impaired.

The question still remains why early CTL responses are so efficient in controlling viral replication, whereas CTL responses generated later in infection appear impaired. During initial infection, the sequence of transmitted virus is largely homogenous but eventually, sequence mutations begin to affect the affinity by which these peptides bind to MHC class I, resulting in escape from $CD8^+$ T cell recognition. Because of these ongoing recombination and mutations, HIV-1 escapes recognition by $CD8^+$ T cell responses in the host. However, it has been suggested that the ability of HIV-1 to escape virus-specific immunity is finite and comes at a fitness cost to the virus (Brumme, Brumme, et al. 2008; Brumme, Tao, et al. 2008; Jones et al. 2004; Boutwell et al. 2013; Koup 1994; Schneidewind et al. 2009). This might play a unique role in the acute phase of infection, when the virus has not diversified as much as it has by the chronic phrase of infection. Therefore, early $CD8^+$ T cell responses might have an advantage in skewing the virus to lower viral fitness.

The preserved functionality of $CD8^+$ T cells seen in patients with low viremia could represent either the cause of viral control, or the product of having a low antigen load and therefore optimal $CD4^+$ T cell help (Imami et al. 2013). Such changes are however not always observed in CMV, EBV and influenza specific $CD8^+$ T cells within the same patient. (Streeck, Brumme, et al. 2008). In addition, there is partial improvement of HIV-1-specific $CD8^+$ function when viremia is suppressed by cART, implying that persistent antigenic stimulation by HIV-1 may be responsible for the decline in $CD8^+$ T cell function (Oxenius et al. 2000; Rehr et al. 2008). Genetic factors such as HLA type can also influence what epitopes become immunodominant and the time it takes for the first T cell responses to become targeted to conserved epitopes (Westrop et al. 2017).

1.8.4 The $CD4^+$ T cell response

HIV-1-specific $CD4^+$ T cell responses emerge simultaneously or even earlier than $CD8^+$ T cell responses during primary HIV-1 infection but decrease after the first months of infection (Maenetje et al. 2010; Rosenberg et al. 1997). This contraction of the $CD4^+$ T cell response pool has been suggested to be due to preferential infection by HIV-1, as HIV-1 infects and depletes $CD4^+$ T cells, especially those that are specific for HIV-1 proteins (Douek et al. 2002; Sleasman et al. 1996). Cell death may occur either as a consequence of the cytopathic effects of the virus or following targeting by $CD8^+$ T cells and NK cells. Bystander killing of uninfected cells has also been reported, mediated by mechanisms such upregulation of death receptors by Nef and by the enhanced expression of ligands to death receptors on other activated cells (Cummins and Badley 2010; Doitsh et al. 2014; Ji et al. 2007; Nie et al. 2009).

Given the crucial role that $CD4^+$ T cells play in shaping the quality of the ensuing immune responses, the rate of $CD4^+$ T cell depletion directly correlates with the quality of $CD8^+$ T cell response and the humoral response (Sun and Bevan 2003). $CD4^+$ T cells are better known for

their helper function through production of cytokines but more recently, granzyme and perforin positive $CD4^+$ T cells that are cytotoxic *in vitro* have been described in HIV-1 infected individuals (Sacha et al. 2009; Appay et al. 2008; Appay et al. 2002; Norris et al. 2004). The frequency of such cytotoxic $CD4^+$ T cells is reportedly higher in patients with good control of viremia (Appay et al. 2002).

The presence of robust polyfunctional $CD4^+$ T cell responses is an important hallmark that distinguishes nonpathogenic HIV-2 infection from pathogenic HIV-1 infection (Duvall et al. 2006). Moreover, a robust $CD4^+$ T cell response in acute HIV-1 infection has been associated with subsequent control of viral replication, and viral escape from $CD4^+$ T cell–targeted epitopes has been observed (Erdmann et al. 2015). However, whether the presence of HIV-1-specific $CD4^+$ T cells is the consequence of low viremia or effectively contributes to viral suppression remains unclear. Interestingly, the immunogenicity data from the Thai RV144 trial suggest that the vaccine induced both antibody responses and robust $CD4^+$ T cell responses (Rerks-Ngarm et al. 2013).

Moreover, although studies show that HIV-1 replication can be predominantly controlled by polyfunctional $CD8^+$ T cells, the effectiveness of these $CD8^+$ T cell responses appears to be affected by the presence or absence of fully functional proliferative $CD4^+$ T helper cells (Kemball et al. 2007; Matloubian, Concepcion, and Ahmed 1994; Sun and Bevan 2003; Imami et al. 2013). Interestingly, antigen-specific $CD8^+$ T cells can be generated in the absence of $CD4^+$ T cell help, but the secondary expansion on antigen reencounter is inefficient and likely dependent on help provided by CD4 T cells (Frohlich et al. 2016; Janssen et al. 2003; Sun, Williams, and Bevan 2004). Antigen-specific CD4 T cells can be preserved through the initiation of cART during primary HIV-1 infection (Rosenberg et al. 1997).

The rationale underlying the general exclusion of $CD4^+$ T cells from vaccine design strategies that seek to induce protective T cell responses originated from other studies showing that HIV-1 preferentially infects HIV-1-specific $CD4^+$ T cells, as a vaccine candidate boosting these responses could in theory enhance viral replication.

1.8.5 B cell responses

Although there is barely any evidence that HIV-1 can productively replicate in B cells *in vivo*, there is strong evidence that HIV-1 binds to B cells *in vivo* through interactions between the complement receptor CD21 which is expressed on most mature B cells, and complement proteins bound to HIV-1 virions that are circulating *in vivo* (Kacani et al. 2000; Moir et al. 2000). Such immune-complex-based interactions might provide stimulatory signals to B cells, although the low frequency of B cells directly interacting with HIV-1 virions would predict that this is a minor activating pathway. However, through this interaction B cells can facilitate cell-to-cell transmission of HIV-1 (Banki et al. 2005; Delibrias, Kazatchkine, and Fischer 1993). Additional potential HIV-1-binding receptors have been identified on B cells, including DC-specific ICAM-3-grabbing non-integrin (DC-SIGN), other C-type lectin receptors and surface immunoglobulins of the variable heavy chain 3 (VH3) family (Berberian et al. 1993; He et al. 2006; Rappocciolo et al. 2006). Several cytokines and growth factors directly or indirectly trigger the activation of B cells in HIV-1-viremic individuals including IFNα, TNFα, IL-6, IL-10, CD40 ligand and B-cell-activating factor (BAFF) (Diop et al. 2008; He et al. 2006; Mandl et al. 2008; Muller et al. 1998; Ng 1996; Rieckmann et al. 1991; Weimer et al. 1998).

These factors are associated with B-cell hyperactivation in HIV-1-viremic individuals characterized by several features including hypergammaglobulinaemia, increased polyclonal B-cell activation, increased cell turnover, increased expression of activation markers, increase in the differentiation of B cells to plasmablasts, increased production of autoantibodies and an

increase in the frequency of B-cell malignancies (Lane et al. 1983; Moir et al. 2001; Moir et al. 2004; Shirai et al. 1992).

The importance of the humoral immune responses towards HIV-1 is clear from studies that demonstrate that passive therapy with broadly neutralizing monoclonal antibodies against the HIV-1 envelope can protect monkeys from SHIV challenge (Hessell et al. 2009; Parren et al. 2001). The first antibodies against HIV-1 can be detected in plasma as immune complexes containing IgG and IgM antibodies specific for gp41 and gp120, while free HIV-1-specific IgG later become detectable in plasma with the sequential appearance of gp41, p55, p66, gp120, p17 and p31-specific antibodies (Fiebig et al. 2003). In contrast to the rapid development of HIV-1-specific antibodies, antibodies that can neutralize autologous virus are first detected 3 months post infection, but these drive escape mutants (Bhiman et al. 2015; Piantadosi et al. 2009). Broadly neutralizing antibodies (bNAb) that can inhibit infection by heterologous strains are only developed by a few HIV-1-infected patients, typically comprise only a small fraction of the total plasma antiviral antibodies and come too late in infection to be of any help to the patient (Bhiman et al. 2015; Landais et al. 2016; Landais and Moore 2018; Walker et al. 2011).

bNAbs have the potential to provide complete protection from HIV-1 infection, however there are considerable challenges in inducing these antibodies by vaccination. The conserved epitopes to which they are directed are masked, heavily glycosylated and poorly immunogenic, and these antibodies are often characterised by high levels of somatic hypermutation, a long heavy chain complementarity-determining region 3 (HCDR3), not always derived from a cognate naïve B cell, are often autoreactive and potentially deleted by self-tolerance mechanisms and are therefore generated through complex maturation pathways that are not well understood (Frost et al. 2005; Havenar-Daughton et al. 2018; MacLeod et al. 2016; Moore et al. 2006; Moore et al. 2009; Rantalainen et al. 2018; Ward and Wilson 2017). There is hope that an antibody based vaccine or cure may be attainable from recent advances in the isolation

and characterization of broadly neutralizing antibodies from HIV-1-infected subjects, resolution of the structure of the HIV-1 envelope glycoprotein and in improved understanding of the immunological pathways leading to bNAb elicitation (Bhiman et al. 2015; Lee, Ozorowski, and Ward 2016; MacLeod et al. 2016; Sullivan et al. 2017; Walker and Burton 2018). Multiple studies including the RV144 HIV trial that showed modest protection suggest that in addition to the ability to neutralize a broad range of HIV-1 variants, antibodies will need to have enhanced FcR function and longer half-lives to successfully protect against infection with HIV-1 (Hessell et al. 2007; Willey and Aasa-Chapman 2008).

1.8.6 Chronic immune activation and exhaustion

One hallmark of HIV-1 infection is chronic activation of the immune system that not only increases the number of activated $CD4^+$ target cells but also directly impairs the immune system through activation-induced cellular exhaustion. Lymphocyte responses primed in acute HIV-1 infection have a better metabolic starting position than those generated under persistent viral infection with abundance of antigen (Streeck, Li, et al. 2008; Wherry 2011). When naive T cells and B cells recognize their antigen, they mature to effector cells, recognizing and killing the respective target cells and producing relevant antibodies respectively. After clearance of an acute viral infection, these populations contract, and only a minor fraction of the effector cells develop into a long-lived memory pool. However, in chronic persistent infections and under persistent levels of antigen, these cells become progressively exhausted (De Milito 2004; Imami and Gotch 2002; Khaitan and Unutmaz 2011).

Although the immune system has developed several strategies to counteract this abundant activation, it has been shown to be one of the strongest contributors to $CD4^+$ T cell loss in the case of HIV-1 infection. One specific mechanism of evasion from hyperactivation is a specific

expansion of inducible $FoxP3^{+}CD25^{+}$ regulatory T (T-reg) cells after acute HIV-1 infection (Blackburn et al. 2009; Blackburn and Wherry 2007; Weimer et al. 1998).

This exhaustion follows a clear hierarchical pattern. The cells first lose the ability to proliferate, to secrete different cytokines and chemokines, and their cytolytic activity, and finally they enter a stage of full exhaustion (Streeck, Brumme, et al. 2008; Streeck, Li, et al. 2008; Wherry et al. 2007). This metabolic loss of functional abilities is followed by physical deletion. The different stages of exhaustion are reflected by the up-regulation of different inhibitory molecules on the cell surface, such as programmed death 1 (PD-1), CTLA-4, KLRG1, TIM-3, or CD160 (Porichis and Kaufmann 2012; Blackburn et al. 2009; Bui and Mellors 2015; Moir and Fauci 2014; Rosignoli et al. 2009; Wherry et al. 2007).

Chronic immune cell activation and exhaustion is also associated with increased KIR expression on T cells. Indeed, the iKIR/HLA receptor-ligand system has similarities to the PD-1/PDL1/2 system in that both iKIR and PD-1 are inhibitory receptors that interfere with proximal T cell receptor signaling and are up-regulated in the context of chronic viral infection and on tumor infiltrating lymphocytes (Boelen et al. 2018). Both are also expressed on late stage differentiated memory T cells, typically in a mutually exclusive manner.

Overall, HIV-1-specific lymphocytes generated in the chronic phase of infection face an immune system that is inclined to reduce and regulate rather than activate immune responses. Thus, the ability of these cells to decrease the level of viral replication more efficiently might be impaired.

1.9 Thesis hypothesis and aims

Undoubtedly, the control of HIV-1 viremia and preservation of CD4 T cells involves a complex and multifactorial interplay between the host genetic background, biological characteristics of the transmitted/founder virus, the early immune response and a host of other factors that are not well understood. Thus, the precise pathogenic mechanisms involved are still not well understood. The overall hypothesis of this work is that transmitted/founder viruses that are associated with faster disease progression have unique genotypic characteristics, cause a more exacerbated but dysfunctional immune response, infect a broader range of target cells and are of higher replicative capacity. These characteristics work in synergy to deplete $CD4^+$ T cells and cause immune dysregulation, hence leading to worse disease outcome. Thus, investigations described in this thesis examine selected phenotypic and genotypic characteristics of the virus populations that establish HIV-1 infection in a subset of patients drawn out of a well characterised cohort, and possible mechanisms by which these virus characteristics influence HIV-1 pathogenesis. The specific aims were as follows:

Chapter 3

1. To identify the near full-length genotype of the transmitted founder HIV-1 in a well characterised cohort of men who have sex with men (MSM).
2. To assess the transmission bottleneck in this MSM cohort compared to published heterosexual cohorts.
3. To investigate the relationship between the HIV-1 transmission bottleneck and disease progression.

Chapter 4

1. To determine the replicative capacity of the transmitted/founder virus as defined by the transmitted *gag.*
2. To evaluate cell-to-cell spread of transmitted/founder viruses of known replicative capacity.
3. To assess how *in vitro* replicative capacity and mode of spread between cells influences disease trajectory.

Chapter 5

1. To investigate a role for transmitted/founder virus genotypic and phenotypic characteristics identified in Chapters 3 and 4 above in immune responses.

Chapter 2: Materials and methods

2.1. Study subjects

HIV-1^+ samples were drawn from a large well characterized prospective multicentre HIV-1 infection incidence study (IAVI protocol C) (Amornkul et al. 2013; Kamali et al. 2015; Prentice et al. 2016). All samples used in this study were from antiretroviral therapy naïve patients. Genetic data such as HLA type as well as clinical data such as peak viral load, longitudinal viral loads and $CD4^+$ T-cell count data were also available. Patients received HIV prevention counselling and when HIV-1 infected were referred for HIV-1 treatment as per the national guideline, which was to initiate treatment at CD4 T cell counts ≤350 cells/mm^3 blood.

2.1.1. The KEMRI MSM acute HIV patients

In 2005, a prospective study of men and women considered to be at high risk of HIV acquisition was established at the Kenya Medical Research Institute (KEMRI) in coastal Kenya as part of IAVI's protocol C cohort described above (Sanders 2014; Sanders et al. 2013; Sanders et al. 2015). Adults aged 18–49 years were eligible if they were HIV-1-seronegative and reported transactional sex work, a recent STI, multiple sexual partners, sex with an HIV-1-infected partner or unprotected anal sex.

HIV-1 testing was performed using two rapid test kits (Determine, USA and Unigold, Ireland) in parallel. All HIV-1-negative samples were tested for p24 antigen (Biomérieux Ltd, France), and plasma tested for HIV-1 RNA level (Roche, Switzerland). Individuals who were seronegative at initial screening but tested positive for HIV-1 p24 at subsequent visits were invited to enrol into an acute infection cohort, where blood samples were taken either monthly or quarterly for HIV-1 plasma viral load and CD4 T-cell assessments and are the focus of this study.

The estimated date of HIV-1 infection (EDI) was calculated as 10 days before the sample collection date when the sample had a positive HIV-1 RNA level and negative p24 antigen and HIV-1 serology, 14 days before a positive p24 antigen test or the mid-point between a previously negative and subsequently positive HIV-1 serologic test, in the absence of either a positive HIV-1 RNA level or p24 antigen test (Wahome et al. 2013).

Written informed consent was obtained from all study participants and the study was approved by the ethical review board at the Kenya Medical Research Institute.

2.1.2. The ZEHRP discordant couples

A HIV-1 discordant couples' cohort exists in the Zambia Emory HIV Research Project (ZEHRP) Lusaka Zambia that is also as part of IAVI's protocol C cohort, where both the HIV-1 seropositive patients and their HIV-1 seronegative partners are followed longitudinally in the same manner described for the KEMRI cohort (Trask et al. 2002; Woodson et al. 2018). All participants in this cohort were enrolled in human subjects' protocols approved by both the University of Zambia Research Ethics Committee and the Emory University Institutional Review Board.

Prior to enrolment, individuals received counselling and signed a written informed consent form. Incident infections in the seronegative partner are confirmed to be linked to the seropositive partner by phylogenetic analyses of HIV-1 gp41 sequences (Trask et al. 2002). PBMC were obtained from the patients at the first instance when the HIV-1 p24 antigen was detected before plasma antibodies could be detected. The algorithm used to determine the estimated date of infection (EDI) in this cohort was previously described by Haaland et al (Haaland et al. 2009).

2.1.3. HIV-1 seronegative controls

HIV-1 seronegative buffy coats obtained from the Clinical Laboratory Services (CLS) blood bank at South Africa were used to set up all the experiments, and as healthy controls in the analysis. Informed consent was obtained from all blood donors and approved by the local ethics committee.

2.1.4. Inclusion criteria

Patients were included in this study if they had been enrolled before 45 days after the estimated date of infection and had been followed up for over 3 years whilst antiretroviral therapy naïve. Participants also needed to have a plasma sample available within the first 45 days after the estimated date of infection to allow for definition of the transmitted/founder genome, and PBMC sample at 3, 9 and 24 months after infection to allow for the characterization of immune responses.

2.2. Media and wash buffers

2.2.1. R20

Complete medium (RPMI 1640) (Sigma-Aldrich, UK) containing 20% Fetal Calf Serum (FCS, Sigma-Aldrich), 1% 1M HEPES buffer (Sigma-Aldrich), L-glutamine final concentration 2mM (Sigma-Aldrich), Penicillin-streptomycin final concentration 100IU/ml and 100µg/ml respectively (Sigma-Aldrich) and 1% Sodium Pyruvate (Sigma-Aldrich).

2.2.2. R10

Complete medium (RPMI 1640) (Sigma-Aldrich) containing 10% FCS (Sigma-Aldrich) 1% 1M HEPES buffer (Sigma-Aldrich), 1%L-glutamine (Sigma-Aldrich), 1% Penicillin-streptomycin (Sigma-Aldrich) and 1% Sodium Pyruvate (Sigma-Aldrich).

2.2.3. D10

Complete medium (DMEM) (Gibco, USA) containing 10% FCS (Sigma-Aldrich) 1% 1M HEPES buffer (Sigma-Aldrich), 1% L-glutamine (Sigma-Aldrich), 1% Penicillin-streptomycin (Sigma-Aldrich) and 1% Sodium Pyruvate (Sigma-Aldrich).

2.2.4. MACS buffer

MACS BSA Stock Solution (Miltenyi, Germany) diluted 1:20 with AutoMACS Rinsing Solution (Miltenyi) and filtered before use.

2.3. Amplification and sequencing of near full-length HIV genomes

2.3.1. Viral RNA extraction and cDNA synthesis

RNA was extracted from 140µl of the earliest available plasma, earlier than 45 days after estimated date of infection using the QIAamp Viral RNA Mini Kit (Qiagen, Netherlands) following the manufacturer's instructions. RNA was recovered from spin columns in a final elution volume of 60µl and either frozen at -80°C or immediately used to synthesize cDNA.

RNA was reverse transcribed using either the SuperScript III or Superscript IV cDNA synthesis kit (Life Technologies, USA) according to the manufacturer's instructions and as previously described (Salazar-Gonzalez et al. 2008; Salazar-Gonzalez et al. 2009). First, 11µl RNA was

mixed with 1µl of 2pMol of reverse primer 1.3'3'PlCb or OFM19 (primer sequence detailed in Table 2.1), and 1µl of 40µMol deoxynucleoside triphosphate (dNTP) and incubated for 5 min at 65°C to denature secondary RNA structure. This was followed by addition of 4µl of 5x RT buffer, 1µl of 5mM dithiothreitol, 2µl of the RNase inhibitor RNaseOUT, and 1µl Superscript III enzyme and incubated at 50°C for 1hr after which 1µl of Superscript enzyme was added and the reaction incubated at 55°C for a further 2 hours. After the completion of the reverse transcription step, the reaction was heat inactivated at 70°C for 15 minutes when 1µl of RNase H was added and incubated at 37°C for 20 minutes to degrade any RNA remnants. This cDNA was used as template for single genome PCR immediately or stored at -80°C until it was needed.

2.3.2. Single genome amplification of HIV-1

cDNA was serially diluted in replicates of 8 to 32 PCR wells and subjected to nested PCR amplification with HIV-1-specific primers that yield a 9-kb fragment beginning at the first nucleotide of the U5 region of the 5'long terminal repeat (LTR) and extending to the last nucleotide of the R region of the 3'LTR (Figure 2.1).

First-round PCR was performed in 1x Q5 Reaction Buffer, 1x Q5 High GC Enhancer, 0.35mM of each dNTP, 0.5µM of primers and 0.02 U/µl of Q5 Hot Star High-Fidelity DNA Polymerase (New England Biolabs, USA) in a total reaction volume of 25µl. Cycling conditions for both reactions were 98°C for 30s, followed by 30 cycles of 98°C for 10s, 72°C for 7.5min, with a final extension at 72°C for 10min. Second round PCR was performed in similar conditions as the first round PCR but using the primers 2.3'3' PlCb and 2U5Cc.

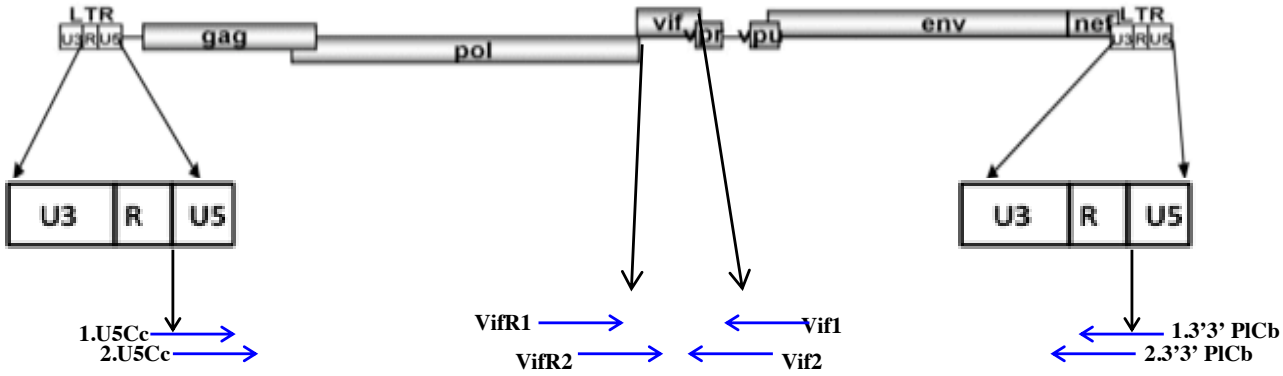


Figure 2.1: Primer position for the amplification of ~9kb near full length HIV-1 genome. The near full-length piece measured ~9kb and spanned from the U5 region of the 5' long terminal repeat region to the 3' long terminal repeat. Where two overlapping halves were amplified, they spanned the same regions but overlapped by the entire *vif* gene.

Where NFLG PCR failed after several attempts, the HIV-1 genomes were amplified in two 5kb half genomes overlapping by the *Vif* gene using the Q5 PCR master mix composition used for NFLG PCR but with internal primers Vif1 and VifR1 for the first round, and Vif2 and VifR2 for the second round.

The amplicons were sized on 1% agarose gel (Invitrogen, USA) and run on either 1x TAE or Lithium Acetate buffer. All products derived from cDNA dilutions yielding <30% PCR-positive wells and ~9-kb in length were further amplified using primers 1.3'3'PlCb and 1.U5Cc with a 20-nucleotide barcode added to the primer, and PCR product purified using the Promega PCR clean up kit as per manufacturer's recommendations (Promega, USA) in preparation for sequencing. All PCR procedures were performed under PCR clean-room conditions. Positive amplicons were quantified using a NanoDrop_ND-1000 Spectrophotometer (Thermo Fisher Scientific, USA).

Table 2.1 Primers used to generate near full length and half genome HIV-1 amplicons.

Primer	Primer sequence	Primer use
OFM19	5' GCACTCAAGGCAAGCTTTATTGAGGCTTA 3’	cDNA synthesis
1.3’3’PlCb	5’ACTACTTAGAGCACTCAAGGCAAGCTTTATTG 3’	Near full-length genome PCR
1.U5Cc	5’CCTTGAGTGCTCTAAGTAGTGTGTGCCCGTCTGT 3’	Near full-length genome PCR
2.3’3’PlCb	5’TAGAGCACTCAAGGCAAGCTTTATTGAGGCTTA 3’	Near full-length genome PCR
2.U5Cd	5’AGTAGTGTGTGCCCGTCTGTTGTGTGACTC 3’	Near full-length genome PCR
Vif1	5’GGGTTTATTACAGGGACAGCAGAG 3’	5’ Half genome PCR
VifR1	5’TTCCTCGTCGCTGTCTCCGCTTCTTCCT 3’	3’ Half genome PCR
Vif2	5'GCAAAACTACTCTGGAAAGGTGAAGGG 3’	5’ Half genome PCR
VifR2	5’GTCCCCTAGTGGGATGTGTACTTCTGAAYTT 3'	3’ Half genome PCR

2.3.3. SMRT sequencing of HIV-1 genomes

Genome libraries containing multiple HIV-1 genome amplicons were constructed by pooling equal amounts of independently amplified single genomes derived from different patients to a final concentration of 3000ng. SMRTbell libraries were then generated for each pool according to protocols from the DNA Template Prep Kit 2.0 (Pacific Biosciences Inc, California, USA).

Specifically, initial repair of the amplicons was done by combining 3000ng of the pooled DNA sample with 5µl of DDR Buffer, 0.5µl of NAD^+, 0.5µl of dNTP (10mM) and 2µl of DNA Damage Repair Enzyme, and incubated at 37◦C for 20 min and then at 4◦C for 1 min. Then the

mixture was subjected to a round of DNA purification using AMPure PB magnetic beads (Pacific Biosciences Inc) and eluted in 30µl of Elution Buffer.

End Repair was done by adding 5µl of Template Prep Buffer, 5µl of ATPHi (10mM), 2µl of dNTP (10mM), 5.5µl of water and 2.5µl of End Repair Mix Enzyme. This mix was incubated at 25^{o}C for 15min and then at 4^{o}C for 1–2 min. Another round of DNA purification using AMPure PB magnetic beads was then performed and the DNA eluted in 30 µl of Elution Buffer. This was followed by Ligation with SmartBell Adaptors. 1µl of blunt adaptors, 4µl of Template Prep Buffer, 2 µl of ATP Low (1mM), 2µl of water and 1µl of T4 Ligase (30U/µl) were added to the mixture and incubated overnight at room temperature and then heated for 10 min at 65^{o}C to inactivate the ligase. 0.75µl of Exonuclease III and 0.75µl of Exonuclease VII were added to the ligation mix and incubated at 37^{o}C for 1 hour in order to remove any unligated DNA. Finally, three rounds of DNA purification using the AMPure PB magnetic beads were performed, eluting in 100µl of Elution Buffer after first and second round, and eluting in 15µl of Elution Buffer after the final round of purification. Figure 2.2 is a summary of the library preparation process.

The quality of the library was then assessed by running the sample in the Agilent 2100 Bioanalyzer system (Agilent Technologies, USA). Primer annealing and P4 polymerase binding to the SMRTbell libraries were then performed, and SMRT sequencing done on the PacBio RSII (Pacific Biosciences Inc) yielding above 20,000 reads of suitable lengths per SMRT library.

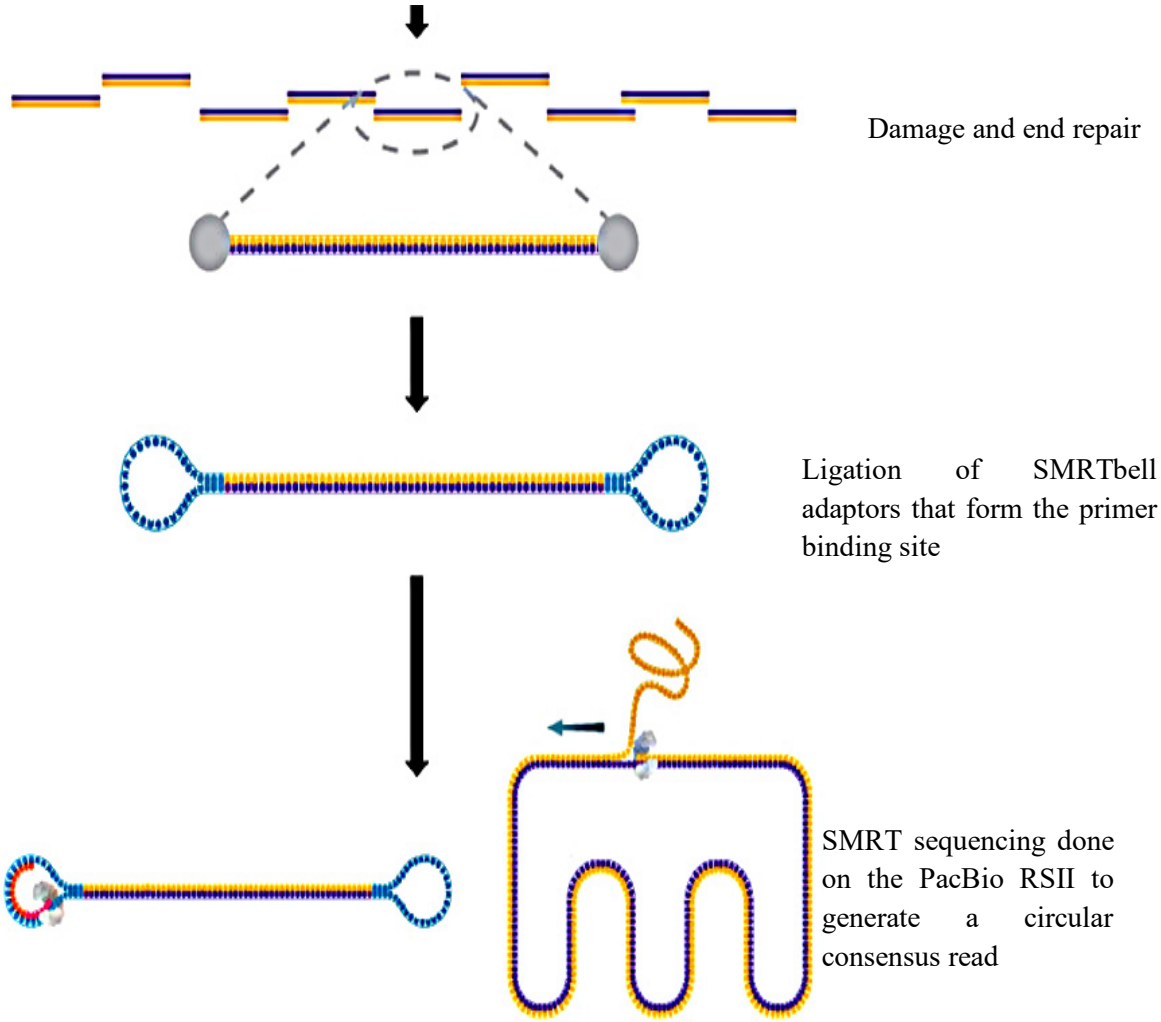


Figure 2.2: Preparation of SMRTBell libraries for PacBio sequencing.

Damage and end repair is followed by ligation of SMRTBell adaptors on which the sequencing primers anneal and initiate the sequencing reaction (modified from (Kong et al. 2017).

2.4. Sequence analysis

2.4.1. Identification of the transmitted/founder virus

Single Molecule, Real-Time (SMRT®) sequencing (Pacific Biosciences Inc) provides deep sequencing and gives the longest continuous DNA sequencing reads currently available (Khiste and Ilie 2017; Yanhu, Lu, and Li 2015). However, the relatively high error rate in the raw read

data requires novel analysis methods to de-convolute sequences derived from complex samples. An algorithm described by Dilernia *et al* that stratifies unique reads from the different genomes and estimates consensus within each genome strata to remove sequencing error was used (Dilernia et al. 2015). A FASTA file containing the initial set of reads to be analysed was generated and using the Quiver algorithm, sequences that likely originated from different genomes stratified and a consensus estimated within each stratum.

The majority of errors in PacBio raw reads are insertions and deletions (INDELs) and so for every non-consensus nucleotide at every position in the alignment, the probability for that nucleotide to be a sequencing error was defined as the complement probability to the binomial cumulative distribution with a 5% uniform rate (expected frequency for noise). Only non-consensus nucleotides with a q-value less than 0.2 were considered likely to be true polymorphisms. Classification of reads was performed by implementing a hierarchical clustering method and the final distance defined as the percentage of differences over the total positions included in the calculation.

In order to derive all of the unique sequences in the mixture, the above steps were repeated until there were no positions with significant diversity remaining within the subgroups. Although each subgroup is homogeneous, some errors in the sequence remain primarily due to bases missed during sequencing. To correct these errors, two error correction algorithms were utilised. Error correction algorithm 1 reanalyses all of the nucleotides initially classified as potentially erroneous insertions in the raw read alignment for evidence of specific nucleotides present at frequencies significantly higher than that expected for noise considering statistically significant any nucleotide having a q -value lower than 0.01. Any nucleotides found to be significantly prevalent are considered real nucleotides missed by the alignment process due to low frequency in the sequencing output data and are consequently inserted in the final consensus sequence. After the analysis, nucleotides that exhibited p -values lower than 0.05

and q -values lower than 0.01 in at least 40 of the 50 replicates are considered true nucleotides and are incorporated into the final consensus sequence. Error correction algorithm 2 explores every single gap in the alignment obtained after correction with algorithm 1 and defines as a 'real' nucleotide any nucleotide type initially classified as potentially erroneous insertions in the raw read alignment that, while exhibiting a significant q -value in at least one of the 50 replicates of algorithm 1, would fill a single gap in the sequence.

2.4.2. Identification of APOBEC mutations

The LANL Hypermut tool was used to perform two types of checks. First, a search was performed for hypermutated sequences, *i.e*., particular sequences that present a statistically significant excess of $G \rightarrow A$ mutations at APOBEC3G/F motifs compared to elsewhere in the sample with a P-value threshold (default $P < 0.1$). The tool then created an artificial sequence which summarizes all mutations found across the sample and is was then tested for APOBEC enrichment via the Hypermut tool. When the latter is significantly enriched, it indicates enrichment across the sample for mutations embedded in the APOBEC motif, in which case we called the sample overall enriched

2.4.3. Classification of circulating HIV-1 subtypes

Virus subtype was determined by phylogenetic clustering with reference genomes from the Los Alamos National database (SEQalign), while recombination patterns were determined using the LANL recombination identification tool (RIP3.0) and SIMPLOT software. Phylogenetic analyses were performed by maximum likelihood parsimony.

A two-step process for the identification of circulating HIV-1 subtypes was used. First, the LANL RIP tool which identifies recombination by calculating similarity to reference genomes of subtype A1, A2, B, C, D, F1, F2, G, H, J and K in a sliding widow was employed. The best

match within each window is qualified as a measure of confidence that represents the best matching reference sequence to the second-best matching reference sequence in a 100-nucleotide sliding window to enable detection of small areas of recombination and probable recombination sites.

Secondly, the patient genomes were aligned to the reference genomes shortlisted in step 1 above and utilizing a sliding window of 400 nucleotides and a bootstrap of 70% eliminated false negative areas of recombination and identified discrete break points.

2.5. Construction of GAG-NL4.3 chimeras

2.5.1. Construction of chimeric plasmid

To study the role of the *gag* gene in viral fitness, chimeric molecular clones were generated on a NL4.3 proviral backbone that only differs by the HIV-1 *gag* gene using a 3-piece modification of the homologous recombination cloning strategy described by Prince *et al* (Claiborne, Prince, and Hunter 2014; Prince et al. 2012).

The first piece was a 1.5kb piece of complete *gag* derived from the transmitted/founder virus sequence starting from the initiation codon of *gag* and extending 142 nucleotides after the gag stop codon and into *pro*. To generate this piece, a PCR was performed using Q5 polymerase and 1µL of the first round near full length or 5' half genome PCR product as template. First-round PCR was performed in 1x Q5 Reaction Buffer, 1x Q5 High GC Enhancer, 0.35 mM of each dNTP, 0.5 µM of primers 5'GagF and Gag 3'Rev1 and 0.02 U/µl of Q5 Hot Star High-Fidelity DNA Polymerase in a total reaction volume of 25µl. Cycling conditions were 98°C for 30s, followed by 30 cycles of 98°C for 10s, 68°C for 30sec and 72°C for 1.5min, with a final extension at 72°C for 10min. Primer sequences for cloning primers are listed in Table 2.2.

The second piece was a 6kb fragment derived from NL4.3 spanning from the start of *pol* to the middle of *env* while the 3rd piece was a 7.4kb pBluescript vector piece containing a fragment of *env* (Figure 2.3). To generate these pieces, two separate PCRs were performed using similar Q5 conditions to those described for the *gag* piece above but using primer GAGPOLFor1 and Rev18Clone for the NL4.3 piece and For18Clone and 5'GagR for the vector piece. Cycling conditions were 98°C for 30s, followed by 30 cycles of 98°C for 10s, 69°C for 30sec and 72°C for 5min, with a final extension at 72°C for 10min. Cloning primers contained a 15bp overhang of the adjacent cloning piece to allow for homologous recombination in subsequent steps. The three cloning pieces were purified by electrophoresis on a 1% agarose gel, cutting out the required band size and further purification using the Promega PCR clean up (Promega, USA) according to manufacturer's recommendation with elution in 40µl of nuclease free water.

Table 2.2 Primer sequences for cloning.

Primer name	**Primer sequence**	**Primer use**
5'GagF	5' TAGAAGGAGAGAGATGGGTGCGAG 3'	Amplification of complete *gag*
Gag 3'Rev1	5' CCTTTAATTGCCCCCCTATCTTTACTG 3'	Amplification of complete *gag*
GAGPOLFor1	5' GGGGGCAATTAAAGGAAGCTCTATTAG 3'	Amplification of *gag* to *env* cloning piece
Rev18Clone	5' ACCACAGCCAATTTGTTATG 3'	Amplification of *gag* to *env* cloning piece
For18Clone	5' CAAATTGGCTGTGGTATAT 3'	Amplification of *env* and vector cloning piece
5'GagR	5' ATCTCTCTCCTTCTAGCCT 3'	Amplification of *env* and vector cloning piece

To generate a replication competent circular plasmid, 100ng of the 3 pieces at a ratio of 3:1:1 were mixed with 2µl of 5x infusion enzyme premix to a total reaction volume of 10µl and incubated at 50Oc for 15 minutes. Stellar competent cells were transformed with 2.5µl of the

ligation products, plated onto LB/agar plates supplemented with 100µg/ml ampicillin and grown at 30°C (Figure 2.3).

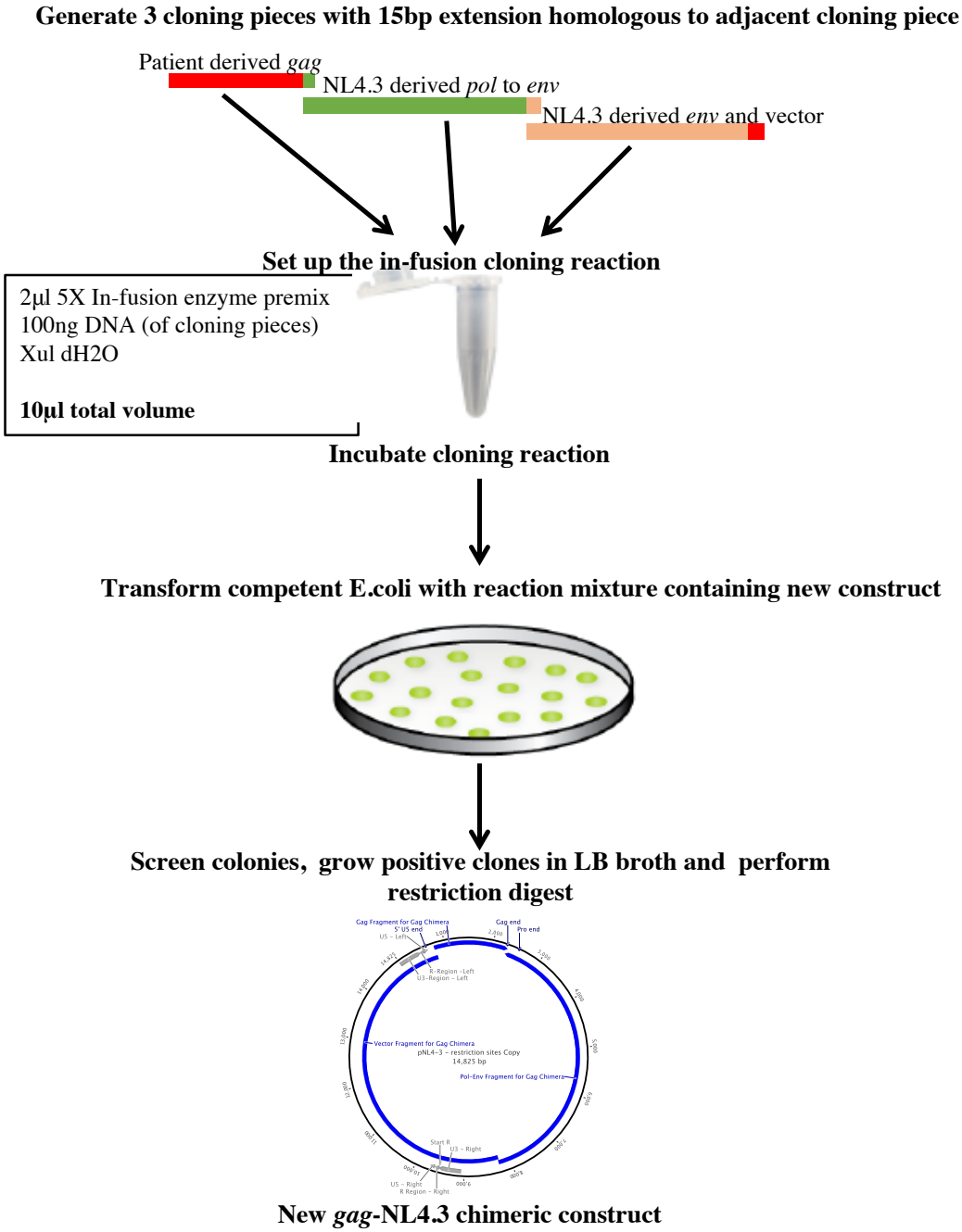


Figure 2.3: Cloning strategy to generate gag-NL4.3 chimeric plasmid.

Three cloning pieces were generated by PCR using primers that added 15bp homologous overhangs and ligated to form a circular plasmid which was used to transform stellar competent cells.

Distinct small white colonies were picked and dissolved in 20µl or LB broth, from which 10µl was heat denatured at 95°C for 10 minutes and 0.5µl of the denatured bacteria used as template to screen for gag insertion by PCR using Gag3 and Gag4 in 1x Q5 Reaction Buffer, 1x Q5 High GC Enhancer, 0.35mM of each dNTP, 0.5µM of primers and 0.02 U/µl of Q5 Hot Star High-Fidelity DNA Polymerase (New England Biolabs, USA) in a total reaction volume of 25µl. Cycling conditions were 98°C for 30s, followed by 30 cycles of 98°C for 10s, 62°C for 15sec and 72°C for 1min, with a final extension at 72°C for 5min to yield a 150kb product.

Colonies that tested positive for Gag were further cultured in 5mL of LB broth with 100µg/ml of ampicillin for 48 hours at room temperature, after which Gag-NL4.3 chimeric DNA was isolated from cultures using the PureYield™ Plasmid Miniprep System (Promega, USA) and restriction digestion performed with Sph1 enzyme (New England Biolabs, USA) which has a restriction site within *gag* to confirm correct plasmid size and successful insertion of patient derived *gag* gene. Three identical independent clones per patient were chosen for replication assays in order to ensure backbone fidelity during the cloning process.

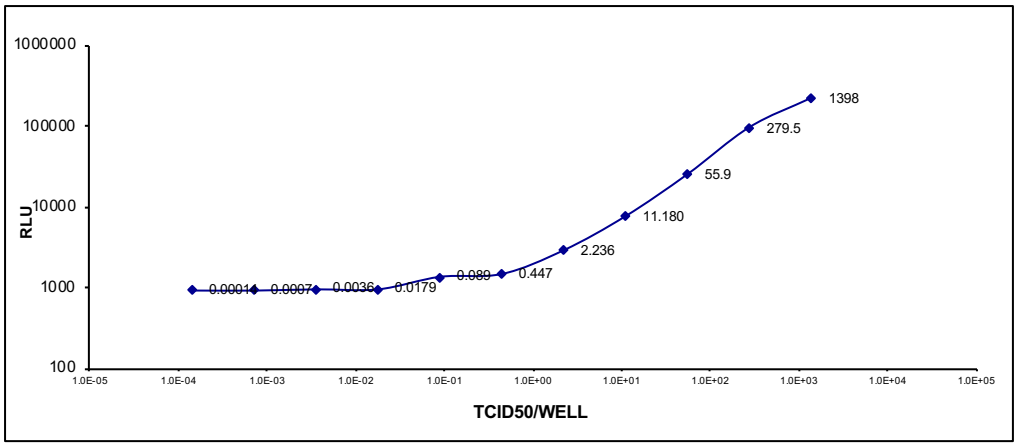
2.5.2. Generation and titration of virus stocks

1.5 mg of proviral plasmid clones were used to transfect 293T cells (NIH AIDS Reagent Program, USA) with Fugene-HD Transfection reagent (Promega, USA) and incubated at 37°C and 5%CO_2. 293T cell supernatants were collected 48 hours after transfection and clarified by centrifugation. Virus stocks were then titered on the Tzm-bl reporter cell line (NIH AIDS Reagent Program) according to standard protocols. Three independently cloned plasmids but of similar sequence were transfected for each patient.

To titre virus stocks, Tzm-bl cells were plated on 96 well flat-bottomed plates (Sigma Aldrich) at a concentration of 1 x 10^4 cells/well in 100 µl D10 media per well and incubated at 37ºC and 5 % CO_2 overnight, after which the media was removed and 200µl of virus stocks that had been

diluted in D10 containing 10 µg/ml of DEAE Dextran was added per well and incubated at 37ºC and 5 % CO_2. Eleven dilutions in quadruplicate were done per virus stock.

48 hours later, Luciferase expression was evaluated by replacing 100µl of supernatant with 100µl of Britelite plus reagent (PerkinElmer, USA), incubating at room temperature for 5 minutes and transferring to 96 well white flat bottom plates (Sigma Aldrich) and measuring luciferase lunimiscence in a Tecan Infinite plate reader (Tecan, Switzerland). TCID50 was calculated for each virus. A titration curve and TCID50 values were calculated for each virus stock. An example is shown on Figure 2.4.



Dilution	# pos	# neg	pos & neg totals		% neg	Dilution	TCID50/Well
row 1	4	0	20	0	0	10	1398
2	4	0	16	0	0	50	279.5
3	4	0	12	0	0	250	55.9
4	4	0	8	0	0	1250	11.180
5	4	0	4	0	0	6250	2.236
6	0	4	0	4	100	31250	0.447
7	0	4	0	8	100	156250	0.089
8	0	4	0	12	100	781250	0.0179
9	0	4	0	16	100	3906250	0.0036
10	0	4	0	20	100	19531250	0.0007
11	0	4	0	24	100	97656250	0.00014
(totals)	20	24					
Dilution at 50% is:		13975					
		TCID50/ml			69,877		

Figure 2.4: TCID50 calculation.

TCID50 per well graphed for each dilution, absolute values are shown on the table below. Eleven serial dilutions were performed in quadruples for each virus stock.

2.6. *In vitro* replicative capacity assay

In order to assess the *in vitro* replicative capacity (RC) of the generated infectious molecular clones, GXR25 cells which are a CXCR4+ CCR5+ CEM cell line derivative (donation from Eric Hunter's Lab, Emory University, USA) were infected at a multiplicity of infection (MOI) of 0.01 and 100 ml of viral supernatants collected at 2-day intervals (Figure 2.5).

Briefly, GXR25 cells and virus were incubated with 5 mg/ml polybrene at 37ºC for 3 hours, washed 5 times with complete R10 media and plated into 24-well plates. Cells were split 1:2 to maintain confluence, replaced with an equal volume of fresh media, and viral supernatants were taken at days 2, 4, 6 and 8 as previously described (Claiborne, Prince, and Hunter 2014).

Virion production was quantified using a colorimetric reverse transcriptase assay (Roche, Switzerland). Forty µl of lysis solution was added to 10µl of culture supernatants from infected cells and standard curve reagent and incubated at room temperature for 30 minutes after which 20µl of a reaction mixture containing dNTP and reverse transcriptase template were added and incubated for 15 hours at 37ºC. Sixty µl of this mixture was incubated in MP modules at 37ºC for 1 hour and washed 5 times with the kit provided wash buffer before addition of anti-DIG-POD working solution and further incubation at 37ºC for 1 hour. The plates were then washed 5 times, 200µl of ABTS substrate solution added per well and the absorbance read at 405nm referencing 490nm. RT activity per supernatant was derived from the standard curve.

Based on values obtained for days 2–8, the optimal window for logarithmic growth for all viruses was determined to be between days 2 and 6, as by day 8 many high replicating viruses had exhausted target cells causing a flattening or decline of the replication curve. Therefore, log10-transformed slopes were calculated based on days 2, 4 and 6 for all viruses. Replication scores were generated by dividing the log10-transformed slope of the replication curve for each Gag-NL4.3 chimera by the log10- transformed slope of wild-type NL4.3. Three independent

Gag-NL4.3 chimera clones per patient recipient were run and their replicative capacity scores averaged.

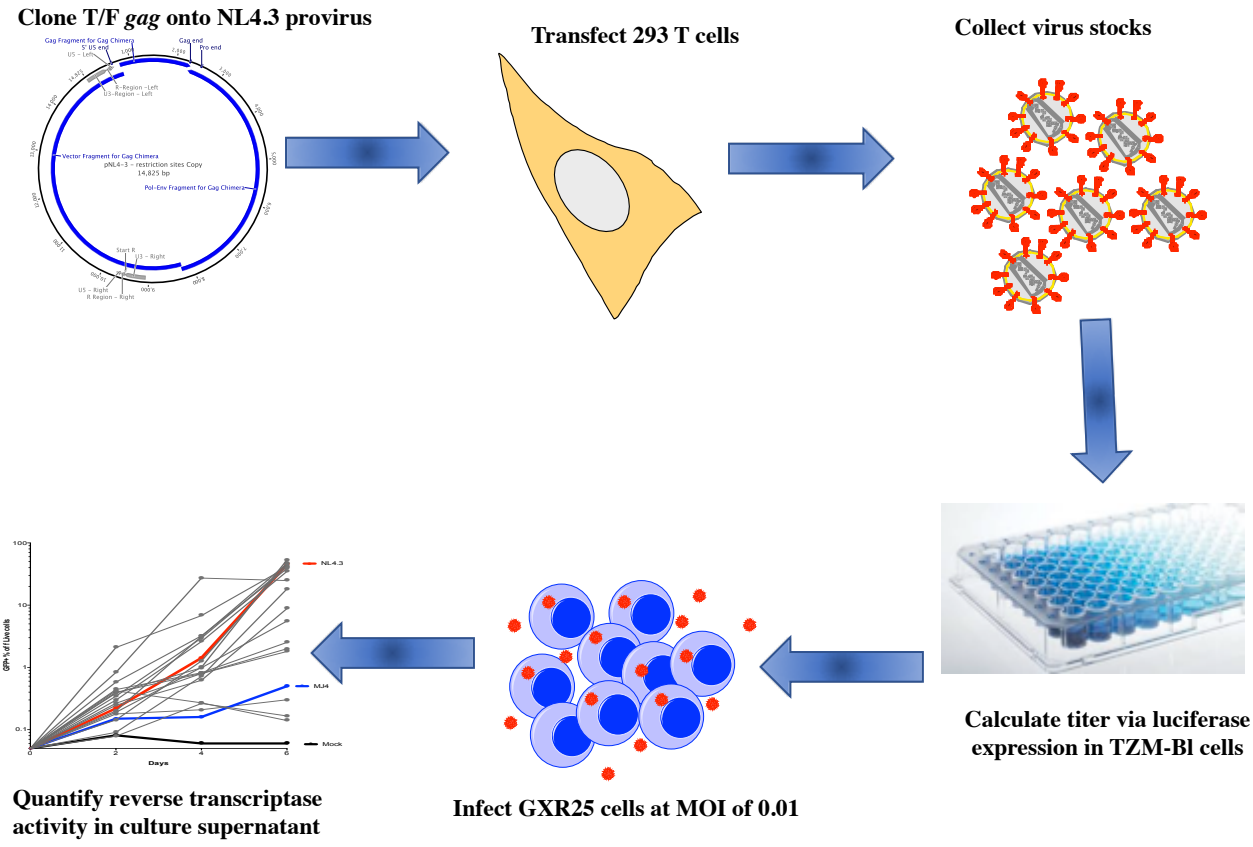


Figure 2.5: *In vitro* assessment of viral replicative capacity.

Patient derived transmitter/founder *gag* was inserted into a NL4.3 backbone to generate chimeric viruses, whose growth curve was quantified using a colorimetric reverse transcriptase assay on culture supernatants, and the growth curve of the chimeric viruses normalised to that of wild type NL4.3 to generate a replicative capacity score.

2.7. Cell-cell transmission experiment

2.7.1. Generation of infected donor cells

$CD4^+$ T cells were isolated from a HIV negative donor using the Miltenyi $CD4^+$ T cell isolation kit. PBMC were pelleted and re-suspended in 40 µl of 0.5% BSA in autoMACS solution and 10 µL of $CD4^+$ T Cell Biotin-Antibody Cocktail per 10^7 total cells. The antibody labelled PBMC were mixed well and incubated for 5 minutes at 2−8ºC, and 30 µL of buffer added before addition of 20 µL of $CD4^+$ T Cell MicroBead Cocktail and incubation for 10 minutes at 2−8ºC. The cell suspension was then added to a buffer washed LS column on a magnetic MACS separator and the flow through containing unlabelled $CD4^+$ T cells collected. The column was washed with 3mL of MCS buffer to collect further unlabelled $CD4^+$ T cells which were then washed once in R10 and counted using the ViCell counter (Beckman Coulter).

Sorted $CD4^+$ T cells were activated by dividing into 3 groups at $4x10^6$ cells/ml and activated using either 5mg, 1mg or 0.5mg of PHA per mL in R10 containing 100U/ml of IL-2 for 72 hours in 24 well plates. Activated cells were counted, pooled in equal numbers, and spinoculated at 1200g for 2 hours with *gag* chimeras of known replicative capacity at a MOI of 0.1 then cultured at 37^OC 5%CO_2 for 5 days with media change at day 3. At the end of the infection culture, infected donor cells were washed 3 times with R10 to eliminate cell free virus, rested for 12 hours and labelled with EF450 dye (ThermoFischer scientific, USA) for 10 minutes at 37ºC (Figure 2.6). Concurrently, a subset was stained for p24 to confirm that the target cells were infected.

2.7.2. Cell-cell infection

At the end of the 6-day infection, fresh autologous $CD4^+$ T cells were isolated from the same donor as described above and labelled with EF670 dye (ThermoFischer scientific) for 10 minutes. $8x10^5$ target cells were co-cultured with $4x10^5$ target cells in a final volume of 500 µL and culture supernatant collected at 8, 24 and 48 hours. At 24 hours, cells were analysed for infection by p24 flow cytometry and culture supernatants for viral loads by qPCR as described in section 2.7.3 and 2.7.4 respectively.

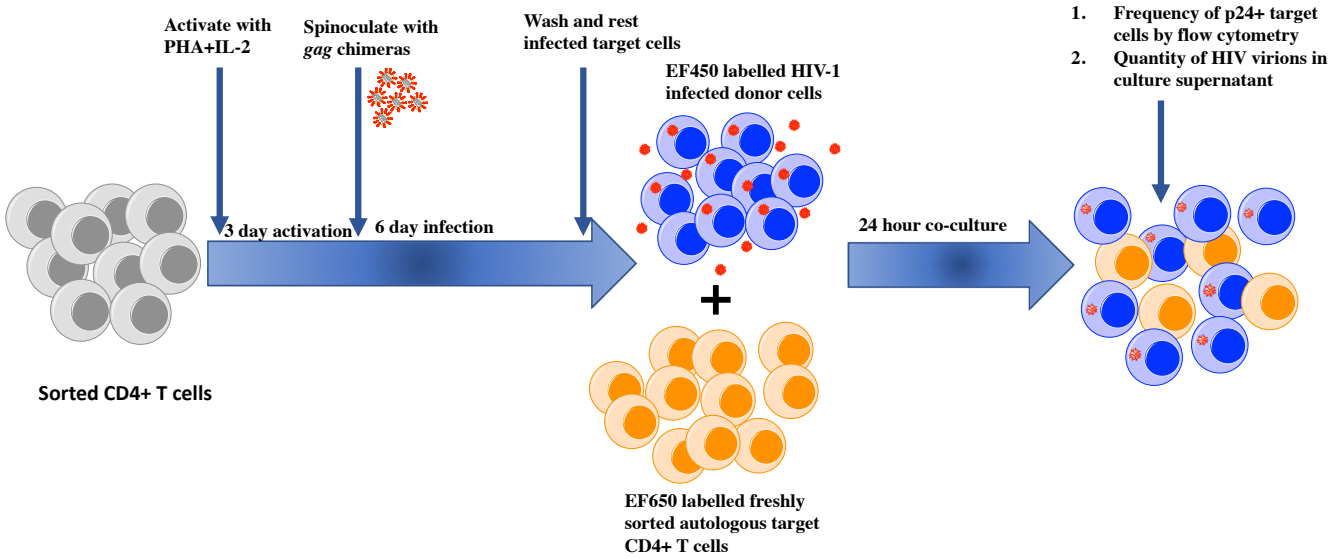


Figure 2.6: Cell to cell experiment.

Primary $CD4^+$ T cells from a healthy donor were activated for 3 days and cultured with *gag* chimeras of known replicative capacity for 6 days to generate infected donor cells. EF450 labelled infected donor cells were used to infect freshly isolated EF670 labelled autologous $CD4^+$ T cells, and infection of target cells measured after 24 hours.

2.7.3. Flow cytometry analysis of infection

After 24 hours of culture, cells were stained with 100 µl per well of a 1/400 dilution of the viability dye (Thermo Fischer Scientific) (prepared by first diluting the stock dye 1:40 in deionized water, and thereafter 1:10 in PBS) and incubated for 20 minutes at room temperature in the dark.

1.25 µl of anti CD3 antibody and 2.5 µl of anti CD4 antibody were added to the cells in 100 µl of PBS (Sigma-Aldrich, UK) and incubated for 20 minutes at room temperature in the dark. The cells were then washed once in PBS and fixed with 200 µl of Cytofix/Cytoperm (BD Biosciences, UK) at $4^{O}C$ for 20 minutes, after which they were stained with 2 µl of anti-p24 antibody for 20 minutes at room temperature in a 50 µl staining volume, washed and acquired on the BD Symphony flow cytometer. The flow cytometry panel is shown in Table 2.3.

Table 2.3 Flow cytometry panel used to identify cell types involved in cell-cell transmission.

Antibody	Origin	Clone	Fluorochrome	Supplier	Volume used (µl)
Viability	Synthetic dye	N/A	Amcyan	Invitrogen	0.25
CD3	Mouse	SK7	APC H7	BD Pharmingen	1.25
CD4	Mouse	RPA-T4	BV 605	Biolegend	2.5
P24	Mouse	FH190-1-1	PE	BD Horizon	2
Donor cells	Synthetic dye	N/A	EF450	Thermofischer	0.5
Target cells	Synthetic dye	N/A	EF650	Thermofischer	0.5

To correct for spectral overlap between fluorochromes, compensation controls for each antibody were prepared by staining anti mouse antibody compensation particles (BD

Biosciences) and negative control particles (BD, UK) with the titrated antibody volumes, incubating at room temperature for 20 minutes and topping up with 200 µl of Permwash (BD Biosciences) for acquisition.

2.7.4. qPCR assay to quantify virus in culture supernatants

The amount of virus in the cell to cell transmission assay culture supernatant was quantified as an indirect measure of the contribution of cell free spread in these cocultures by qPCR in a two-step process.

The first step was a two-step reaction where cDNA was synthesized from purified RNA followed by PCR amplification. 10µl of RNA extracted from culture supernatant of the cell-cell transmission assays was used as template in a master mix containing 12.5µl of 2x Superscript IV buffer (Invitrogen), 0.5µl RNAse out (Thermo Fischer Scientific), 0.6µM RNA primer mix (IDT), 0.5µl Superscript IV enzyme (Thermo Fischer Scientific), 0.75µl DEPC treated water (Qiagen) and 10000ng/µl of tRNA (Sigma-Aldrich) in a cool bucket. A standard curve set up was run concurrently but containing 10µl of serially diluted HIV-1 plasmid as template to generate a standard curve. The cycling conditions were 50^{O}C for 15 minutes, 95^{O}C for 2 minutes, 3 cycles of 95^{O}C for 15 seconds, 52^{O}C for 10 seconds and 60^{O}C for 1 minute, and finally 7 cycles of 95^{O}C for 15 seconds, 56^{O}C for 10 seconds and 50^{o}C for 1 minute.

The second step was a nested qPCR using 8µl of the PCR product generated in step one as template diluted 1 in 10 with 100ng/µL tRNA (Sigma-Aldrich), in a master mix containing 10µl 2x qPCRBIO probe mix (PCR Biosystems), 1µl of 20x dUTP UDGase (PCR Biosystems), 0.4µl of molecular biology grade water (Qiagen) and 0.4µl of a primer-probe mix (Thermo Fischer). The reaction was run on Quantistudio 3.0 software (Thermo Fischer) starting with uracil digestion at 37^{O}C for 10 minutes, initial denaturation of 95^{O}C for 3 minutes and 40 cycles

of 95^{O}C for 15 seconds, 60^{O}C for 30 seconds and 50^{o}C for 1 minute. HIV-1 RNA concentration was calculated against the standard curve.

2.8. Flow cytometry analysis

2.8.1. Thawing and overnight rest of PBMC

Frozen PBMCs were thawed in a 37^{O}C water-bath and washed once in 9 mL of R20 at 400g for 10 minutes. The cells were then rested overnight in 4 mL R20 at 37^{0}C, 5%CO_2, 95% humidity. On the following morning, the cells were counted using the ViCell viable cell counter (Beckman Coulter, UK) and re-suspended in R10 at a concentration of 10^7 viable cells per mL.

2.8.2. Peptide stimulation

PBMC were stimulated for 6 hours using 3 pools of 15mer HIV-1 GAG peptides (NIH reagent repository, USA) alongside a mock and PHA (Sigma-Aldrich) control. The first HIV-1 peptide pool consisted of 32 conserved HIV-1 GAG peptides covering HIV-1 clade A, B, C and D. The second and third peptide pools were derived from amino acid sequences of the global Potential T-cell Epitopes (PTEs) that cover all PTEs with a frequency equal to or greater than 15% in any one of the subtypes A, B, C and non-A, B, C. The peptides were diluted to a concentration of 1µg/Ml in R10 before the assay day and 100 uL of peptide per well frozen on 96 well plates at -80^{o}C until the assay day.

2.8.3. Flow cytometry panels

To test how the replicative capacity of the transmitted/founder virus impacts the early immune response, 5 flow cytometry panels were designed and run on PMBC at 3, 9 and 24 months after infection. Table 2.4 – 2.8 show the details of each panel.

Table 2.4: T cell phenotyping panel comprising of markers of activation, differentiation and exhaustion of $CD4^+$ and $CD8\ T^+$ cells.

Antibody target	Origin	Clone	Fluorochrome	Supplier	Volume used (µl)
Viability	Synthetic dye	N/A	Amcyan	Invitrogen	0.25
CD3	Mouse	SK7	APC H7	BD Pharmingen	1.25
CD4	Mouse	RPA-T4	BV 605	Biolegend	2.5
CD8	Mouse	RPA-T8	BV655	BD Horizon	1
CD45RO	Mouse	UCLH1	PECF594	BD Horizon	2
CCR7	Rat	3D12	APCR700	BD Horizon	1
CD27	Mouse	M-T271	PeCy7	BD Pharmingen	2
HLADR	Mouse	L243	PE	EBioscience	0.5
CD38	Mouse	HB7	APC	BD Horizon	1.5
PD1	Mouse	EH12.1	BV421	BD Horizon	3
Ki67	Mouse	35/Ki67	FITC	BD Horizon	10
CD57	Mouse	HNK-1	PerCP Cy5.5	Biolegend	1

Table 2.5: Cytotoxicity panel comprising of markers of T cell and NK cell cytotoxic degranulation.

Antibody target	Origin	Clone	Fluorochrome	Supplier	Volume used (µl)
Viability	Synthetic dye	N/A	Amcyan	Invitrogen	0.25
CD3	Mouse	SK7	APC H7	BD Pharmingen	1.25
CD4	Mouse	RPA-T4	BV 605	Biolegend	2.5

CD8	Mouse	RPA-T8	PerCP Cy5.5	BD Pharmingen	2
CD16	Mouse	EBioCB16	PECY7	EBioscience	1.25
CD56	Mouse	NCAM16.2	PECF594	BD Horizon	0.5
Perforin	Mouse	B-D48	PE	Biolegend	2.5
Granzyme B	Mouse	GB11	PE CY5	BD Pharmingen	1.5
CD107a	Mouse	H4A3	BV421	Biolegend	1
CD57	Mouse	HNK-1	FITC	BD Pharmingen	5

Table 2.6: B cell phenotype panel to determine whether viral replicative capacity influences B cell maturation, differentiation and class switching.

Antibody target	**Origin**	**Clone**	**Fluorochrome**	**Supplier**	**Volume used**
Dump:					
Viability	Synthetic dye	N/A	Amcyan	Invitrogen	0.25
CD3	Mouse	UCHT1	V500	BD Biosciences	1.25
CD19	Mouse	5526C1	QD 605	BD Horizon	1.25
CD21	Mouse	B-Ly4	PECF594	BD Horizon	2.5
CD27	Mouse	M-T271	PeCy7	BD Horizon	2
CD10	Mouse	H110a	APCH7	Biolegend	0.6
IgM	Mouse	MHM-88	APC H7	Biolegend	0.6
IgD	Mouse	IA6-2	PE	BD Horizon	0.6
CD27	Mouse	M-T271	PeCy7	BD Pharmingen	2
CD86	Mouse	IT2.2	PerCP Cy5.5	Biolegend	1
CD38	Mouse	HB7	APC	BD Biosciences	1.5
PDL1	Mouse	29E.2A3	BV421	Biolegend	5

Table 2.7: Innate cell panel to determine whether viral replicative capacity influences NK cell activation and monocyte subsets.

Antibody target	Origin	Clone	Fluorochrome	Supplier	Volume used (µl)
Viability	Synthetic dye	N/A	Amcyan	Invitrogen	0.25
CD3	Mouse	SK7	APC H7	BD Pharmingen	1.25
CD16	Mouse	EBioCB16	PECY7	EBioscience	1.25
CD56	Mouse	NCAM16.2	PECF594	BD Horizon	0.5
CD14	Mouse	HCD14	A700	Biolegend	2.5
NKG2C	Mouse	134522	FITC	R&D systems	1
NKG2A	Mouse	131411	PE	R&D systems	3
NKp44	Mouse	P44-S	APC	Biolegend	3.5
CCR7	Mouse	150503	BV421	BD Pharmingen	2
HLADR	Mouse	L243	PerCP Cy5.5	Biolegend	1
CD38	Mouse	HIT20	BV605	Biolegend	1.25

Table 2.8: T cell killing panel will reveal whether T cells express markers of cell death.

Antibody target	Origin	Clone	Fluorochrome	Supplier	Volume used (µl)
Viability	Synthetic dye	N/A	Amcyan	Invitrogen	0.25
CD3	Mouse	SK7	APC H7	BD Pharmingen	1.25
CD4	Mouse	RPA-T4	BV 605	Biolegend	2.5
CD8	Mouse	RPA-T8	PerCP Cy5.5	BD Pharmingen	2
CD45RO	Mouse	UCLH1	PECF594	BD Horizon	2

CD27	Mouse	M-T271	PeCy7	BD Pharmingen	2
Caspase 3	Rabbit	C92-605	PE	BD Pharmingen	5
Caspase 1	Peptide	N/A	FITC	ABD Serotec	0.5
CD95	Mouse	DX2	APC	Biolegend	1
PD1	Mouse	EH12.1	BV421	BD Horizon	3
CCR5	Mouse	HEK/1/85a	A700	Biolegend	1.5

2.8.4. Optimizing the flow cytometry panel

To determine the appropriate antibody volumes, antibodies were titrated down starting with volumes twice the manufacturer's recommendation and thereafter 7 serial dilutions. Singlet lymphocytes were then gated out and the fluorescence signal to noise ratio at different antibody concentrations was determined. The antibody concentration that gave the maximum signal to noise ratio was chosen for use (Figure 2.7).

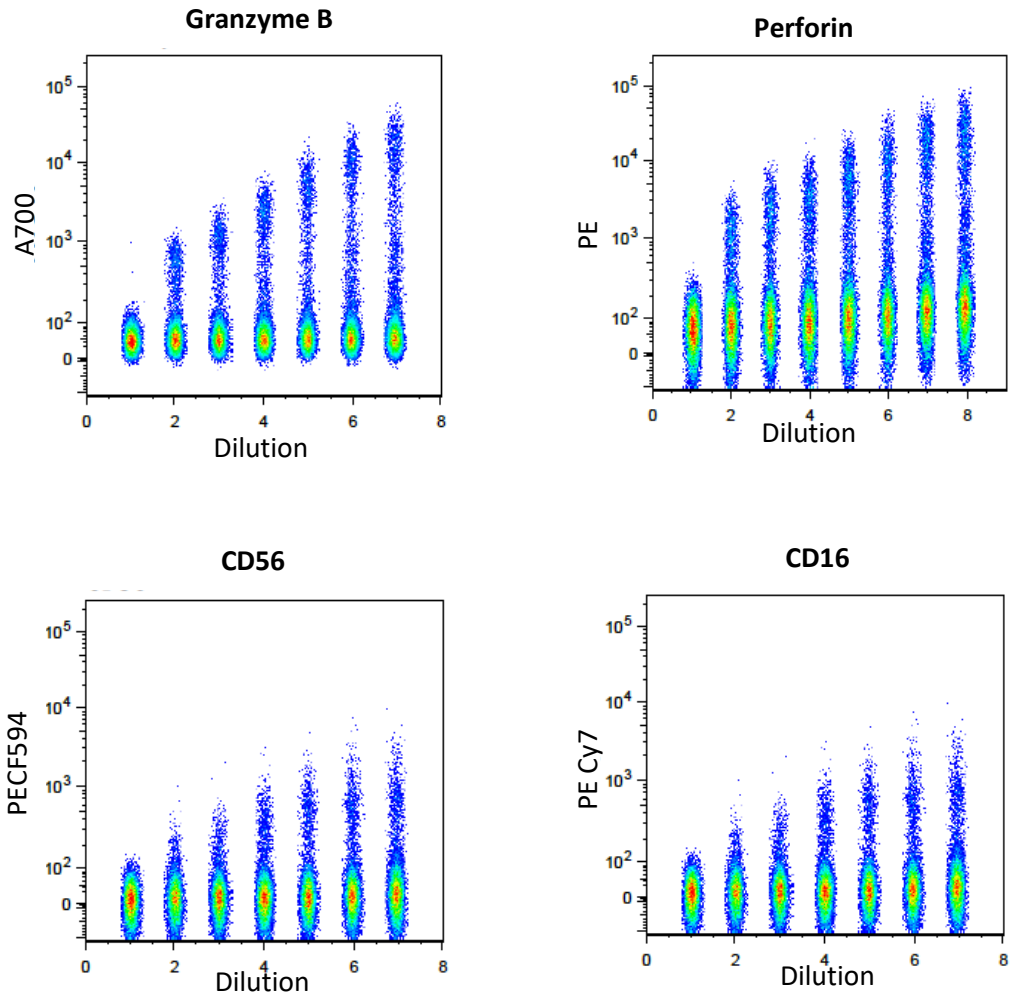


Figure 2.7: A representative of antibody dilution for flow cytometry staining

A representative of antibody dilution for flow cytometry staining starting with twice the manufacturers recommendation and a two-fold dilution thereafter. The dilution that yielded the maximum signal to noise ratio was adopted.

Once optimal antibody concentrations had been established, PBMCs from HIV-1 seronegative controls were stained with the optimized antibody panels, sequentially omitting one fluorochrome-conjugated antibody at a time to visualize the Fluorescence Minus One (FMO). FMOs were used to test how well the antibodies interacted in a panel, the quality of compensation and the position of the negative populations for each marker (Figure 2.8).

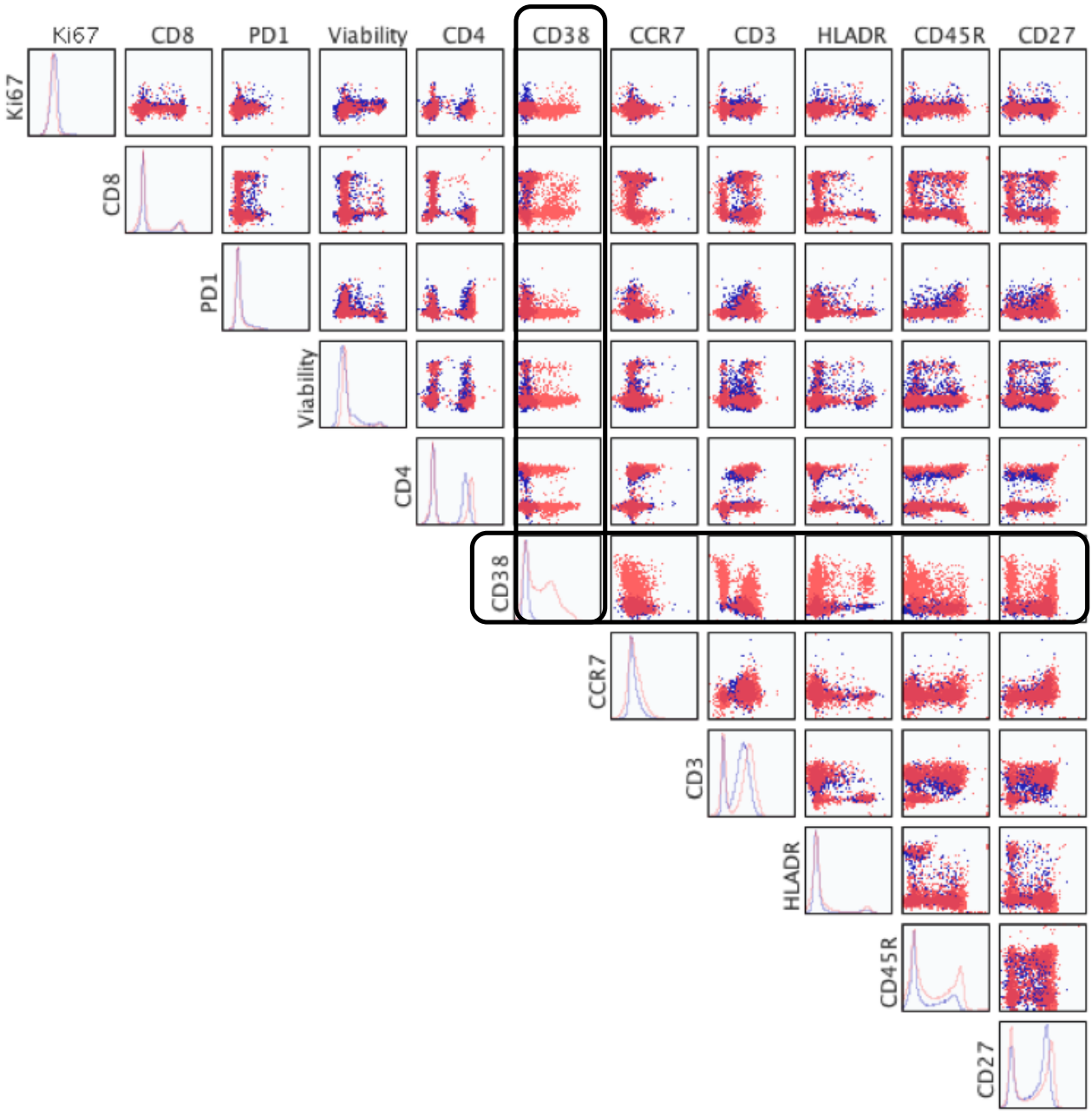


Figure 2.8: An example of fluorescence minus one staining.

N by N plot of the fluorescence minus CD38 APC (blue dots) overlaid on a sample that was fully stained with the T cell phenotyping panel (red dots). The lack of CD38 staining on the fluorescence minus CD38 plot is highlighted.

2.8.5. Flow cytometry staining protocol

All staining for acute samples was done in a biosafety level 3 laboratory room H.2.19 up until the fixation step while staining for the healthy controls was done in a biosafety level 2 laboratory room H.2.17.

10^6 PBMCs per well were transferred to a 96 well plate and washed once with 200 µL of Phosphate Buffered Saline (PBS) (Sigma-Aldrich) before staining for viability. Cells were stained with 100 µL per well of a 1/400 dilution of the viability dye (prepared by first diluting the stock dye 1:40 in deionized water, and thereafter 1:10 in PBS) and incubated for 20 minutes at room temperature in the dark.

CCR7 and CCR5 staining was performed in 50 µL of PBS (Sigma-Aldrich) for 10 minutes at 37^0C before the rest of surface antibodies were added in a staining cocktail and incubated for a further 15 minutes at room temperature in the dark. The cells were then washed once in PBS and fixed with Cytofix/Cytoperm (BD Biosciences) for 20 minutes at 4^0C, after which intracellular staining was performed for 20 minutes at room temperature, washed with Perm wash buffer and acquired on the BD Fortessa flow cytometer.

To correct for spectral overlap between fluorochromes, compensation controls for each antibody were prepared by staining anti mouse antibody BD™ Compensation Particles (BD, UK) and negative control particles (BD Biosciences) with the titrated antibody volumes, incubating at room temperature for 20 minutes and topping up with 200 µL of Permwash (BD, UK) for acquisition.

2.8.6. Flow cytometry analysis

The laser delay, area scaling factors and PMT voltages were optimized every time before samples were run using a protocol adapted from Perfetto *et al* (Perfetto et al. 2012). First,

Rainbow fluorescent particles (BD, UK) that are excited by any wavelength from 365-650nm were used to set the laser timing, synchronize the peak area and height signals and set the voltage to one that gave the maximum Median Fluorescence Intensity (MFI) within its linear range. Once the desired voltages had been obtained, the linearity of the emission spectra was checked using rainbow calibration particles (BD Biosciences) that are dyed to eight different fluorescent intensities and are excited at any wavelength between 365-650 nm.

At least 30,000 viable $CD3^+$ lymphocytes were acquired on the Fortessa flow cytometer using FACSDiva software (BD Biosciences). Analysis was done on FlowJo V10.1.1 (Treestar Inc, USA).

To gate for the different T-cell subsets, $CD3^+$ singlet viable lymphocytes were gated for CD4 and CD8 expression, and single gates for CD38, HLA-DR, CD27, CD45RO, CCR7, CD57, PD-1, Ki67, Granzyme B, Perforin and CD107a carried out. Boolean gates were then created for the populations of interest, and the expression profiles of the different cells analysed on GraphPad Prism V7 (San Diego, USA), Pestle V1.7 (Maryland, USA), Spice V6 (Maryland, USA) and JMP V14 (Marlow, UK). NK cells were defined as $CD3^-$ singlet viable lymphocytes that expressed CD16, CD56 or both. These were then gated for the expression of Granzyme B, Perforin, CD107a, NKG2A, NKG2C, NKp44, CCR7 and CD38.

Monocyte subsets were defined based on their expression of CD16 and CD14 while B cells were defined as $CD3^-$ $CD19^+$ lymphocytes and further characterized for expression of CD21, CD27, CD10, CD38, IgM, IgD, CD86 and PD-L1. The gating strategies and subset definitions will be further discussed in Chapter 5.

Table 2.9: Technical specifications of the special order Fortessa flow cytometer used.

Laser	Excitation (nm)	Power (MW)	Detector	Dichroic (LP)	Filter
Blue	488	100	PerCP Cy5.5	685	695/40
			FITC	505	530/30
Green	532	150	Pe-Cy7	735	780/60
			PerCP Cy5.5	685	695/40
			PE-Cy5	640	660/40
			PeTxRd	600	610/20
			PE		575/25
Red	633	100	APC Cy7	755	780/60
			A700	680	730/45
			APC	Empty	660/20
Violet	405	50	QD705	685	705/70
			QD655	635	660/20
			QD605	595	605/40
			QD565	550	560/40
			Amcyan	505	515/20
			Pacific Blue	Empty	440/40

2.9. Cytokine analysis

2.9.1. Cytokine analysis from culture supernatant

Multiplexed measurements of cytokines and soluble proteins from culture supernatants after PBMC stimulation with GAG peptides described in section 2.8.3 was done using the LEGENDplex human CD8/NK and antiviral kit (BioLegend, USA) according to the manufacturer's instructions.

In 96 well V-bottomed plates, 25µl of Assay Buffer, 25µl of mixed beads and 25µl of diluted standard control or test supernatant were added to each well and shaken at 800 rpm on a plate shaker for 2 hours at room temperature. The plates were washed with 200µl of washing buffer, 25µl of Detection Antibodies added to each well and incubated on a shaker for 1 hour at room

temperature. The plates were washed again with 200µl of washing buffer and resuspended in 150µl of 1X Wash Buffer for acquisition and analysis of PE and APC fluorescence on the BD Fortessa flow cytometer (Figure 2.9). Cytokine concentration per well was then calculated form the standard curve.

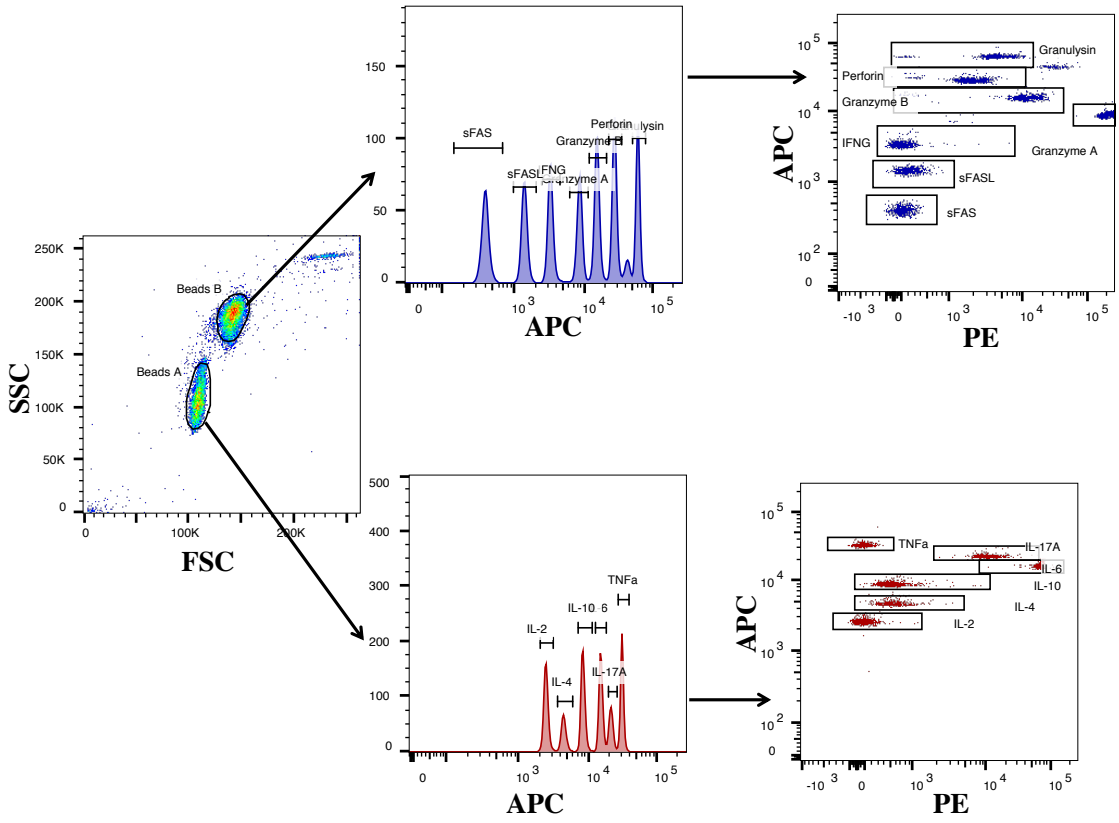


Figure 2.9: Gating strategy to quantify cytokines and soluble proteins in culture supernatant.

Gating strategy to quantify cytokines and soluble proteins in culture supernatant following peptide stimulation of PBMC. Beads were gate on forward and side scatter, followed by gating for analyte specific beads based on internal APC fluorescence, and subsequent analysis of the analytes of interest's PE fluorescence intensity.

This assay tested for concentrations of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IFN-α2, IFN-β, IFN-γ, IFN-λ1, IFN-λ2/3, TNF-α, IP-10, GM-CSF, soluble Fas, soluble FasL, granzyme A, granzyme B, perforin and granulysin in culture supernatant which were then correlated with expression of the degranulation marker CD107a in $CD4^+$ and $CD8^+$ T cells and NK cells.

2.9.2. Plasma cytokine levels

Plasma cytokine levels at acute infection were evaluated using V-Plex kits on the Meso Scale Discovery (MSD) platform (MSD, USA). Plasma samples were thawed on ice and diluted before plating 25 µL on to blocked capture antibody coated MSD plates and incubating for 2 hours on a shaker at room temperature. Eight standard curve dilutions were performed in duplicate on every plate. Plates were washed 3 times with the kit provided wash buffer, 25 µL of electro chemiluminescent labelled detection antibody added and incubated for 2 hours at room temperature with shaking. The plates were washed 3 times, read buffer added and plates read on an MDS reader within 10 minutes. The calculations to establish calibration curves and determine concentrations were carried out using the MSD discovery workbench analysis software (Meso Scale Diagnostics, USA).

Analytes tested included IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-12/IL-23p40, IL-13, IL-15, IL-16, IL-17A, IL-21, IL-22, IL-23, IL-27, IL-31, IFN-γ, TNF-α, TNF-β, GM-CSF, VEGF-A, Eotaxin-1, Eotaxin-3, IP-10, MCP-1, MCP-4, MDC, MIP-1α, MIP-1β, MIP-3α and TARC.

2.10. Cell death pathway profiling

An aliquot of donor and target cells from the cell-cell transmission experiments described in section 2.7.2 above was used to test the pathways involved in the depletion of $CD4^+$ T cells by viruses of different replicative capacities and cell-cell transmission characteristics.

Total RNA was extracted from freshly cultured cells using the Qiagen Allprep kit according to manufacturer's recommendations (Qiagen). Cells were pelleted and lysed in buffer RLT substituted with beta-mercaptoethanol (Sigma-Aldrich) and the lysate bound on to RNeasy spin columns, washed twice and eluted in 50µl of nuclease free water (Sigma-Aldrich).

cDNA was synthesized using the RT^2 first strand synthesis kit (Qiagen) starting with genomic DNA elimination by incubation of RNA with buffer GE at 42^OC for 5 minutes. Ten µL containing 5x buffer BC3, reverse transcriptase master mix, control P2 and RNase free water was then added and incubated at 42^OC for 15 minutes after which the reaction was stopped by incubating at 95^OC for 5 minutes.

To perform the RNA array PCR, 91 µL of RNase free water was added to each cDNA sample. For each 96 well plate, 102 µL of cDNA was mixed with 1350 µL of 2x RT^2 SYBR Green Mastermix (Qiagen) and 1248 µL of RNase free water, then 25 µL transferred per well and tightly sealed with optical adhesive tape. Real time PCR was performed using a Roche LightCycler 480 for 10 minutes at 95^OC followed by 45 cycles of 15 sec at 95^Oc and 1 minute at 60^oC. Threshold cycle C_T and dissolution (melting) curves were calculated for each well representing a unique analyte. Analysis was performed using the SABiosciences PCR array data web-based analysis using uninfected cells as baseline.

2.11. Statistical Analysis

Statistical analysis and graphical presentation were performed using GraphPad Prism version 7.0 software (GraphPad Software) and JMP statistical software version 14 (SAS, USA). Statistical analysis was performed using the Mann-Whitney U test for nonparametric unpaired observations or with the Kruskal-Wallis test when multiple sets of nonparametric unpaired observations were compared. The Wilcoxon matched pairs test was used to compare statistical difference between two sets of paired nonparametric data.

All bivariate continuous correlations were performed using standard linear regression. One-way comparison of means was performed using Student's t test, and one-tailed P values are reported. Kaplan–Meier survival curves and Cox proportional hazards models were performed using an endpoint defined as a single $CD4^+$ T-cell count reading less than 400, unless otherwise specified, and statistics reported for survival analyses were generated from the log-rank test. A generalized linear model as applied in IMB SPSS was used to test for the independent contribution of each parameter when multiple parameters had been identified to influence the rate of CD4+ T cell decline.

Where adjusted p values are shown, they have been corrected for the false discovery rate using the Benjamini-Hochberg technique which is less stringent than the Benferroni technique as applied in JMP software version 14. In the Benjamini-Hochberg technique, instead of controlling for whether any differences were falsely declared significant, the criteria are changed to controlling for the rate at which declarations of significance were false (Benjamini 1995). Therefore, p-values are sorted from high to low. For the highest p-value, the false discovery rate rate of 0.05 is used. For each next highest p-value, the criterion shrinks a little, subtracting 0.05/m, forming a ramp. At the bottom of the ramp is the criterion for the smallest p-value, which is 0.05/m, the Bonferroni criterion. Any p-values that fall under that ramp are

declared significant, and Benjamini-Hochberg proved that for independent tests, this method could correct for the false discovery rate to yield adjusted p values.

Principal component analysis was performed using the JMP version 11 statistical package. Missing values were imputed using multiple linear regression models, and individuals for whom more than three parameters were missing were excluded.

P values of <0.05 were considered to be of statistical significance.

Chapter 3: Genotypic characteristics of HIV-1 transmitted/founder virus in Men who have sex with Men (MSM)

3 Genotypic characteristics of HIV-1 transmitted/founder virus in Men who have sex with Men (MSM)

3.1 Background and aims

The early interaction between HIV-1 and the human host is a complex and dynamic environment that sets the stage for disease trajectory. The outcome of this early interaction is shaped by both the virus characteristics and multiple host factors which when favourably aligned result in initial control of viremia and preservation of $CD4^+$ T cell counts, albeit to different levels in different patients. However, variations in disease progression have been reported despite the presence of favourable host genetics including HLA types and protective host genetics that are associated with viral control, underscoring the importance of viral characteristics in influencing disease trajectory (Cohen et al. 2011; Haase 2011; O'Brien 1998; Payne, Branch, et al. 2014; Yue et al. 2015).

HIV-1 transmission is associated with a severe bottleneck in which a very limited number of HIV-1 variants from genetically diverse quasi-species in the transmitting partner establish infection (Tully et al. 2016; Oberle et al. 2016; Carlson et al. 2014; Salazar-Gonzalez et al. 2008; Salazar-Gonzalez et al. 2009). Founder viruses are likely to present characteristics that favour onward transmission, which are rarely similar to those that are associated with adaptation to the chronic transmitting patient's immune defences (Ashokkumar et al. 2018; Parrish et al. 2013; Fenton-May et al. 2013; Salazar-Gonzalez et al. 2009; Keele et al. 2008). An accurate characterization of transmitted/founder virus characteristics is crucial to understanding of the role of virus characteristics in HIV-1 pathogenesis and shaping disease progression, and in identifying target immunogens and novel vaccine strategies.

While most studies agree that transmission selects for ancestral variants with more consensus like amino acids residues and less glycosylated envelopes that are sensitive to antibody

neutralization and use CCR5 to gain entry into cells (Parrish et al. 2013; Parrish et al. 2012; Salazar-Gonzalez et al. 2009; Keele et al. 2008; Carlson et al. 2014), some characteristics such as increased replicative capacity and resistance to type 1 interferons are only observed in some cohorts but not others (Fenton-May et al. 2013; Deymier et al. 2015; Iyer et al. 2017; Ochsenbauer et al. 2012), partially owing to differences in cohorts and experimental methodologies used. Whereas some cohorts draw their conclusion from comparing variants present near the time of transmission to those that emerge in chronic infection, others draw their comparisons from non-transmitted variants present near the time of transmission in epidemiologically-linked transmission pairs.

Additionally, most reports study single genes. For instance, the fast disease progression attributed to subtype C compared to subtype A and D is derived from studies of partial sequences of the *pol* or *env* genes but unique recombinant forms of HIV-1 exist and often have unique breakpoints that cannot be inferred from partial sequencing (Sanders et al. 2017; Kiguoya et al. 2017; Amornkul et al. 2013; Kaleebu et al. 2001).

Moreover, the role of the route of transmission in susceptibility to HIV-1 infection has been previously studied and some routes shown to be associated with increased odds of transmission and increased likelihood of multivariant infection (Sanders et al. 2013; Dosekun and Fox 2010; Sagar, Kirkegaard, et al. 2004; Grobler et al. 2004). However, few studies have described a role for multi-variant infection in HIV-1 pathogenesis or disease progression. It is not known whether variants transmitted in multivariant infection have similar characteristics or are a result of a less stringent transmission bottleneck that allows any reasonably fit virus to establish infection. An accurate description of transmitted/founder virus traits is paramount to understanding the role of the founder virus in HIV-1 pathogenesis and in the design of intervention strategies.

Our cohort of longitudinally followed MSM was one of the earliest MSM cohorts to be identified in East Africa and presents a unique opportunity to characterize transmitted/founder viruses in a group where disease outcome is known (Sanders et al. 2017; Amornkul et al. 2013; Powers et al. 2018).

We hypothesize that transmitted/founder viruses that are associated with faster disease progression have unique and identifiable genotypic characteristics that allow for their successful transmission. However, as we do not have access to the transmitting partners of these MSM, comparisons will be drawn from published literature. Thus, investigations described in this chapter examine selected genotypic characteristics of the virus populations that establish HIV-1 infection including the number of transmitted/founder variants, accurate subtype, enrichment for APOBEC induced hypermutation, HLA class I preadaptation and transmitted drug resistance mutations

The specific aims of this chapter are:

1. To identify the near full-length genotype of the transmitted founder HIV-1 through single genome PCR close to the transmission event.
2. To assess the transmission bottleneck in this MSM cohort compared to published heterosexual cohorts.
3. To investigate the relationship between the HIV-1 transmission bottleneck and disease progression.

3.2 Results

3.2.1 Cohort characteristics

Thirty-eight patients met the eligibility criteria in the KEMRI MSM group while thirty-seven were included in the ZEHRP discordant couple group. All subjects were within Fiebig stage I/II at screening having detectable HIV-1 RNA in plasma, with or without a positive p24 antigen test. The median duration after the estimated date of infection for the KEMRI and ZEHRP groups was 21 days (range 12-59 days) and 46 days (range 19-60 days) respectively. All subjects were treatment naïve for the duration of study. Transmitted/founder virus sequencing from patients in the ZEHRP study group had already been performed by Professor Eric Hunter's lab at Emory University, and the genotypic characteristics described in this chapter will therefore focus on MSM patients from the KEMRI cohort using similar methodologies. A summary of key demographic and clinical data for the two cohorts is shown in Table 3.1.

Table 3.1: Key demographic data of study participants.

	KEMRI	**ZEHRP**
Risk group	Men who have sex with men (MSM)	Discordant couples
Gender composition: Male	38/38 (100%)	18/37 (49%)
Female	0/38 (0%)	19/37 (51%)
Median days post infection	21 (12-59)	46 (19-60)
Median viral load at first detection (copies/ml plasma)	433,000 (5160-19840000)	44,500 (424-1922000)
Median absolute CD4 T cell count at first detection (copies/mm^3 blood)	472 (186-824)	486 (258-984)
Median set point viral load (copies/ml plasma)	55,639 (67-269,195)	41,365 (365-445,685)

3.2.2 Identification of transmitted/founder virus

Using the earliest available plasma samples, amplification of near full-length (~9kb) or two overlapping (~5kb) HIV-1 genomes from thirty-eight men who have sex with men (MSM) from the KEMRI cohort was done using methods that have been previously described (Salazar-Gonzalez et al. 2008; Prince et al. 2012; Deymier et al. 2014).

A total of 120 near full-length genome amplicons that represent 91 unique sequences, and 537 half genome amplicons representing 415 unique sequences were generated. The near full-length HIV-1 genome contained all the HIV-1 genes but with the 5' LTR lacking the U3 and R region, and the 3' LTR lacking the U5 region. Because genomic viral RNA is flanked by the R region of the LTR, only partial LTR sequences and near-complete viral genomes could be obtained. Specifically, the internal nested 5'primer corresponds to the first 30 nucleotides of U5, and the internal nested 3'primer corresponds to the last 30 nucleotides of the 3'R region, thus rendering these two short stretches of 30 nucleotides primer derived rather than authentic virus sequences. Although the 5' and 3' half genomes overlapped by the entire *vif* gene, they were treated as independent half genomes and not assembled into near full-length genomes due to the extreme homogeneity within *vif* sequences early in infection. Figure 3.1 shows ~9kb and ~5kb HIV-1 genomes resolved on 1% agarose gel.

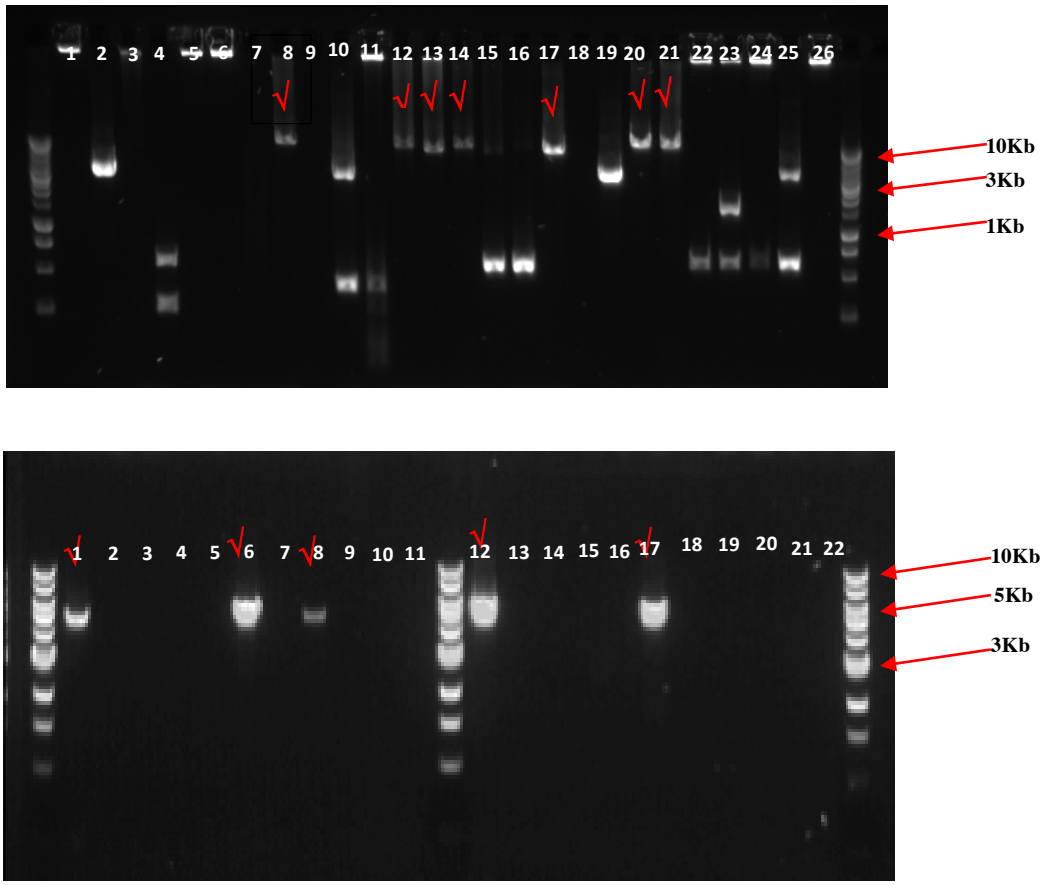


Figure 3.1: Gel images showing PCR product from single genome amplification.

~9kb near full length (above) and ~5kb half genome (below) single genome PCR product.

Amplicons of required band size are shown in red ticks.

The sequences were examined by using neighbour-joining (NJ) phylogenetic tree methods together with a novel sequence visualization tool (LANLHighlighter) which provides a visually informative representation comparing each sequence to a selected reference sequence and highlights individual nucleotide polymorphisms. There was remarkable within-patient homogeneity of sequences, with most genomes differing by less than 10 nucleotides across the entire ~9kb HIV-1 genome. The median number of sequences analysed per patient was 15

(range, 6–42) with the within-patient diversity ranging from 0.05% to 0.3%. In all cases, mutations were spread across the whole genome with little evidence of selection pressure and the sequence that was least mutated and over represented was presumed the founder virus sequence. An example is shown in Figure 3.2.

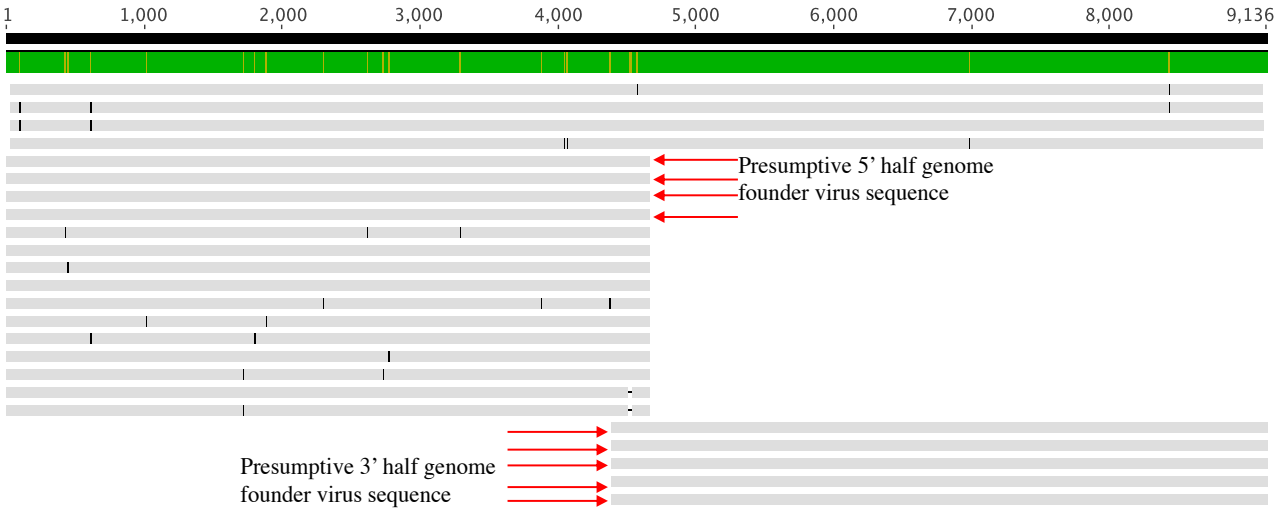


Figure 3.2: Alignment of near full-length and half genome sequences.

Near full-length and half genome sequences obtained from the same patient. Red arrows point to sequences that are identical to the consensus sequence and presumed to represent the founder virus sequence. Grey areas represent sequence regions that are identical to that patient's consensus sequences while black tick marks denote nucleotides that are different from the consensus sequence. Shown in green at the top is coverage on the consensus sequence.

For each subject, the frequency distribution of all intersequence Hamming distances (defined as the number of base positions at which two genomes differ) was determined including whether or not the observed sequences evolved under a star-phylogeny model (i.e. all evolving sequences are equally likely, and all coalesce at the founder). Three subjects had an overall

enrichment for G-to-A hypermutation patterns with APOBEC3G/F signatures. In all three of these cases, the evidence for hypermutation was only evident across a subset of sequences.

3.2.3 Multiplicity of infection

HIV-1 transmission is accompanied by a genetic bottleneck characterized by the transmission of only one variant in a majority of transmissions. A multi-step approach for resolving the multiplicity of transmission variants by combining previously published methods was applied (Shirreff et al. 2013; Abrahams et al. 2009; Keele et al. 2008; Shaw and Hunter 2012).

First, the presence and number of phylogenetically distinct clusters of recipient sequences arising from distinct source variants was inferred using maximum likelihood (ML) trees to categorize distinct sets of identical or nearly identical sequences within the same patient with bootstrap resampling with 1000 replicas to assess the robustness of the phylogenetic trees. Next, the distribution of pairwise viral diversity and highlighter plots was analysed. Given previous work demonstrating an intra-host evolutionary rate of 0.015 (range 0.0017–0.05) substitutions/site/year (Alizon and Fraser 2013), a maximum diversity cut-off of twice the maximum measured evolutionary rate (>0.10 substitutions/site) to conservatively identify individuals with multiple founders was chosen.

Two measures of diversity were used: a categorical measure that distinguishes between subjects with a single founder variant or multiple founder variants and a continuous measure of diversity which corresponds to the mean pairwise diversity among sequences from a subject. An infection was classified as occurring from a single founder when all HIV-1 sequences from the recipient formed a monophyletic clade with boot-strap support >70 percent. In all cases, clones from each patient clustered together and separately from any clones from other patients (Figure 3.3).

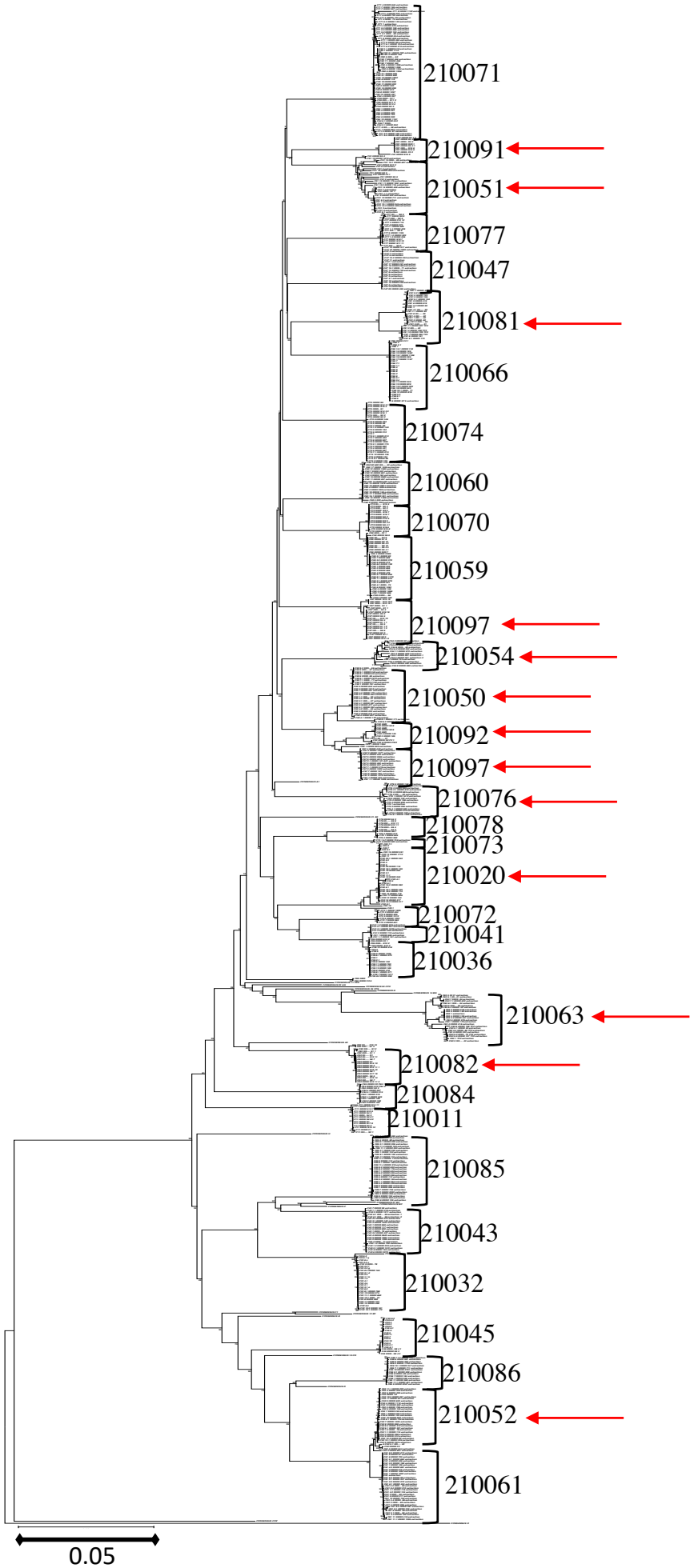

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Figure 3.3: 5' half genomes derived from 33 patients.

Unique reads from the different genomes were then stratified and phylogenetic analysis done by maximum likelihood parsimony. Intra-patient sequences were homogeneous and clustered in distinct monophyletic branches separate from other patients. Using this approach, we could identify patients who were infected with more than one founder variant denoted with red arrows.

Using this approach, 39% (15 out of 38) of these MSM volunteers were infected with multiple founder viruses (2-12 founder viruses), much higher than has been described for heterosexual transmission. Each lineage contained a unique set of identical or near identical sequences suggesting that these subjects were infected by very closely related viruses, most likely from one partner as opposed to a single virus that evolved into two distinct lineages in the brief period preceding peak viremia, or superinfection. Multivariant transmission was evident by the phylogenetic tree topology (Figure 3.3), the distribution of pairwise distances (Figure 3.4) and on highlighter plot alignments (Figure 3.5 C and D). Single variant infection was characterised by homogeneity of all intra-patient sequences with only a few unique differences at the nucleotide level (Figure 3.5 A and B).

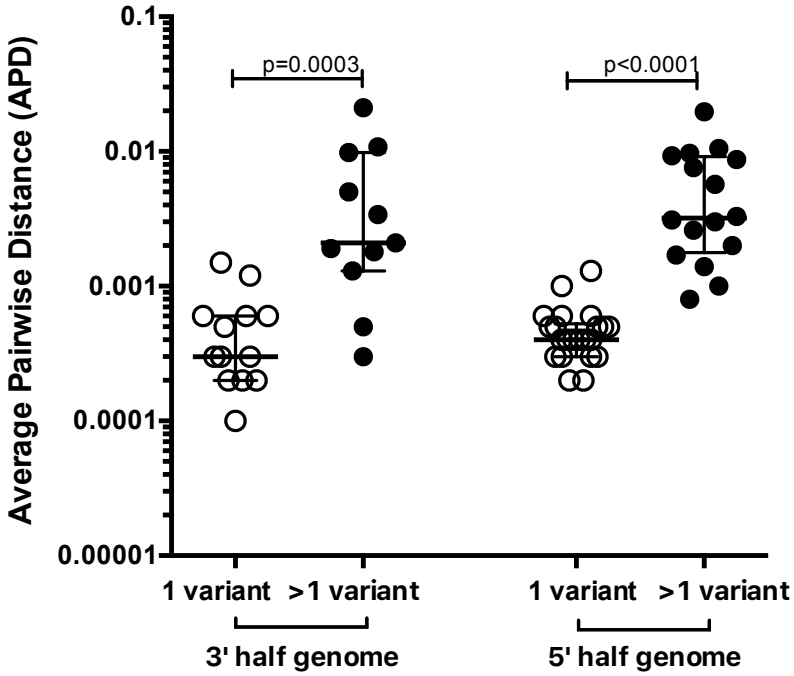


Figure 3.4: A comparison of pairwise distance in patients infected with a single vs multiple transmitted/founder virus on both the three prime and five prime half genome. Pairwise distances were higher in patients infected with multiple founder viruses ($p<0.0001$, student's *t* test)

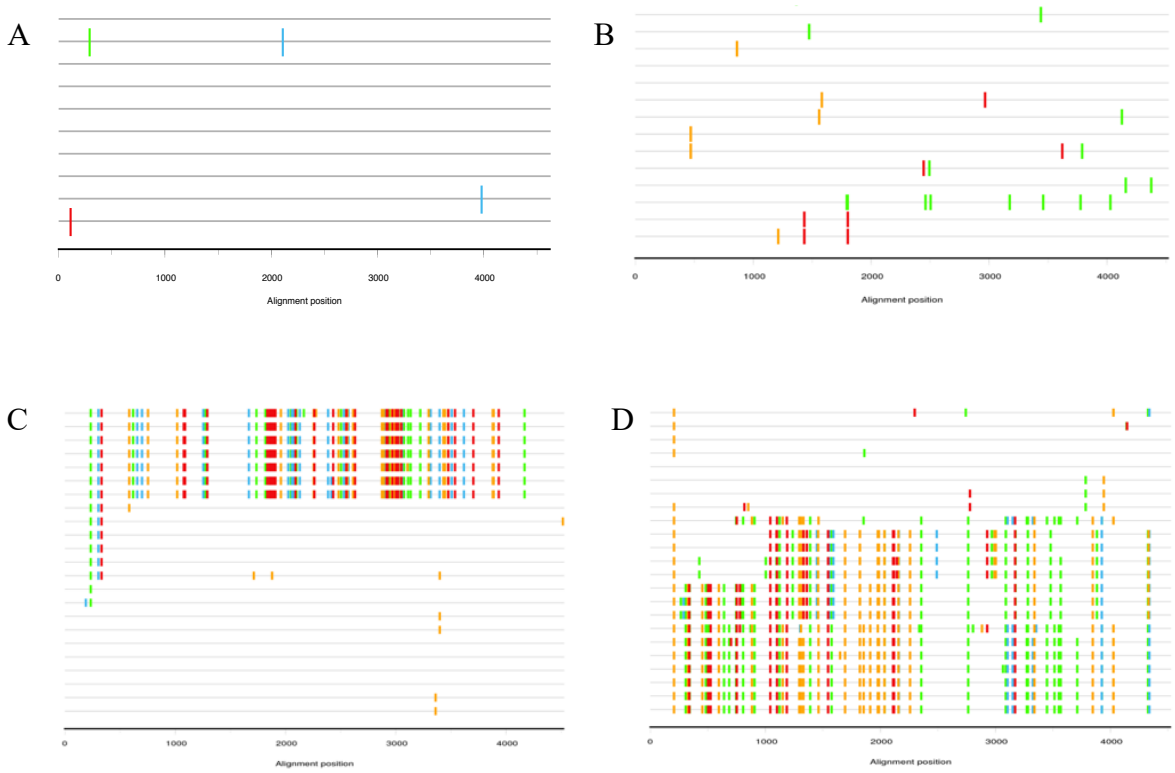


Figure 3.5: Highlighter plots showing heterogeneity of founder viruses.

Infection with single homogenous (A), single heterogenous (B), multiple homogenous (C) and multiple heterogenous (D) HIV-1 populations. Each alignment shows sequences derived from the same patient. Figures represent 5 prime half genomes as representatives. In cases of multivariant infection, there was evidence of recombination between the two infecting variants.

In the infections in which a multiple founder variant was inferred, the proportional representation of this variant within that patient's sampled HIV-1 population was examined by estimating the relative frequencies of each variant and ranking them based on proportional representation within the viral population. Variants were conservatively defined as minority variants when they represented <10 percent of the observed viral population. The proportions of T/F viruses in each individual were highly variable, with major T/F viruses as high as 96% and minor T/F viruses as low as 3% (Figure 3.6). The highly unequal proportions of T/F viruses

at acute infection suggest different replication advantages among T/F viruses in the same HIV-1-infected individuals.

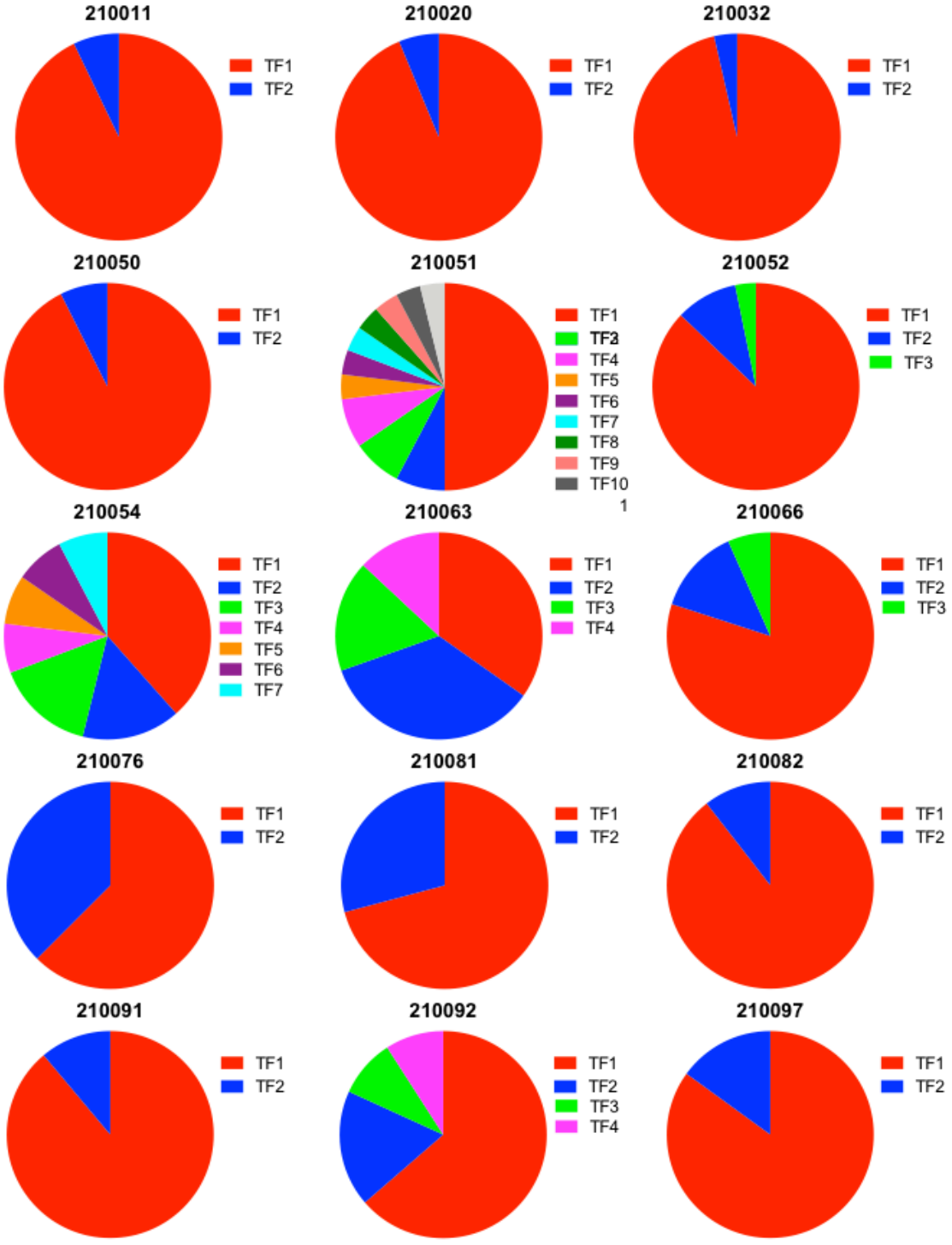


Figure 3.6: Proportion representation of multiple transmitted/founder viruses.

In all cases, there was unequal representation of variants, with major variants representing as much as 96% of the virus populations and minor variants representing as low as 3% of the virus population sampled.

Next an investigation of whether infection with multiple founder variants resulted in different disease outcome using CD4 T cell count and viral load measurements as a measure of disease outcome was done. Infection with multiple founder viruses resulted in lower CD4 T cell counts in the first 5 years of infection (Figure 3.7 A), with patients infected with multiple founder viruses reaching CD4 T cell counts under 400 cells/mm^3 faster than those infected with a single variant (Figure 3.7 B).

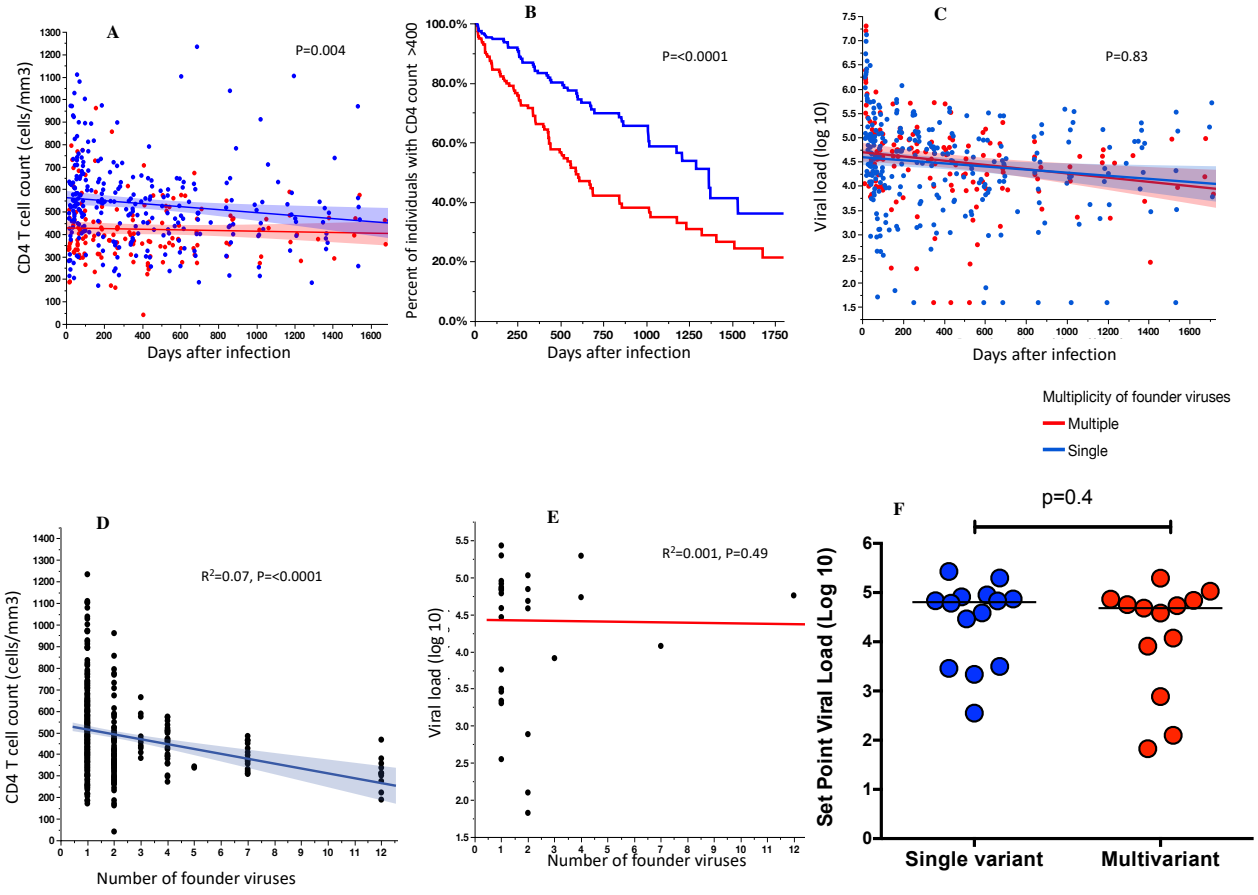


Figure 3.7: Trends on disease progression based on multiplicity of infection.

A comparison of CD4 T-cell count, viral load and viral load set point between patients infected with one founder virus (blue) and those infected with multiple founder viruses (red) within the first five years of infection. Patients infected with multiple founder variants had lower CD4 counts (A, B and D) but no differences were observed in viral load over time (C) or set point viral load (E and F).

There was an inverse correlation between the number of founder virus variants and CD4 T cell counts at all time points (Figure 3.7 D) but no difference in longitudinal viral load measurements over time except when the analysis was limited to those VL measurements taken within the first month of diagnosis, and viral load set points were similar regardless of the number of infecting founder virus variants (Figure 3.7 C and E). These results suggest that greater heterogeneity in the HIV-1 founder population of recently infected individuals is associated with a worse disease outcome in a manner that is independent of antigen load in blood within the timepoints tested and further provides evidence that pathogenic events at acute HIV-1 infection disproportionately contribute to disease progression even after control of viremia.

3.2.4 Virus subtypes based on near full-length HIV-1 genomes

Understanding of the differences in the clinical outcome of infection with the different HIV-1 subtypes relies upon accurate classification of the infecting virus. All sequences were analysed for evidence of inter-subtype recombination by the recombination identification program (RIP3.0) and the REGA HIV-1 subtyping tool (StanfordREGA). Inter-subtype recombinant viruses were confirmed by boot-scanning analysis using SimPlot v3.5 and the RDP3 tool.

A HIV-1 sequence was considered a recombinant if the recombination signal was supported by at least two methods. Figure 3.8 shows an example of recombination analysis for a pure and

recombinant sequence respectively. Similar analyses were performed for all thirty-eight subjects.

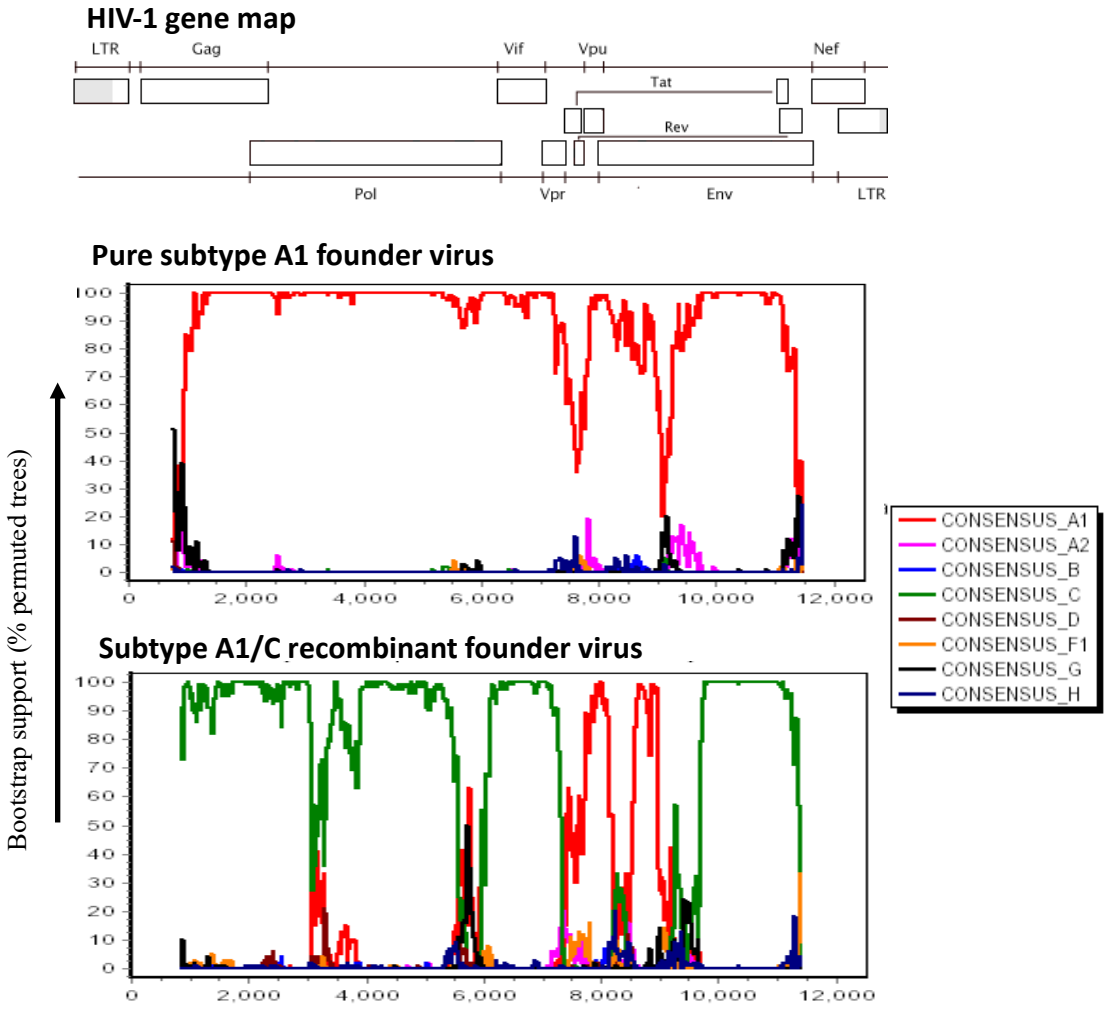


Figure 3.8: Recombination analysis plot for a pure and recombinant sequence. Regions were considered recombinant if there was clear distinction in windows above 100 nucleotide supported by bootstrap values above 70%.

60.5% of the patients were infected with the following pure subtypes: 50% (19 out of 38) subtype A, 2.6% (1 out of 38) subtype C, 5.2% (2 out of 38) subtype D and 2.6% (1 out of 38) subtype G. The 39.5% (15 out of 38) who were infected by recombinant viruses represented

the following intra-subtype recombinant viruses: A1/A2, A1/A2/D, A1/B, A1/C, A1/D, AI/G, C/F, and D/G. Subtype A, which was the most frequently occurring pure subtype, was also the most commonly represented subtype among the recombinant viruses, with 13 out of 15 (87%) of the recombinant viruses having one or more genes that were of subtype A. The most frequently occurring recombination was of type A1 and D (5 out of 17, i.e. 29% of the recombinant viruses). In all cases, all sequences emanating from the same patient were of the same subtype. Traditional subtyping utilizes the *pol* gene. In these patients, subtyping based on the *pol* region alone underestimates the frequency of circulating recombinants significantly (Figure 3.9).

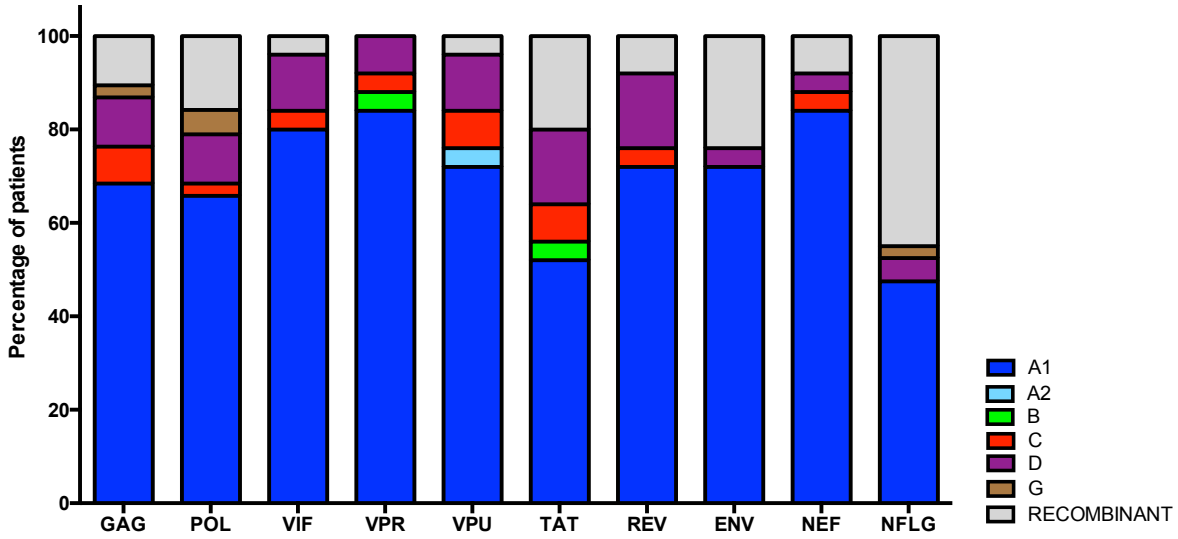


Figure 3.9: Subtype distribution patterns in all 9 HIV-1 genes.

Subtype classification by partial gene sequencing was inaccurate at determining subtype. No gene was identified that if sequenced could accurately depicts the near full-length genome.

We hypothesized that recombination was a random event in which the larger genes were more likely to recombine. The viral quasispecies from the subjects with evidence for inter-subtype recombination were therefore analysed for the distribution of recombination breakpoints in all

the genes (Figure 3.10). Based on the distribution of recombinant breakpoints there were four regions in which recombination breakpoints were enriched. These were within *gag* and *pol* (5/11), within p*ol* (6/11), within *tat* (5/11) and within *tat* and *env* (5/11) (Figure 3.10). In all sequences that were analysed, one or more recombination breakpoints occurred in one of these four regions. The frequency of recombination correlated closely with gene size except in the *tat* gene in which recombination was 5-fold what would be expected for its gene size if recombination was a random event (Figure 3.11). *Tat* is therefore likely a HIV-1 recombination hotspot.

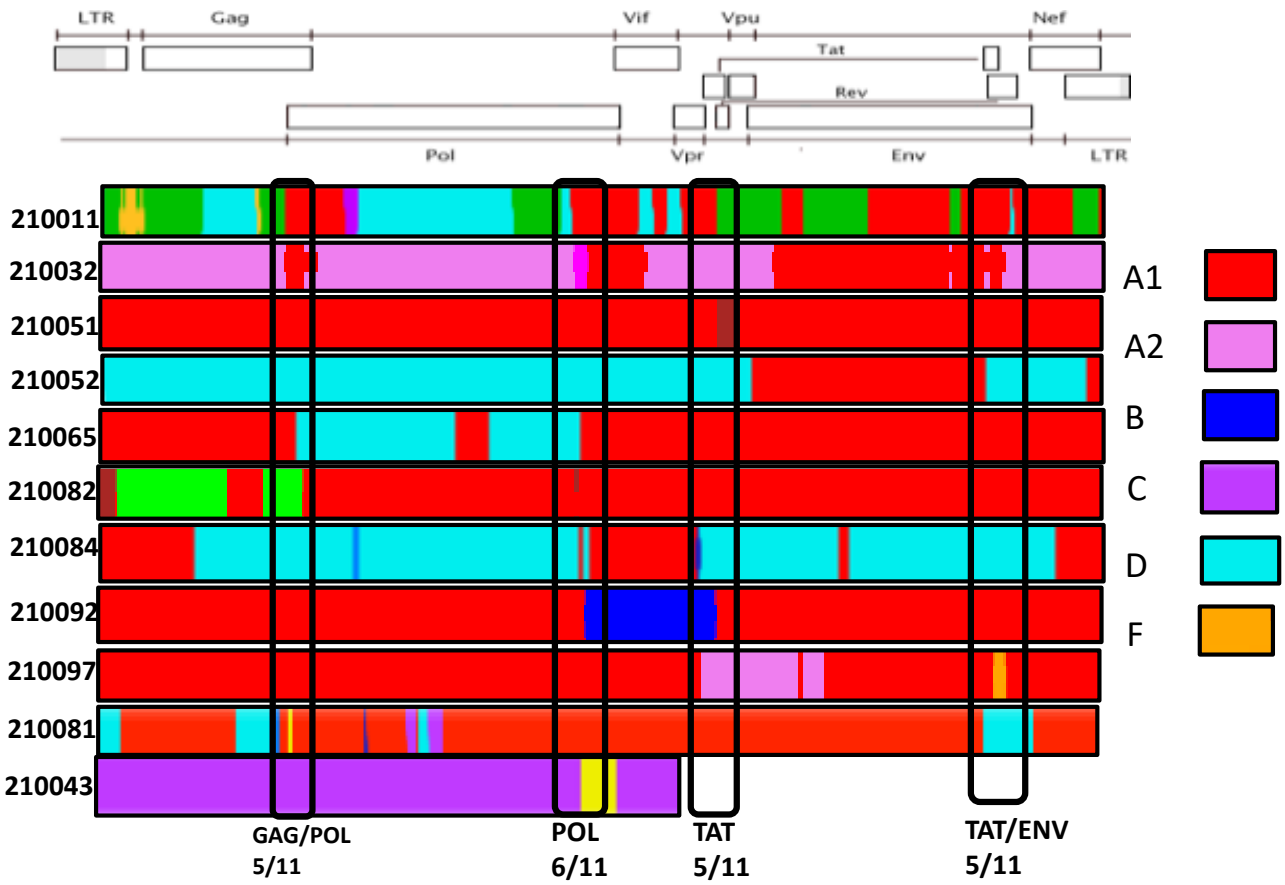


Figure 3.10: Recombination plots showing the recombination pattern of near full length or half genomes in the patients in whose inter-subtype recombination was detected.
In all sequences that were analysed, one or more recombination breakpoints occurred in one of these four regions - *gag* and *pol* (5/11 sequences), within p*ol* (6/11 sequences), within *tat* (5/11 sequences) and within *tat* and *env* (5/11 sequences).

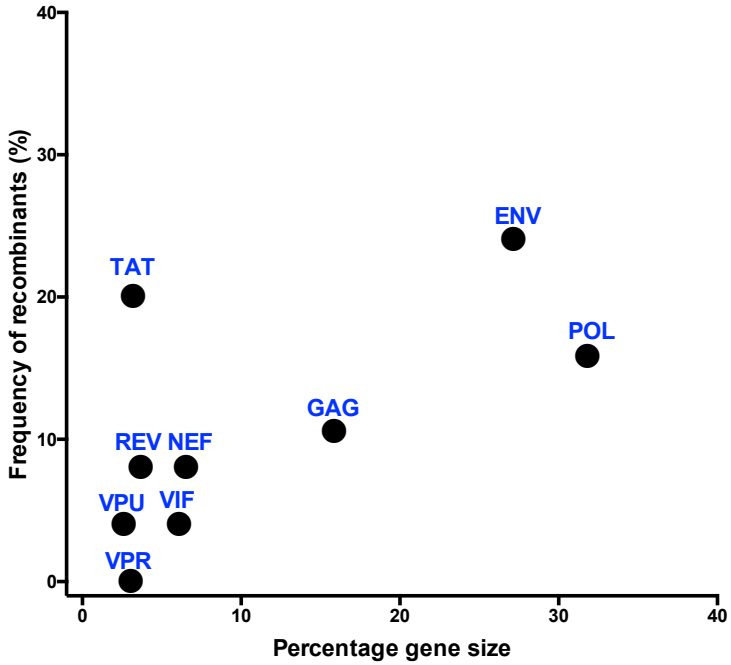


Figure 3.11: Correlation of frequency of HIV-1 recombination in each gene with gene size. The frequency of recombination correlated closely with gene size except in the *tat* gene in which recombination was 5-fold what would be expected for its gene size if recombination was a random event.

There is evidence that different HIV-1 subtypes differ in pathogenicity (Amornkul et al. 2013; Baeten et al. 2007; Chopera et al. 2012; Easterbrook et al. 2010). However, most studies are based on partial HIV-1 subtyping using partial or complete *pol* or *env* genes. A more accurate

characterisation based on multiple genes is therefore required. Patients infected with pure subtype D viruses lost their CD4 T cells fastest (p=0.0014, Log rank) but surprisingly also had the lowest plasma viral loads over time. Recombination did not offer any protection from CD4 T cell decline even though patients infected with recombinant viruses had lower plasma viral loads (Figure 3.12).

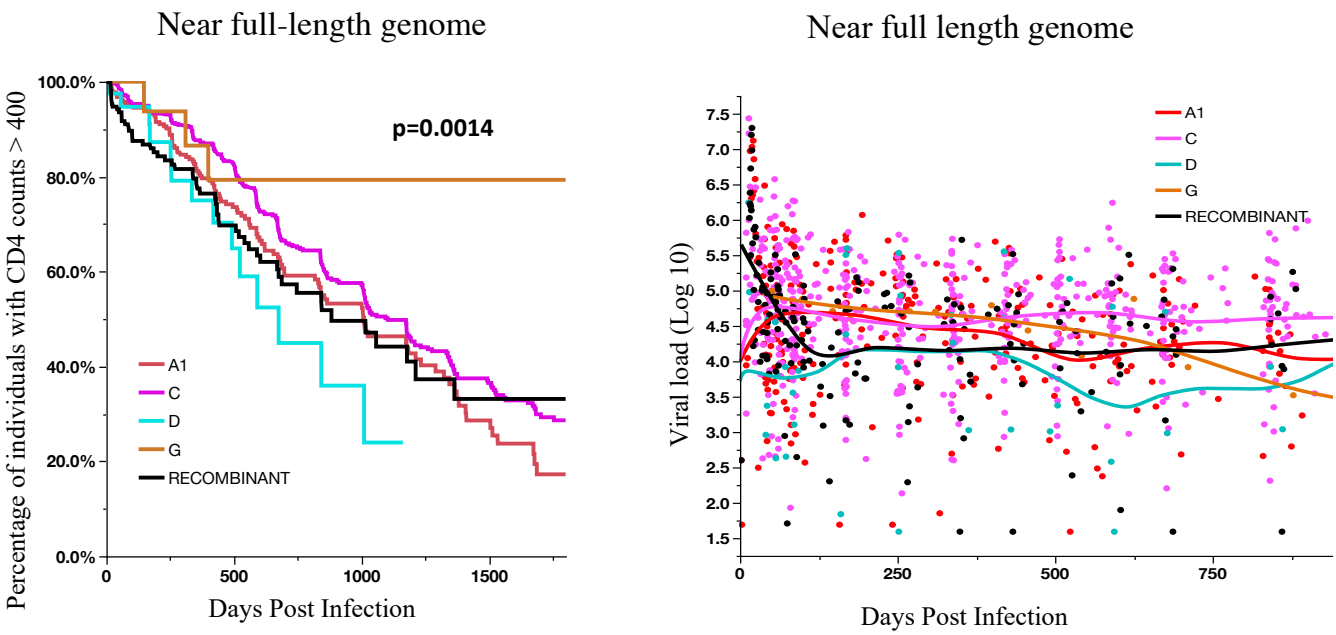


Figure 3.12: CD4 T cell decline (left) and plasma viral load (right) in patients infected with different HIV-1 subtypes and recombinant forms.

Patients infected with pure subtype D viruses lost their CD4 T cells fastest (p=0.0014, Log rank). Recombination did not offer any protection from CD4 T cell decline even though patients infected with recombinant viruses had lower plasma viral loads.

It is not well known whether recombination within the different genes of HIV-1 contributes equally to disease outcome. Thus, trends in plasma viral loads and CD4 T cell decline in patients who were infected with unique recombinant forms, compared to those infected with pure subtypes in each gene were investigated. However, apart from subtype A, there weren't enough patients per pure subtype to make statistical inferences and the trends observed should be interpreted with caution.

Recombination offered protection from CD4 T cell decline in *tat, vpu, env, rev* and *nef* and had the opposite effect in *pol* and *vif* (Figure 3.13). Similarly, lower plasma viral loads were observed in recombinant *tat, vpu, env* and *rev* but surprisingly not in *nef*, and higher viral loads in recombinant *gag* and *vif* (Figure 3.14). Overall, recombination was advantageous to the patient except in *vif*, *pol* and *gag* where it was associated with faster CD4 T cell decline and/or high viral loads.

Amongst the pure subtypes within each gene, we were unable to make any conclusion on the effect of subtype A2, B, C, D and G genes as there were very few patients whose founder virus possessed these subtypes in their pure form within a whole gene. However, in 4 genes (*gag, pol, vpu* and *env*), subtype A was associated with the fastest decline of CD4 T cells but preservation of CD4 T cells was observed in *vpr* (Figure 3.13). Within *vpr* and *vif,* the lowest viral loads were observed in subtype A whereas subtype A was associated with highest viral load in *vpu* and *env* (Figure 3.14).

However, it should be noted that there were very few patients with subtype B, C or G in any one gene as these are extremely rare in the sample geographic location, and statistical analysis to compare these subtypes to subtype A, D or recombinants was therefore not performed.

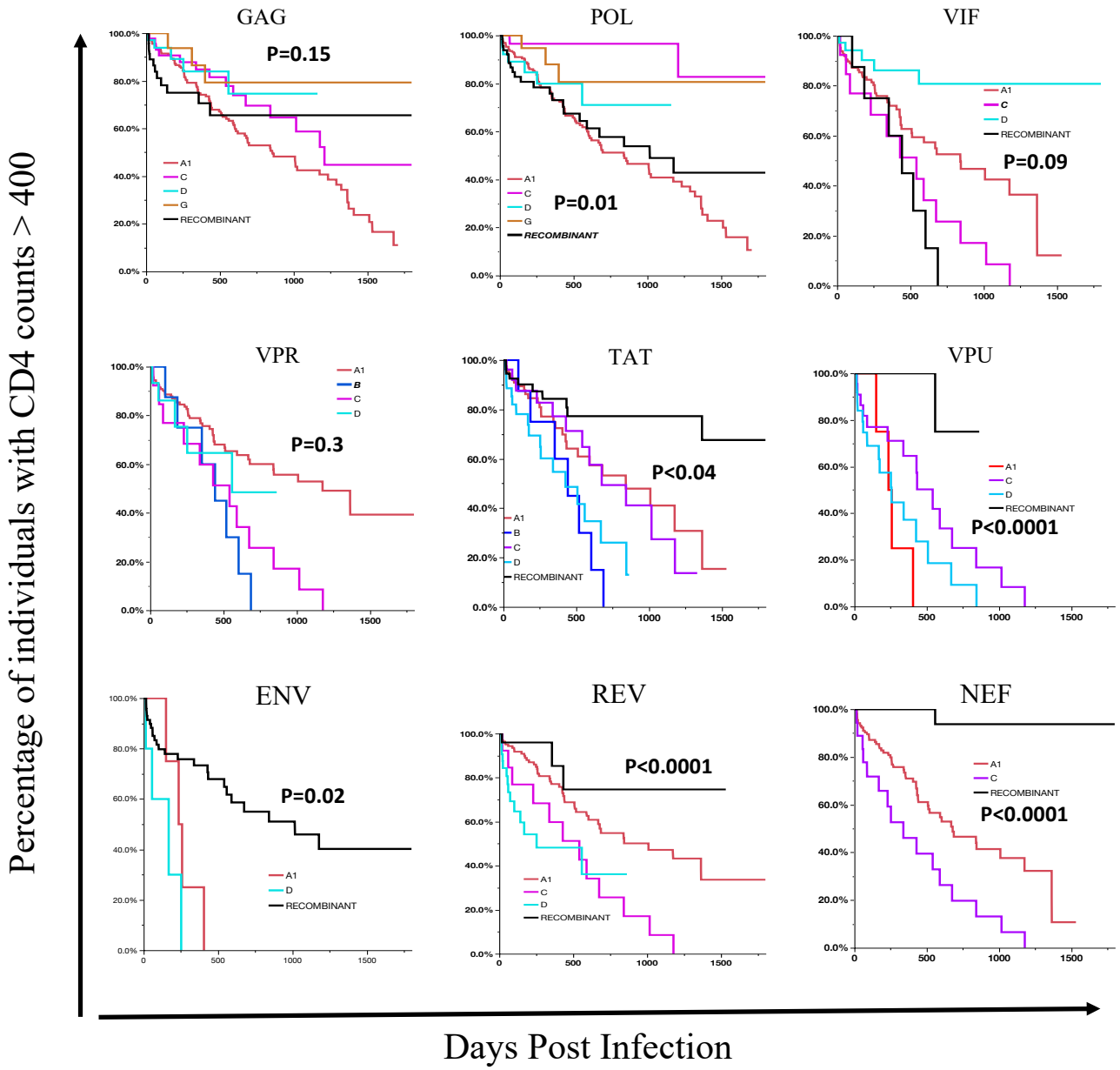


Figure 3.13: Trends in CD4 T cell decline in patients infected with different subtypes. Recombination was associated with preservation of CD4 T cell count when it occurred in *nef, rev, env, vpu and tat* but not in *gag, pol* or *vif*. There was no recombination within *vpr*. Associations are derived from Wilcoxon log rank tests with an endpoint of CD4 counts >400 cells/mm^3.

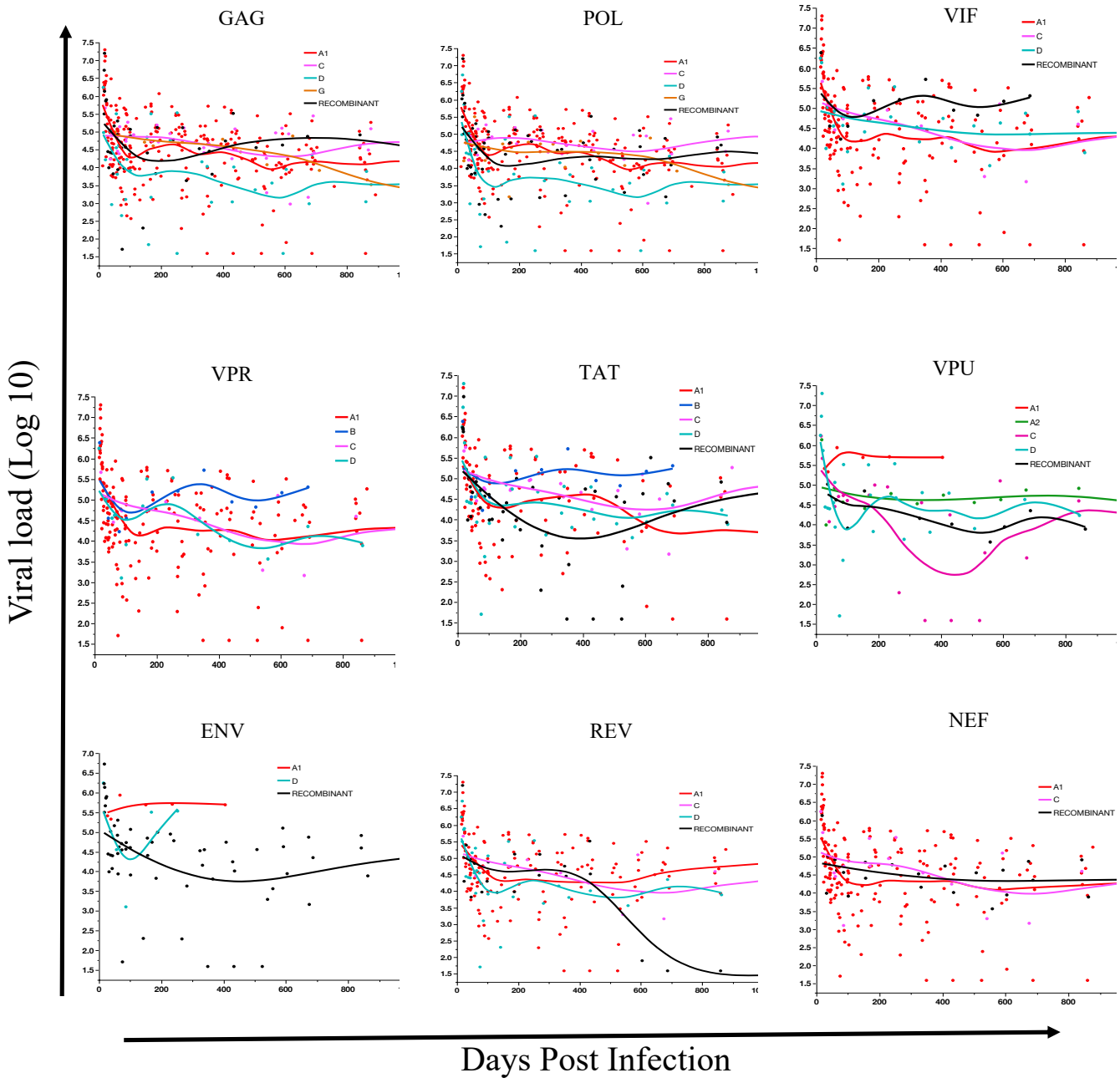


Figure 3.14: Trends in plasma viral loads in patients infected with different subtypes. Recombination was associated with higher viral loads when it occurred within *vif* but lower viral loads when it occurred within Env.

3.2.5 HLA escape mutations within *gag*

HLA-associated polymorphisms in HIV-1 *gag* were identified according to a published reference database that contains information about sequence variants and mutations of HIV-1 CTL epitopes (LANLEpitope). Only variants identified in studies that tested for immunological response were included and variants were omitted if the study only provided sequence or statistical data.

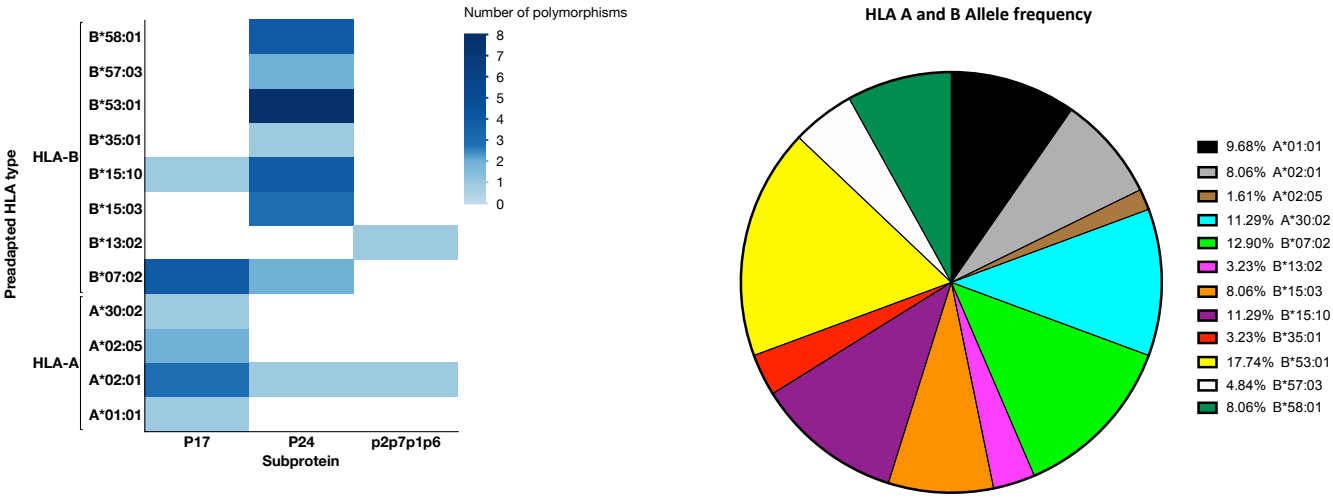
Estimation of the degree of adaptation of the transmitted *gag* to the HLA class I (A, B and C) alleles present in the newly infected patient's HLA-class I type could be inferred from a comparison of the HLA-class I driven polymorphisms present in the transmitted/founder virus to the HLA-class I reservoir of the patient. Only mutations that were present in all the sequences obtained per patient were selected, as this suggests that they were transmitted from a pre-adapted virus as opposed to rapidly selected in the new patient. This proportion of preadapted sites was used in the analyses described below to determine the impact of transmitted preadaptation on viral control and disease progression.

The identified polymorphisms were linked to a wide variety of HLA-A and B alleles, but none was identified within HLA-C. Overall, most were linked to HLA-B alleles (73%), although 27% of them were associated with HLA-A. Polymorphisms were distributed across all of the proteins encoded by the *gag* gene including p24, p17, p7, p2, p6 and p1 (Figure 3.15).

Transmission of polymorphisms was not homogeneous between all the proteins encoded by *gag* ($p < 0.0001$, Kruskal-Wallis test), with p17 and p24 transmitting a larger proportion of polymorphisms than p15. In addition, preadapted residues were particularly frequent in certain epitopes. 24% of the observed polymorphisms were in the PY9 epitope (p24, HXB2 position 122-130), 13.5% were in the RY11 epitope (p17, HXB2 position 76-86), 10.8% were in the RM9 epitope (p17, HXB2 position 22-30), and 10.8% were in the YL9 epitope (p24, HXB2

position 164-172). This suggests that these positions constitute hot spots of immune recognition. The full list of identified polymorphisms is detailed in Table 3.2.

Figure 3.15: Distribution of HLA class I polymorphisms in gag and allele frequency of these



HLA-A and HLA-B alleles.

HLA class I associated polymorphisms were enriched in p17 and p24. Majority of the polymorphisms in p17 were associated with HLA-A while those on p24 were associated with HLA-B.

A HLA-class I preadaptation score was obtained by counting the number of HLA-class I adapted polymorphisms present within each patient's repertoire of HIV-1 sequences, where the highest possible number was 6 in which case the patient was infected with a virus that had escape mutations that conferred some degree of preadaptation to both alleles of HLA-A, B and C, and a score of zero represented a lack of polymorphism linked to any of that patient's HLA-class I type. Surprisingly, there was no correlation between preadaptation score and either viral load or the rate of CD4 T cell decline.

Table 3.2: List of HLA-class I linked polymorphisms that were identified in the cohort.

Patient ID	Patient's HLA type	Epitope name	Subprotein (HXB2 location)	Epitope amino acid sequence	Variant (protein)	Mutation type description*	Method and reference
210020	A*02:05	SL9	p17(76-86)	SLYNTVATLY	Y79F	SF/A/P	Intracellular cytokine staining, Sequence, TCR binding (Varela-Rohena et al. 2008; Lazaro et al. 2011)
210032	B*53:01**	PY9	p24(122-130)	PPIPVGEIY	E128D	A/P	Intracellular cytokine staining (Tenzer et al. 2014)
210036	A*02:01	RY11	p17(76-86)	RSLFNTVATLY	R76K	DHB/DR	CD8 T-cell IFNy Elispot, HLA binding (Tenzer et al. 2009)
	B*07:02	RM9	p17(22-30)	RPGGKKHYM	M30K	E/RCR	Other (Kloverpris, Cole, et al. 2015)
		GL9,	p24(223-231)	GPSHKARVL	S357G	SF	CD8 T-cell Elispot - IFNy (Keane et al. 2012)
210037	B*15:10	YL9	p24(164-172)	YVDRFFKTL	F301Y	LE, SF	CD8 T-cell Elispot – IFNy (Novitsky, Flores-Villanueva, et al. 2001)
210041	A*02:01	RY11	p17(76-86)	RSLFNTVATLY	R76K/Y79F	DHB/DR	CD8 T-cell IFNy Elispot, HLA binding (Tenzer et al. 2009)
210045	B*58:01	TW10	p24(108-117)	TSTLQEQIGW	A248G	A/P	Intracellular cytokine staining (Tenzer et al. 2014)
		QW9	p24(176-184)	QATQDVKNW	T310S	E	Other (Kloverpris, McGregor, et al. 2015)
210047	B*07:02	RM9	p17(22-30)	RPGGKKHYM	M30R	E/RCR	Other (Kloverpris, Cole, et al. 2015)
210050	B*53:01	PY9	p24(122-130)	PPIPVGEIY	E128D	A/P	Intracellular cytokine staining (Tenzer et al. 2014)
210051	B*15:10	YL9	p24(164-172)	YVDRFFKTL	F301Y	LE, SF	CD8 T-cell IFNy Elispot, Sequence. (Novitsky, Rybak, et al. 2001)
210052	B*15:10	YL9	p24(164-172)	YVDRFFKTL	F301Y	LE, SF	CD8 T-cell Elispot - IFNy, Sequence (Novitsky, Rybak, et al. 2001)
210054	B*13:02	R19	p2p7p1p6(66-74)	RQANFLGKI	I437V, I437V/K436R	A/E	CD8 T-cell IFNy Elispot (Tenzer et al. 2009)

	A*01:01	GY9	p17(71-79)	GSEELKSLY	K76R	SF	CD8 T-cell IFNy Elispot (Schweighardt et al. 2010)
210057	B*57:03	KF11	p24(30-40)	KAFSPEVIPMF	V168I	SF	CD8 T-cell IFNy Elispot, Chromium release, Tetramer binding (Gillespie et al. 2002)
210059	A*02:01	Gag 386	p24(230)-p2p7p1p6(7)	VLAEAMSQV	V370T	SF	CD8 T-cell IFNy Elispot, HLA binding (McKinney et al. 2004)
	B*07:02	RM9	p17(22-30)	RPGGKKHYM	M30R	E, RCR	Other (Kloverpris, McGregor, et al. 2015)
	B*53:01	PY9	p24(122-130)	PPIPVGEIY	E128D	A/P	Intracellular cytokine staining (Tenzer et al. 2014)
210060	A*30:02	RY10		RLRPGGKKK	K28R	SF/DR	Flow cytometric T-cell cytokine assay, Intracellular cytokine staining (Sanchez-Merino, Nie, and Luzuriaga 2005; Casazza et al. 2005)
	B*07:02	GL9	p24(223-231)	GPSHKARVL	S357G	SF	CD8 T-cell IFNy Elispot (Keane et al. 2012)
	B*57:03	ISW9	p24(14-23)	AISPRTLNAW	I147L	LE	Sequence (Payne, Muenchhoff, et al. 2014)
210063	B*53:01	PY-9	p24(122-130)	PPIPVGDIY	D128E	A/P	Intracellular cytokine staining (Tenzer et al. 2014)
210065	B*53:01	PY9	p24(122-130)	PPIPVGEIY	E128D	A/P	Intracellular cytokine staining (Tenzer et al. 2014)
210073	A*02:01	Gag 271	p24(118-126)	MTSNPPIPV	S252G	SF	CD8 T-cell Elispot - IFNy, HLA binding (McKinney et al. 2004)
210074	B*15:10	YL9	p24(164-172)	YVDRFFKTL	F301Y	LE, SF	CD8 T-cell Elispot - IFNy, Sequence (Novitsky, Rybak, et al. 2001)
	B*35:01	PY9	p24(122-130)	PPIPVGEIY	E128D	A/P	Intracellular cytokine staining (Tenzer et al. 2014)
210076	B*53:01	PY9	p24(122-130)	PPIPVGEIY	E128D	A/P	Intracellular cytokine staining (Tenzer et al. 2014)

210077	A*30:02	RY11	p17(76-86)	RSLFNTVATLY	Y79F	SF	CD8 T-cell Elispot - IFNy, HLA binding (Tenzer et al. 2009)
210080	B*15:03	VF-9	p24(24-32)	VKVVEEKAF	V27I	A/P	Intracellular cytokine staining (Tenzer et al. 2014)
	B*53:01	PY9	p24(122-130)	PPIPVGEIY	E128D	A/P	Intracellular cytokine staining (Tenzer et al. 2014)
210082	B*07:02	RM9	p17(22-30)	RPGGKKHYM	M30R	E, RCR	Other (Kloverpris, McGregor, et al. 2015)
	B*15:03	VF-9	p24(24-32)	VKVVEEKAF	I27V	A/P	Intracellular cytokine staining (Tenzer et al. 2014)
210084	B*58:01	ISW9	p24(14-23)	AISPRTLNAW	I147L	LE	Sequence (Payne, Branch, et al. 2014)
		QW9	p24(176-184)	QATQDVKNW	T310S	E	Other (Kloverpris, Cole, et al. 2015)
210085	A*02:01	RY11	p17(76-86)	RSLFNTVATLY	K79F	DHB/DR	CD8 T-cell Elispot - IFNy, HLA binding (Tenzer et al. 2009)
	B*15:03	VF-9	p24(24-32)	VKVVEEKAF	V27I	A/P	Intracellular cytokine staining (Tenzer et al. 2014)
210086	B*15:10	RY11	p17(76-86)	RSLFNTVATLY	K79F	DB	CD8 T-cell Elispot - IFNy, HLA binding (Tenzer et al. 2009)
210097	A*02:05	SL9	p17(76-86)	SLYNTVATLY	Y79F	SF/A/P	Intracellular cytokine staining, Sequence, TCR binding (Varela-Rohena et al. 2008; Lazaro et al. 2011)
	B*53:01	PY9	p24(122-130)	PPIPVGEIY	E128D	A/P	Intracellular cytokine staining (Tenzer et al. 2014)

Mutation type description*: A (HLA Association), **DHB** (Diminished HLA binding or increased off rate), **DR (**Diminished response), **E** (Escape), **LE** (Literature escape), **SF** (Susceptible form), **P** (Processing), **RCR** (Replicative capacity reduced)

Next, we investigated whether individual escape mutations influenced the rate of disease progression by comparing longitudinal CD4 T cell counts and viral load in patients in whom HLA-class I linked polymorphisms were observed. Mutations were described as influencing disease progression when the median CD4 T cell count and/or viral load differed from that of study participant possessing the wild type epitope with a $p<0.05$.

9 HLA-class I associated polymorphisms in the GA271, RY10, SL9, K76R, PY9, RM9, GAG386, TW10 and QW9 epitopes were associated with low CD4 T cell counts (Figure 3.16). Possession of mutations in both the PY9 and YL9 epitope was the only polymorphism that conferred protection from CD4 T cell decline. Polymorphisms within the TA10, and NY10 conferred lower viral loads while those within PY9 and/or YL9 were associated with increased viral loads.

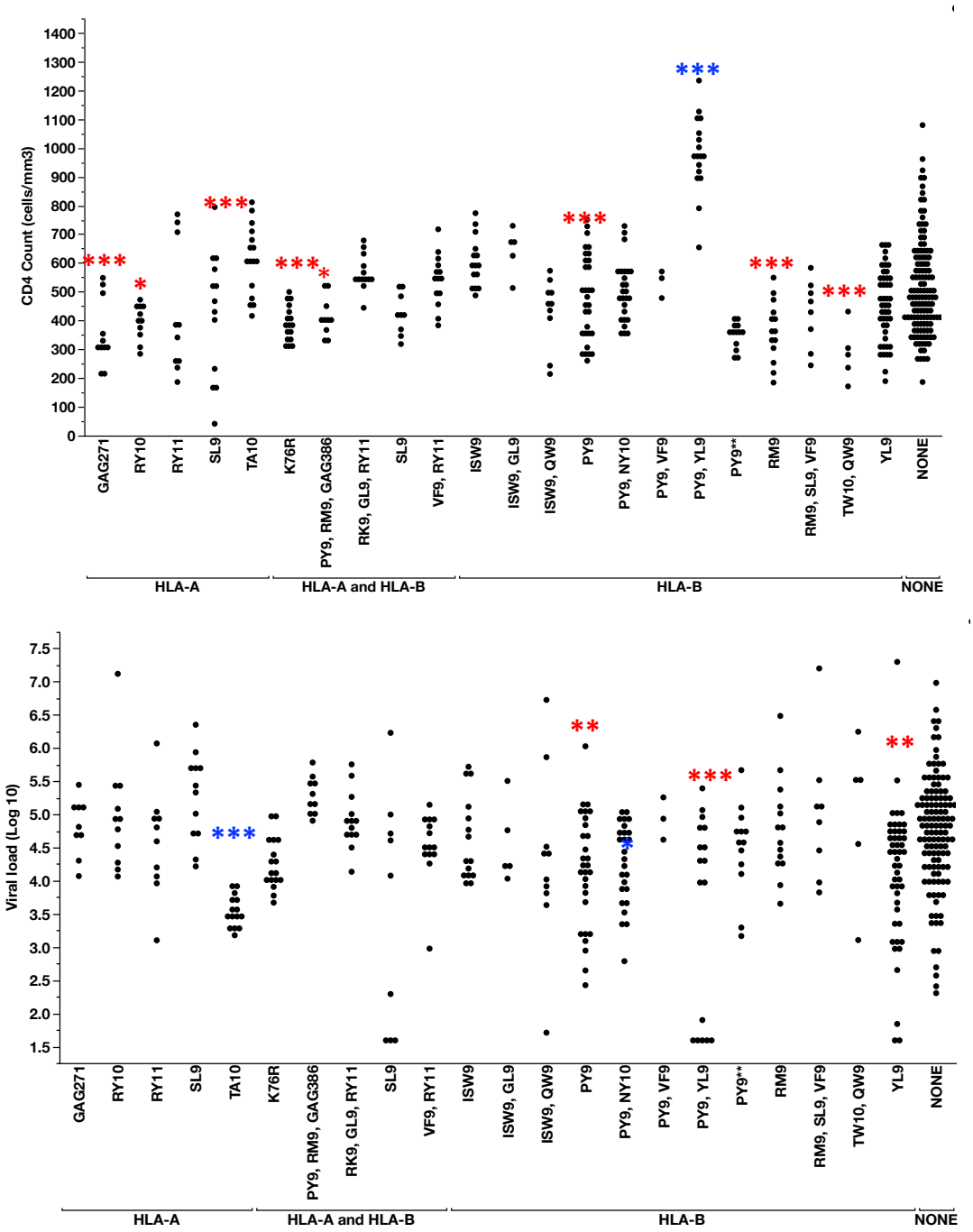


Figure 3.16: CD4 T cell counts and viral loads in patients possessing HLA-class I linked polymorphisms within *gag*.

CD4 T cell counts (top) and viral loads (bottom) in patients possessing HLA-class I linked polymorphisms within *gag*. Mutations that were significantly associated with changes in CD4 count or viral load are denoted with blue asterisks for protective polymorphisms and red for detrimental polymorphisms, where *, ** and *** represents p values between 0.05 and 0.01,

0.009 to 0.001 and 0.0009 to <0.0001. p values have been corrected using the Benjamini-Hochberg technique as described in the materials and methods.

3.2.6 Transmitted drug resistance mutations

The emergence of resistance mutations within the reverse transcriptase (*RT*) and protease genes during antiretroviral therapy has been shown to be associated with a reduction in viral fitness (Nicastri et al. 2003). Drug resistance mutations (DRM) were identified through the Stanford HIV database for transmitted drug resistance version 6.0 (StanfordTDRM/CPR) against the World Health Organization surveillance of transmitted DRM list.

Overall, the prevalence of non-nucleoside reverse transcriptase inhibitors (NNRTI), protease inhibitors (PI) and integrase inhibitors (INIs) transmitted drug resistance mutations in the 38 sequenced patients was 21% (n=8), 7.8% (n=3), and 10% (n=4) respectively (Figure 3.17). No NRTI mutations were observed. Cross-class resistance (involving two or three classes) was also not observed.

The most prevalent NNRTI mutation was E138A (n=6) that reduces susceptibility to etravirine (ETR) and rilpivirine (RPV) about 2-fold. Other NNRTI mutations present included K101E (n=1) associated with reduced susceptibility to nevirapine (NVP) by 3 to10 fold, ETR and RPV by about 2 fold; K103N (n=1) that reduces NVP and efavirenz (EFV) susceptibility by about 50 and 20-fold respectively, and V179T that appears to be associated with minimal reductions in ETR and RPV susceptibility and does not appear to interfere with the response to a first-line EFV containing regimen.

Similarly, the following mutations that confer resistance to integrase inhibitors were detected: E138K (n=1) that alone does not reduce INIs susceptibility but in combination with Q148 mutations reduce raltegravir (RAL) and elvitegravir (EVG) susceptibility >100-fold and dolutegravir (DTG) susceptibility up to 10-fold and T97A (n=3) that reduces EVG

susceptibility by ~3-fold, however has not been shown to reduce the efficacy of first-line EVG-containing regimens. Alone, it has minimal if any effect on RAL or DTG susceptibility, but it contributes to markedly reduced RAL and DTG susceptibility when combined with other INIs-resistance mutations. The only protease inhibitor resistance detected was K20T (N=1) that is associated with reduced susceptibility to each of the PIs except darunavir (DRV) and tipranavir (TPV). Most of these drugs are however not used in first line treatment of HIV-1 except nevirapine, efavirenz and dolutegravir, to which only 4 of the patients showed a resistant genotype.

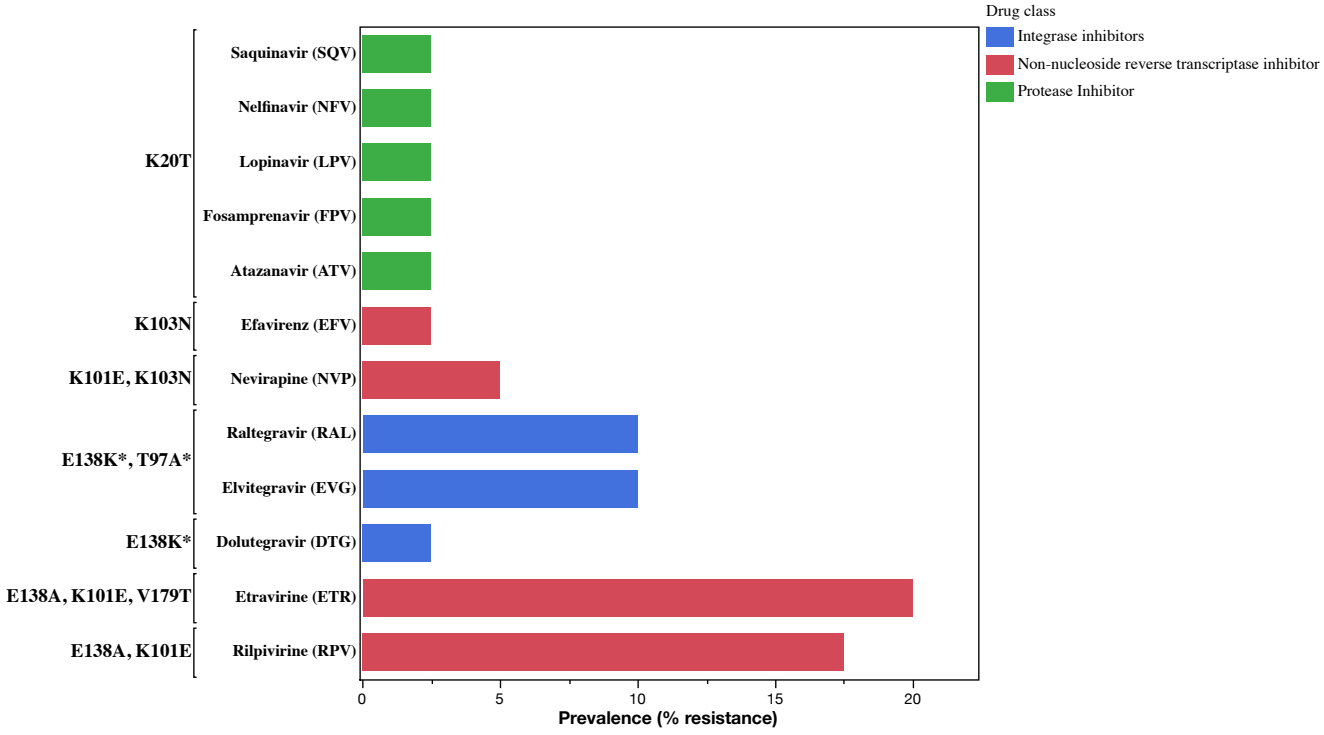


Figure 3.17: List of mutations in the cohort that conferred transmitted drug resistance. The highest prevalence of TDR was to non-nucleoside reverse transcriptase inhibitors (in red), followed by integrase inhibitors (in blue) and the lowest prevalence was of protease inhibitors (in green). Where multiple mutations are shown they all confer resistance to the same drug, whereas an asterisk denotes that the mutation was only present in a subset of that patient's sequences. Most of these drugs are however not used in first line treatment of HIV-1 except

nevirapine, efavirenz and dolutegravir, to which only 4 of the patients showed a resistant genotype.

Infection with a founder virus that possessed transmitted drug resistance mutations was not associated with differences in CD4 T cell counts over time (P=0.06), although patients who were infected with a virus that already possessed a drug resistance genotype had higher plasma viral load set points (P=0.02), suggesting that mutations that confer transmitted drug resistance may confer increased replicative fitness (Figure 3.18). The patients were however not tested for drugs to verify their ART naïve status.

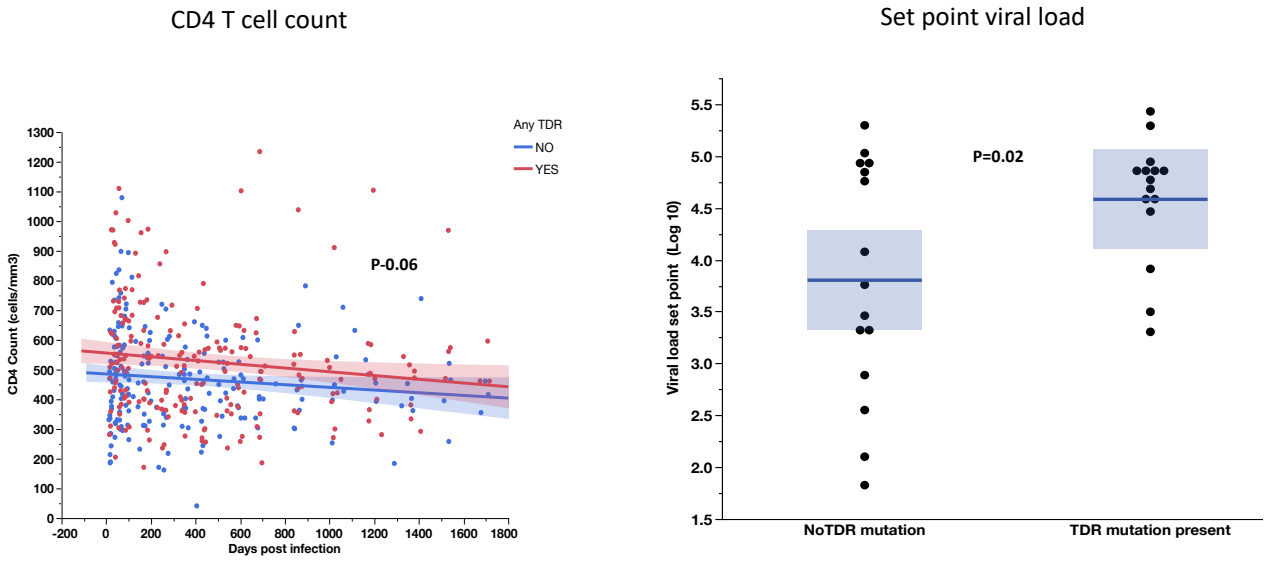


Figure 3.18: CD4 T cell counts and plasma viral load set point in patients infected with founder viruses that possessed drug resistance mutations. Transmitted drug resistance mutations were associated with higher set point viral load.

3.3 Discussion

A primary goal of AIDS vaccine development is to prevent acquisition of HIV-1 at mucosal surfaces. Therefore, it is critical to determine whether newly transmitted HIV-1 strains share traits that provide novel targets for vaccine design. This chapter builds on a large body of published work on acute HIV-1 infection by shedding new light on the genetic properties of HIV-1 near the moment of transmission and in the critical period of virus replication and diversification leading to peak viremia and seroconversion. The study of full-length transmitted/founder HIV-1 sequences is essential because they contain the complete genetic information of viruses that successfully establish infection.

By analysing viral sequences from early time points in primary HIV-1 infection (median 21 days post infection) as well viral sequences from the donors of linked sexual and mother-to-child transmissions, studies have demonstrated that the virus population in the newly infected individual is much less diverse than that in the transmitting partner or mother, sometimes a minor variant in the blood of the transmitters (Wolinsky et al. 1992; Wolfs et al. 1992; Zhu et al. 1993; Tully et al. 2016; Naidoo et al. 2017; Masharsky et al. 2010; Haaland et al. 2009). The use of end-point dilution PCR (single genome amplification or SGA) to amplify sequences from multiple single genomes present in plasma very early after primary infection followed by direct sequencing of the DNA amplicon avoids sequencing errors introduced by the Taq polymerase, *in vitro* recombination induced by template switching during the PCR reaction, and non-proportional representation of sequences as a result of template resampling (Salazar-Gonzalez et al. 2008; Ochsenbauer et al. 2012).

Using this approach, near full-length and overlapping half genomes were sequenced from 38 acutely infected MSM and revealing remarkable within-patient homogeneity of sequences, with some of the genomes being identical, and most differing by less than ten nucleotides across the

entire ~9kb HIV-1 genome. In most cases, mutations were spread across the whole genome. This confirms that these patients were indeed very early in infection and enables precise identification of the founder virus. Furthermore, it has been possible to define the number of T/F variants, and the nucleotide sequence of each variant.

In this chapter, four findings were made that advance previous understanding of the role of HIV-1 founder virus in driving disease progression by confirming earlier studies done on different HIV-1 subtypes and geographical locations, and at the same time providing additional novel insights. Firstly, MSM infection is associated with increased multiplicity of founder viruses, and that this negatively impacts disease outcome. At least 37% (14 out of 38) had evidence of infection by two or more HIV-1 variants compared with ~20% as has been previously described in heterosexual transmission cohorts (Baalwa et al. 2013; Carlson et al. 2014; Janes et al. 2015). This was however lower than has been reported in earlier studies that have demonstrated high multiplicity of infection in Men who have sex with men (Chaillon et al. 2016; Li et al. 2010). Each lineage contained a unique set of identical or near identical sequences suggesting that these subjects were infected by very closely related viruses, most likely from one partner as opposed to a single virus that evolved into two distinct lineages in the brief period preceding peak viremia, or superinfection. Receptive anal intercourse has a reported hazard of 10 times that of penile-vaginal intercourse (Dosekun and Fox 2010), and in these patients who practice it more often than heterosexual cohorts this is accompanied by a slightly less stringent genetic bottleneck. It is noteworthy that the MSM in this cohort also report transactional sex, intravenous drug use, a high number of sex partners and increased prevalence of STI, factors which in combination with the fragility of the rectal mucosa are likely to increase both the odds of exposure to a HIV-1 infected person, and a more permissive microenvironment contributing to the increased multiplicity of infection.

It is not known whether the genetic transmission bottleneck in transmission by receptive anal intercourse selects for founder viruses of a different phenotype than those transmitted by the penile-vaginal route. Differences in risk behaviour, routes of virus transmission (receptive vs insertive anal intercourse), clinical stage of and viral load of the source of infection, and presence of STI in recipient may have influenced the numbers of viruses transmitted and subsequent disease natural history. Our findings regarding the number of viruses leading to productive clinical infection are minimal estimates, and additional viruses could have been transmitted but not sufficiently propagated *in vivo* to allow detection within the timing of our sampling or our depth of sequencing. In the context of multivariant infection, the proportions of T/F viruses in each individual were highly variable, with major T/F viruses as high as 96% and minor T/F viruses as low as 3%. The highly unequal proportions of T/F viruses at acute infection suggest different replication advantages among T/F viruses in the same HIV-1-infected individuals.

Multivariant infection resulted in lower CD4 T cell counts in the first 5 years of infection with patients infected with multiple founder viruses reaching CD4 T cell counts under 400 cells/mm^3 much faster than those infected with a single variant and an overall inverse correlation between the number of founder virus variants and CD4 T cell counts at all time points. There was no significant difference in longitudinal viral load measurements over time except in the first month of diagnosis unlike in the study done by Chaillon et al where infection with multiple founder variants was associated with high viral load (Chaillon et al. 2016), and viral load set points were similar regardless of the number of infecting founder virus variants suggesting that greater heterogeneity in the HIV-1 founder population of recently infected individuals is associated with a worse disease outcome in a manner that is independent of viral load within the timepoints tested. This is in contrast to the observation from both the STEP and RV144 HIV-1 vaccine trials that when vacinees got infected, infection with more diverse HIV-1 populations in early infection was associated with

significantly higher viral load one year after HIV-1 diagnosis (Janes et al. 2015), but further confirming an earlier heteroduplex mobility assay-based report (Sagar, Lavreys, et al. 2004). Without mucosal samples taken at the time of transmission, the possibility that the major T/F variants were clonally amplified at the donor mucosa at the time of transmission cannot be ruled out, although downstream bottlenecks may reduce or obscure any transmission advantage associated with viral frequency in the transmission fluid. Nonetheless, these results demonstrate that greater heterogeneity in the HIV-1 founder population of recently infected individuals is associated with a worse disease outcome in a manner that is independent of viral load within the timepoints tested. The one individual who had potent broadly neutralising antibodies in this study group had multiple founder viruses, although there were too few patients with both sequence data and neutralisation antibody data to make a comparison. Nonetheless, there is need to investigate whether infection with multiple founder viruses is associated with increased breadth of B cell and T cell responses, and whether this can explain their preserved CD4 T cell counts despite no differences in set point viral load.

Few studies employ near full length genome sequencing as this is extremely labor and time consuming expensive and technically challenging (Rousseau et al. 2006; Rousseau et al. 2007). This study demonstrates the extent to which HIV-1 subtyping using partial sequences is inaccurate at determining HIV-1 subtype, and that sequencing of multiple genes is essential to understand the role of HIV-1 subtype in HIV-1 disease progression. Previous studies based on partial *pol* or *env* sequences demonstrated that subtype C and D infected patients progress faster than subtype A to all three endpoints: $CD4^+$ T cell decline, viral load and duration to onset of AIDS, and that individuals with subtype A were more likely than individuals with subtypes C or D to report acute retroviral syndrome symptoms (Eduard Sanders, personal communication), with each individual symptom other than rash being more prevalent in subtype A than in subtype C or D (Amornkul et al. 2013; Baeten et al. 2007; Easterbrook et al. 2010; Kaleebu et al. 2001; Vasan et al. 2006; Kiwanuka et al.

2008; Sanders et al. 2017). However, it is now well established that recombination between the two RNA copies that a HIV-1 virion carries is a common occurrence and can give rise to unique inter-subtype recombinants, with little consensus on the existence and location of recombination hotspots (Turk and Carobene 2015; Nikolaitchik et al. 2015; Arenas, Lorenzo-Redondo, and Lopez-Galindez 2016). Understanding of the differences in the clinical outcome of infection with the different HIV-1 subtypes relies upon accurate classification of the infecting virus.

This study demonstrates that in settings where inter-subtype recombinants exist, the use of specific HIV-1 genes as opposed to the full HIV-1 genome to subtype the virus underestimates the frequency of recombinant forms. 42.5% (17 out of 40) of our study participants were infected with unique recombinant forms, with subtype A being the most frequently occurring pure subtype and also the most commonly represented subtype among the recombinant viruses, with 88% of the recombinant viruses having one or more genes that were of subtype A. The most frequently occurring recombination was of type A1 and D (5 out of 11, i.e. 45% of the recombinant viruses) which is not unexpected as both subtypes are prevalent in East Africa. In this cohort, the subtyping based on the *pol* region alone underestimates the frequency of circulating recombinants by two thirds.

Regions were identified in *gag, pol, tat* and *env* in which recombination breakpoints were enriched. *Tat* was further identified as a potential recombination hot spot as the occurrence of recombinant *tat* was 5-fold of what would be expected for its gene size if recombination was a random event. In an analysis of HIV-1 recombination in *gag* and *pol* in T cells, recombination was favoured at a number of recombination hot spots, where recombination occurs six times more frequently than at corresponding cold spots (Smyth, Davenport, and Mak 2012; Smyth et al. 2014). However, hotspots and cold spots were spread evenly across the genome and occurred near important features of the HIV-1 genome, although not at sites immediately around protease inhibitor or reverse transcriptase inhibitor drug resistance mutations (Cromer et al. 2016).

Overall, possession of recombinant viruses did not offer any protection from CD4 T cell decline by itself even though patients infected with recombinant viruses consistently had lower plasma HIV-1 RNA loads. However, having specific subtypes on some of the genes was shown to be advantageous with recombination offering protection form CD4 T cell decline in *tat, vpu, env, rev* and *nef* and having the opposite effect in *pol* and *vif*. Similarly, lower viral loads were observed in recombinant *tat, vpu, env* and *rev* but surprisingly not in *nef*, and higher viral loads in recombinant *gag* and *vif*. Amongst the pure subtypes within each, no conclusion was reached on the effect of subtype A2, B, C, D and G genes as there were very few patients whose founder virus possessed their subtype in their pure form within a whole gene. However, in 4 genes (*gag, pol, vpu* and *env*), subtype A was associated with the fastest decline of CD4 T cells but preservation of CD4 T cells was seen in *vpr*. Within *vpr* and *vif,* the lowest viral loads were observed in subtype A whereas subtype A was associated with highest viral load in *vpu* and *env*. These data suggest that the effects of HIV-1 subtypes in driving disease progression should be viewed in the context of the characteristics of each gene as some subtypes may be fitter in some genes but not others, and further poses an additional challenge to vaccine design. Sequencing variants present at the viral load set point in patients infected with multiple founder viruses of different *in vitro* replicative capacities will reveal whether any of the founder viruses has an *in vivo* fitness advantage.

Thirdly, this chapter is consistent with studies done in HIV-1 subtype C and in non-MSM patients that infected individuals can receive a pre-adapted founder virus variant and that preadaptation in some epitopes sends the patient on a trajectory of fast depletion of CD4 T cells. If transmitted preadapted polymorphisms affect the capacity of the recipient to mount an immune response against the transmitted/founder virus, the degree of preadaptation found in the transmitted virus will have a significant impact on the course of the infection in the newly infected individual. This advantage is thought to arise because being more similar to the consensus sequence produces viruses with elevated infectiousness and is further supported by

the observation that when low-fitness CTL escape mutants are transmitted, they can revert to the consensus sequence at that site (Woodman and Williamson 2009; Treurnicht et al. 2010; Monaco et al. 2016; Carlson et al. 2016; Brockman et al. 2010). Therefore, the influence of individual escape mutations on the rate of disease progression was investigated by comparing longitudinal CD4 T cell counts and plasma viral load in patients in whom HLA-class I linked polymorphisms were observed.

9 HLA-class I associated polymorphisms in the GA271, RY10, SL9, K76R, PY9, RM9, GAG386, TW10 and QW9 epitope were associated with low CD4 T cell counts with possession of mutations in both the PY9 and YL9 epitope conferring protection from CD4 T cell decline. Polymorphisms within the TA10 and NY10 conferred lower viral loads while those within PY9 and/or YL9 were associated with increased viral loads. Surprisingly, even though the PY9 and YL9 when occurring separately conferred higher viral loads, this effect was not seen in patients who harboured both polymorphisms, possibly due to a lack of statistical power. In addition, when the PY9 mutation occurred together with the NY10, patients had lower viral loads, suggesting that the NY10 polymorphisms was responsible for this protection but we cannot confirm that as no patient possesses the NY10 polymorphism in isolation. This finding that most polymorphisms only had an impact on the rate of CD4 T cell decline is consistent with findings that newly infected individuals can receive a pre-adapted variant that leads to an accelerated disease progression (faster CD4 T cell decline) without showing a significant effect on set-point VL (Monaco et al. 2016; Carlson et al. 2016). Transmission of polymorphisms was not homogeneous between all the proteins encoded by *gag* with p17 and p24 transmitting a larger proportion of polymorphisms than p2p7p1p6. Unexpectedly, there was no correlation between preadaptation score and either viral load or the rate of CD4 T cell decline.

Fourthly, the prevalence of transmitted drug resistance mutations in these patients was quantified. The scale up of antiretroviral therapy amongst HIV-1-infected patients alongside

changes in policy that recommend that all patients be started on ART as soon as they are diagnosed with HIV-1 has led to unprecedented control of viremia in chronic HIV-1 patients (Woodson et al. 2018; Schuetz et al. 2014; Azzoni et al. 2015). In addition, antiviral agents are currently in use as PrEP for men and women at high risk of HIV-1 infection with a combination of tenofovir disoproxil fumarate and emtricitabine approved for this purpose in several developed countries but access outside elsewhere is largely limited to clinical trials or demonstration projects (Zhang et al. 2018; Shrestha and Copenhaver 2018; Pintye et al. 2018; Eaton et al. 2018). Consequently, the emergence of resistance mutations is on the rise and has been shown to vary from 0.5% to 15% in different cohorts (Van Vaerenbergh 2001; Rhee et al. 2015; Onywera et al. 2017; Myers et al. 2012; Kiptoo et al. 2013; Hassan et al. 2013).

The observed prevalence of NNRTI, PI, INI and NRTI transmitted drug resistance mutations in the 38 sequenced patients was 15.7% (n=6), 2.5% (n=1), 10% (n=4) and 0% respectively and no resistance to multiple classes of drugs was observed within the same individual . As a result, unless these mutations revert to a susceptible form due to a lack of drug pressure in the newly infected patients, these patients are likely to be resistant to a broad range of antiretroviral drugs including etravirive, rilpivirine, nevirapine, efavirenz, raltegravir, elvitegravir and dolutegravir. Of these, 4 patients (10%) would be predicted to have some degree of resistance WHO approved first line treatment for adults (WHO 2016; Mbuagbaw et al. 2016; Corado and Daar 2017; Cohen et al. 2008). Drug resistance mutations found in reverse transcriptase (*RT*) and protease genes have been shown to be associated with a reduction in viral fitness (Nicastri et al. 2003), which contradicts the observation that infection with a founder virus that possessed transmitted drug resistance mutations had higher viral load set points.

Without mucosal samples taken at the time of transmission, the emerging picture is that transmission occurs in a multiple-step process that probably involves physical stochastic bottlenecks that limit transmission of all viruses and selective bottlenecks that select for

biological properties. Nonetheless, the genotypic characteristics of the founder virus play a significant role in shaping disease progression. In the next chapter, selected phenotypic characteristics of the founder virus that may influence disease progression will be explored.

Chapter 4: Phenotypic characteristics conferred by the transmitted Gag

4 Phenotypic characteristics conferred by the transmitted Gag

4.1 Background and aims

To date, most research has focused on host factors to explain the variation in HIV-1 pathogenesis between individuals. The most important host genetic determinant of disease progression is HLA, implicating both innate and cellular immune responses as being important in controlling the virus (Fellay et al. 2007; Pereyra et al. 2010). However, HLA only accounts for about 20% of the variation in disease progression, leaving other host factors and viral factors as potential contributors to the rate of disease progression (O'Brien, Gao, and Carrington 2001; Amornkul et al. 2013; Chopera et al. 2012).

Soon after transmission, there is an initial period of limited host response where the phenotypic characteristics of the transmitted virus could impact the quality of the initial interaction between the virus and the host (Carlson et al. 2014; Keele et al. 2008). This in addition to the transmission bottleneck that accompanies HIV-1 infection means that the initial virus–host battle is carried out with a relatively homogeneous viral population, involving viruses carrying pre-existing mutations carried over from the donor viral population (Salazar-Gonzalez et al. 2009; Yue et al. 2015). A case for studying viral phenotypic characteristics is made by several studies that show that the viral load set point is partially heritable as evidenced by a trend for transmission pairs to have more similar viral load set point than if there was no transmission link (Boeras et al. 2011; Carlson et al. 2014; Prince et al. 2012), suggesting that transmitted viral factors also play an important role in disease progression and that they are potentially underestimated. In addition, HLA sharing between the source and recipient of a HIV-1 infection predisposes the newly infected patient to worse disease outcome.

Although disease progression as defined by the loss of CD4 T cells largely correlates with set point viral load, studies now show that only a small percentage of the variability of CD4 T cell

loss can be directly attributed to set point viral load (Kiepiela et al. 2007). Some variants of HIV-1 are less pathogenic than others, yet only recently have studies sought to determine whether reductions or increases in HIV-1's ability to reproduce (i.e. replication capacity) can be measured accurately and if these measurements could provide a useful tool for monitoring HIV-1 progression and the effectiveness of therapy (Weber et al. 2017; Prince et al. 2012; Deymier et al. 2015; Sarmati et al. 2004).

Viral replicative capacity as measured in infectious molecular clones and chimeric viruses derived from heterosexual transmission, haemophiliacs and mother-child transmission has been shown to influence the rate of disease progression (Claiborne et al. 2015; Kouyos et al. 2011; Prince et al. 2012). Claiborne *et al*. showed that, overall, there is a reduced rate of CD4 T cell loss in subjects with viruses whose Gag proteins conferred reduced replicative capacity *in vitro,* especially those with the lowest third of fitness values in his assay (Claiborne et al. 2015). In a multivariable analysis, the magnitude of the protective effect (i.e. reduced rate of CD4 T cell loss) in the group with the lowest fitness values was comparable in magnitude although independent of the protective effect of the "protective" HLA alleles B*57:01 and B*58:01, although these variables were additive in their protective effects. They also found that viral DNA levels were higher in both naive and central memory CD4 T cell subsets in subjects infected with high replicative capacity viruses. This observation led them to suggest that people with high replicative capacity virus may have a larger latent reservoir, which is thought to reside in the central memory CD4 T cell subset. However, low fitness variants can be transmitted, raising the question of their contribution to different aspects of viral pathogenesis and disease progression. In addition, most groups have studied subtype C viruses, and a role for replicative capacity in other subtypes that differ in the rate of disease progression is not clear (Amornkul et al. 2013; Chopera et al. 2012).

Furthermore, it is likely that viruses that differ in disease progression profiles also differ in the mechanisms by which they induce CD4 T cell death. HIV-1 induced CD4 T cell death occurs in both uninfected and infected T cells (Church 2014). Productively infected CD4 T cells expressing HIV-1 peptides on the surface in the context of MHC molecules are targeted by immunity and mainly die by apoptosis. However, bystander killing of non-productively infected CD4 T cells also occurs and has been shown in resting CD4 T cells that take in HIV-1 but are not productively infected due to their quiescent state (Doitsh et al. 2014; Monroe et al. 2014). Such resting cells represent the main target encountered by HIV-1 and die by alternative bystander mechanisms of cell death. As opposed to apoptosis, which is immunologically silent, these cells may die in highly inflammatory modes of cell death such as pyroptosis, mediated by a distinct set of caspases and intracellular signalling molecules. CD4 T cells release their cytoplasmic contents including inflammatory cytokines and fragments of Pathogen Associated Molecular Patterns (PAMPS) to the extracellular milieu (Cummins and Badley 2010). CD4 T-cell death by bystander mechanisms *in vivo* would therefore be accompanied by increased inflammation, generalized immune activation and possibly exhaustion that is characteristic of high replicative capacity infection. It is unclear whether some viruses induce bystander killing better than others, but recent data shows that pyroptosis occurs in quiescent cells that are infected through virological synapse-induced cell-to-cell transmission in resting CD4 T cells as opposed to cell free transmission in activated CD4 T cells (Galloway et al. 2015; Monroe et al. 2014; Munoz-Arias et al. 2015).

A systematic characterization of the ability of founder viruses from patients who differ in disease progression, and of known replicative capacity to enter cells through cell-cell transmission as well as the likelihood that they may be better at bystander killing of CD4 T cells has yet to be performed. In the Zambian Subtype C infected patients characterized by Claiborne *et al*., the detrimental effects of infection with high replicative capacity viruses were

independent of viral load. Together, these data suggest that high replicative capacity viruses induce CD4 T cell decline through a bystander mechanism, and the degree to which bystander killing and consequently generalized bystander immune activation is triggered early in infection by viruses of different replicative capacities may explain the varying rates of disease progression among these individuals, and potentially the varying rates of disease progression between subtype A and C patients (Bachtel et al. 2018; Ende et al. 2018; Kiguoya et al. 2017).

In this study, using non-clade C viruses isolated from acutely infected men who have sex with men, the impact of early phenotypic viral characteristics on subsequent viral load and CD4 T cell decline will be examined. We hypothesise that high viral replicative capacity in subtype A infected MSM is associated with fast disease progression. The relationship between viral replicative capacity and disease progression has not been shown in men who have sex with men, whom previous studies suggest may have a less stringent transmission bottleneck. In addition, we hypothesise that high replicative capacity is a result of increased viral spread by cell-cell transmission as opposed to cell free spread.

The specific aims of this chapter are as follows:

1. To generate subtype A patient derived founder virus *gag* chimeras on an NL4.3 backbone.
2. To measure the *in vitro* viral replicative capacity conferred by subtype A *gag* and determine a role for disease progression, drawing comparisons with previous work in subtype C chimeras. Subtype C *gag* chimeras had been previously cloned and replicative capacity determined by Dr Eric Hunter's lab.
3. To characterize potential pathogenic mechanisms of high replicative capacity founder viruses including cell-cell transmission and mechanism of CD4 T cell decline for both subtype A and C *gag* chimeras *in vitro*.

4.2 Results

4.2.1 Generation of *gag*-NL4.3 chimeras

From a total of thirty-eight acute infected patients whose founder virus had been sequenced and characterized as discussed in Chapter 3, 33 individuals from the KEMRI cohort whose founder virus could be unambiguously identified were selected. From these 48 founder *gag* sequences were cloned into a NL4.3 backbone. These represented 48 founder variants, 24 drawn from single variant infection in 24 patients and 24 drawn from multivariant infection in 9 patients. All *gag* sequences cloned represented the consensus *gag* sequence from that patient.

Since the study participants were all recently infected with HIV-1 with relatively homogeneous virus populations and sequences had been derived using a single genome amplification method, a direct cloning strategy was employed that allows for the introduction of the entire *gag* gene into a replication competent lab adapted provirus. A homologous recombination method was employed to fuse the NL4.3-LTR-U5 sequence with the transmitted *gag* sequence. This ensures that the *cis*-acting sequences upstream of *gag*, which may influence expression levels, are constant throughout all constructs. The resulting chimeras include the entire transmitted *gag* sequence from the initiation codon to the end of *gag*. For each newly infected individual, at least three independent *gag-NL4.3* chimeras were assayed for replicative capacity. An analysis of variation of replicative capacity between the two independent clones derived from each newly infected linked recipient had previously shown to be 8.5% (Claiborne, Prince, and Hunter 2014). Testing three independent clones, therefore, ensured that the observed replicative capacity was not due to the confounding effect of backbone mutations that might have arisen during the cloning process and provides an estimate of experimentally induced variation. Figure 4.1 shows the titers of the three independently cloned but identical *gag-NL4.3* chimeras from the 48 chimeric viruses studied which ranged from a TCID of 85 to 241,474,164 IU/Ml.

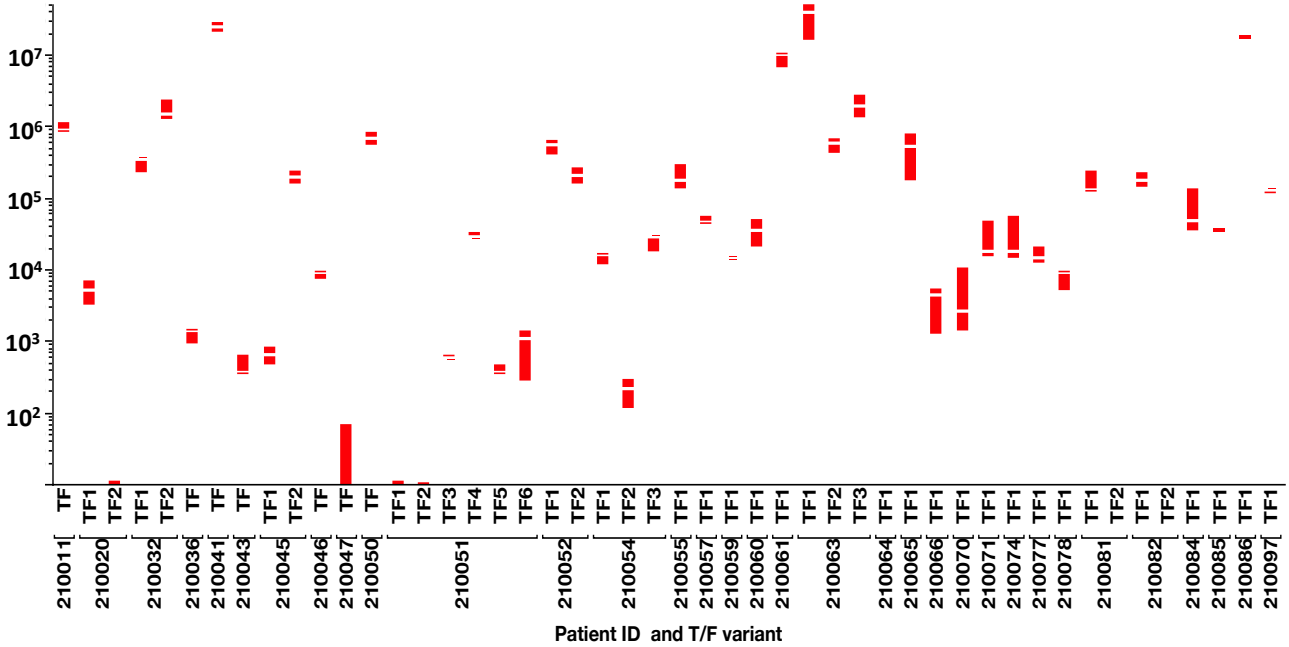


Figure 4.1: Titers (TCID50) of 48 *gag*-NL4.3 chimeras.

Each bar represents the range of three independently cloned and titered virus stocks that are identical in sequence because they were cloned from amplicons that all represented the consensus sequence.

4.2.2 Measurement of viral replicative capacity

To determine the suitable multiplicity of infection that would allow for the detection of logarithmic growth for most chimeras at the same multiplicity of infection (MOI), the wildtype NL4.3 virus was titrated and a MOI of 0.01 chosen as it resulted in logarithmic grown in the first 6 days of culture. Figure 4.2 shows the titration series while Figure 4.3 shows typical replicative capacity growth curves for all *gag*-NL4.3 chimeras with wild type NL4.3 and mock control depicted in red and blue respectively. In initial replication assays previously performed in order to test assay precision, wild-type lab adapted viruses exhibited an intra-assay variability of 10.4% and an inter-assay variability of 8.7% (Claiborne, Prince, and Hunter 2014). Due to

this large variability, all *gag* chimeras were tested for replicative capacity in one batch. The growth curve for each *gag-NL4.3* chimera was then normalized to that of wild-type NL4.3 to derive an absolute replicative capacity value.

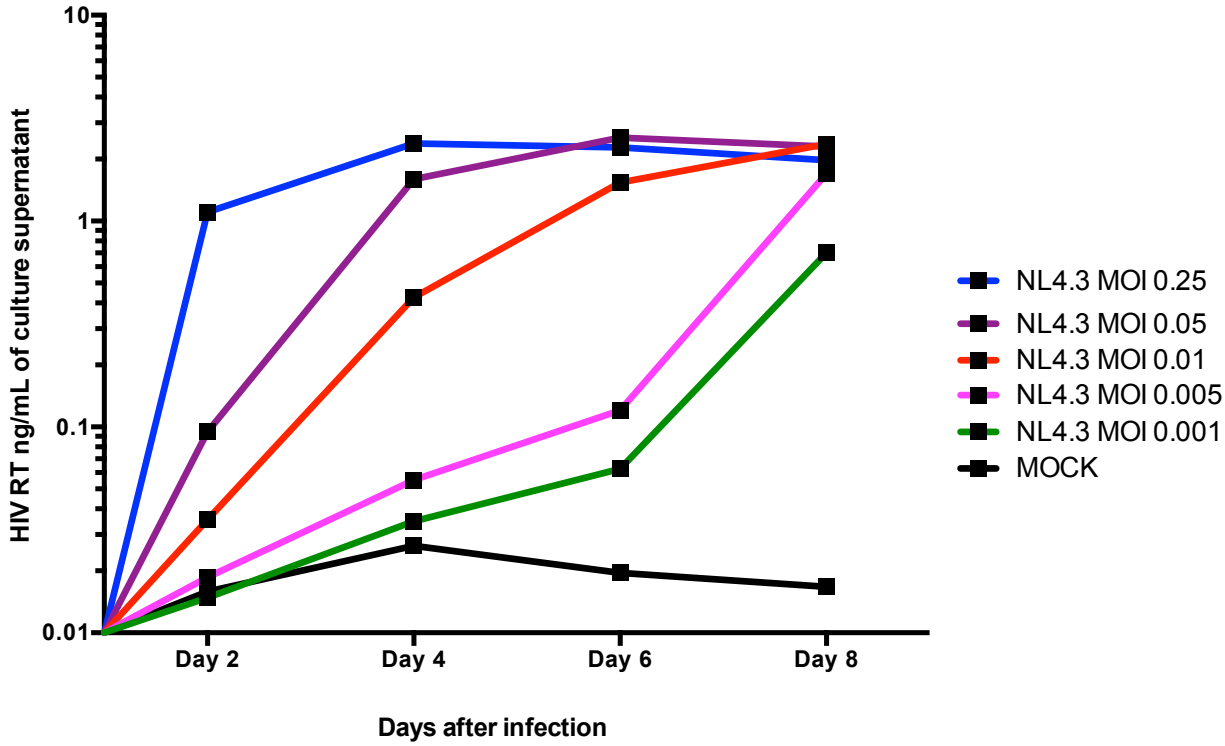


Figure 4.2: Titration of wild type NL4.3 to determine multiplicity of infection (MOI).
A MOI of 0.01 was chosen as it showed logarithmic growth in the first 6 days of culture.

Insertion of patient derived *gag* genes on to NL4.3 drastically altered the replicative capacity of NL4.3, with only 6 out of 48 chimeras increasing the replicative capacity of NL4.3 and the rest reducing its replicative capacity by up to 10-fold (Figure 4.3).

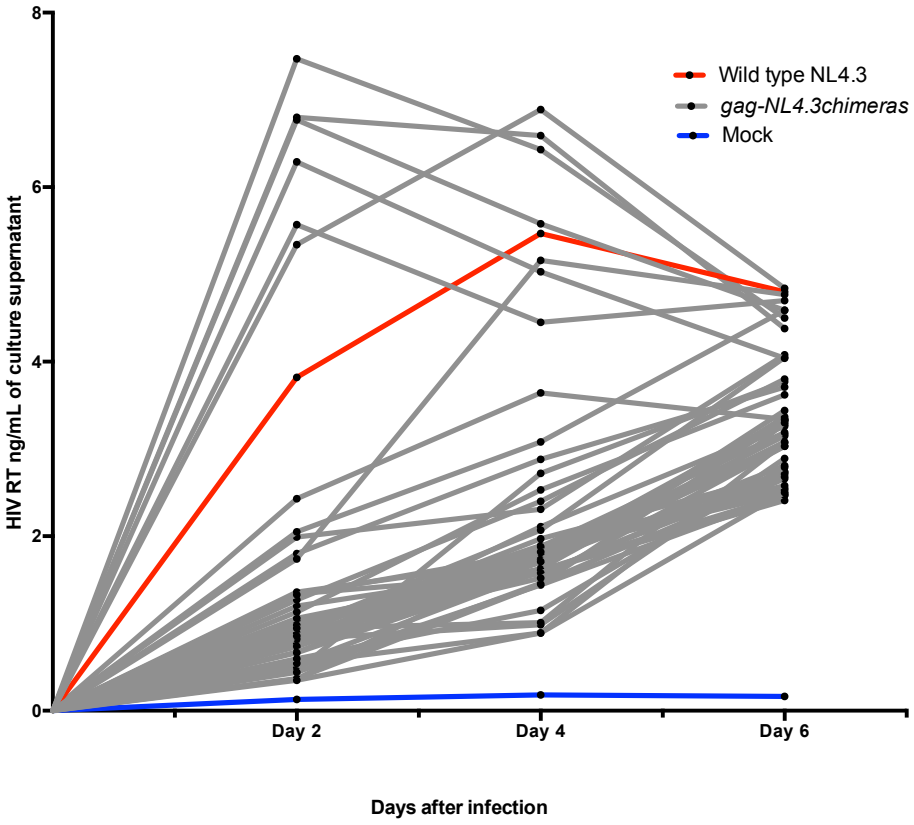


Figure 4.3: Typical replicative capacity growth curves for all gag-NL4.3 chimeras.

Replicative capacity growth curves for all *gag-NL4.3* chimeras with wild type NL4.3 and mock control depicted in red and blue respectively. Only 6 out of 48 patient derived *gag* sequences conferred increased replicative capacity to wild type NL4.3.

The replicative capacity was derived using both area under the curve and growth curve slope measurements, with both measurements showing close correlation (Figure 4.4a; Spearman correlation r^2=0.5, $p<0.0001$). The lack of perfect correlation may be explained by the observation that the chimeras that grew very quickly depleted the reservoir of susceptible cells in culture and stopped producing new virions, and therefore had an area under the curve that was different from the slope.

The CEM-based GXR25 cell line used for these replication studies is one of the few established T cell lines able to support entry and replication of CCR5-tropic strains of HIV-1. This has been achieved by retroviral transduction to allow stable expression of human CCR5 (Brockman et al. 2006). This cell line naturally expresses CXCR4 and will support replication of CXCR4-tropic HIV-1, such as the laboratory-adapted strain NL4-3. The cell line also expresses GFP under a Tat promoter when infected by HIV-1 allowing for infection to be monitored by flow cytometry. Other groups have measured replicative capacity based on radiolabelled reverse transcriptase activity in culture supernatant (Claiborne, Prince, and Hunter 2014; Deymier et al. 2014; Prince et al. 2012). The use of radiolabelled substances requires special imagers and containment levels and therefore both intracellular GFP expression as well as a colorimetric reverse transcriptase assay were employed to quantify virus growth, and averaged the replicative capacity derived from the two methods to generate a replicative capacity score for each chimera. The replicative capacity derived through both methods correlated closely (Figure 4.4b; Spearman correlation $r = 0.61$, $p =<0.0001$). The averaged normalized replicative capacity values of the chimeras ranged from 0.05 to 1.2 showing a narrower range than has been reported for *gag* chimeras on the subtype C MJ4 backbone, with most viruses replicating worse than NL4.3 and demonstrating that substitution of *gag* can have an impact on the ability of the virus to replicate in cells.

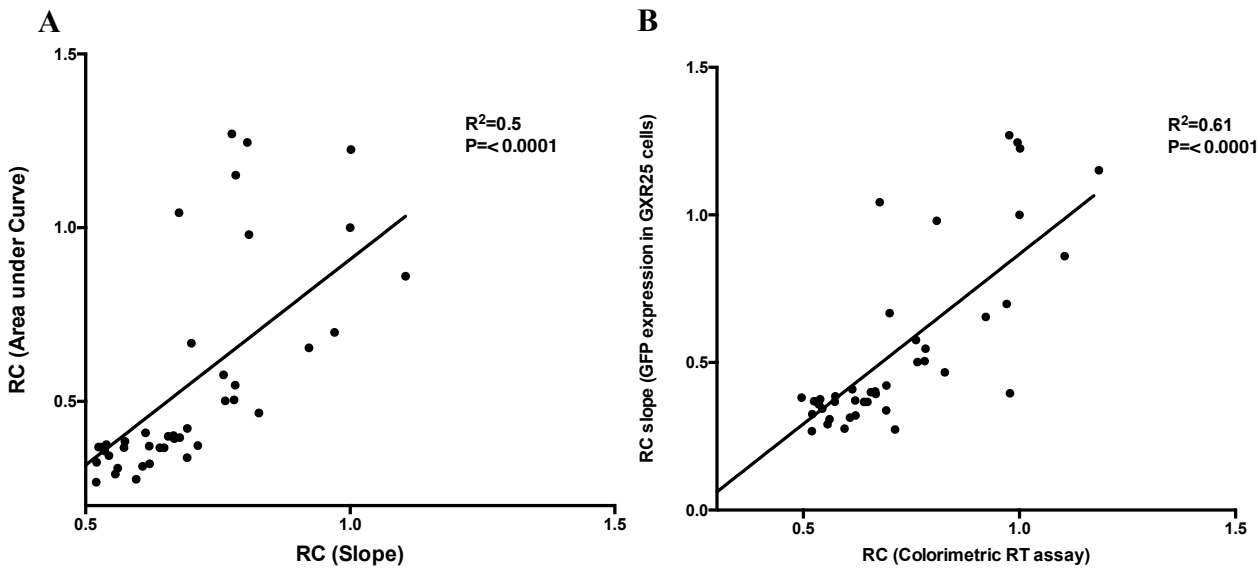


Figure 4.4: A comparison of various ways of measuring replicative capacity.

A comparison of replicative capacity defined by slope vs area under the curve and (A) and a comparison of replicative capacity defined by GFP expression compared to reverse transcriptase activity (B).

4.2.3 Replicative capacity in multivariant infection

Some studies into transmission pairs have suggested that founder viruses possess greater fitness than non-transmitted variants, but some groups find no evidence for increased fitness or particle infectivity (Deymier et al. 2015; Biesinger et al. 2010; Naidoo et al. 2017; Oberle et al. 2016; Parrish et al. 2013; Iyer et al. 2017). Moreover, the host factors that select for founder viral replicative capacity or allow for propagation of one variant over the other are not well understood. Replicative capacity in cases of multivariant infection was therefore examined to elucidate whether the transmission bottleneck within an individual patient would select for variants of similar replicative capacity traits. There was a wide range of replicative capacity of intra patient founder viruses and no evidence for selection of similar replicative capacity (Figure 4.5), suggesting that any reasonably fit virus could be transmitted. Replicative capacity values above the cohort median were defined as high replicative capacity while those below the cohort

median were defined as low replicative capacity. Surprisingly, there was no correlation between the number of sequences obtained per founder variant and the replicative capacity (Figure 4.6; Spearman correlation r = 0.06, p =<0.28), suggesting that the overrepresentation of some sequences within the patient were not a result of higher replicative capacity of that virus, and may have been a result of either a high inoculation dose of that variant, or high fitness of genes outside of *gag*.

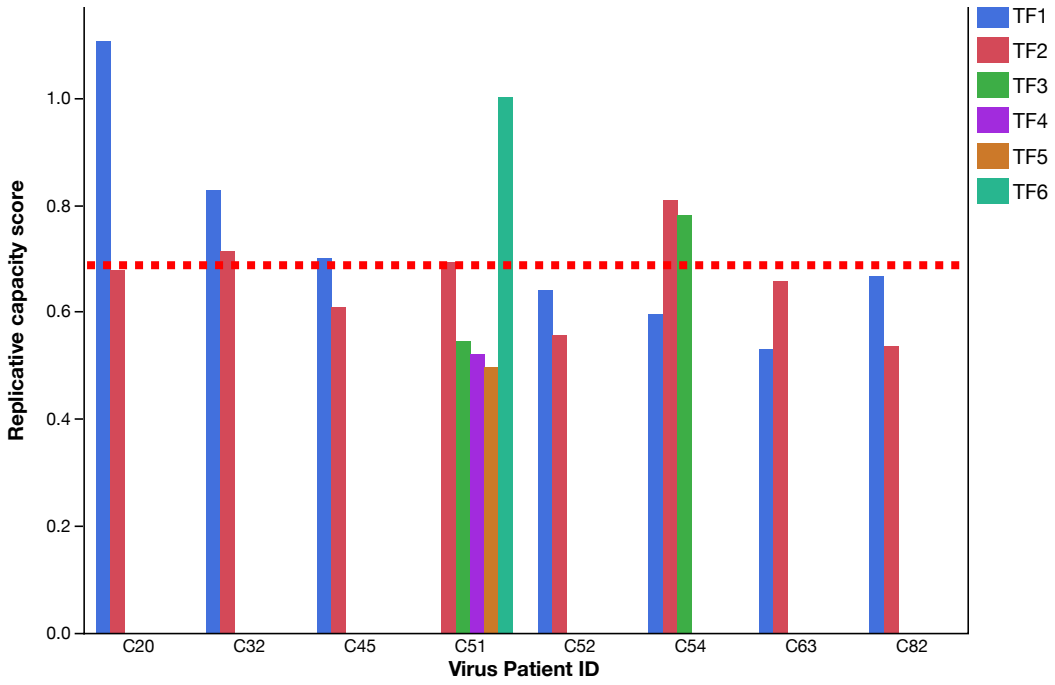


Figure 4.5: Replicative capacity profiles in multivariant infection.

There was a wide range of replicative capacity of intra patient founder viruses and no evidence for selection of similar replicative capacity. The red line indicates the cohort median replicative capacity score.

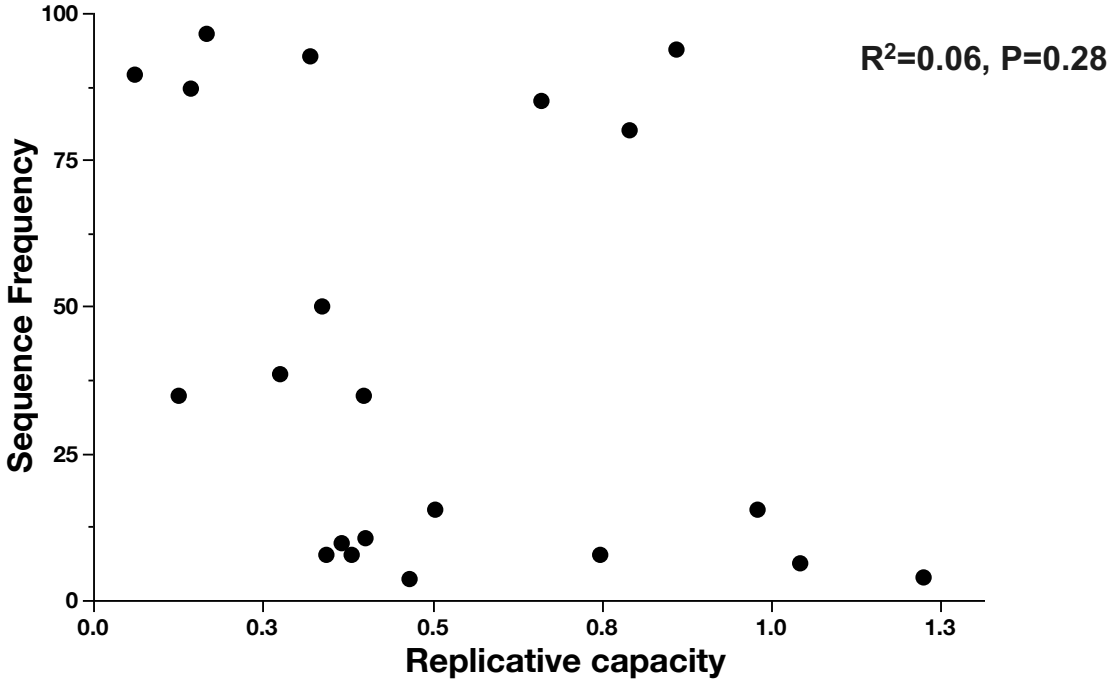


Figure 4.6: Correlation between frequency of a variant's sequences and replicative capacity. Founder viruses that were overrepresented were not necessarily the highest in replicative capacity.

4.2.4 Replicative capacity conferred by subtype A *gag* influences disease progression

Previously, our work, and that of others, showed that transmitted viral characteristics in subtype C infection significantly correlate with early set point viral load (SPVL) (Fraser et al. 2014; Goepfert et al. 2008; Yue et al. 2015; Yue et al. 2013) as well as CD4 T-cell decline even in the context of viral control by previously identified host factors, such as protective HLA alleles (Prince et al. 2012). However, the role of viral replicative capacity in subtype A disease progression and in different risk groups including MSM has not been adequately studied. An examination of the replicative capacity of subtype A transmitted *gag* allowed us to determine the role of viral replication in defining set point viral load in acutely infected MSM before

significant viral adaptation to immune pressure of the host had taken place, which might confound the relationship of replicative capacity to viral load. The role of *in vitro* replicative capacity in disease progression in subtype A infection was therefore investigated by comparing replicative capacity scores with longitudinal CD4 T cell counts, viral loads and set point viral load.

There was a significant correlation between the average CD4 T cell counts and the replicative capacities of Gag-NL4.3 chimeras. At all the time points, patients infected with high replicative capacity virus had lower CD4 T cell counts (Figure 4.7a, p=0.0031) and also had lower nadir CD4 counts (Figure 4.7b, p=0.0186). In a Kaplan-Meier survival analysis with an end point of CD4 T cell count <400 cells/mm^3 there was a difference in the number of individuals that maintained CD4 counts >400 cells/mm^3 blood between those infected with viruses that replicate very poorly and those infected with highly replicating viruses within the first 5 years of infection, demonstrating a median difference of 360 days before falling below the CD4$^+$ count cut off between individuals infected with low and high replicating viruses (Figure 4.7c, p = 0.001).

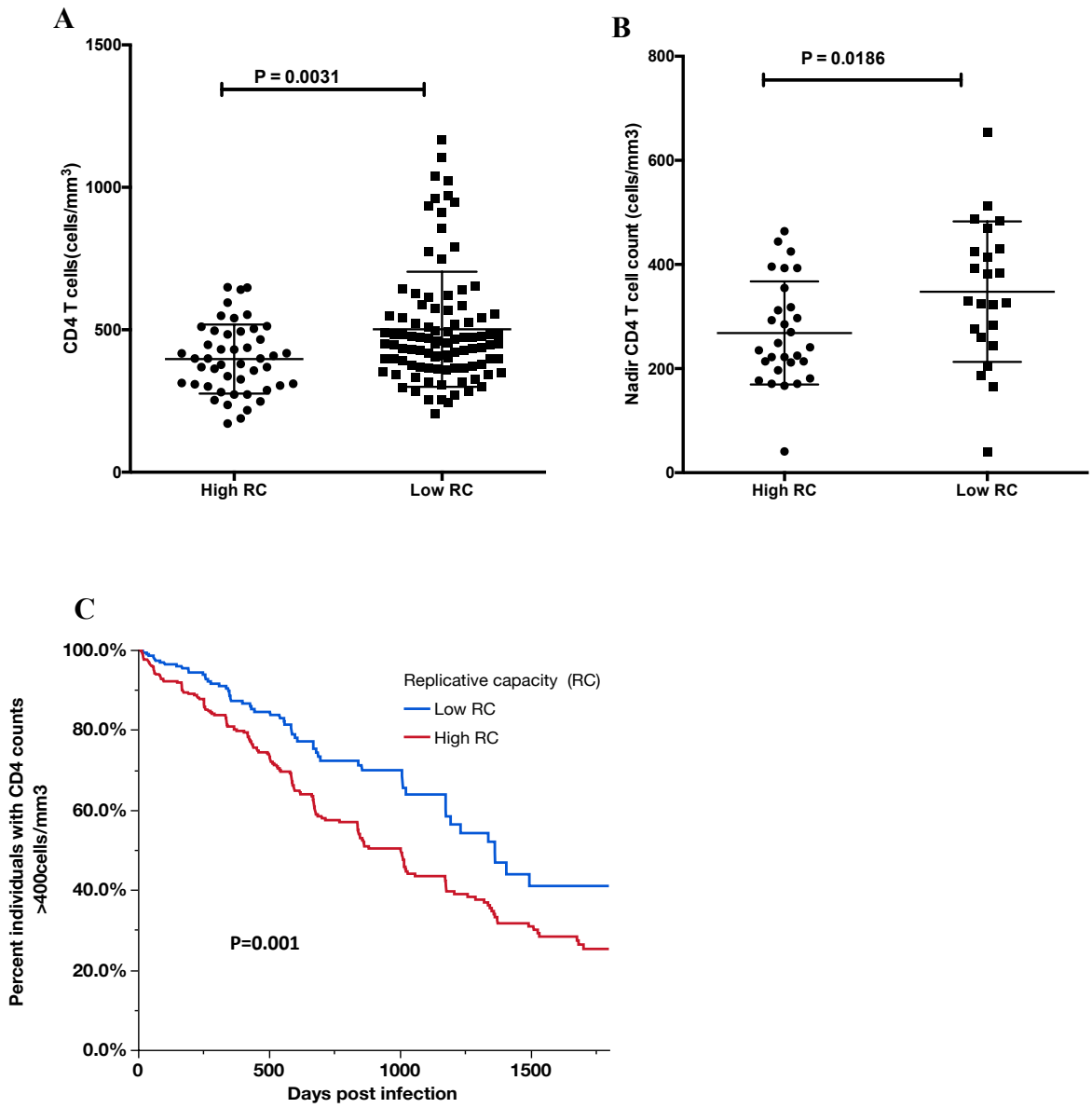


Figure 4.7: Replicative capacity influenced disease progression.

High replicative capacity viruses were associated with lower CD4 T cell counts (A), lower nadir CD4 T cell counts (B) and faster decline to CD4 counts under 400 cells/mm^3 (C).

We sought to more definitively determine if the replication capacity defined by the *gag* gene affected CD4 T cell decline in a manner linked to, or independent of the well documented effect of early set point viral load on subsequent disease progression. Overall, as expected there was an inverse relationship between CD4 T cell count and viral load regardless of the replicative capacity of the infecting virus, although the association was stronger in high RC infection

(Figure 4.8a, High RC R^2=0.27 P<0.001, Low RC R^2=0.21 p=<0.001). Moreover, the relationship between CD4 T cell count and set point viral load was only observed in high replicative capacity (Figure 4.8b, R2=0.07 P<0.001) but not low replicative capacity infection (Figure 4.8b, R2=0.0 P=0.5).

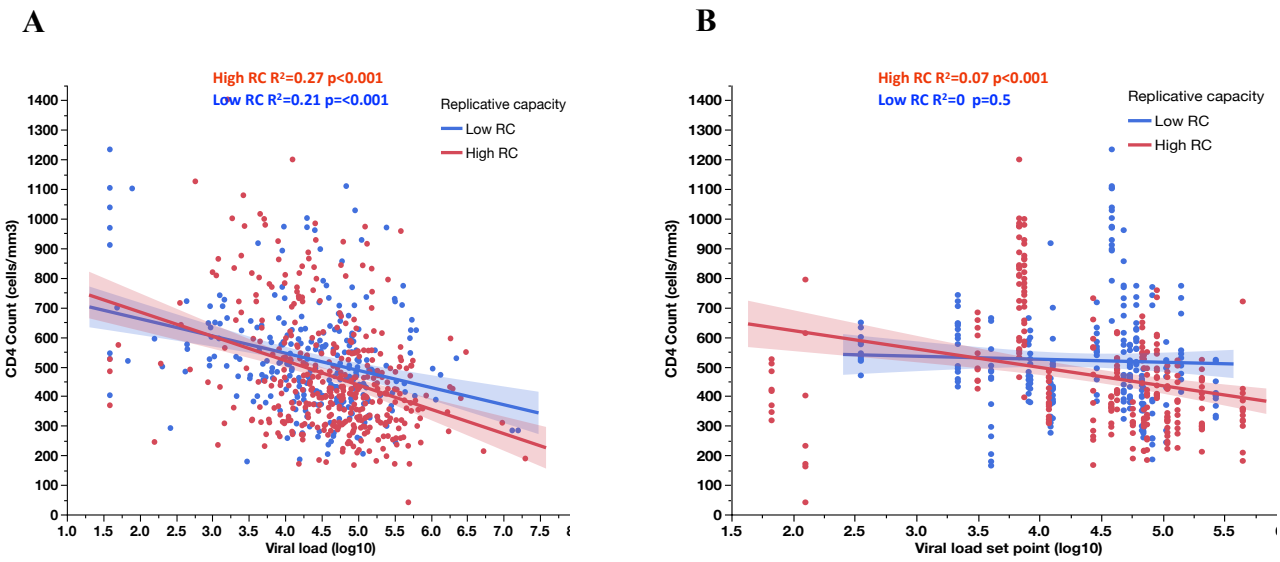


Figure 4.8: CD4 T cell count inversely correlated with viral load (A) and set point viral load (B). The relationship between CD4 T cell count and set point viral load was only observed in high replicative capacity (R^2=0.07 P<0.001) but not low replicative capacity infection (R^2=0.0 P=0.5).

Set point viral load >10^5 RNA copies/mL plasma was associated with poor outcomes for all volunteers as expected (Figure 4.9a, P < 0.0001). However, replicative capacity significantly dichotomized the trajectory of viral load (Figure 4.9b, P < 0.001) and CD4 T cell decline (Figure 4.7c, p<0.0001) in all individuals, more so in individuals with SPVLs <10^5. This suggested independent, but additive effects, of both replicative capacity and set point viral load on HIV-1 disease progression and demonstrates that infection with attenuated viruses may impart some survival benefit to newly infected individuals. This early benefit is not transient and quickly

lost but persisted over the first 5 years of infection. Taken together, these data firmly establish replicative capacity of the founder virus as a distinct contributor to HIV-1 disease progression in subtype A infection.

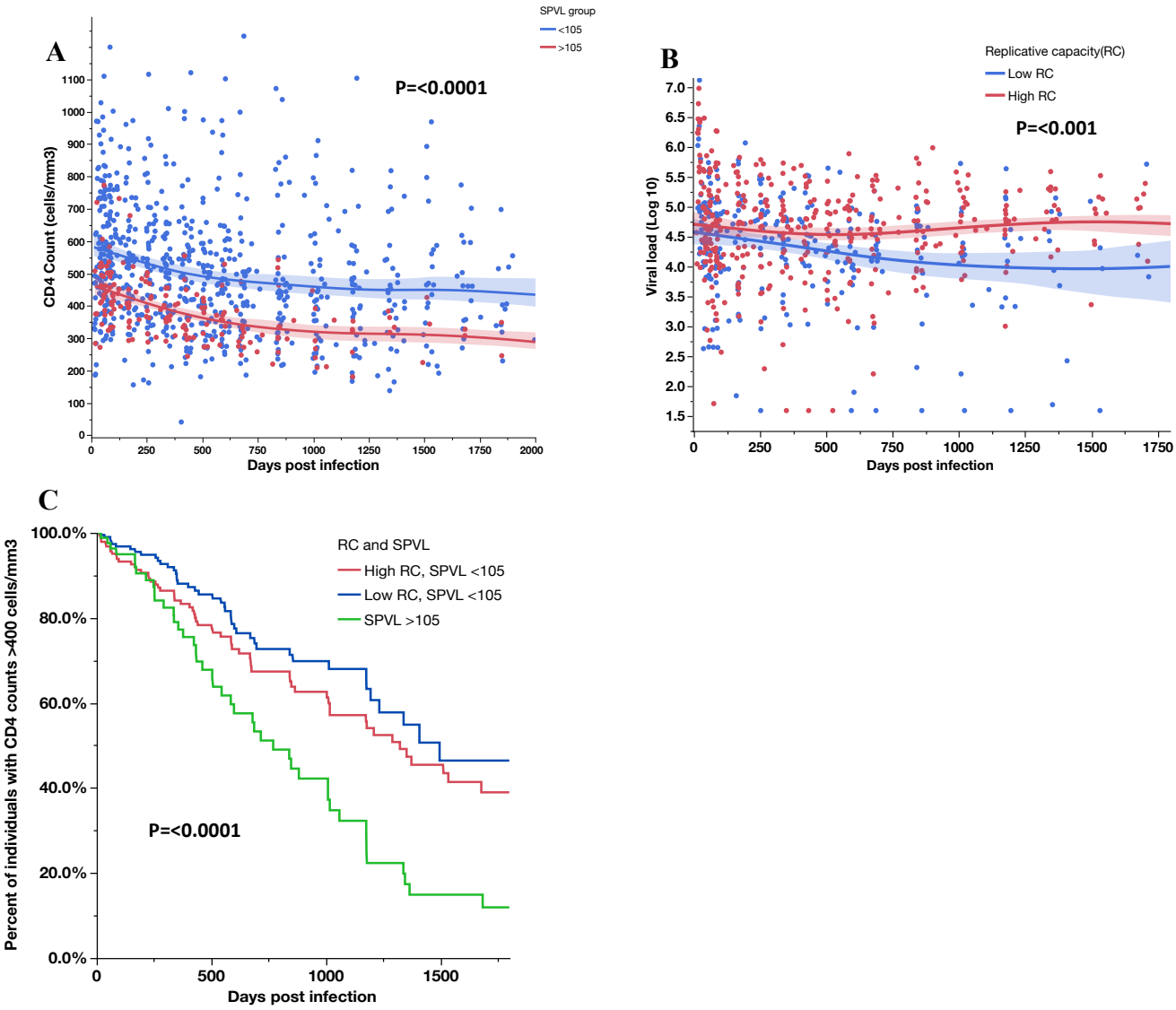


Figure 4.9: CD4 T cell counts were lower over time in patients infected with high replicative capacity virus (A), while viral load was higher over time (B).

Patients with a set point viral load above 10^5 progressed faster than those with set point viral load above 10^5 (C), and a clinical benefit of low replicative capacity virus could be observed in patients with set point viral load above 10^5.

4.2.5 HLA associated polymorphisms in *gag* associated with changes in viral replicative capacity

Uncovering sites of vulnerability in HIV-1 is a high priority for the informed design of an effective HIV-1 vaccine (Goulder and Watkins 2004; Smith 2004). Therefore, all 48 *gag* sequences and their replicative capacity were examined using an exploratory pairwise analysis to uncover residues that significantly affect the *gag* chimeras' ability to replicate *in vitro*.

Seven polymorphisms were associated with high replicative capacity and 10 with low replicative capacity. Ten of these polymorphisms were HLA-B linked while only 4 were HLA-A associated and 3 were associated with both HLA-A and -B. Polymorphisms that were associated with increased replicative capacity include mutations on the GAG271, K76R, SL9 and QW9 epitopes while mutations on the RY10 epitope were only found in low replicative capacity chimeras (Figure 4.10). All the other observed polymorphisms occurred in different combinations as shown in Figure 4.10 making it difficult to quantify the net effect of each polymorphism on replicative capacity. This is consistent with the fact that not all HLA associated polymorphisms within a particular Gag sequence will necessarily reduce fitness (Brener et al. 2018). Therefore, it is likely the balance and interaction of both fitness increasing and fitness decreasing polymorphisms within a particular sequence that ultimately determines the replicative capacity of the virus.

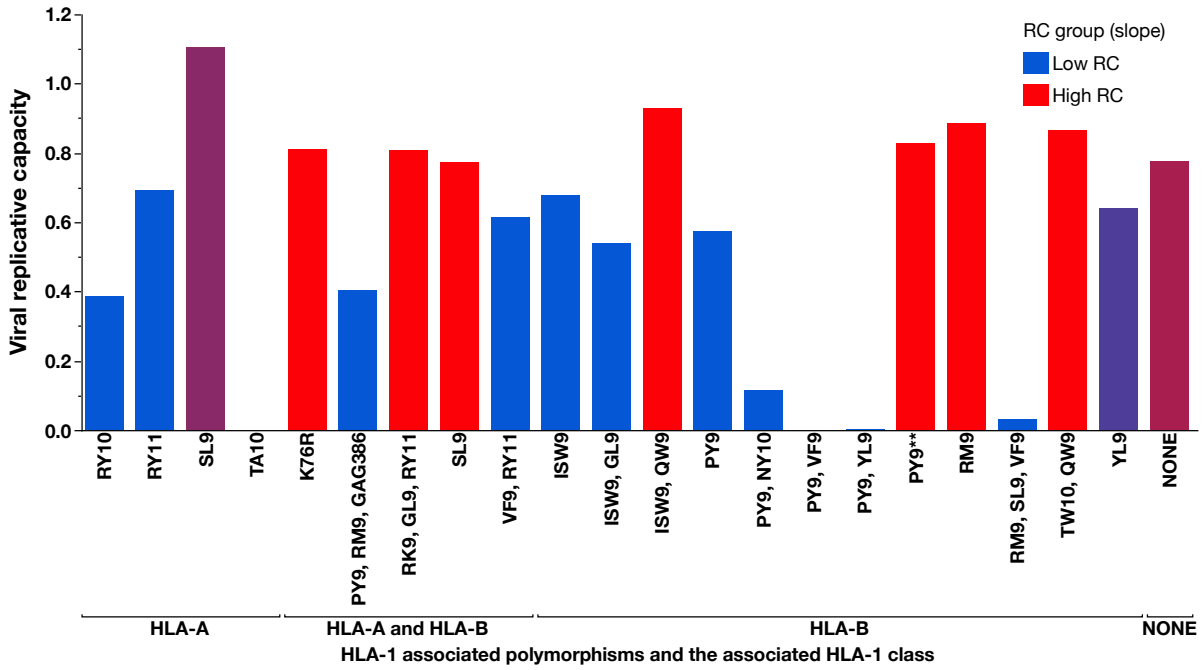


Figure 4.10: HLA associated polymorphisms influenced viral replicative capacity.

Some polymorphisms were associated with increased replicative capacity (red) and some with reduced replicative capacity (blue), with the purple colour denoting both opposing effects of that polymorphism.

The locations of all HLA associated polymorphisms along with their effects on replicative capacity was compared to the median replicative capacity of all viruses, and results are plotted linearly on a graphical representation of the Gag protein (Figure 4.11). Residues that modulate replicative capacity were enriched in p17 (Fisher's exact test, p=0.0001), consistent with previous reports that determinants underlying subtype differences in the replicative capacity conferred by Gag are located outside of the p24 capsid protein (Kiguoya et al. 2017).

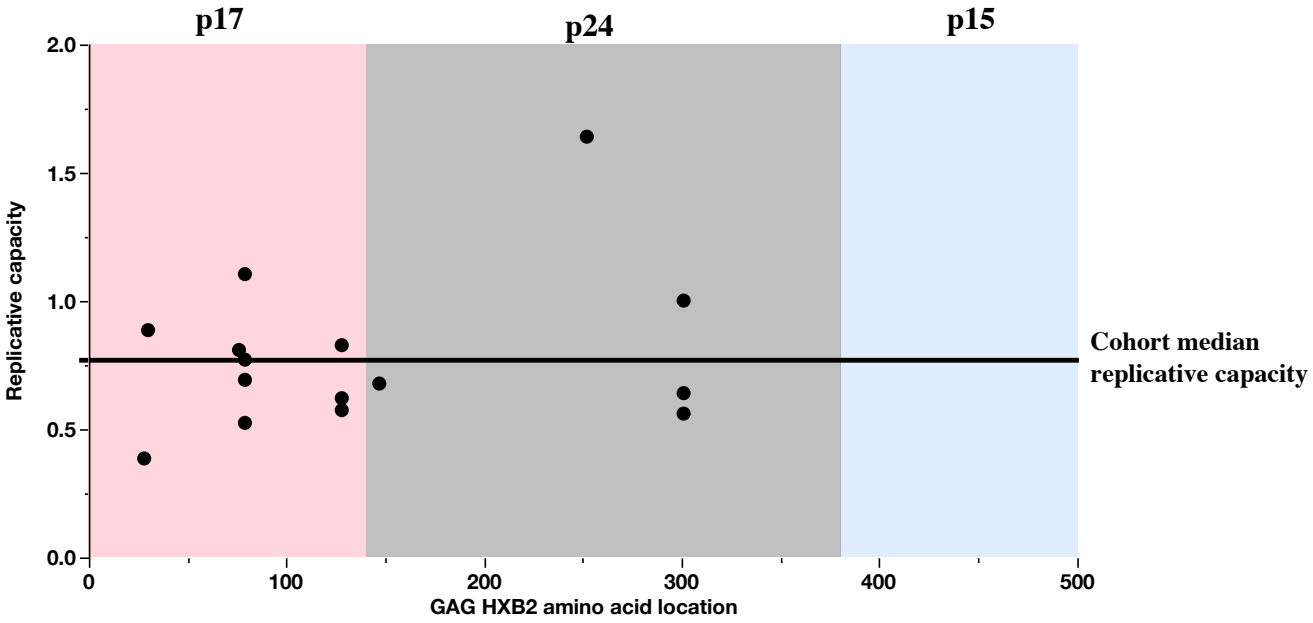


Figure 4.11: Locations of all HLA associated polymorphisms along with their effects on replicative capacity. Polymorphisms that modulate replicative capacity were enriched in p17.

4.2.6 Measurement of HIV-1 spread between cells

Having confirmed a role for viral replicative capacity in HIV-1 subtype A pathogenesis, the underlying mechanisms by which replicative capacity of transmitted HIV-1 impacts the trajectory of CD4 T cell decline were evaluated. As the role of replicative capacity was independent of but additive with that of viral load, high replicative capacity viruses may have been better spread by cell-cell transmission, which is a more efficient mode than cell free infection and could potentially account for the incomplete association with of CD4 T cell decline with viral load.

To understand the relative contribution of cell contact to viral dissemination, the mode of virus spread between cells was evaluated by comparing direct-contact routes to cell-free routes as described in the methods chapter. The donor cells were either Jurkat or purified primary CD4

T cells labelled with fluorescent EF670 dye and the target cells were either Jurkat or purified primary human CD4 T cells labelled with fluorescent EF450 dye, either in contact to measure cell-cell transmission or in transwell plates to measure cell free infection. Production of p24 in target cells was measured by flow cytometer 24 hours after infection, which several groups have shown to be the optimal time to measure cell-cell transmission, without the interference of cell free infection (Mohammadi et al. 2013; Boulle et al. 2016; Gupta et al. 1989; Law et al. 2016; Zhong, Agosto, Ilinskaya, et al. 2013).

Even though all donor cells were initially infected at the same MOI, there was a strong correlation between the number of infected donor and target cells for both cell free and cell-cell transmission (Figure 4.12a and b; cell free R^2=0.53 p=<0.0001, cell-cell R^2=0.24 p=0.0004). Therefore, to correct for the differences in donor cells infection, an infectivity index was derived by normalising the number of p24 positive target cells to the number of p24 positive donor cells. The infectivity index was higher in cell-cell transmission compared to cell free spread in both CD4 and Jurkat cells (Figure 4.12c; p<0.0001and p<0.0001 respectively). These results are consistent with other findings that during culture, the levels of cell-free viral transfer are very small relative to those of cell-to-cell transfer (Jolly and Sattentau 2004; Sourisseau et al. 2007).

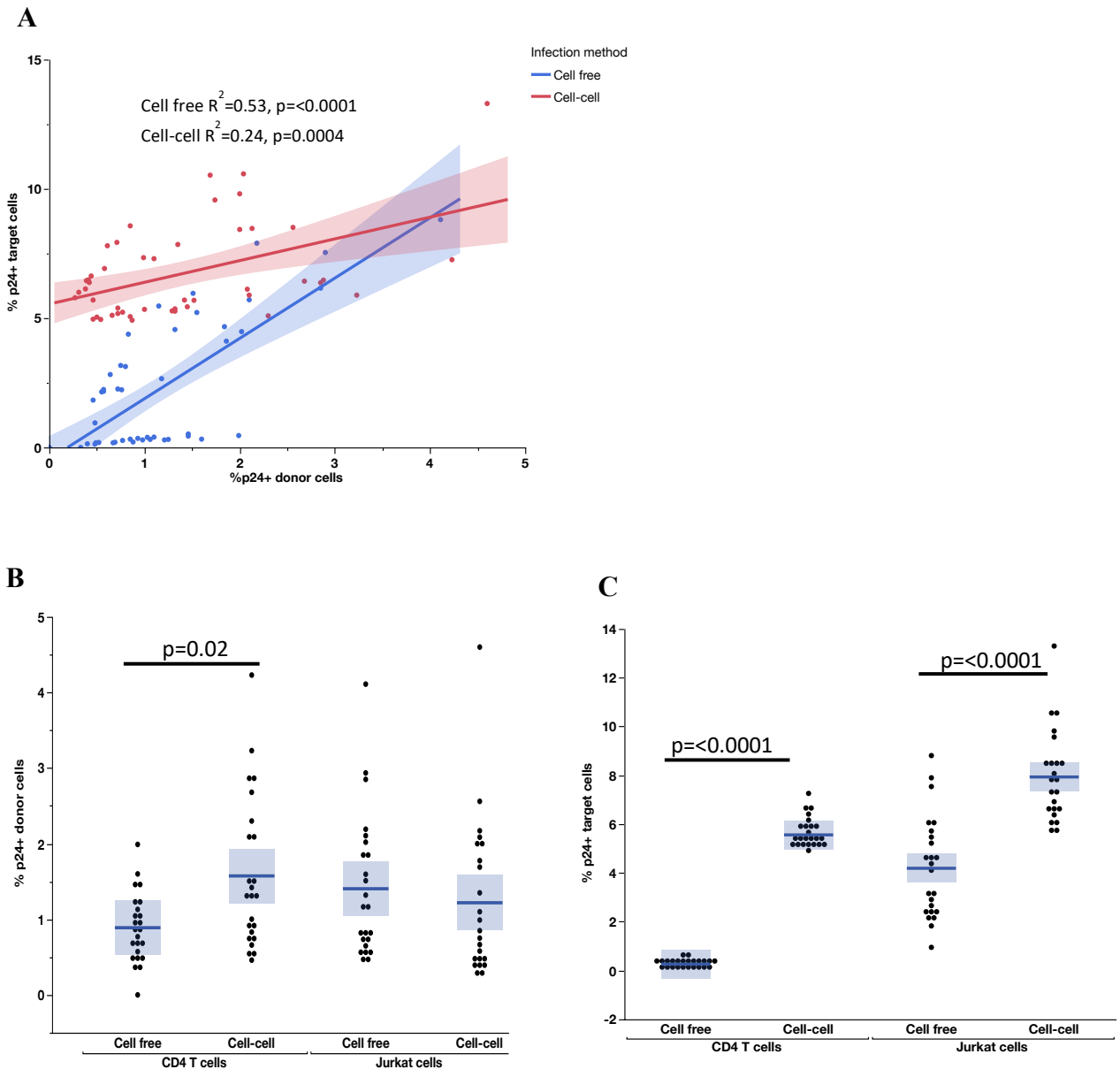


Figure 4.12: Measurement of cell-cell and cell-free spread of gag-NL4.3 chimeras.

Measurement of cell-cell and cell-free spread of *gag*-NL4.3 chimeras from infected donor cells to target cells. The number of infected donor cells and infected target cells correlate in both cell free and cell-cell transmission (A and B). A higher infectivity index was observed in cell-cell transmission.

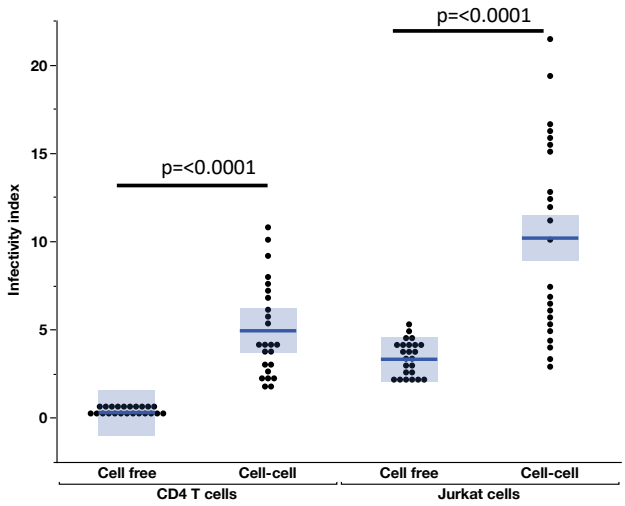


Figure 4.13: Infectivity index was higher in cell-cell transmission in both sorted CD4 T cells and Jurkat cell line. Infectivity index was a measure that had been derived by normalising the number of p24 positive target cells to the number of p24 positive donor cells to correct for the differences in donor cells infection. The infectivity index was higher in cell-cell transmission compared to cell free spread in both CD4 ($p<0.0001$) and Jurkat cells ($p<0.0001$)

4.2.7 Does cell-cell transmission drive replicative capacity?

Having measured cell-cell transmission, a relationship between the infection index in cell-free vis-a-vis cell-cell transmission with replicative capacity was investigated. Replicative capacity did not correlate with infectivity index in cell free infection, both in the Kilifi (subtype A *gag* on NL4.3 backbone) and Zambian cohort (subtype C *gag* on MJ4 backbone).

In contrast, high replicative capacity *gag* conferred a striking increase in cell-cell infectivity on subtype A *gag* on an NL4.3 backbone, but not on subtype C *gag* on MJ4 backbone (Figure 4.14). The difference in the relationship between viral replicative capacity and cell-cell infectivity is likely a result of the differences in the proviral backbones used. While MJ4 is a

clade C R5 virus, NL4.3 is a clade B X4 and highly pathogenic strain that is well known to be capable of spreading through virological synapses. Nonetheless, this data suggests that at least on an NL4.3 backbone, the mechanisms that confer increased replicative capacity overlap with those involved in cell-cell transmission, but not cell free transmission.

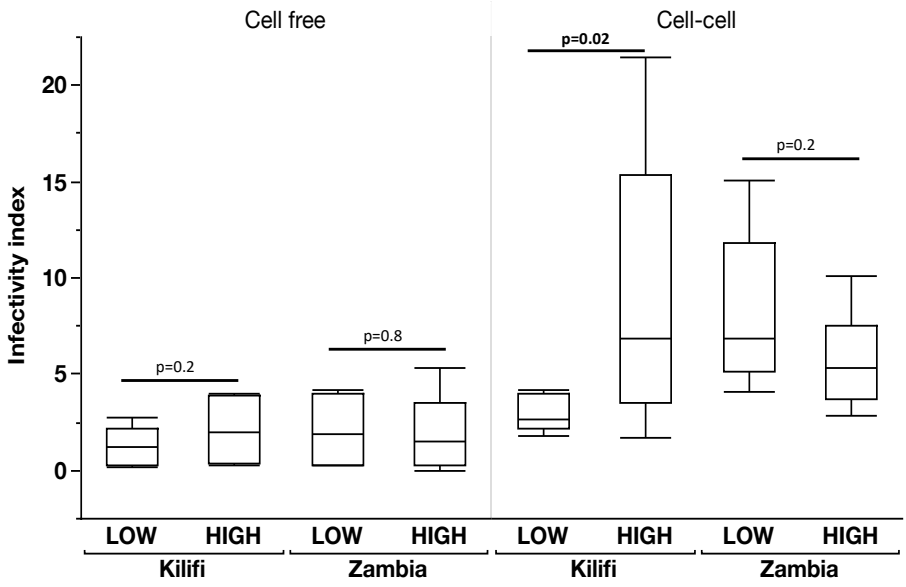


Figure 4.14: A comparison of cell-cell and cell-free spread between cells infected with *gag*-chimeras of high and low replicative capacity.

A comparison of cell-cell and cell-free spread between cells infected with *gag*-chimeras of high and low replicative capacity, in the Kilifi (subtype A *gag* on NL4.3 backbone) and Zambian cohort (subtype C *gag* on MJ4 backbone).

To establish whether the increased cell-cell infectivity in high replicative capacity *gag* on NL4.3 was associated with increased release of virus, virus concentration in culture supernatant drawn from cell-cell transmission experiments in high and low replicative capacity was compared. There was no difference in the virus quantities in culture supernatant after 24 hours of culture in both the Kilifi and Zambia cohort (Figure 4.15). As it was difficult to establish

whether the detected virus had been produced by donor or target cells, this virus could in part be due to increased release by the infected donor cells, but also points to the possibility of a faster life cycle in the target cells infected with high replicative capacity viruses.

Increased cell-cell transmission in the Kilifi cohort over the Zambian cohort may therefore represent the result of subtype differences in *gag* but is more likely to be the result of the differences in pathogenicity of NL4.3 and MJ4. Nonetheless, within the Kilifi cohort where *gag* sequences were cloned on a backbone that is well known to effectively spread by cell-cell transmission, the result of high replicative capacity *gag* on cell-cell spread was evident.

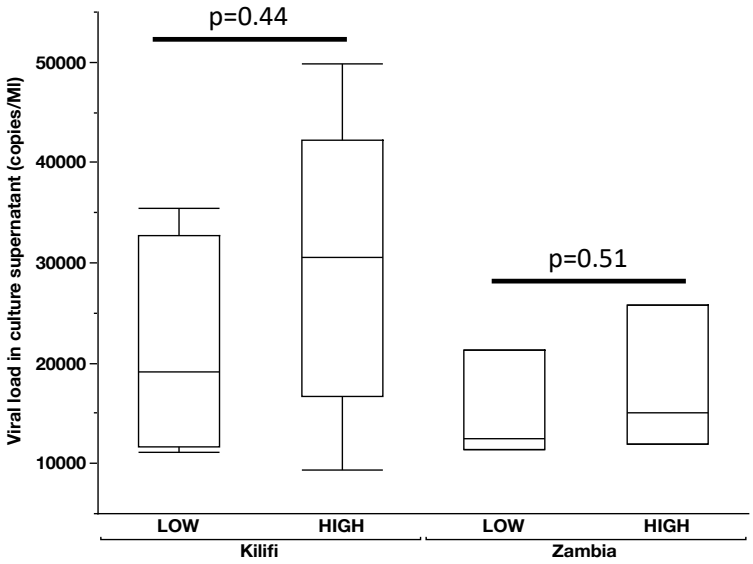


Figure 4.15: Virus concentration in culture supernatant of cell-cell transmission experiments. No difference in Zambian *gag* chimeras (subtype C *gag* on MJ4 backbone) however I observed a trend towards increased cell-free virus in high replicative capacity cultures of the Kilifi cohort (subtype A *gag* on NL4.3 backbone).

4.2.8 Does viral replicative capacity define mechanisms of CD4 T cell death?

4.2.8.1 Expression of cell death receptors

Research from multiple groups suggests that the majority of CD4 T cells that die in lymph nodes during HIV-1 infection are cells that would normally be refractory to productive HIV-1 infection and are therefore not productively infected. Such cells die by caspase 1 mediated cell death. It is possible that transmitted/founder virus factors that influence the kinetics of productive infection and cellular permissiveness to HIV-1 infection such as replicative capacity and cell-cell transmission that are also associated with faster decline of CD4 T cell counts may be associated with differential pathways of CD4 T cell death. The expression of caspase 1, caspase 3 and CD95 (FAS-R) which are mediators of pyroptosis, apoptosis and FAS-L mediated cell death respectively in CD4 T cells from patients infected with founder viruses of known replicative capacity was therefore measured. While the expression of caspase 3 and CD95 was similar in multiple memory subsets regardless of replicative capacity, there was higher expression of caspase 1 in central memory CD4 T cells during acute infection ($p=0.02$), 6-9 ($p=0.04$) and 24-30 ($p=0.01$) months after infection in patients infected with high replicative capacity founder viruses, and in naïve CD4 T cells at acute infection (Figure 4.16, $p=0.05$). These differences were not observed in effector memory or terminal effector CD4 T cell subsets.

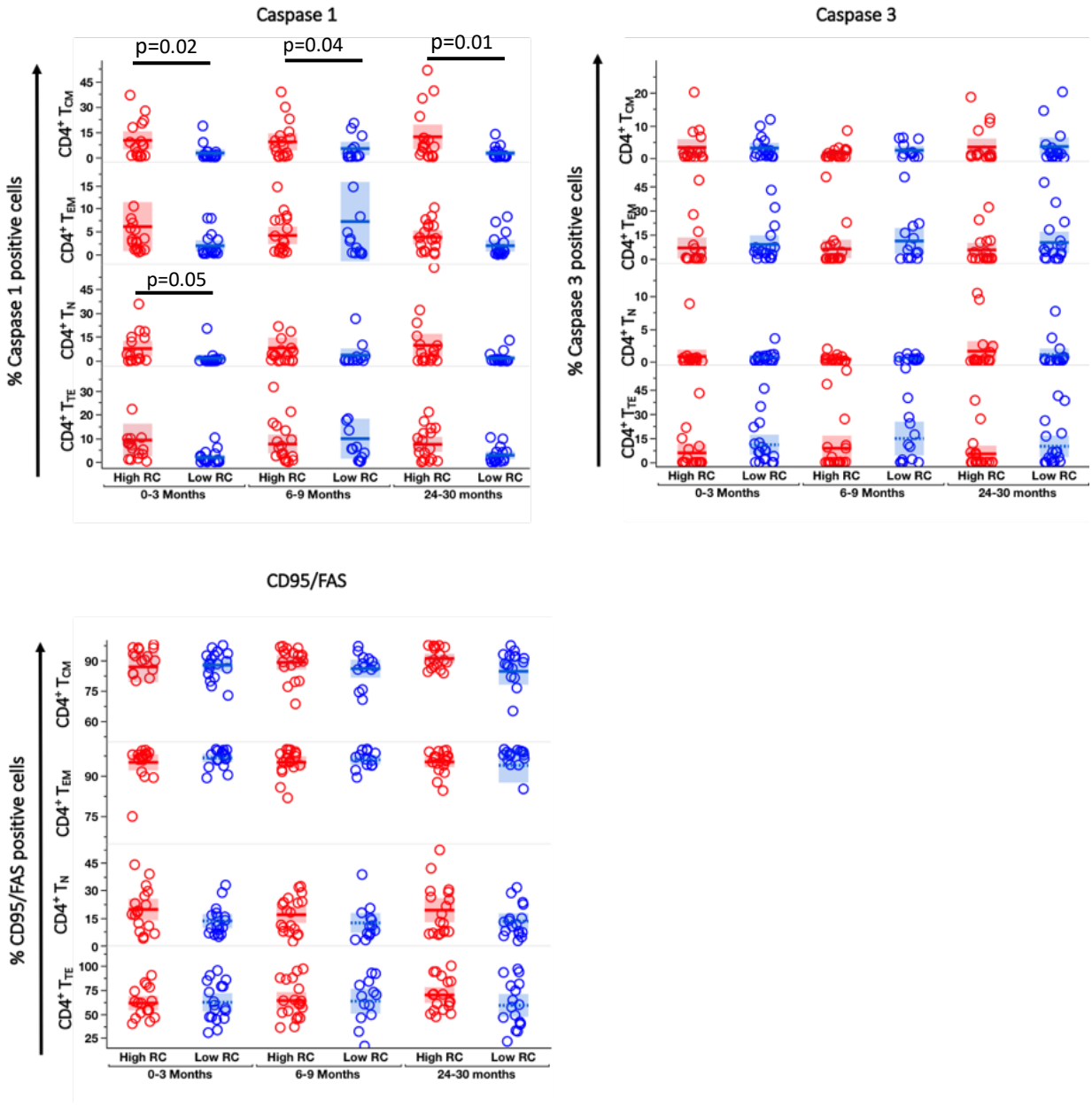


Figure 4.16: Expression of Caspase 1, Caspase 3 and CD95 in CD4 T cell memory subsets.

While the expression of caspase 3 and CD95 was similar in multiple memory subsets regardless of replicative capacity, there was higher expression of caspase 1 in central memory CD4 T cells during acute infection (p=0.02), 6-9 (p=0.04) and 24-30 (p=0.01) months after infection in patients infected with high replicative capacity founder viruses, and in naïve CD4 T cells at acute infection (p=0.05). p values have been corrected using the Benjamini-Hochberg technique as described in the materials and methods.

4.2.8.2 RNA related to cell death

Next, a relationship between increased replicative capacity and increased cell-cell transmission and the expression profile of transcriptomes of even more mediators of cell death was investigated by measuring the expression of mRNA encoding protein that are associated with apoptosis, necrosis and autophagy in sorted cell aliquots from the cell-cell transmission assay described in Section 2.7 and 4.2.6, and in cryopreserved PBMC drawn from patients infected with high vs low replicative capacity virus within the first 3 months of infection.

Overall, there was higher expression of mRNAs involved in cell death pathways in cultured CD4 T cells compared to PBMC (Figure 4.17). Within the *in vitro* cell-cell transmission assay set-up, the highest expression of pro-apoptotic, autophagy and necrosis markers was observed in high replicative capacity viruses from both Kilifi and Zambia, and in high cell-cell transmission, further suggesting that the cell death pathways in high replicative capacity infection and cell-cell transmission overlap. The cell-death RNA profile in low replicative capacity infection resembled that of cell free infection and close to that of HIV-1 uninfected cells, which is consistent with *in vivo* findings that these patients had preserved CD4 T cell counts. There were no differences in the expression of mRNA that are specifically related to either apoptosis, necrosis or autophagy as a result of replicative capacity.

Of note, RNA encoding the anti-apoptotic protein NOL3 that has been shown to down-regulate the enzyme activities of caspase 2, caspase 8 and tumour protein p53 (Templin et al. 2017) was only upregulated in increased cell to cell transmission both *in vitro* and *ex vivo* but not in high replicative capacity infection (Figure 4.18). Likewise, CTSS which encodes the poly(ADP-ribosyl) transferase-like 2 protein (Pawar et al. 2016) and BMF which encodes the E1-like activating enzyme that is essential for autophagy and cytoplasmic to vacuole transport (Liew et

al. 2014; Oudenaarden, van de Ven, and Derksen 2018) were only upregulated in high replicative capacity infection, both *in vitro* and *ex vivo*.

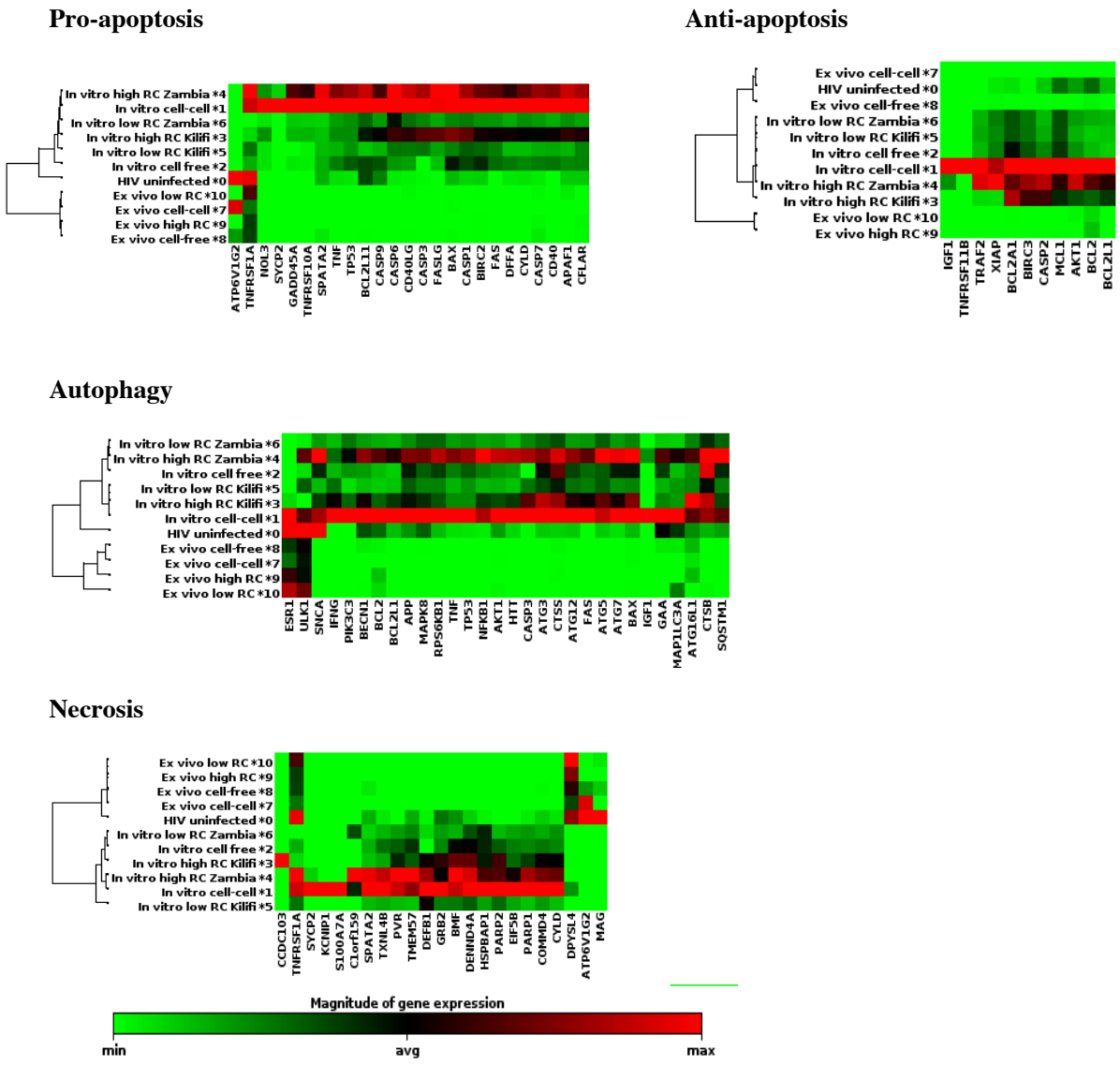


Figure 4.17: *In vitro* and *ex vivo* expression of RNA encoding mediators of apoptosis, autophagy and necrosis in infection with viruses of high vs low replicative capacity represented as fold increase over uninfected cells.

There was higher expression of mRNAs involved in cell death pathways in cultured CD4 T cells compared to PBMC. Within the *in vitro* cell-cell transmission assay set-up, the highest

expression of pro-apoptotic, autophagy and necrosis markers were observed in high replicative capacity viruses from both Kilifi and Zambia, and in high cell-cell transmission.

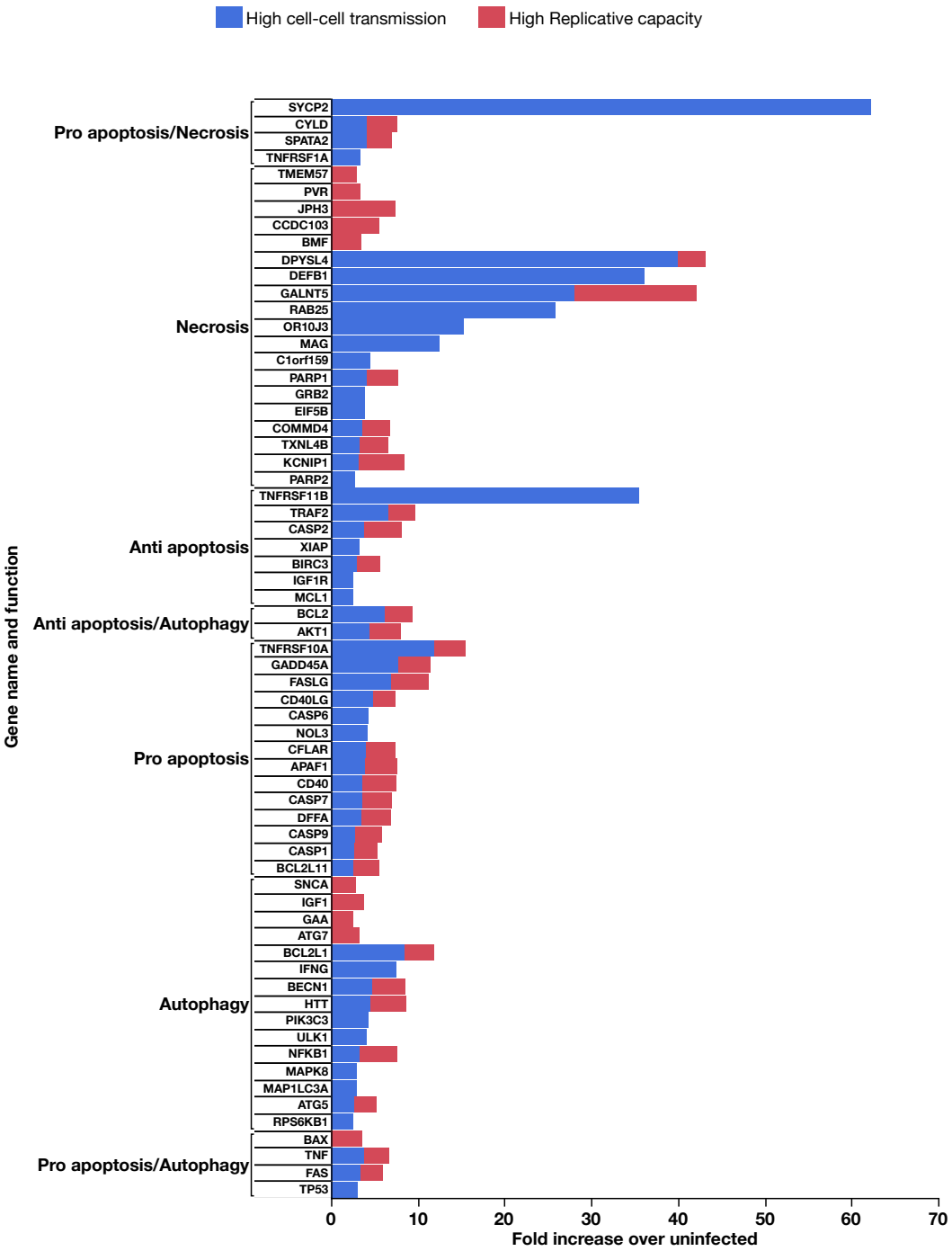


Figure 4.18: Fold increase in the expression of mRNAs encoding proteins related to cell death pathways.

Fold increase in the expression of RNA related to cell death pathways in *in vitro* cultures of sorted CD4 T cells infected with *gag* chimeras of high repliactive capacity, and in cell-cell treansmission.

Within sorted CD4 T cells where the highest expression of RNA was observed, high replicative capacity infection was associated with upregulation of RNA related to the regulation of necrosis including the transmembrane protein 57 (TMEM57), PVR which encodes a transmembrane glycoprotein belonging to the immunoglobulin superfamily, Juctophilin 3 (JPH3) which encodes a component of junctional complexes with specific affinity for plasma membranes, CCDC103 which is also associated with dyskinesia and BMF apoptosis regulators in the BCL2 family (Anding and Baehrecke 2015; Green and Llambi 2015; Romero et al. 2017; Torphy, Schulick, and Zhu 2017).

Of note, expression of the apoptosis executioner caspase 3 that activates caspases 6, 7, and 9 (Cummins and Badley 2010; Julien and Wells 2017) was upregulated 1025-fold in *in vitro* cell-cell transmission in sorted CD4 T cells but only 2.8-fold in *in vitro* high replicative capacity infection in sorted CD4 T cells (not shown in graph). This is especially surprising as the intracellular expression of active caspase 3 in any of the memory CD4 T cell subsets was not increased. Likewise, expression of ESR1 which encodes estrogen receptor 1 that plays a role in regulation of autophagy (Lacroix 2006; Suba 2017) was upregulated 101-fold in cell-cell transmission but not in high replicative capacity infection.

4.2.9 Multivariate analysis of viral predictors of disease progression

Having identified infection with multiple founder variants, APOBEC induced hypermutation, the degree of HLA preadaptation and replicative capacity conferred by the transmitted Gag as viral characteristics that contribute to disease progression in univariate analysis, we employed multivariate methods assess the impact of each of these variables on the rate of $CD4^+$ T cell

decline, while adjusting for host genetic and viral characteristics known to influence this rate. This also allowed us to identify possible interactions between the contributors.

Generalized linear models (IBM SPSS 21) were applied to test for interactions between these parameters including infection with multiple founder variants, APOBEC induced hypermutation, viral replicative capacity and HLA preadaptation. $CD4^+$ T cell count in the first month of infection was chosen as an endpoint as it is a well-known predictor of disease progression. Including other well-known predictors of disease progression including set point viral load and HLA B*57 (n=3) and B*58 (n=5) allowed us to interpret the data with these interactions considered.

Multivariant infection and high replicative capacity remained significantly associated with a low $CD4^+$ T cell count in the first month of infection (p=0.0002 and 0.003 respectively). However, even though patients who were infected with multiple founder variants of high replicative capacity also had lower $CD4^+$ T cell counts in the first month of infection (p=0.015), their $CD4^+$ T cell counts were not as low as when multivariant infection and high replicative capacity were considered in isolation. This reflected our earlier observation that multivariant infection was characterized by lower replicative capacity reflective of a less stringent transmission bottleneck, and infection with multiple founder variants of high replicative capacity was a rare occurrence.

A high HLA-1 preadaptation score (defined as the number of HLA-1 alleles that the founder virus was already preadapted to, with a score of zero denoting no preadaptation and a score of 6 denoting preadaptation to both HLA-1 alleles, both HLA-B alleles and both HLA-C alleles) was also associated with low $CD4^+$ T cell counts in the first month of infection. This suggests that T/F escape from CTL recognition may have been a contributor to the observed decline of

$CD4^+$ T cells. Enrichment for APOBEC induced hypermutation was not predictive of a low $CD4^+$ T cell count in the first month of infection unlike in previous univariate analysis.

Table 3.4: Multivariable model evaluating the role of founder virus characteristics in determining early CD4+ T cell count. A generalized linear model (IBM SPSS21) was used to analyze the independent contribution. Of each of the variables including multivariant infection, replicative capacity, HLA preadaptation and APOBEC induced hypermutation.

Feature	**β**	**95% CI (Lower – Higher)**	**Wald Chi-Square**	**p value**
Early $CD4^+$ T cell count				
Single T/F	394.9	(602.4-187.4)	13.91	0.0002
High RC	505.8	(733.3-278.3)	9.1	0.003
Single TF*High RC	459.0	(89.6-828.3)	5.93	0.015
HLA-1 preadaptation score	93.2	(-12.3-198.8)	2.99	0.044
APOBEC hypermutation	83.5	(7.1- 159.9)	4.59	0.32

The association between number of founder virus and replicative capacity with disease outcome was further confirmed in a multivariable Cox proportional hazards model assessing the relative risk of replicative capacity and multivariant infection to the rate of $CD4^+$ T cell decline to <350 cells/μl in the context of viral load and canonical protective HLA class I alleles B*57 (n=3) and B*58 (n=5) which are well-established predictors of HIV disease progression. Using a backward stepwise deletion approach, we found that multivariant infection, replicative capacity, peak viral load, and the number of HLA escape mutations in the founder variant were each highly significant independent predictors of the rate of $CD4^+$ T cell decline to <350 cells/μl

(p=0.028, 0.049, 0.034 and 0.039 respectively) (Table 3.5). Of note, the protective effect of being infected with a single founder variant and low RC variants was higher than that of having a low peak viral load, as evidenced by the higher hazard ratios (Table 3.5). Taken together, these data firmly establish viral replicative capacity and the number of founder variants as a distinct contributor to HIV disease progression in addition to the well-known influence of immune escape and viral load.

Table 3.5: Multivariate cox proportional hazards model evaluating the role of viral characteristics in independently predicting the rate of CD4+ T cell decline to <350 cells/ul. A cox regression was used to analyze the independent contribution of multivariant infection and high replicative capacity variants in defining the rate of $CD4^+$ T cell decline. Replicative capacity was dichotomized on the median cohort replicative capacity, while peak viral load was defined as the highest viral load observed in the first 45 days of infection. The number of escaped epitopes is the sum of individual CTL escape mutations that were present within Gag.

Feature	**HR**	**95% CI (Lower – Higher)**	**Wald Chi-Square**	**P Value**
Risk of CD4 count <350 cells/ul				
Multiple founder variants	3.317	(1.148-12.008)	4.800	0.028
High RC	7.706	(0.791-75.106)	3.090	0.049
Peak Viral load	2.507	(1.071-5.866)	4.487	0.034
Number of escaped epitopes	1.729	(1.027-2.910)	4.246	0.039

CI, Confidence interval; HR, Hazard ratio

4.3 Discussion

As HIV-1 rapidly evolves following transmission, it is crucial for pathogenesis studies to elucidate the properties of viruses collected soon after transmission, prior to extensive adaptation in the new host. The viral phenotypic properties that are selected for during transmission as well as their impact on disease progression remain unclear.

Some studies have suggested that HIV-1 transmission selects for viruses with high infectivity and replication capacity, and that replicative capacity of the founder virus can predict the rate of subtype C disease progression (Deymier et al. 2015; Prado et al. 2010; Prince et al. 2012; Claiborne et al. 2015; Wright et al. 2012). To assess the impact of the transmitted *gag* sequence on replicative capacity, the *gag* gene which encodes the major structural proteins within the virion was amplified from plasma virus during acute infection time points (median 21 days after estimate date of infection), replication-competent virus produced by cloning the *gag* gene into the lab adapted proviral backbone NL4.3, and replicative capacity measured in an *in vitro* cell culture assay as described previously. When the chimeric virus replicated more slowly, this was taken as a sign of reduced replicative capacity associated with the Gag protein. Previous studies in HIV-1 subtype C had shown that the replicative capacity conferred by *gag* correlates with that of the infectious molecular clone (Claiborne et al. 2015; Prince et al. 2012). This indicates that although other genes undoubtedly play a role in defining *in vitro* HIV-1 replicative capacity, the contributions of *gag* are a significant component of the replicative capacity of the full-length virus, and *gag* chimeras present an important tool in defining the role of *gag* in the overall viral fitness.

Previous studies investigating the role of Gag in viral fitness employed a recombination approach in which sequences are PCR amplified as a bulk population, allowed to recombine into a *gag* deleted *NL4-3*, and resulting viruses propagated in permissive cells (Claiborne,

Prince, and Hunter 2014). In this method, there is no control over the sites of recombination, and it requires the outgrowth of virus which may select for the most fit virus in the population and could also select for sequence changes. In order to avoid these limitations and because study participants were recently infected with HIV-1 where the population is generally homogeneous, a direct cloning method that allows for the introduction of the entire *gag* gene into a replication competent and well characterized provirus, NL4.3, was employed. A homologous recombination approach was employed to fuse the *NL4.3-LTR-U5* sequence with the transmitted *gag* sequence.

Molecular cloning with large (>10kb) retroviral plasmids has traditionally been difficult because large plasmids reduce transformation efficiency of competent bacterial strains (Inoue, Nojima, and Okayama 1990), while retroviral inserts which contain long terminal repeat (LTR) sequences reduce stability of the plasmid and compromise replication fidelity of the plasmid DNA within the bacterial host leading to deletions of the retroviral genome (Bichara et al. 2000). Bacteria transformed with NL4.3 or Gag-NL4.3 plasmid products were grown at 30°C rather than the traditional 37°C. Recovery steps after heat shock transformation, growth of transformed bacteria on agar plates, and growth of bacterial colonies in liquid culture were also performed at 30°C. This lower temperature reduces the growth rate of the bacteria and thus helps to ensure replication fidelity of the plasmid. Due to these limitations, purified plasmid products should always be checked for correct plasmid size by restriction enzyme digestion. Once successful generation of chimeric Gag-NL4.3 plasmids has been accomplished, virus was generated via transfection of 293T cells, titered on an indicator TZM-bl cell line, and replication capacity measured using a CEM-based T cell line. A limitation of this approach is that the use of a cell line to measure viral replicative capacity rather than use of primary cells may bias towards growth characteristics in a physiological environment that is different from the *in vivo* environment.

One of the disadvantages of using the NL4.3 subtype B proviral backbone with subtype A derived sequences is that there is a risk of suboptimal gene pairing between subtype B proteins with subtype A *gag* genes. However, even if subtype matching was performed, a certain amount of within-clade diversity exists as evidenced by the clustering of HIV-1 sequences by country or region even when found within the same subtype.

Replicative capacity was compared in cases of multivariant infection to elucidate whether the transmission bottleneck within an individual patient would select for variants of similar replicative capacity traits. Our data suggests that any reasonably fit virus could get transmitted, as variants isolated from the same patient often differed in replicative capacity. The lack of correlation between the frequency of the occurrence of a particular variant in the patient's plasma and replicative capacity also suggests that replication capacity may not have be responsible for that variant's dominance over other minor variants within that patient. As the patients were identified very early in infection, it is possible that the proportions of virions in the patient's plasma may represent the ratio in which the variants occurred in the donor/transmitting patient and were present in the inoculum. Alternatively, there may have been increased outgrowth of the major variant as a result on fitness conferred by genes outside of *gag* and therefore not captured in our replicative capacity assay.

This study demonstrates that the presence of a virus with high replicative capacity during early infection contributes significantly to disease progression, both by increasing the viral load and by accelerating CD4 T cell decline. Individuals harbouring HIV-1 with reduced replicative capacity had a slow rate of disease progression determined by time to a CD4 T cell count below 400 cells per mm^3 of blood, and lower plasma HIV-1 RNA load. This supports the hypothesis that the replicative capacity of the transmitted virus determines the extent of damage in the newly infected individual before the onset of an adaptive immune response and viral escape. The effect of replicative capacity can be seen in the incomplete link between SPVL and the rate

of $CD4^+$ T-cell decline in the study cohort: when SPVL was very low ($<10^5$ copies of viral RNA per mL of plasma), CD4 T-cell loss was very slow; when SPVL was very high ($>10^5$copies per mL), cell loss was rapid; however, in patients whose SPVL was under 10^5 copies/ml, having a low RC was protective from CD4 T cell loss.

HLA associated polymorphisms that were associated with increased replicative capacity were identified including mutations on the GAG271, K76R, SL9 and QW9 epitopes while mutations on the RY10 epitope were only found in low replicative capacity chimeras. All other observed HLA-associated polymorphisms occurred in different locations making it difficult to approximate the net effect of each polymorphism on replicative capacity, but with residues that modulate replicative capacity being enriched in p17. This is consistent with the fact that not all HLA associated polymorphisms within a particular Gag sequence will necessarily reduce fitness and that the balance and interaction of both fitness increasing and fitness decreasing polymorphisms within a particular virus ultimately determines the replicative capacity of the virus (Carlson et al. 2016; Monaco et al. 2016).

As the role of replicative capacity was independent of but additive to viral load, the possibility that high replicative capacity viruses were better spread by cell-cell transmission, which is a more efficient mode than cell free infection and could potentially account for the incomplete association of CD4 decline with viral load was explored.

Viruses can spread by infecting cells in a cell-free form or via cell-cell contacts (Law et al. 2016; Phillips 1994; Zhong, Agosto, Munro, et al. 2013). Both modes of transmission offer distinct advantages and disadvantages for viral spread (Boulle et al. 2016; Zhong, Agosto, Ilinskaya, et al. 2013; Schiffner, Sattentau, and Duncan 2013; Russell et al. 2013). Given the high mutation rate of HIV-1 and the resulting increased capacity to adapt, HIV-1 attempts to balance out the advantages and disadvantages of either mode of transmission and efficiently

spread from cell to cell, tissue to tissue, and person to person. While the contribution of both modes to virus spreading in virus is unclear, there is overwhelming evidence that HIV-1 spreads more efficiently by utilizing direct cell-cell contact *in vitro*. In tissue culture, contact-mediated spread of HIV-1 is more efficient than cell-free transmission (Law et al. 2016; Duncan et al. 2014; Russell et al. 2013; Kolodkin-Gal et al. 2013; Rudnicka et al. 2009; Piguet and Sattentau 2004). This contact-dependent mode of transmission, known as cell-to-cell transmission, involves the formation of a virological synapse between an infected donor cell and an uninfected target cell.

Previous studies had used mobile T cell cultures to monitor cell-cell transmission of HIV-1 (Sourisseau et al. 2007), but the infectivity of viruses produced by mobile lymphocytes is dramatically reduced and the amount of envelope protein present decreased (Chazal et al. 2014), suggesting that inefficient HIV-1 replication in mobile lymphocytes in this experimental system is not only due to avoidance of viral cell-to-cell transfer but also to the loss of infectivity of the viral particles due to the alteration of the composition and functionality of the particles produced by these lymphocytes. To quantify cell-cell transmission of *gag*-NL4.3 chimeras, previously described methods were adapted to allow for infection of donor cells with viruses of known characteristics and quantify the ability of those cells to infect target cell, through the measurement of p24 protein in target cells 24 hours after infection.

Even though all donor cells were initially infected at the same MOI, the number of infected target cells and infected donor cells for both cell free and cell-cell transmission correlated strongly, giving rise to a need to normalise the infection rate of target cells to the frequency of infected donor cells so as to derive a standard infectivity index. As expected, cell-cell transmission was better at spreading HIV-1 between cells, in both Jurkat and sorted CD4 T cells. However, we did not investigate the possibility that virions may be trapped on the

transwell membrane in the cell free experiments, which would further reduce the infection of target cells in the transwell experiments.

Replicative capacity was associated with high cell-cell but not cell free spread, but even so, only in subtype A *gag* sequences on a NL4.3 backbone. NL4.3 is well known to effectively spread by cell-cell transmission, in both CCR5 and CXCR4 expressing cells. A NL4.3 backbone containing NL4.3 genes except *gag* may therefore have had increased cell-cell transmission, making it possible to observe any changes to cell-cell transmission that may have been a result of the insertion of high replicative capacity *gag*.

It is important to note that even though donor cells were thoroughly washed and rested before being used to infect target cells, there was still some amount of virus detectable in culture supernatant 24 hours after coculture. There was a trend towards high virus titres in the culture supernatants of cultures with high replicative capacity virus, but only on the samples of the virus on a NL4.3 backbone. This may represent the result of subtype differences in *gag*, high replicative capacity with increased virus turnover in both donor and target cells, or the result of the differences in pathogenicity of NL4.3 and MJ4. Nonetheless, within the Kilifi cohort where *gag* sequences were cloned on a backbone that is well known to effectively spread by cell-cell transmission, the result of high replicative capacity *gag* on cell-cell spread was evident.

The virus that was detected in cultures from cell-cell transmission experiments may have been produced by donor or target cells, and therefore this virus could in part be due to increased release by the infected donor cells, but also points to the possibility of a faster life cycle in the target cells infected with high replicative capacity viruses.

In HIV-1 subtype C infection, fast replicating viruses are associated with faster loss of CD4 T cells in a manner that is independent of viral load and HLA type (Claiborne et al. 2015; Prince et al. 2012), suggesting that the pathway of CD4 T cell depletion in infection with high

replicative capacity viruses is distinct from what low replicative capacity viruses employ. The expression of caspase1, caspase 3 and CD95 in CD4 T cell memory subsets was compared, and RNA involved in cell death pathways in cell lysates from high and low cell-cell transmission, and in high and low replicative capacity virus infection evaluated. The expression of RNA involved in cell death pathways in PBMC derived at 3 months post infection from patients infected with virus of known replicative capacity was also measured.

Overall, there was higher expression of RNA involved in cell death pathways in cultured CD4 T cells compared to PBMC. This may be because of the high multiplicity of infection used in culture compared to natural infection, or because the of the purity of sorted CD4 T cell population which presents the major target of HIV-1 infection and pathogenesis compared to PBMC which include cellular subsets that are not productively infected by HIV-1 and therefore allows for cleaner investigation of HIV-1 mediated cell death. The extracellular environment would also be very different in vivo and vivo, including levels of cytokines, receptor ligand interactions with other cell types not present in culture and changes in the immune microenvironment architecture.

Within the sorted CD4 T cells, the highest expression of pro-apoptotic, autophagy and necrosis markers was observed in high replicative capacity viruses from both Kilifi and Zambia, and in high cell-cell transmission but not low replicative capacity or cell-free infection, further suggesting that the cell death pathways in high replicative capacity infection and cell-cell transmission overlap. The cell-death pathway RNA profile in low replicative capacity infection resembled that of cell free infection and was close to that of HIV-1 uninfected cells, which is consistent with *in vivo* findings that these patients had preserved CD4 T cell counts. There were no differences in the expression of RNA related to either apoptosis, necrosis or autophagy as a result of replicative capacity. It is noteworthy that as these experiments were performed in pooled cells that likely represent different phenotypes, our approach is a blunt tool that only

captures a cross section of the cell death pross in cells at different stages of their life cycle and may miss out on the complete picture of the pathways involved.

Expression of the apoptosis executioner caspase 3 that activates caspases 6, 7, and 9 was upregulated 1025-fold in *in vitro* cell-cell transmission in sorted CD4 T cells but only 2.8-fold in high replicative capacity infection. Likewise, expression of ESR1 which encodes estrogen receptor 1 that plays a role in regulation of autophagy was upregulated 101-fold in cell-cell transmission but not in high replicative capacity infection. Other genes that were differentially upregulated include NOL3 which was only upregulated in cell-cell transmission and CTSS, TMEM57, PVR, JPH3 and CCDC103 which were only upregulated in infection with high replicative capacity virus.

These results suggest that infection with a low replicating virus confers clinical benefit outside of the effect of replicative capacity on set point VL, and that the kinetics of viral replication early in infection can ultimately dictate long-term pathogenesis in HIV-1 subtype A. Moreover, they suggest that replicative capacity may modulate innate immune events very early after infection, which could alter both the establishment of an inflammatory state and the development of an effective adaptive immune response capable of controlling viremia.

To further test this hypothesis, early immune responses and levels of circulating inflammatory cytokines, immune activation, and exhaustion in T cell, B cell, NK cell and monocyte compartments were evaluated.

Chapter 5: Role of virus characteristics in shaping the immune response to HIV-1

5. Role of virus characteristics in shaping the immune response to HIV-1

5.1 Introduction

To date, research has focused on identifying host factors that contribute to viral control and favourable disease outcomes, whereas viral characteristics have received less scrutiny. The observation that not all individuals harbouring protective HLA class I alleles go on to become long-term non-progressors (Chahroudi et al. 2012; Nissen et al. 2018; Brener et al. 2015) suggests that other factors outside of host immunogenetics play a role in defining disease progression. Transmitted viral characteristics have indeed been shown to impact viral load within heterosexual transmission pairs (Carlson et al. 2014; Claiborne et al. 2015; Ende et al. 2018; Monaco et al. 2016; Prince et al. 2012; Archary et al. 2010; Katoh et al. 2016) suggesting that viral characteristics are heritable and can impact disease severity and the body's initial attempt to control HIV-1 infection. Indeed, in the previous chapters, we showed that factors related to the HIV-1 transmission event such as multiplicity of founder variants as well as genotypic and phenotypic characteristics of the founder virus such as accurate subtype, degree of HLA preadaptation, transmitted drug resistance and replicative capacity as defined by *gag* can influence disease progression.

Not much is known about how the genetic and biological characteristics of the founder virus shape the early immune response. As the HIV-1 quasispecies that arise following a mucosal infection are usually derived from a single transmitted virus (Abrahams et al. 2009; Baalwa et al. 2013; Salazar-Gonzalez et al. 2009; Bar et al. 2010; Carlson et al. 2014) the first immune response should be effective at controlling infection before the well-known challenge of HIV-1 diversity arises, but instead drives the selection of virus escape mutations and bystander immune activation (Ndhlovu et al. 2015; Boasso and Shearer 2008; Sauce, Elbim, and Appay 2013; Sauce et al. 2011; Kijak et al. 2017). Indeed, multiple studies are in full support of

immune activation being better than viral load set point at predicting $CD4^+$ T-cell loss and AIDS (Lubaki et al. 1999; Benecke, Gale, and Katze 2012; Boasso and Shearer 2008; Brenchley and Douek 2008; Moir and Fauci 2014; Liovat et al. 2012). Activation of innate cells and adaptive cells is a striking feature of acute HIV-1 infection of humans and SIV infection of rhesus macaques, persists to a varying degree into chronic infection and is not limited to cells that are infected by or are specific for HIV-1 (Burgers et al. 2009; Duvall et al. 2006; Hardy, Imami, and Gotch 2002; Benecke, Gale, and Katze 2012; Liovat et al. 2009). Although the direct contribution of HIV-1 replication to chronic immune activation is well recognized, lines of evidence indicate that high levels of HIV-1 replication are neither sufficient nor necessary to induce pathological levels of immune activation. These include the observations that: (i) virologic non progressors (VNPs) maintain a high and remarkably stable $CD4^+$ T-cell count for many years despite levels of viral replication similar to those found in non-controllers, but show a profile of non-activation (Anderson, Ascher, and Sheppard 1998; Deeks et al. 2004; Giorgi et al. 1999; Rotger et al. 2011; McGary et al. 2014); (ii) successfully CART-treated patients with undetectable viremia can still show higher levels of T-cell activation than healthy controls, with the extent of this residual immune activation being associated with increased morbidity and mortality (Aiuti and Mezzaroma 2006; Hunt et al. 2003; Sauce et al. 2011); (iii) amongst elite controllers, those with higher T-cell activation display slow but progressive $CD4^+$ T-cell loss and can develop AIDS (Cortes et al. 2018; Crowell and Hatano 2015; Jacobs et al. 2017; Olson et al. 2018); (iv) the frequency of activated T cells largely exceeds the frequency of HIV-1-infected $CD4^+$ T cells and HIV-1-specific $CD4^+$ and $CD8^+$ T cells (Doisne et al. 2004; Selliah et al. 2003; Doitsh and Greene 2016); (v) other cell types that do not become infected with HIV-1 including B cells, NK cells, pDCs, and monocytes show increased levels of activation, turnover and cell death (Brown, Trichel, and Barratt-Boyes 2007; Burdo et al. 2011; Hasegawa et al. 2009); and (vi) chronic immune activation is not observed in naturally SIV-infected sooty

mangabeys, in which the infection rarely progresses to AIDS, despite high levels of virus replication and acute $CD4^+$ T cell depletion (Chahroudi et al. 2012; Liovat et al. 2009; Chakrabarti 2004; Silvestri 2005). Residual chronic immune activation referred to as immune activation set point persists even in HIV-1-infected patients in which viral replication is successfully inhibited by antiretroviral therapy, is established early in HIV-1 infection and determines the rate at which CD4 T cells are lost over time (Aiuti and Mezzaroma 2006; Benito et al. 2005; Deeks et al. 2004; Paiardini and Muller-Trutwin 2013).

Certain HIV-1 gene products, such as Env, Tat, and Nef, have been previously proposed to be involved in HIV-1-induced immune activation (Mlcochova et al. 2015; Wei et al. 2003; Kikuchi et al. 2015). For instance, Nef proteins of SIV-infected sooty mangabeys and African Green Monkeys (AGMs) that do not show immune activation can downmodulate the CD3-TCR complex from the cell surface, thus making infected $CD4^+$ T cells more refractory to further antigenic stimulation, contrary to the Nef protein of HIV-1 that has lost the ability to effectively downmodulate the CD3-TCR complex from the surface of infected T cells (Munch et al. 2007; Schindler et al. 2006; Toussaint et al. 2008).

In this chapter, possible contribution of virus characteristics in driving immune activation, differentiation and exhaustion will be evaluated. Since the detrimental consequences of multivariant infection, high replicative capacity variants and subtype differences unique to each gene that were observed in the previous chapters were partially independent of although additive to set point viral load (SPVL) but still drove CD4 T cell decline, we hypothesise that these virus characteristics are also associated with mechanisms that might initiate irreversible pathogenic events early in infection, including exacerbated immune activation and inability to resolve inflammation to a low immune activation set point, which might then dictate the kinetics of subsequent disease progression.

The specific aims are to:

1. Describe T cell, B cell, NK cell and monocyte subsets observed at 3 months (0-3 months), 9 months (6-9 months) and 24 months (24-30 months) post infection.
2. Explore a role for virus characteristics in shaping the T cell, B cell, NK cell and monocyte phenotypes including chronic immune activation.

5.2 Results

5.2.1 Gating strategy for different cell subsets

To define the different T cell subsets, cells were gated based on forward scatter height (FSC-H) and forward scatter area (FSC-A) to exclude doublets (Figure 5.1). The side scatter area (SSC-A) versus the FSC-A was used to identify lymphocytes based on their size and granularity. After gating on CD3+, CD3+CD4+ and CD3+CD8+ cells, further single, quadrant or Boolean gates were set for each of the respective populations based on fluorescence minus one controls (FMO) controls for each marker. Lineage differentiation markers CD45RO, CD27 and CCR7 were used to subdivide CD4+ T cells and CD8+ T cells into subpopulations of naïve, central memory, effector memory, transitional memory, terminal effector and effector subsets. In addition, activation markers HLA-DR and CD38 were used to subdivide define activation status, granzyme B, perforin and CD107a to define cytotoxicity status, PD-1, CD57 and Ki67 to define exhaustion, senescence and proliferation respectively (Figure 5.1).

The following B cell subsets were identified based on indicated surface markers: immature/transitional, CD19+CD10+CD38++CD27-; naive, CD19+CD27-CD21+; tissue-like memory, CD19+CD27-CD21-; resting memory, CD19+CD27+CD21+; activated mature, CD19+CD27+CD21-; plasmablasts, CD19+CD27++CD38+++; unswitched resting memory, CD19+CD21+CD27+IgD+; and switched resting memory, CD19+CD21+CD27+IgD-. The expression of CD86 was also evaluated (Figure 5.2).

Likewise, CD3- singlet live lymphocytes were gated for CD16 and CD56 to identify NK cells (Figure 5.3). Three NK cell subsets were identified based on the expression of CD16 and CD56; Anergic NK cells, CD56-CD16+; Cytolytic NK cells, CD56+CD16+ and CD56 bright, CD16+/-CD56+++ NK cells. These subsets were then further characterized for cytotoxicity based on their expression of perforin, granzyme B and CD107a, senescence based on the

expression of CD57 and the expression of NK cell receptors NKG2A and NKG2D (Figure 5.3). Finally, the three monocyte populations (classical, non-classical and intermediate) monocytes were gated based on their expression of CD14 and CD16 in a monocyte population defined on forward and side scatter (Figure 5.4).

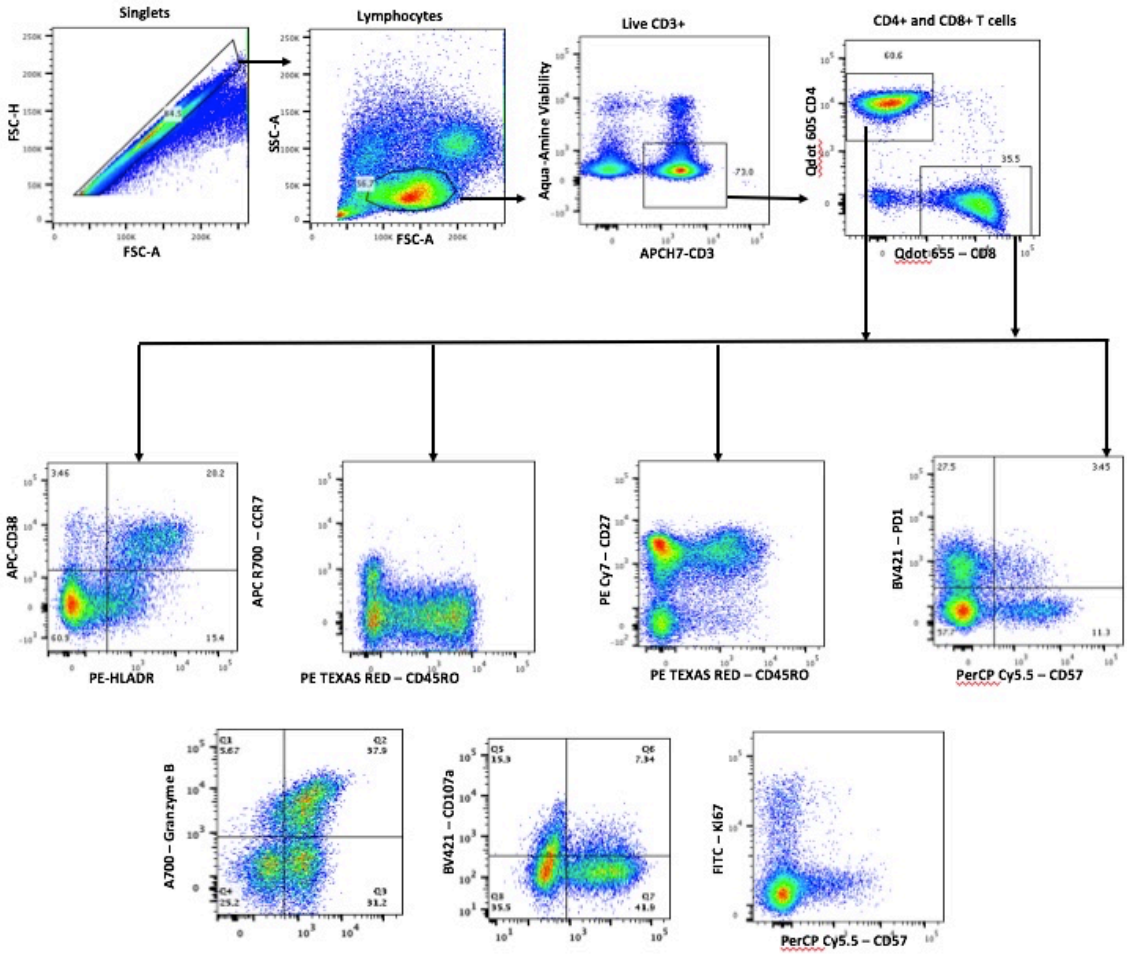


Figure 5.1: Gating strategy to define T cell subsets.

Singlet lymphocytes were gated for CD3 expression on live cells, followed by gating of the various cellular subsets of interest within CD4 and CD8 T cells as earlier described.

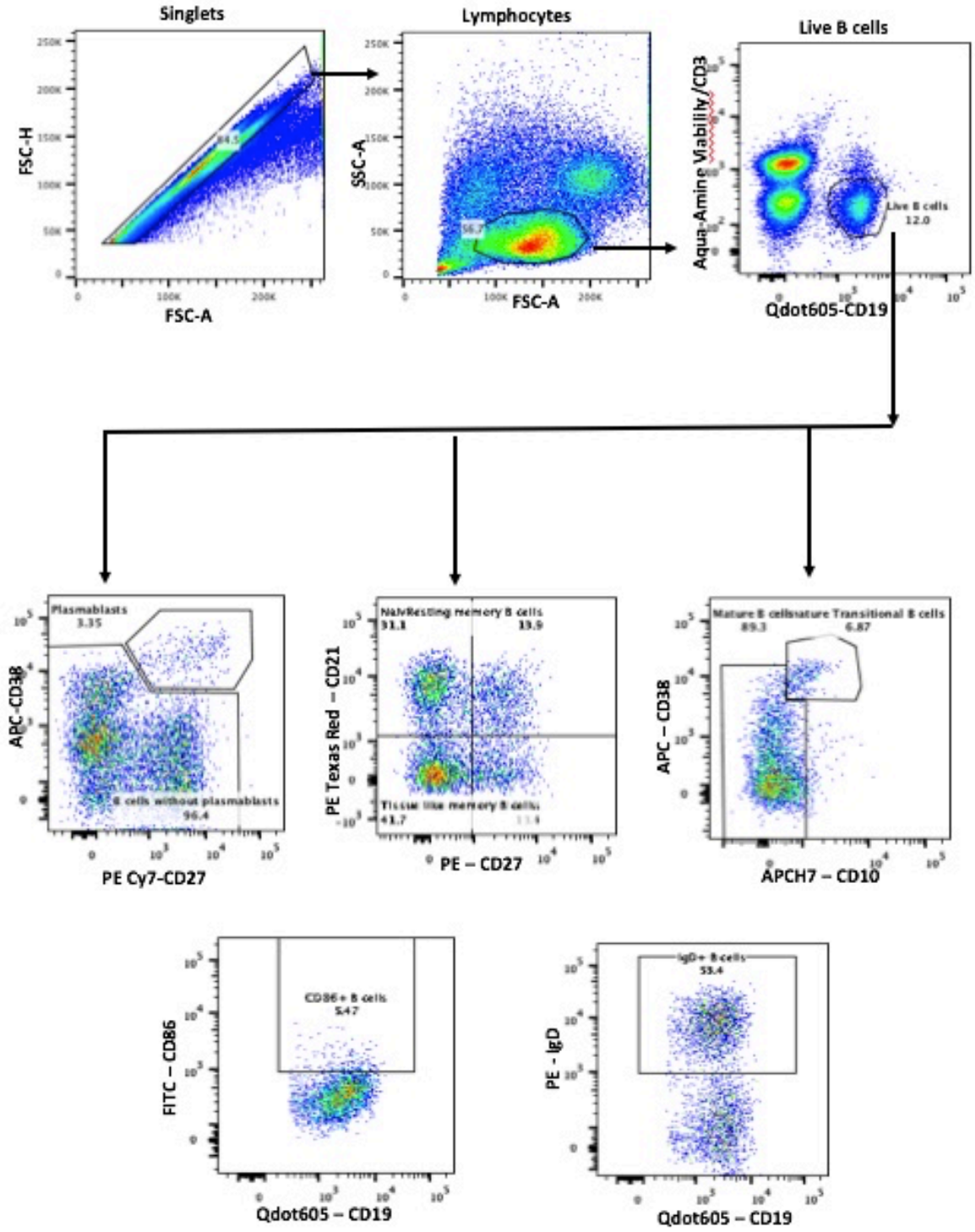


Figure 5.2: Gating strategy to identify B cell subsets.

Singlet lymphocytes were gated for CD19 expression on live CD3- cells, followed by gating of the various B cell subsets of interest as earlier described.

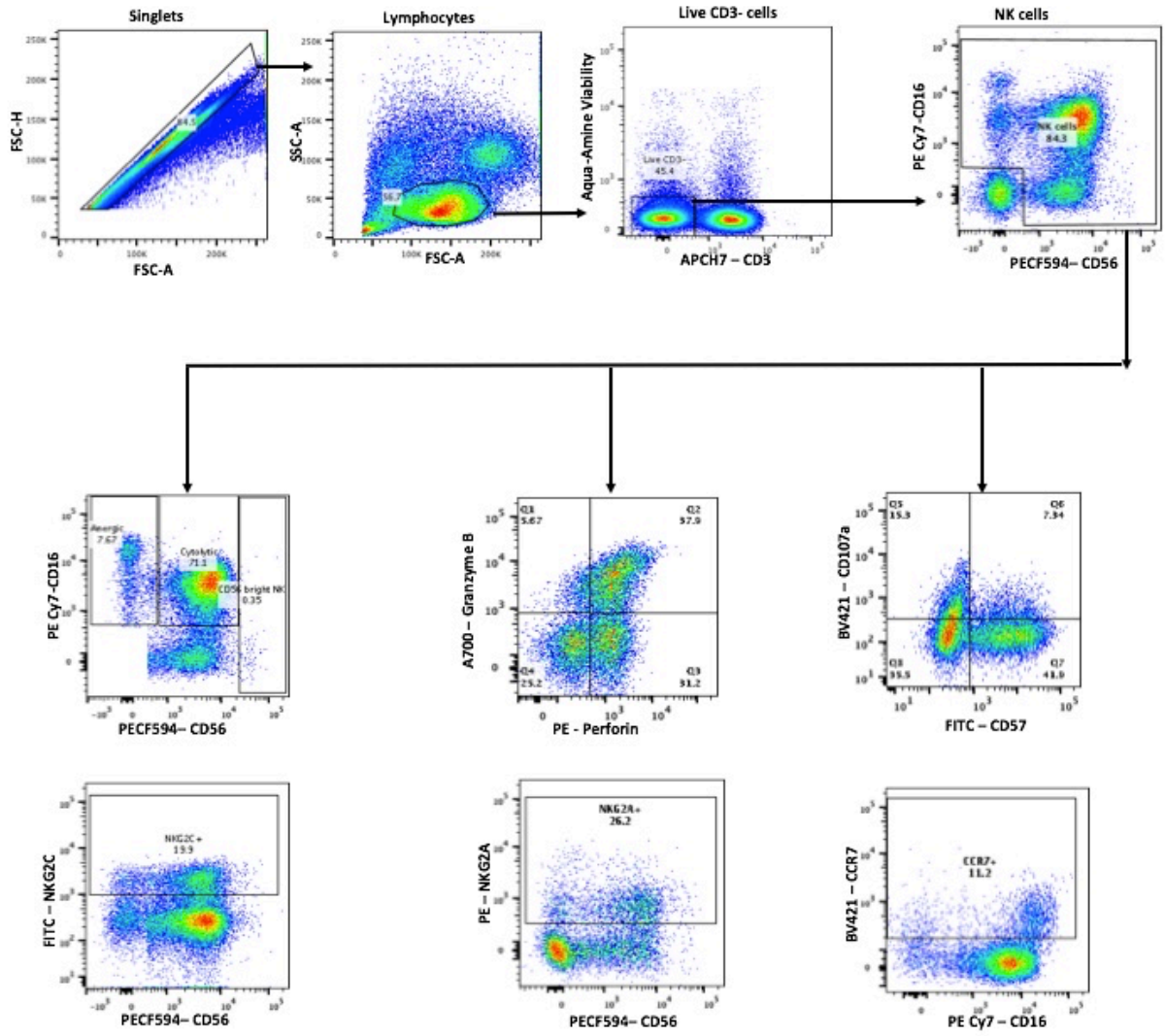


Figure 5.3: Gating strategy to identify NK cell subsets.

Singlet lymphocytes were gated for CD16 and CD56 expression on live CD3- cells, followed by gating of the various NK subsets of interest as earlier described.

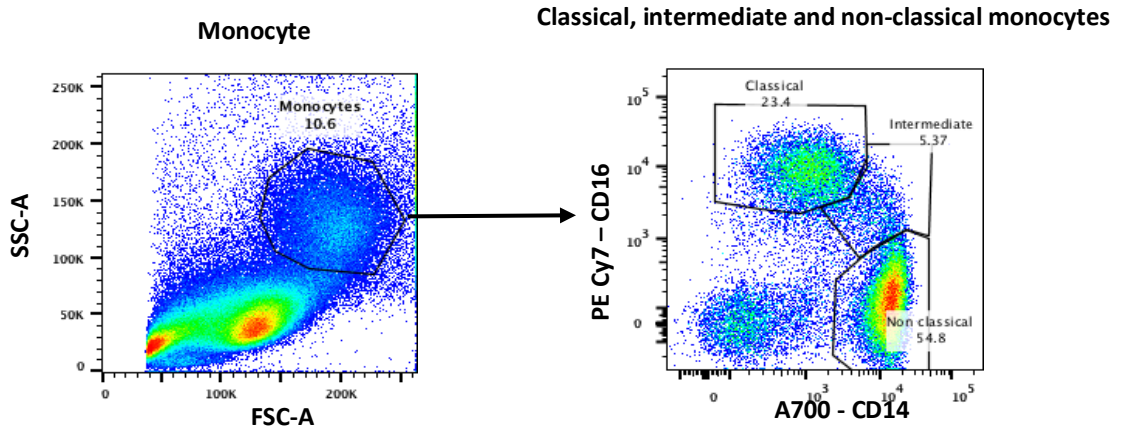


Figure 5.4: Gating strategy to identify monocyte subsets.

Monocytes were gated on forward and side scatter and the 3 subsets defined based on their expression of CD14 and CD16 as earlier described.

5.2.2 Principal component analysis

Since acute HIV-1 infection is associated with complex changes in different cellular subsets over time, distinct phenotypic "profiles" of T cells, B cells, NK cells and monocytes that could explain the impact of viral characteristics were defined by using an unsupervised data reduction tool, principal component analysis (PCA), which groups linear variables into combinations termed principal components (PCs) as has previously been applied to biological data (Giuliani 2017; Jolliffe and Cadima 2016). Principal component 1 (PC1), which describes the greatest variation in the dataset is by definition uncorrelated with principal component 2 (PC2) which describes the second-greatest variation in the data. Principal components with the highest eigenvalues and thus the highest contribution to the variation in the analysed cell types were identified, and the contributions of each measured phenotype to each principal component (PC loadings) analysed. To identify virus characteristics that drove the variability in each principal

component, Student's *t* tests were performed between the identified principal component profiles and the previously identified founder virus characteristics.

5.2.3 CD4 T cell principal components

The top three CD4 T cell principal components (PC) could explain 55% of the variation in CD4 T cell subsets, with PC1, PC2, PC3 and PC4 accounting for 17.8%, 15%, 13.7% and 8% of the observed variation respectively. The total number of CD4 T cell principal components alongside their cumulative contribution to CD4 T cell variation are shown in Figure 5.5.

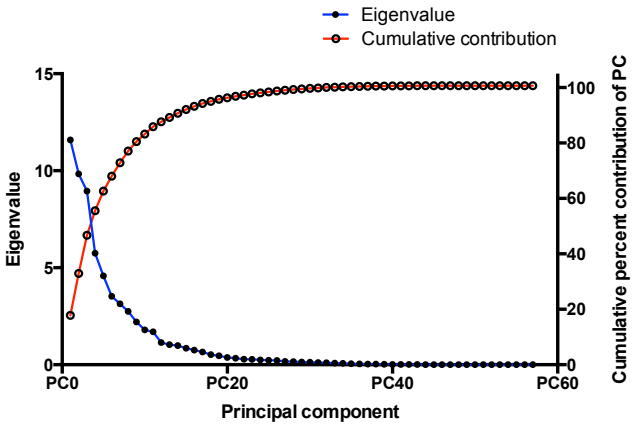


Figure 5.5: Scree plot showing all the identified CD4 T cell principal components. Eigenvalue loadings and cumulative percent contribution of each principal component have been plotted on the right and left Y axis respectively..

The CD4 T cell Principal component 1 (PC1) profile comprised patients with increased proliferation and subsequent differentiation of CD4 T cells at all time points measured, but lower residual activation. Their CD4 T cells showed increased proliferation as shown by increased frequencies of $Ki67^+$ cells in multiple CD4 T cell subsets including naïve (T_N), central

memory (T_{CM}), transitional memory (T_{TM}), effector memory (T_{EM}) and terminal effector (T_{TE}) at all three timepoints measured (0-3 months, 6-9 months and 24-30 months after infection) (Figure 5.6A). These patients also showed increased differentiation with increased proportions of T_{EM}, T_{CM} and T_{TM} cells, and fewer T_N cells. There was increased senescence and exhaustion throughout the sampled time points as shown by the increase in PD-1^+ and $CD57^+$ cells. During chronic infection (24-30 months), these patients had fewer cytotoxic (Granzyme B^+ $Perforin^+$ $CD107a^+$) CD4 T cells. CD4 T cells that were not activated at chronic 24-30 months ($CD38^-$ HLA-DR^-) contributed negative loadings to PC1 (Figure 5.6A).

Principal component 2 (PC2) profile comprised patients with high frequencies of activated CD4 T cells at acute infection, with eventual exhaustion and senescence in chronic infection (Figure 5.6B). Their CD4 T cells exhibited increased activation ($CD38^+$ HLA-DR^+) in multiple subsets including T_{CM}, T_{TE}, T_{EM}, and T_{TM}, with loss of CD38 but persistence of HLA-DR expression ($CD38^-$ HLA-DR^+) in T_{TE}, T_{CM}, T_{TM} and T_{EM} persisting to 6-9 months post infection (Figure 5.6B). These patients' CD4 T cells also showed increase in frequencies of exhausted PD-1^+ cells in memory subsets at acute infection including T_{CM}, T_{EM} and T_{TM}, which was resolved in T_{TM} and T_{CM} but persisted in T_{TE} up to 9 months post infection as has been previously observed in HIV-1 infection (Banga et al. 2016; Lee and Lichterfeld 2016). As infection progressed, their CD4 T cells became senescent ($CD57^+$) starting from 6-9 months. However, by 24-30 months post infection, their CD4 T cells had recovered and increased in proliferative (Ki67 expression) and cytotoxicity (CD107a expression) abilities.

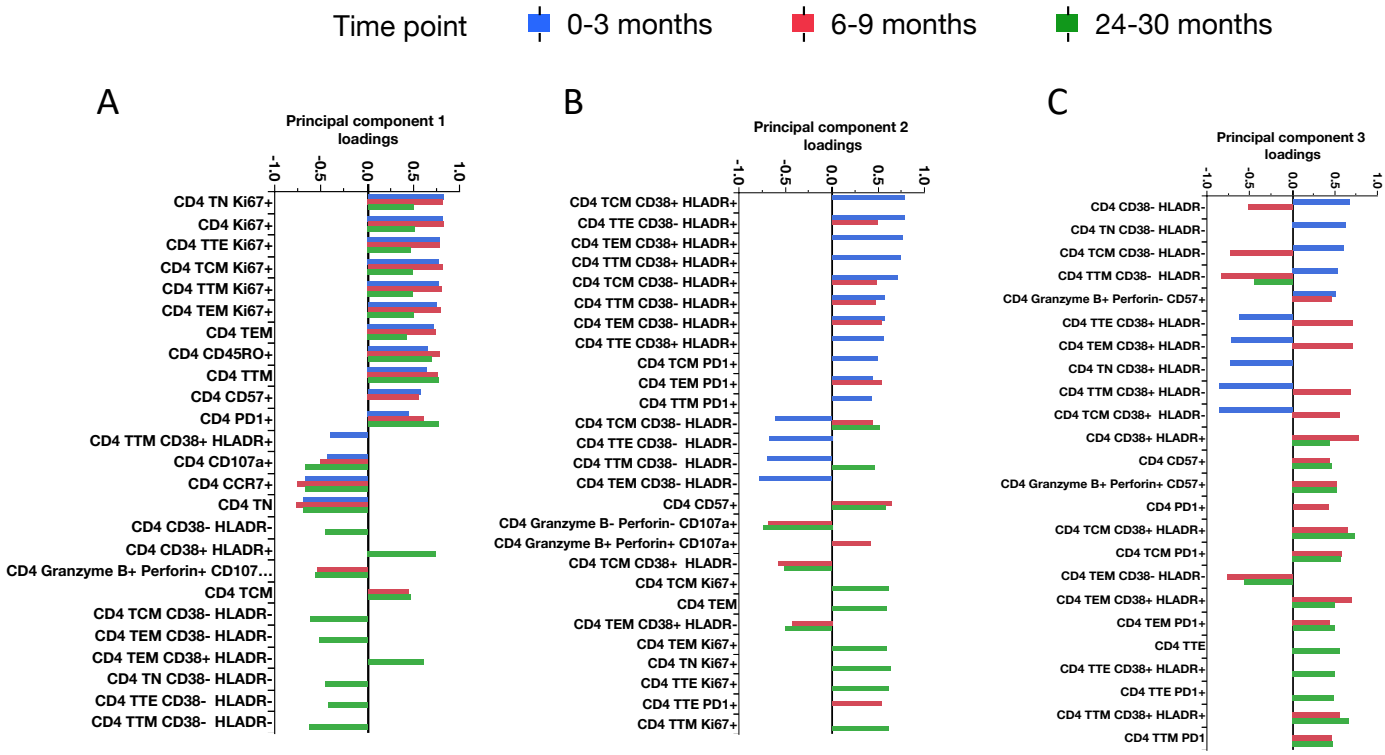


Figure 5.6 A-C: The constituents of various CD4 T cell principal component profiles.

PC1 was mainly defined by frequencies of proliferating cells at acute infection and lack of activation at 24-30 months, PC2 was defined by activation at acute infection and proliferation at 24-30 months of infection while PC3 was defined by lack of activated CD4 T cells at acute infection and increased exhaustion at later time points.

The principal component 3 (PC3) profile comprised patients whose CD4 T cells showed increased frequencies of activated CD4 T cells at acute infection, with activation lingering up to 9 months post infection in some memory subsets (Figure 5.6C). Similarly, there was increased exhaustion (PD-1 expression) in memory subsets, persisting up to 9 months in T_{TE}, even though there was an increase in cytotoxicity at 30 months.

5.2.4 Role of virus characteristics in driving CD4 T cell responses

Having identified principal components within CD4 T cells that could explain the largest variation in this compartment, associations between any of the principal components with the virus characteristics identified in Chapters 3 and 4 as important for either CD4 T cell decline or set point viral load were tested. The top ten principal components were tested for association with the viral characteristics that were previously measured, and only components that showed significant association with disease progression are shown.

Replicative capacity contributed to the largest variation in T cell activation with high replicative capacity being associated with principal component 1, 2 and 4 as follows. Patients infected with founder viruses whose *gag* conferred a replicative capacity above the cohort median had higher loadings for principal component 1 at 24-30 months (p=0.03), higher loadings for principal component 3 at 6-9 months (p=0.04) and 24-30 months (p=0.05) and higher loadings for principal component 4 at 30 months (p=0.04) (Figure 5.7). Thus, at 9 months there was increase in proportions of highly activated ($CD38^+HLA\text{-}DR^+$) and exhausted ($PD\text{-}1^+$) CD4 T cell subsets which was also observed at 24-30 months, but increased proliferation was only observed at 24-30 months and not at 0-3 or 6-9 months after infection.

In contrast, multivariant infection was only associated with changes in principal component 1 loadings at 3 months after infection and not thereafter (Figure 5.7). There were lower loadings of 0-3-month PC1 loadings (P=0.05), representing an increased proportion of highly activated ($CD38^+HLA\text{-}DR^+$), cytotoxic ($CD107a^+$) and CCR7 expressing CD4 T cells. High SPVL was associated with low loadings for PC2 at 24-30 months (P=0.03), therefore increased proportion of degranulated CD4 T cells (Granzyme B^- $Perforin^-$ $CD107a^+$) and CD38 expressing ($CD38^+$ $HLA\text{-}DR^-$) T_{CM} and T_{EM} at 24-30 months but not at 0-3 or 6-9 months.

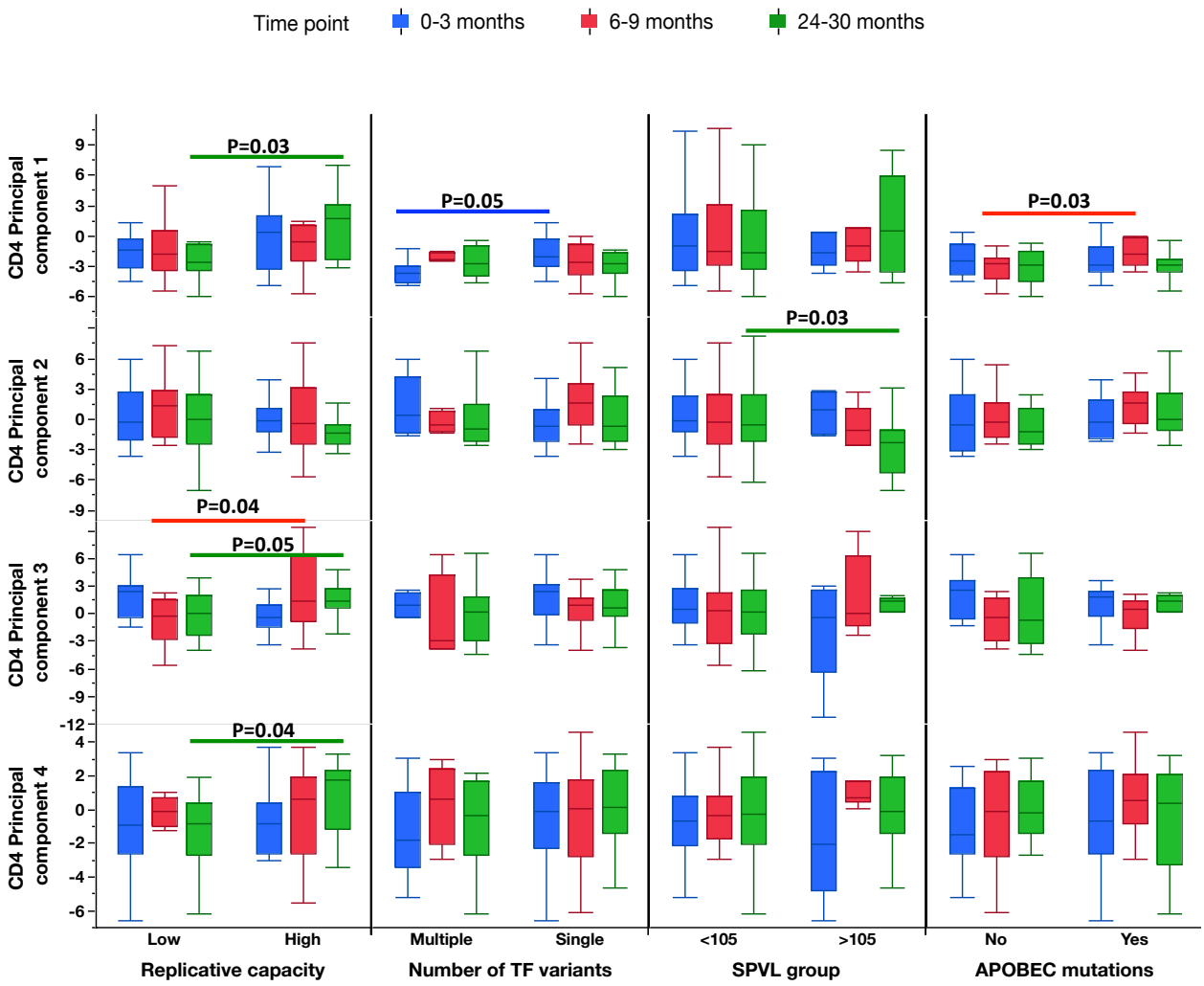


Figure 5.7: Association between virus characteristics and CD4 principal component loadings.

High replicative capacity infection was associated with changes in CD4 principal component 1, 2 and 4 while multivariant infection was only associated with changes in principal component 1 loadings at 3 months after infection and not thereafter. High SPVL was associated with low loadings for PC2 at 24-30 while APOBEC mutations were associated with higher loadings for principal component 1 at 6-9 months. p values have been corrected using the Benjamini-Hochberg technique as described in the materials and methods.

Unlike high SPVL, APOBEC mutations were associated with higher loadings for principal component 1 at 6-9 months (P=0.03), which represented increased proportion of proliferating

CD4 T cells in multiple subsets including T_N, T_{TE}, T_{CM}, T_{TM} and T_{EM}, higher proportions of T_{TM}, increased exhaustion (PD-1 expression) and senescence (CD57 expression) at 6-9 months but not at 0-3 or 24-30 months.

There was no detectable influence of HLA preadaptation or drug resistant mutations on the composition of CD4 T cells.

5.2.5 CD8 principal components

The top four CD8 T cell principal components could explain 59% of the variation in CD8 T cells, with PC1, PC2, PC3 and PC4 accounting for 23.1%, 18%, 9.6% and 8.1% of the observed variation respectively. The total number of CD8 T cell principal components alongside their cumulative contribution to CD8 T cell variation are shown in Figure 5.8.

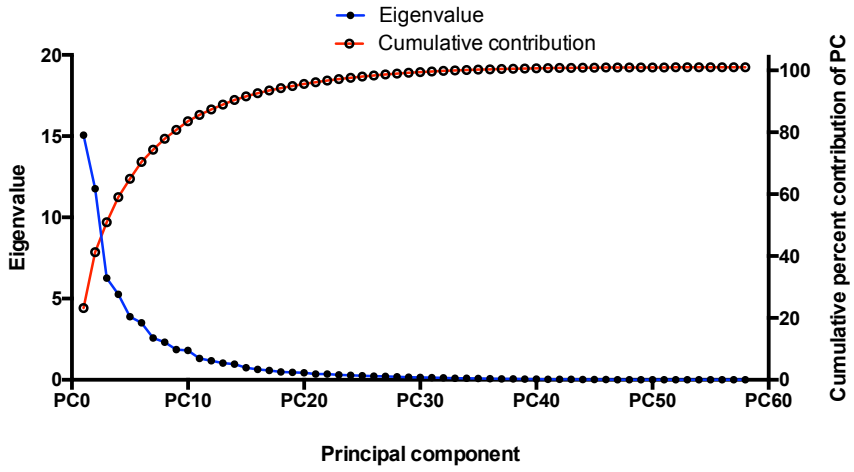


Figure 5.8: Scree plot showing all the identified CD8 T cell principal components.

Scree plot showing all the identified CD8 T cell principal components, eigenvalue loadings and cumulative percent contribution of each principal component.

The CD8 PC1 profile consisted of patients whose CD8 T cells showed increased senescence ($CD57^+$) and cytotoxicity ($CD107a^+$) but reduced proliferation ($Ki67^+$) in multiple CD8 T cell subsets at acute infection, but with recovery at 6-9 months, whereby there was increase in proliferation in all CD8 T cell subsets, and increased activation ($CD38^+HLA\text{-}DR^+$) of transitional memory and effector memory CD8 T cells (Figure 5.9A).

On the other hand, PC2 comprised CD8 T cells that showed increased activation ($CD38^+HLA\text{-}DR^+$) at all time points measured, all through from acute infection to 24-30 months of infection, with corresponding increase in frequencies of cytotoxic cells ($CD107a^+$) at acute infection (Figure 5.9B). T_{CM} and T_{TM} cells of these patients showed exhaustion ($PD\text{-}1^+$) at acute infection, with T_{CM} continuing to show exhaustion even at 24-30 months. There were fewer T_N and increased T_{CM} at 24-30 months post infection, with the T_N showing reduced proliferation.

The PC3 profile comprised patients with increased frequencies of cytotoxic ($CD107a^+$) and proliferating ($Ki67^+$) CD8 T cells at acute infection, and a corresponding reduction of senescent ($CD57^+$) and exhausted ($PD\text{-}1^+$) cells at that time point (Figure 5.9C). As infection progressed, they maintained high frequencies of cytotoxic ($CD107a^+$) CD8 T cells, had increased frequencies of T_{EM} and T_{TE} CD8 T cells, and an increase in HLA-DR expressing but $CD38^-$ CD8 T cells.

On the other hand, PC4 was characterised by increase in CD8 T cells that expressed CD38 without HLA-DR in multiple subsets at acute infection and at 6-9 months (Figure 5.9D). At 24-30 months these patients showed increased exhaustion in T_{EM}, T_{TE} and T_{TM}.

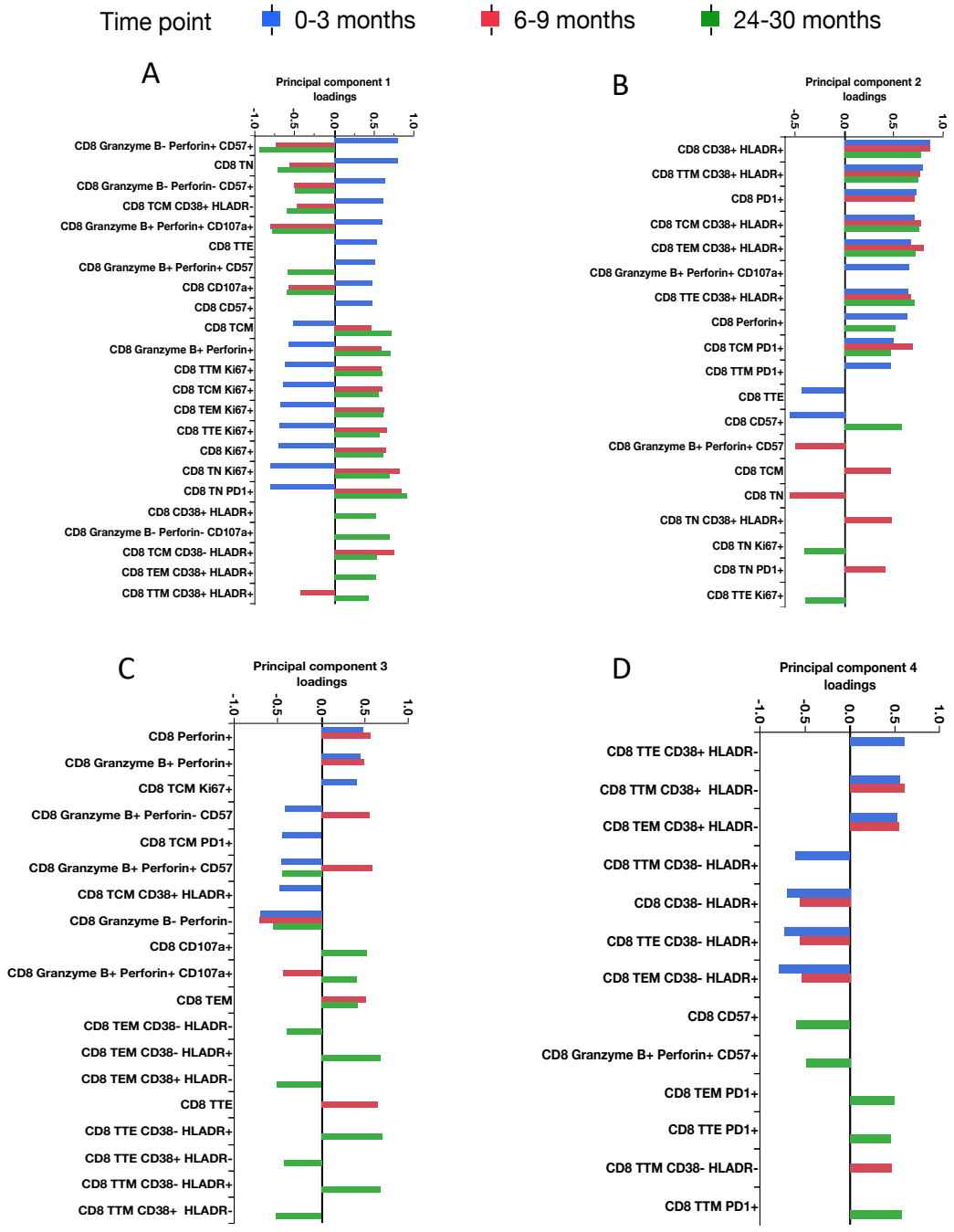


Figure 5.9 A-D: The constituents of various CD8 T cell principal component profiles.

PC1 was mainly defined by frequencies of cytotoxic CD8 T cells at acute infection and proliferating cells throughout infection while PC2 was defined by CD8 T-cell activation at all measured time points. PC3 was defined by the expression of granzymes B, perforin and cellular activation while PC4 was defined by differential expression of CD38 and HLA-DR at 0-9 months post infection, and exhaustion at 24-30 months after infection.

5.2.6 Role of virus characteristics in driving CD8 T cell responses

Like CD4 T cells, the largest variation in CD8 T cells was a result of viral replicative capacity. High replicative capacity infection was accompanied by lower loadings for PC1 at 3 months after infection (p=0.04), but higher loadings at 24-30 months after infection (p=0.02), and higher PC2 loadings at 9 months after infection (p=0.01) (Figure 5.10). Thus, they had increased proliferation (Ki67 expression) of multiple CD8 T cells subsets during acute infection, increased activation ($CD38^+HLA\text{-}DR^+$) and exhaustion ($PD\text{-}1^+$) at 6-9 months with chronic infection at 24-30 months characterised by continued activation and exhaustion with increased proliferation at 30 months. The only observed influence of multivariant infection was a trend towards higher loadings for PC3 at 9 months (p=0.08, Figure 5.10), showing increase in CD57 and granzyme B expression with or without perforin. There was also increased differentiation to T_{EM} and T_{TE} CD8 T cells, but that did not carry on to chronic infection. Low set point viral load was characterised by a trend towards lower PC3 loadings at 9 months (p=0.08) but higher PC4 loadings at 30 months (p=0.06, Figure 5.10). Thus, there was increased cytotoxicity (Granzyme B^+ $Perforin^+$ $CD107a^+$) at 6-9 months and increased exhaustion in T_{EM}, T_{TE} and T_{TM} at 24-30 months. Presence of APOBEC mutations was associated with a trend towards lower loadings for both PC3 (p=0.06) and PC4 (p=0.07) at 3 months post infection (Figure 5.10), i.e. increased $Granzyme^+$ $CD57^+$ CD8 T cells with or without perforin, activation ($CD38^+$ $HLA\text{-}DR^+$) and exhaustion ($PD1^+$) of T_{CM}, and expression of HLA-DR without CD38 in T_{EM}, T_{TE} and T_{TM}.

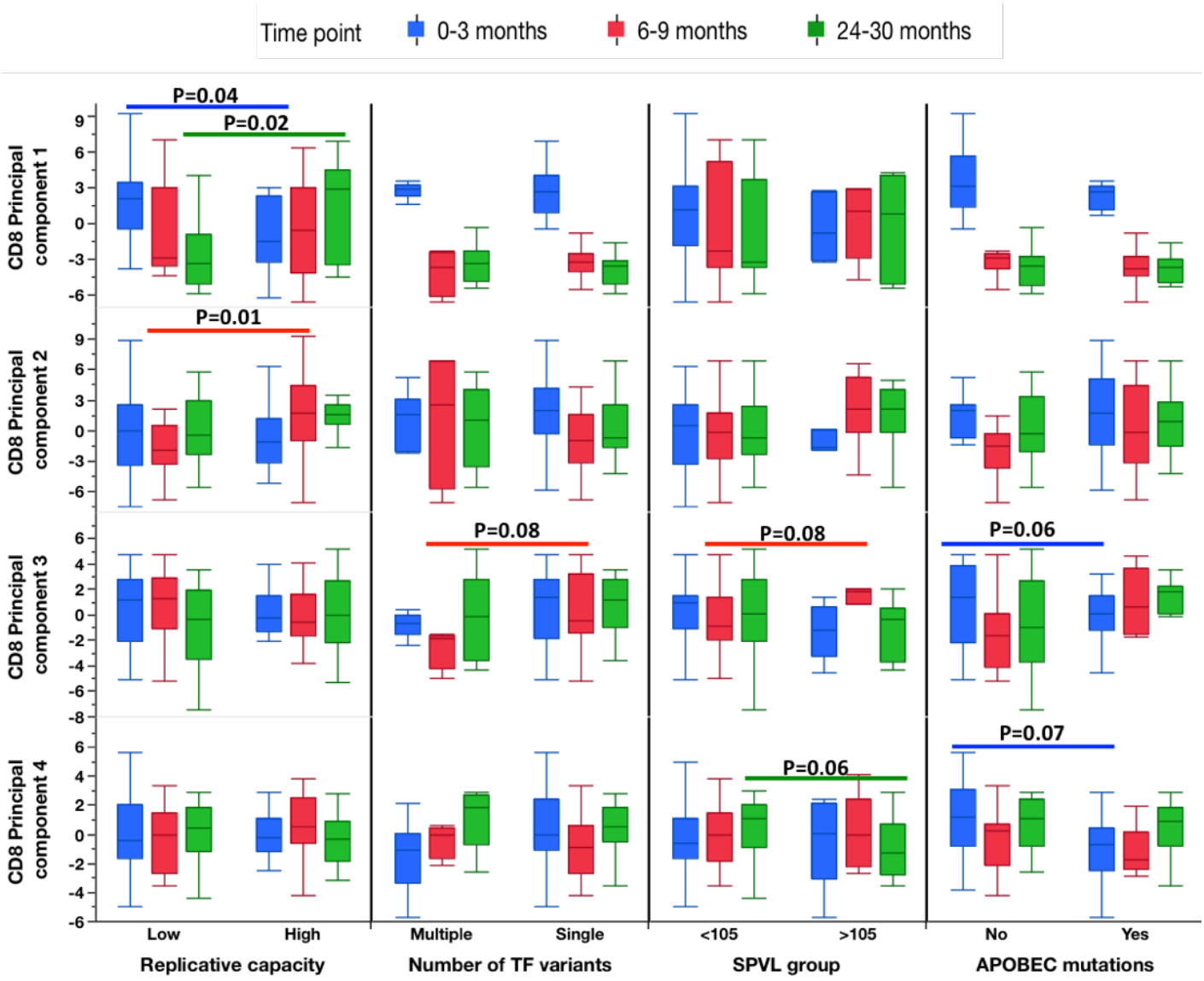


Figure 5.10: The association between virus characteristics and CD8 T-cell principal component loadings.

High replicative capacity infection was accompanied by changes in PC1 at 0-3 and 24-30 months, and PC2 loadings at 9 months. The only observed influence of multivariant infection was a trend towards higher loadings for PC3 at 6-9 months while low set point viral load was characterised by changes in PC3 loadings at 6-9 months and PC4 loadings at 24-30 months. Presence of APOBEC mutations was associated with lower loadings for both PC3 and PC4 at 3 months post infection. p values have been corrected using the Benjamini-Hochberg technique as described in the materials and methods.

5.2.7 B cell principal components

The top four B cell principal components could explain 63.6% of the variation in B cell phenotype, with PC1, PC2, PC3 and PC4 accounting for 23.4%, 17.3%, 14.5% and 8.4% of the observed variation respectively. The total number of B cell principal components alongside their cumulative contribution to B cell variation are shown in Figure 5.11.

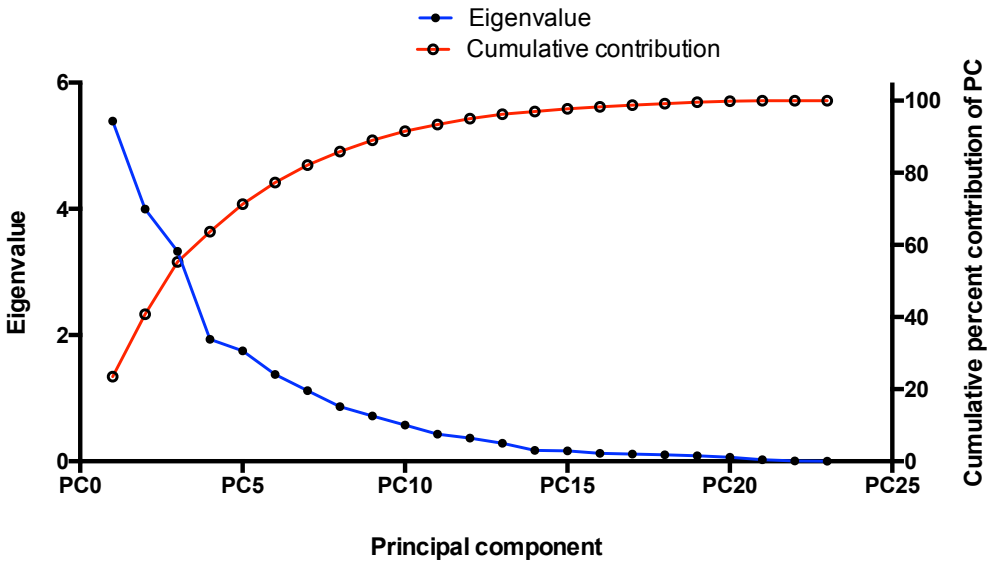


Figure 5.11: Scree plot showing all the identified B cell components.

Scree plot showing all the identified B cell components, eigenvalue loadings and cumulative percent contribution of each principal component.

B cells in PC1 had increased expression of CD86 in multiple subsets at all measured time points, except in plasmablasts which only had increased expression at 24-30 months post infection (Figure 5.12A). There was increased differentiation to activated memory, resting memory and plasmablasts, and fewer tissue like memory B cells, and immature transitional B cells starting at 6-9 months and detectable at 24-30 months after infection. At 6-9 months there was a reduced frequency of IgD^+ B cells, likely representing increase in class switching.

The PC2 profile was of patients whose B cells showed increased frequencies of IgD^+ resting memory and naïve B cells, as well as plasmablasts, but had fewer tissue like memory B cells at acute infection (Figure 5.12B). At 9 months, they had fewer frequencies of CD86 expressing immature transitional and tissue like memory B cells, even though there was increase in the frequency of all immature transitional and tissue like memory B cells and consequent decrease in plasmablasts and resting memory B cells. However, the B cell compartment had recovered at 24-30 months, as there was increased frequency of useful subsets including plasmablasts, mature B cells and resting memory, and fewer transitional B cells.

The PC3 profile comprised patients with increased frequencies of immature transitional B cells and fewer mature B cells at the 0-3- and 6-9-month time points after infection and increase in resting memory and CD86 expressing plasmablasts at the 0-3-month time point, in resting memory B cells at the 24-30 month time point and in activated mature B cells and tissue like memory B cells at all time points. There were more IgD^+ naïve and immature B cells, but fewer IgD^+ plasmablasts (Figure 5.12C).

B cell PC4 patients had increased frequencies of IgD expressing activated memory, resting memory, mature B cells and tissue like memory B cells at the early time points, but fewer immature B cells at 0-3 and 6-9 months, and fewer activated mature B cells at 6-9 and 24-30 months (Figure 5.12D).

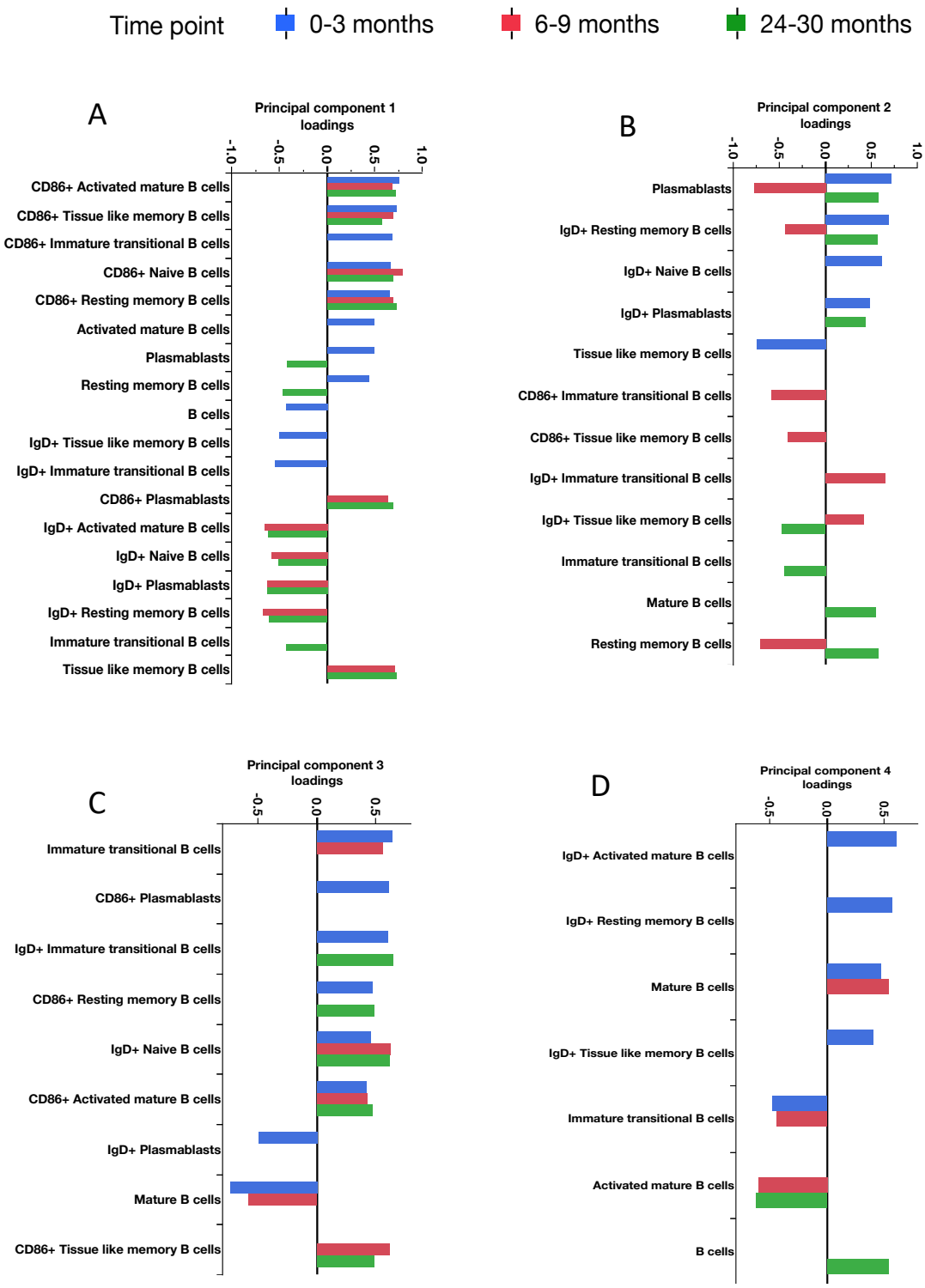


Figure 5.12 A-D: The constituents of various B cell principal component profiles.

All principal component profiles were mainly defined by the expression patterns of CD86 and IgD at all time points.

5.2.8 Role of virus characteristics in driving the B cell response

The largest variation in B cells (PC1) could be explained in part by replicative capacity and the presence of APOBEC mutations. Patients infected with high replicative capacity founder viruses had lower PC1 loadings 0-3 months after infection (p=0.03) and a trend towards lower loadings for PC2 at 0-3 (p=0.08) and 24-30 months (p=0.07) after infection, but higher loadings for PC3 at 0-3 (p=0.03), and 24-30 (p=0.02) months after infection. Thus, at 3 months, they had more unswitched IgD^+ naïve, tissue like memory and immature transitional B cells, fewer tissue like memory B cells, increased expression on CD86 in plasmablasts, resting memory and activated mature B cells. At 24-30 months after infection, they had more IgD^+ tissue like memory B cells, IgD^+ naïve B cells, immature transitional cells, $CD86^+$ resting memory, tissue like memory B cells and activated mature B cells.

Enrichment for APOBEC mutations was associated with perturbations at 6-9 and 24-30 months after infection, in the form of high loadings for PC1 at 6-9 (p=0.03) and 24-30 (p=0.003) months, but lower loadings for PC2 only at 6-9 months (p=0.009) but higher at 24-30 months (p=0.06). This constituted an increase in CD86 expressing activated mature, tissue like memory, naïve, resting memory B cells and plasmablasts at both time points, and increased frequency of tissue like memory B cells at these timepoints.

Multivariant infection was associated with higher loadings for both principal components 3 (p=0.01) and 4 (p=0.03) at 6-9 months only, symbolising increase in mature and immature transitional B cells, increase in IgD expressing naïve B cells and CD86 expressing activated mature and tissue like memory B cells.

Patients with high set point viral load had higher loadings for principal component 3 (p=0.03) but lower loadings for principal component 4 (p=0.002) at 6-9 months, but trend at 0-3 months

(p=0.09) representing a similar profile to multivariant infection, except that they had fewer immature transitional and activated mature B cells.

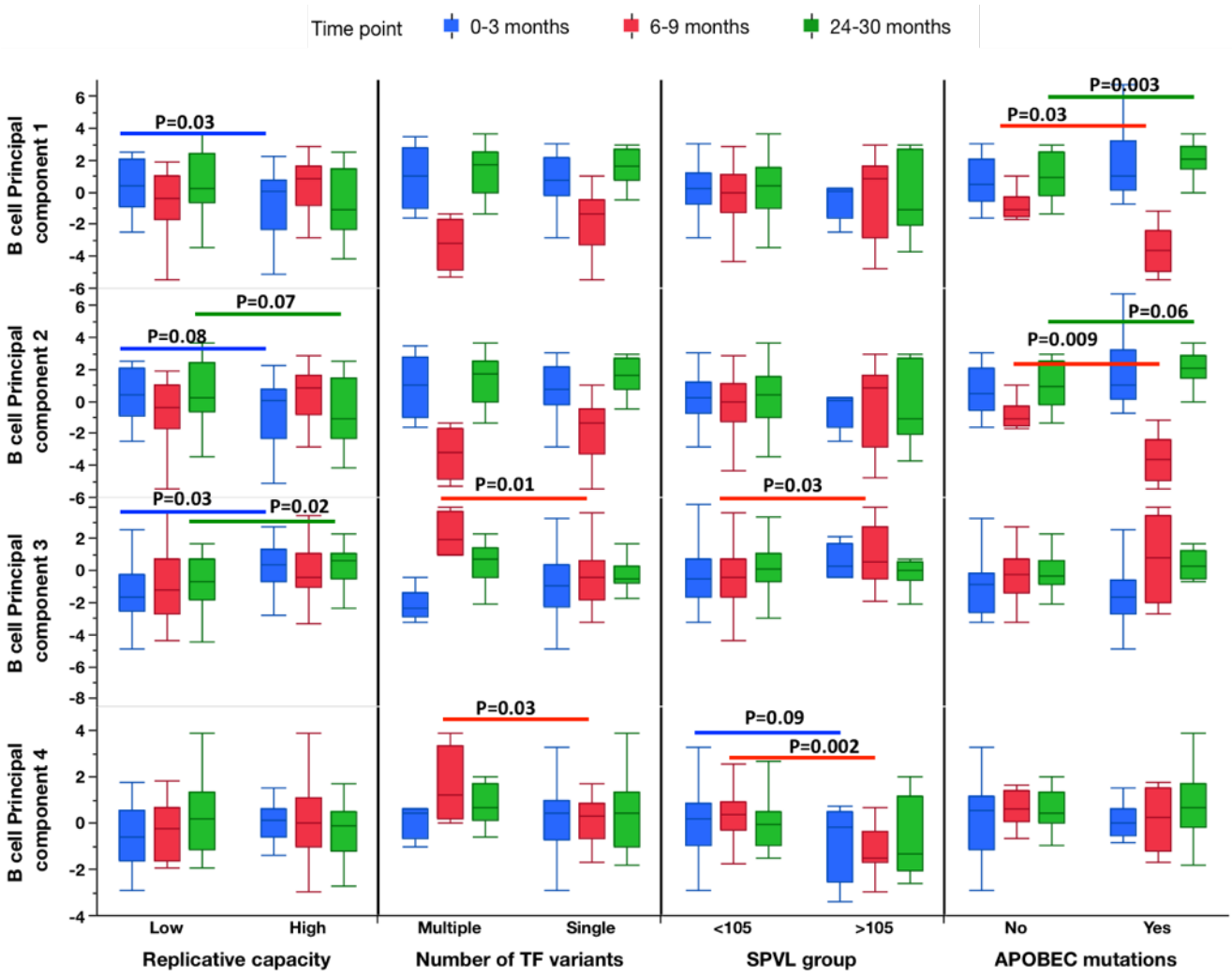


Figure 5.13: The association between virus characteristics and B cell principal component loadings identified.

High replicative capacity infection was accompanied by changes in PC1 at 0-3 months and PC2 and PC3 at 0-3 and 24-30 months. Multivariant infection was associated with higher loadings for PC3 and PC4 at 6-9 months while high set point viral load was characterised by changes in PC3 and PC4 loadings at 6-9 months. Presence of APOBEC mutations was associated with changes in loadings for PC1 at 6-9 months and 24-30 months and PC3 at 6-9 months. p values have been corrected using the Benjamini-Hochberg technique as described in the materials and methods.

5.2.9 NK cell principal components

The top four NK cell principal components could explain 60.7% of the variation in NK cell phenotype, with PC1, PC2, PC3 and PC4 accounting for 33.6%, 12.1%, 7.8% and 7.2% of the observed variation respectively. The total number of NK cell principal components alongside their cumulative contribution to NK cell variation are shown in Figure 5.14.

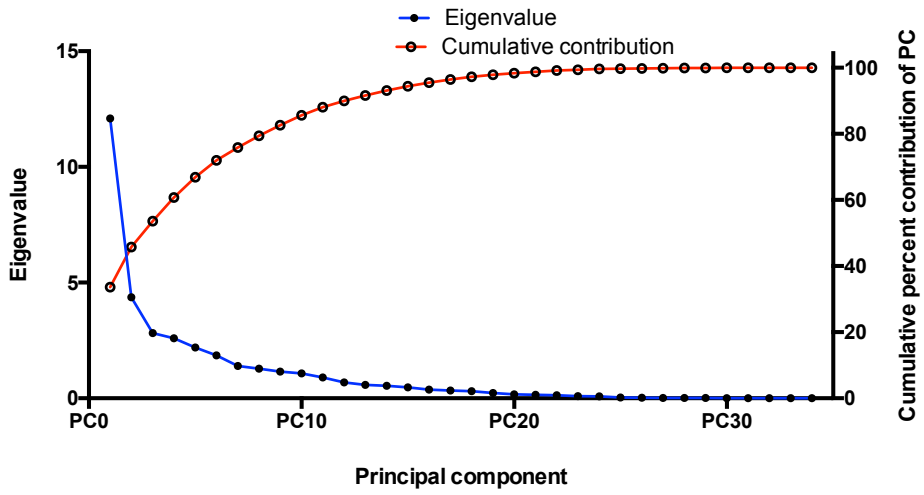


Figure 5.14: Scree plot showing all the identified NK cell components.

Scree plot showing all the identified NK cell components, eigenvalue loadings and cumulative percent contribution of each principal component.

NK cell PC1 comprised patients whose NK cells had increased frequencies of CD57 expressing non-cytotoxic NK cells at all time points measured, and a corresponding reduction in degranulating ($CD107a^+$) NK cells with or without granzyme B and perforin (Figure 5.15A). By 24-30 months post infection, their NK cells had increased frequencies of $CD38^+$ and $NKp44^+$ cytolytic NK cells. Increased frequencies of $CCR7^+$ NK cells were also seen at all time points.

PC2 comprised patients with increased proportions of $CD57^+$ NK cells at acute infection, with or without granzyme B and perforin (Figure 5.15B). There was also increase in $NKG2C^+$ and

decrease in NKG2A$^+$ and CD38$^+$ NK cells at acute infection but which persisted at all measured time points. At acute infection there was increase in granzyme B$^+$ CD107a$^+$ but perforin- NK cells and at the 9-30-month timepoints there was increase in anergic NK cells, NKp44$^+$ cytolytic NK cells, HLA-DR$^+$ NK cells, CCR7$^+$ cytolytic cells and fewer cytolytic NK cells.

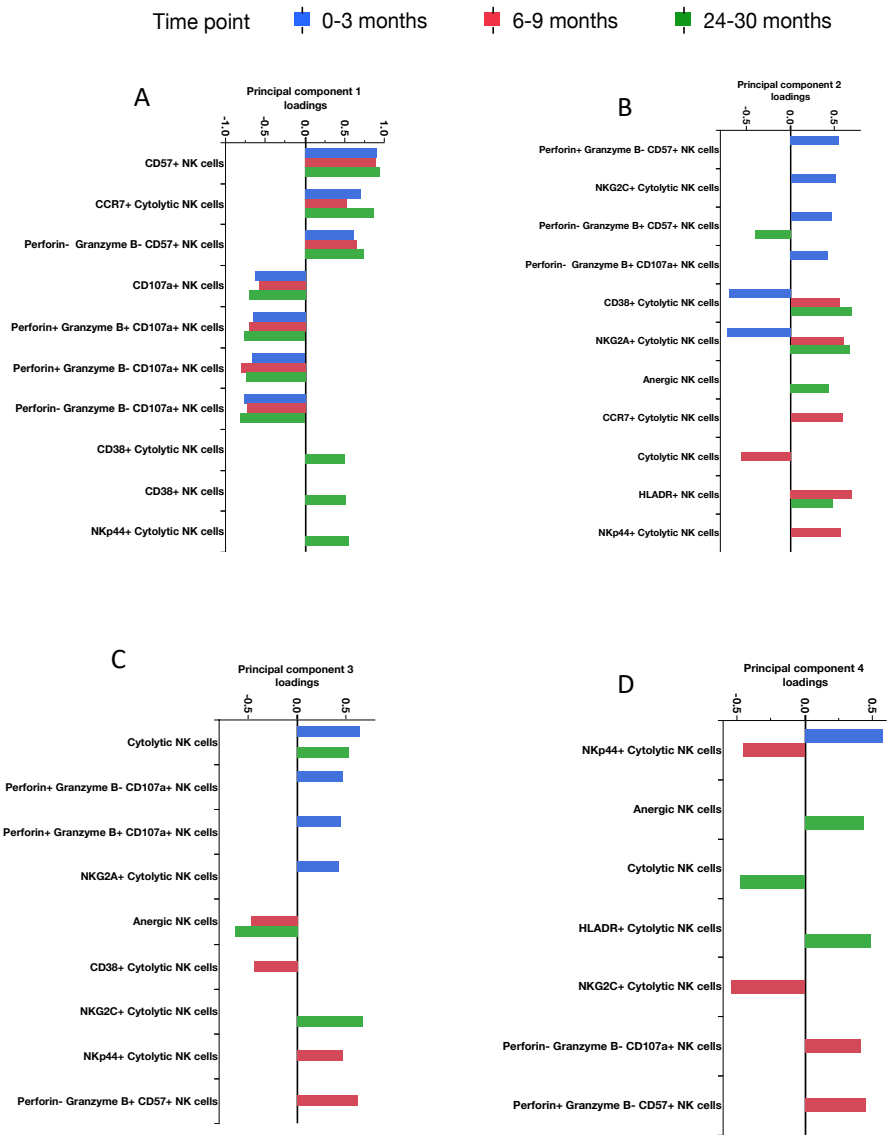


Figure 5.15: The constituents of various NK cell principal component profiles.

All principal components were defined by patterns in cytotoxicity as defined by the expression profile of granzyme B, perforin, CD107a and frequencies of cytolytic NK cells.

PC3 patients had increase in proportions of cytolytic NK cells, NKG2A$^+$ cytolytic NK cells, and CD107a$^+$ Perforin$^+$ but Granzyme- NK cells at 0-3 months (Figure 5.15C). However, by 6-9 months and at 24-30 months, there was an increase in NKG2C$^+$ and NKP44$^+$ NK cytolytic cells, and CD57$^+$ Granzyme$^+$ Perforin- NK cells.

PC4 was defined by the chronic time points. There was increased NKP44$^+$ cytolytic NK cells at acute infection which was decreased by 6-9 months (Figure 5.15D). However, at 6-9 months there was increase in anergic and HLA-DR$^+$ cytolytic NK cells, but fewer cytolytic NK cells overall. At 24-30 months, there was increase in CD107a$^+$ granzyme and perforin negative NK cells, CD57$^+$ Granzyme$^+$ Perforin- NK cells and fewer NKG2C$^+$ NK cells.

5.2.10 Role of virus characteristics in driving the NK cell response

The largest variation in NK cell phenotype could be partially explained by replicative capacity and enrichment for APOBEC mutations. High replicative capacity infection was associated with high PC1 loadings at 3 months (p=0.02), low PC3 loadings at 30 months (p=0.04), and high PC4 loadings at 9 months (p=0.01). These comprised increased CD57 expression in NK cells, especially in the Granzyme B- Perforin- subset, increase in CCR7$^+$ cytolytic NK cells, increase in anergic NK cells, and increase in CD57$^+$ and CD107a$^+$ Granzyme- NK cells with or without Perforin at 9 months. Enrichment for APOBEC mutations was associated with lower PC1 loadings (p=0.001) and PC3 loadings (p=0.05) at 30 months, representing increase in CD107a expression with or without granzyme B and perforin, and increased anergy. High SPVL was associated with high PC4 loadings at 9 months (p=0.003), showing increase in CD57$^+$ and CD107a$^+$ Granzyme- NK cells with or without Perforin. Even though it wasn't statistically significant, there was a trend towards increased loadings for PC2 (p=0.07) at 3

months in multivariant infection, suggesting an increase in cytolytic, NKG2A^{+} cytolytic and CD107a^{+} Perforin^{+} NK cells with or without Granzyme B.

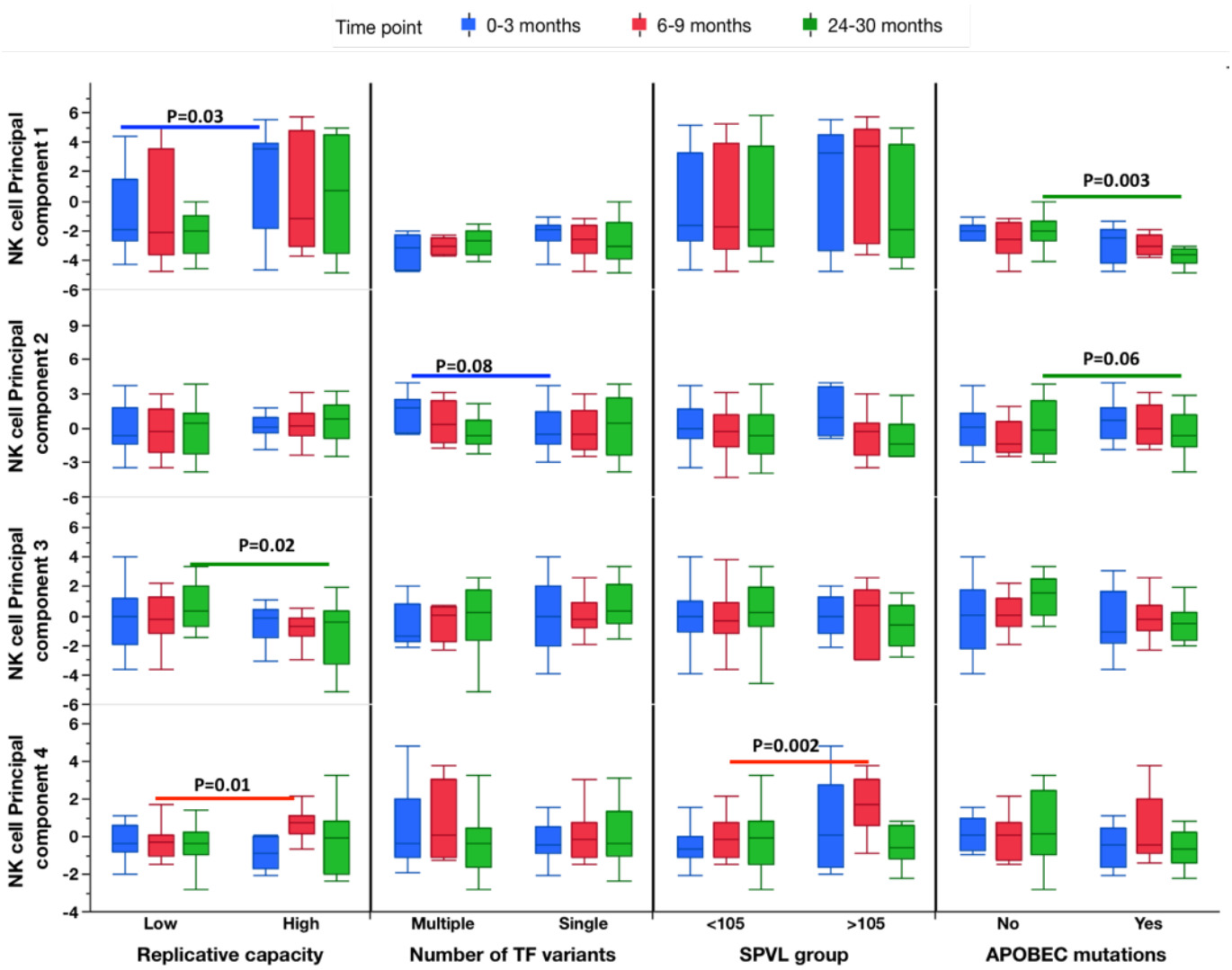


Figure 5.16: The association between virus characteristics and NK cell principal component loadings identified.

High replicative capacity infection was associated with changes in PC1, PC3 and PC4 loadings at 0-3 months, 24-30 months and 6-9 months respectively. Enrichment for APOBEC mutations was associated with changes in PC1 and PC3 loadings at 30 months while high SPVL was associated with high PC4 loadings at 9 months. There was a trend towards increased loadings for PC2 at 3 months in multivariant infection. p values have been corrected using the Benjamini-Hochberg technique as described in the materials and methods.

5.2.11 Role of virus characteristics in driving the monocyte response

Three monocyte subsets were identified based on their expression of CD14 and CD16. The largest contributors to monocyte variation were replicative capacity and SPVL. At acute infection, high RC patients had higher frequencies of non-classical monocytes (p=0.03) and a corresponding trend towards fewer classical monocytes (p=0.09) (Figure 5.17).

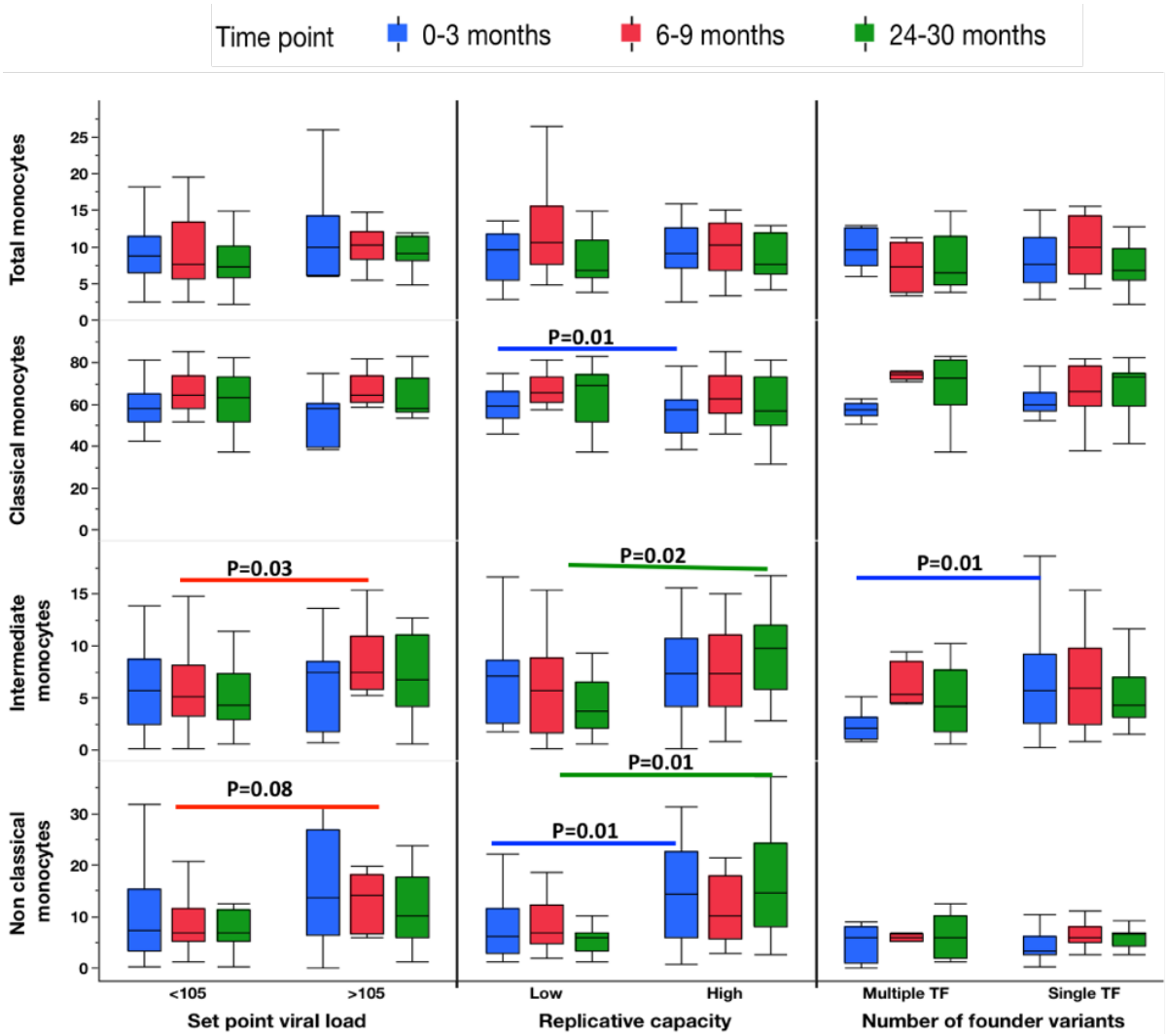


Figure 5.17: Association between virus characteristics and monocyte subsets.

Although the frequency of total monocytes was not affected by virus characteristics, the distribution of classical, intermediate and non-classical monocytes was associated with SPVL, replicative capacity and the number of founder viruses. p values have been corrected using the Benjamini-Hochberg technique as described in the materials and methods.

As infection progressed to 24-30 months, there were still increased frequencies of non-classical monocytes ($p=0.001$) but also intermediate monocytes ($p<0.0001$). Differences brought about by SPVL were seen at 6-9 months post infection, when patients with a high set point viral load had higher proportions of both non-classical ($p=0.05$) and intermediate ($p=0.01$) monocytes. Multivariant infection was associated with higher frequencies of intermediate monocytes ($p=0.06$) at the 3-month time point only.

5.2.12 Role of virus characteristics in driving the early cytokine response

One of the earliest hallmarks of the early immune response to HIV-1 is the acute cytokine storm. A possible contribution of the virus characteristic that had been previously identified as important determinants of disease progression contributed towards skewing the composition of the early cytokine response was therefore evaluated. Figure 5.18 shows the degree to which virus characteristics skewed the early cytokine response in HIV-1 infection. Cytokine levels were not measured in HIV uninfected individuals, and comparisons presented are drawn from comparing cytokine levels in patients with divergent viral characteristics.

HIV controllers had significantly lower levels of the apolipoprotein serum amyloid A (SAA, $p=0.05$), the lymphotoxin tumour necrosis factor β (TNFβ, $p=0.05$), the angiogenesis stimulating factor basic fibroblast growth factor (bFGF, $p=0.03$), the endothelial activation marker vascular cell adhesion marker-1 (VCAM1, $p=0.04$) and the Th_{17} cytokine interleukin 17A (IL-17A, $p=0.01$). On the other hand, high levels of the class II interferon interferon-gamma (IFNγ, $p=0.04$) were observed in patients with high SPVL. High RC was associated with higher levels of IL-15 ($p=0.05$). APOBEC mutations were associated with higher levels of the granulocyte growth factor granulocyte-macrophage colony stimulating factor (GM-CSF,

p=0.01) while multivariant infection was associated with lower levels of the inflammatory cytokine Interleukin 1 alpha (IL-1α, p=0.04). Data are summarised in Figure 5.19.

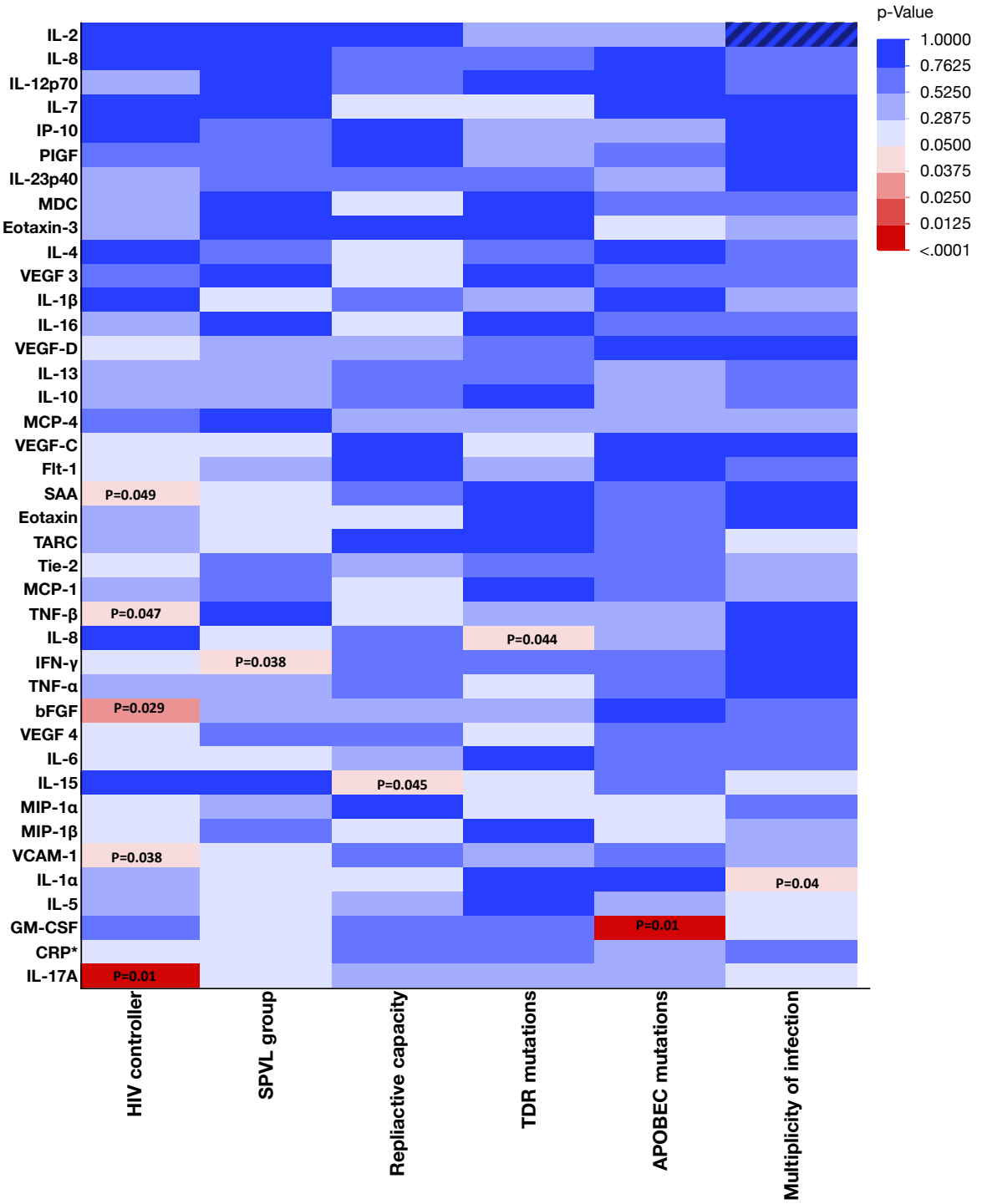


Figure 5.18: Association between virus characteristics and plasma cytokine concentration at acute HIV-1 infection. p values have been corrected using the Benjamini-Hochberg technique as described in the materials and methods.

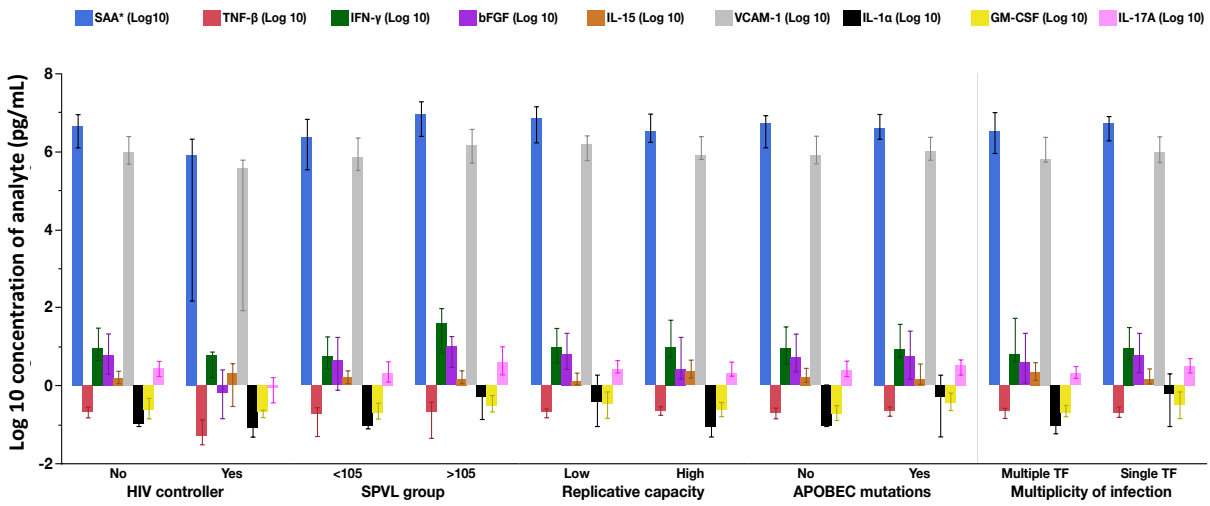


Figure 5.19: Median concentration of plasma cytokines that showed an association with transmitted/founder virus characteristics.

HIV controllers had lower levels of SAA, TNFβ, bFGF, VCAM1 and IL-17A. High levels of IFN were observed in patients with high SPVL while high RC skewed towards higher levels of IL-15. APOBEC mutations were associated with higher levels of GM-CSF while multivariant infection was associated with lower levels of IL-1α.

5.3 Discussion

The causes of HIV-1-associated immune activation established in early HIV-1 infection are not clearly defined. Multiple related events contribute to such activation, including direct viral infection of immune cells, pro-inflammatory cytokine production by innate cells (which drives both direct and bystander activation of other immune cells), translocation of microbial products into the blood through damaged intestinal epithelium, loss of virally infected regulatory T cells and chronic mycobacterial and viral co-infections (Appay and Kelleher 2016; Bandera et al. 2017; Hileman and Funderburg 2017; Moir and Fauci 2017; Paiardini and Muller-Trutwin 2013; Younas et al. 2016; Liovat et al. 2012). Consistent with all these findings, it has been confirmed that viral load is only an indirect contributor to the rate of progression to AIDS, that immune activation predicts changes in $CD4^+$T cells more accurately and independent of viral load, and that the effect of anti-retroviral therapy in increasing $CD4^+$ T-cell counts better correlates with the decrease in immune activation than the suppression of viral load (Liovat et al. 2012; Lubaki et al. 1999; Benecke, Gale, and Katze 2012; Boasso and Shearer 2008; Brenchley et al. 2003). That natural hosts are able to resolve inflammation, antiviral drugs do not target immune activation and that a residual immune activation persists despite successfully suppressed viremia highlights the importance of identifying, and then targeting, the mechanisms that cause immune activation (Lubaki et al. 1999; Burgers et al. 2009; Sauce, Elbim, and Appay 2013; Sauce et al. 2011; Hunt et al. 2003).

A principal component approach was employed to investigate whether levels of the activation and differentiation process of T cells, B cells, NK cells and monocytes, and the early cytokine response would be affected by selected virus characteristics and found evidence that viral characteristics perturb phenotypic characteristics of all cell types characterised to varying degrees, and at various stages of disease.

Multivariant infection had previously been associated with higher peak viral load and lower CD4 T cell counts throughout infection in Chapter 3. Consistent with that, differences in immune responses brought about by multivariant infection were restricted to the earliest time point measured, with only a few changes detectable at 9 months. During early infection, multivariant infection was associated with increased CD4 T cell and NK cell activation and cytotoxicity, with higher frequencies of NK cells expressing the inhibitory receptor NKG2A. There were increased frequencies of intermediate monocytes, and lower levels of IL-1α. By the 6-9-month time point, CD4 T cells, NK cells and monocytes in patients with multivariant infection were similar to those infected by a single founder virus. However, there was an increase of CD8 T cell differentiation to terminal effector CD8 T cells and increased senescence. The increased B cell turnover evidenced by increased frequency of immature transitional and mature B cells was accompanied by increased expression of CD86 in the activated mature B cells and in tissue like memory B cells. Consistent with the observation that patients infected with more than one founder virus had higher peak viral load but no differences in viral load past the second month of infection including SPVL, differences observed as a result of multivariant infection at acute infection when viral load was higher have been previously associated with viremia (Audige et al. 2010; Chen et al. 2017; Krzysiek et al. 1998; Ma et al. 2017; Mitchell et al. 2016; Nicholas et al. 2013; Zulu et al. 2017). For instance, increased cellular activation and differentiation in T cells, B cells, NK cells and monocyte are a hallmark of fast HIV-1 progression (Moir and Fauci 2014; Malaspina et al. 2002; Alter and Altfeld 2009; Hasegawa et al. 2009; Ndhlovu et al. 2015; Liovat et al. 2009). The mechanisms of immune perturbation as a result of multivariant infection however appears to be reversible by normalisation of viral load, with no differences between patients of single vs multiple variant infection after two years of infection.

On the other hand, the effects of viral replicative capacity on levels of cellular immune activation and differentiation were distinctly different from those of multivariant infection and persisted at all time points measured. CD4 T cells in high replicative capacity infection were phenotypically like those of low RC infection at acute infection, but there were increased frequencies of activated CD4 T cells at 6-9 and 24-30 months post infection, and increased proliferation and exhaustion at 24-30 months. A similar profile was seen in CD8 T cells, except that at acute infection there was increased proliferation. Indeed, viral replicative capacity has been previously correlated with increase expression of CD38 and HLA-DR on CD8 T cells in subtype C infection at acute infection (Claiborne et al. 2015). However, this chapter demonstrates that this is accompanied by increased exhaustion and persists past 24 months of infection. CD8 T cell exhaustion is characteristic of chronic pathogenic SIV/HIV infection, and levels of PD-1 expression are predictive of disease progression (Porichis and Kaufmann 2012; Blattman et al. 2009; Rosignoli et al. 2009), a feature that was observed at 6 and 24 months of infection. B cells in high RC infection expressed increased CD86 and showed reduced class switching at all time points. There were fewer tissue like memory B cells at acute infection, but higher frequencies at 24-30 months, as well as activated memory B cells. This increased activation and differentiation of B cells has been previously described in treated viraemic and untreated viraemic patients and associated with fast progression (Lane et al. 1983; Moir et al. 2001; Moir and Fauci 2017; Portugal et al. 2017; Shirai et al. 1992; Buckner et al. 2016) but to the best of our knowledge this is the first time that vRC is associated with defects in the B cell compartment. Similarly, the increased anergy that was observed in NK cells starting at 6-9 months accompanied by increased senescence has been previously described in viraemic and untreated patients but has not been previously linked to vRC (Alter et al. 2006; Alter et al. 2005; Mikulak et al. 2017).

The observation that there was an increase in non-classical monocytes up to 9 months, which have previously been shown to be increased in both acute and chronic HIV-1 infection (Mitchell et al. 2016; Han et al. 2009), and which are functionally different into classical and intermediate monocytes (Ancuta et al. 2009; Chen et al. 2017; De Pablo-Bernal et al. 2016; Tippett et al. 2011; Wong et al. 2012) provides additional insights on how viral replicative capacity modifies the early immune milieu. The intermediate monocyte subset was increased during chronic infection with high vRC virus and has been previously inversely correlated with a decrease in $CD4^+$ T-cell counts and shown to secrete sCD163, whose plasma levels are an independent marker of the progression to death and AIDS (Burdo et al. 2011; Knudsen et al. 2016; Liang et al. 2015; Moller 2012; Tippett et al. 2011). The specific roles of the three monocyte subsets in Th cell differentiation have not been fully characterized in HIV-1-infected patients. In some studies, classical and intermediate monocytes promoted Th1 and Th17 development, which in turn negatively regulated interleukin IL-17 and T-reg induction (Chen et al. 2017; Rossol et al. 2012; Zhong et al. 2012). In contrast to the non-classical and classical monocyte subsets, the frequency of the intermediate monocytes has been positively associated with the frequency of IFN-γ and IL-4 producing $CD4^+$ T cells in HIV-1-infected patients (Chen et al. 2017).

Recent studies have shown that IL-15 is produced during acute HIV and SIV infection expands different $CD4^+$ T cell subpopulations than IL-2 in SIV-infected animals and may therefore be useful for the restoration of effector memory $CD4^+$ T cells that are depleted early in HIV-1 and SIV infection (Calarota et al. 2008; d'Ettorre et al. 2012; Manganaro et al. 2018; Swaminathan et al. 2016). Data also suggest that IL-15 acts not only on $CD8^+$ T cells and natural killer cells, but also on effector memory $CD4^+$ T cells (Bayard-McNeeley et al. 1996; Garrido et al. 2018; Li et al. 2015; Mastroianni et al. 2004; Mueller et al. 2003; Mueller et al. 2008; Younes et al. 2016). Although the role of intrinsic IL-15 during chronic infection is much less defined, administration of IL-15 during acute SIV infection dramatically increases viral set point and

markers of inflammation (Bayard-McNeeley et al. 1996; Mueller et al. 2003; Garrido et al. 2018). There were higher levels of IL-15 during early HIV-1 infection in the contect of high vRC variants, which ties in with the increase in immune activation and higher viral load observed.

Human APOBEC3 proteins are cytidine deaminases that contribute broadly to innate immunity through the control of retrovirus replication by inducing extensive guanosine-to-adenosine (G-to-A) mutagenesis and inhibiting synthesis of nascent human HIV-1 cDNA, and contributing to sequence diversification and adaptation of the viral genome (OhAinle et al. 2006; Refsland, Hultquist, and Harris 2012; Ross 2009; Sheehy et al. 2002). Lethal APOBEC induced HIV-1 mutagenesis will be selected against *in vivo*, and moderate APOBEC mutagenesis appears to induce variation that fuels viral heterogeneity and increased fitness (Kim et al. 2014). This hypothesis is further supported by the observation that in these patients, surprisingly enrichment for APOBEC mutations within *gag* was associated with increased replicative capacity.

Consistent with the effects of epitope diversification and immune selection, amino acid changes accumulated at the APOBEC editing contexts are typically located within HLA-1 appropriate epitopes that are known or predicted to enable peptide binding (Addo et al. 2002; Armitage et al. 2014; Kim et al. 2014). Thus, APOBEC activity may induce mutations that influence the HIV-1 recognition by various immune cells. Indeed, patients whose TF sequences had enrichment for APOBEC mutations exhibited perturbations in all subsets measured except monocytes. At acute infection, CD4 T cells, B cells and NK cells were phenotypically similar regardless of enrichment for APOBEC mutations. However, there was increased CD8 T cell activation, exhaustion and senescence, perturbations which were only observed in CD4 T cells at 6-9 months after infection. APOBEC induced mutations have previously been shown to be enriched in CTL epitopes of HIV-1 and result in diminished T cell responses against previously antigenic epitopes (Casartelli et al. 2010; Monajemi et al. 2014). This is further supported by

evidence that when antigen presenting cells are infected, the introduction of mutations may generate truncated or misfolded proteins, which are degraded by the proteasome and provide MHC class I epitopes leading to increased CTL responses and destruction of infected cells (Ogg et al. 1998; Shankar et al. 2000; Deng et al. 2015), suggestive of their ability to modulate T cell activation and exhaustion.

The different APOBEC3 proteins are expressed to varying levels in hematopoietic cell populations, including $CD4^+$ and $CD8^+$ T cell subsets (e.g. naïve and memory), B cells, and myeloid cells (Chiu and Greene 2009; Malim 2009). Certain cells, such as B cells, shed APOBEC in exosomes, which can be transmitted to virus-infected cells which then produce dead or attenuated viruses (Khatua et al. 2009; Martin, Johnson, and D'Aquila 2011). Such B cells have been shown to be defective in their ability to produce neutralising antibodies and undergo somatic hypermutation in Friend murine leukaemia virus infection (Santiago et al. 2008; Tsuji-Kawahara et al. 2010). There was increased expression of CD86 in multiple B cell subsets, and an overall expansion of the tissue like memory B cell compartment which has been previously described as exhausted, suggesting overall dysfunction of B cell function in high APOBEC activity. APOBEC has also been shown to induce increased NKG2D ligand expression, NK-mediated killing of infected cells and increased destruction of HIV-1 infected cells by natural killer cells (Borrow, Shattock, and Vyakarnam 2010; Croxford and Gasser 2011; Iwatani 2011; Lavender et al. 2016; Norman et al. 2011). NK cells were phenotypically similar at acute and up to 9 months after infection regardless of the presence of APOBEC mutations, but there were increased frequencies of anergic and cytotoxic NK cells, two opposing phenotypes but which together symbolise another antiviral function of APOBEC. It is unclear why APOBEC mutations were associated with high levels of plasma GM-CSF at acute infection. However, loss of peripheral blood MAIT cells and associated shifts in tissue

homing receptor expression and GM-CSF production may contribute to an immune environment that is permissive to HIV-1 infection (Juno et al. 2018).

Having identified a role for viral characteristics in shaping the early immune response, we investigated whether the virus induced perturbations overlapped with those involved in control of viremia. No cellular characteristics were consistently associated with HIV controllers. However, HIV controllers consistently had lower levels of various cytokines and soluble proteins including SAA, TNFβ, bFGF, VCAM1 and IL-17α, supporting the observation by several groups that HIV controllers are better able to control inflammation, and suggesting that these soluble proteins may have been produced by cells and/or tissues that were not sampled (Autran et al. 2011; Buranapraditkun et al. 2017; Card et al. 2012; Cortes et al. 2018; Gonzalo-Gil, Ikediobi, and Sutton 2017; Platten et al. 2016).

Similarly, there were no associations between the measured cellular phenotypes during early infection and SPVL. However, at 6-9 months post infection, high SPVL was associated with increased degranulation in CD8 T cells and NK cells, culminating in increased CD8 T cell exhaustion at 30 months post infection. In addition, there was increased B cell maturation and activation but less class switching, and monocyte differentiation to classical and intermediate phenotypes 6-9 months post infection, with no differences at 24-30 months post infection. Thus, the only cellular perturbations that were observed to correlate with both T/F virus characteristics and SPVL were increased senescence exhaustion at chronic infection, which is likely a result of increased antigenic load and chronic stimulation.

Taken together, these data demonstrate that individuals infected with high RC viruses are either unable to control immune activation and inflammation or driven towards increased immune activation even after the establishment of SPVL, have higher immune exhaustion and anergy, have more differentiated T cells, and B cells, thus positioning these as a link between vRC and

disease progression. Multivariant infection perturbs the immune system at acute infection but most of the perturbations are reversible upon normalisation of viral load even though the effects on CD4 T cell decline persist.

Chapter 6: Final discussion and future work

6. Final discussion and future work

6.1 Generation of *gag*-NL4.3 chimeras

The extent to which differences in experimental approach can impact the interpretation of virus complexity must not be underestimated and was highlighted in a study of acute and early HIV-1 clade C infection (Salazar-Gonzalez et al. 2008). Several groups have used the heteroduplex tracking assay (HTA) method to define the multiplicity of HIV-1 infection (Ritola et al. 2004; Salazar-Gonzalez et al. 2008), but this has been demonstrated to be inferior to the single genome amplification (SGA) direct sequencing of long continuous reads approach that was adopted (Keele et al. 2008; Parrish et al. 2013). First, SGA-direct sequencing eliminates *Taq* polymerase errors in finished sequences because such base substitutions are essentially random in distribution and any one substitution is present in exceedingly low proportions in the uncloned amplified product (McInerney, Adams, and Hadi 2014; Salazar-Gonzalez et al. 2009). Since the amplified product is directly sequenced, such mutations are not evident on sequence chromatograms unless they occur in the initial PCR amplification cycles, in which case they are identified as double peaks and can be confirmed by our barcoding approach. Second, SGA eliminates both template switching between genetically distinct viral genomes and template resampling, because amplification is initiated from single genomes (Potapov and Ong 2017). Third, SGA-direct sequencing avoids misrepresentation of target sequence frequencies because of unequal cloning (Salazar-Gonzalez et al. 2009; Shaw and Hunter 2012). In a phylogenetic tree containing all the patient derived sequences, viral sequences representing intra-host HIV-1 quasispecies could be identified as distinct monophyletic groups.

In generating chimeric viruses that express patient derived *gag*, some groups electroplate patient plasma derived *gag* amplicons containing the whole diversity of quasispecies present within the patient at the sampling point together with a linearized *gag*-deleted backbone, into

GXR cells and rely upon homologous recombination and outgrowth of correctly ligated chimeras (Kiguoya et al. 2017; Ojwach et al. 2018; Wright et al. 2010; Wright et al. 2011). While this strategy better represents the whole array of virus populations that are present in the patient, it requires the outgrowth of viral quasispecies, which may select for the most fit virus, and in some cases, amino acid changes in the viral stocks that are not present in the individual from which they were derived. Our cloning method for generating Gag-NL4.3 chimeric viruses used clonal PCR product and did not rely on viral outgrowth to generate clonal virus stocks which allowed for the characterization of each transmitted/founder variant in isolation and therefore more precisely.

Molecular cloning with large retroviral plasmids has traditionally been difficult for a variety of reasons (Oh et al. 2002). Large plasmids reduce transformation efficiency of competent bacterial strains (Hanahan, Jessee, and Bloom 1991; Horton et al. 1989; Inoue, Nojima, and Okayama 1990), while retroviral inserts which contain long terminal repeat (LTR) sequences reduce stability of the plasmid, and compromise replication fidelity of the plasmid DNA within the bacterial host leading to deletions of the retroviral genome (Bichara et al. 2000; Bichara, Wagner, and Lambert 2006).

The impact of engineering foreign *gag* sequences into NL4.3 on virus replication was significant, with many of the chimeras exhibiting growth lower that wild type NL4.3 which is a great replicator. Previous studies using backbones that replicate less efficiently such as MJ4 have demonstrated greater than a hundred-fold higher replication of *gag* chimeras than wild-type MJ4 (Claiborne, Prince, and Hunter 2014; Claiborne et al. 2015; Prince et al. 2012). This indicates that substitution of Gag can drastically alter the life cycle of the virus when all other viral components are constant. Multiple intra-molecular contacts as well as host protein interactions in Gag are necessary for effective intracellular Gag trafficking (Spearman et al. 1997), particle formation (Ganser-Pornillos et al. 2004), budding (Weiss and Gottlinger 2011),

maturation (von Schwedler et al. 1998) and disassembly (Forshey et al. 2002). Therefore, immune mediated adaptation of this functionally constrained protein that also has a role in viral susceptibility to host cell intrinsic antiviral defences could have clear consequences for viral replication through disruption of these many interactions.

6.2 What TF characteristics influence disease pathogenesis?

The most severe bottleneck occurs during HIV-1 transmission at the genital tract of the recipient, where the viral diversity present in the transmission fluid is typically low and eventually reduced to a single genotype which initiates systemic infection (Carlson et al. 2014; Joseph and Swanstrom 2014; Kariuki et al. 2017; Shaw and Hunter 2012; Tully et al. 2016). The migration of HIV-1 from the blood into the transmission fluid is likely to be greatly influenced by the trafficking of infected immune cells and free viruses from the donor's blood into their genital tract and/or rectum. However, it is also possible that viruses replicate locally in the genital tract (Joseph and Swanstrom 2014). Therefore, the composition of transmitted/founder variants is likely to represent the result of the dynamics of local replication and trafficking of free virus and/or infected cells from the donor's blood and genital tract to the recipient's genital tract and blood. Nonetheless, the period soon after infection when virus sequences are relatively homogenous presents an opportunity to study characteristics of the transmitted founder virus that influence disease progression, as well as the long-term consequence of a less stringent transmission bottleneck. In addition, the probability of transmission per contact is closely related to the donor's viral load, with the recipient's peak viral load being related to the size of HIV-1 reservoir and sanctuary sites, and early damage to the immune system, some of which is not reversible by viral suppression through combination ART (Boily et al. 2009; Dosekun and Fox 2010; Fox and Fidler 2010; Jin et al. 2010; Mastro

et al. 1994; Shaw and Hunter 2012; Vittinghoff et al. 1999). Therefore, this period is not only important for HIV-1 infectiousness but also sets the stage for disease progression.

6.3 Genotypic characteristics influence HIV-1 pathogenesis

6.3.1 Multiplicity of infection

Our observation of a low number of transmitted viruses in most patients but with the occurrence of multivariant infection in our MSM cohort being higher than has been shown in heterosexual cohorts is consistent with epidemiological observations of the relative inefficiency of virus transmission by most sexual routes (Shattock and Moore 2003; Wawer et al. 2005). Keele and colleagues showed that 78 out of 102 subjects with acute subtype B HIV-1 infection had evidence of systemic infection by a single virus, while the remaining 24 had been infected by approximately two to five viruses (Keele et al. 2008). Applying this same method to subtype A and C heterosexual transmission pairs for whom multiple sequences from both partners were derived, Haaland *et al* determined that a single T/F virus established infection in 90% of cases (Haaland et al. 2009), while an analysis of 69 newly infected subtype C individuals from South Africa showed that 78% involved single variant transmission (Abrahams et al. 2009; Lee et al. 2009; Chaillon et al. 2016; Li et al. 2010).

Furthermore, it has been possible to define the near full-length of the transmitted/founder virus to the nucleotide sequence for each variant. It is clear from our study and others, that in situations where multiple viruses initiate infection, the number of infecting variants does not follow a Poisson distribution, with a majority involving only two or three variants but occasionally more than five (Abrahams et al. 2009; Haaland et al. 2009; Keele et al. 2008; Salazar-Gonzalez et al. 2008). This is consistent with each variant being transmitted

independently with low probability. Most likely, in these cases factors such as sexually transmitted infections, intravenous drug use and high donor viral load potentially lowered the barrier to transmission (Haaland et al. 2009; Sagar 2010; Sagar, Kirkegaard, et al. 2004; Sagar, Lavreys, et al. 2004).

Previous studies of infections established by multiple transmitted/founder (T/F) viruses revealed viral profiles that included: a) low-level persistence of minor T/F variants, b) replacement of the major T/F by a minor T/F, and c) an initial expansion of the minor T/F followed by a quick collapse of the same minor T/F to low frequency (Henn et al. 2012; Kijak et al. 2017). In most participants, CTL escape was first detected at the end of peak viremia downslope, proceeded at higher rates than previously measured in HIV-1 infection, and usually occurred through the exploration of multiple mutational pathways within an epitope (Fischer et al. 2010; Henn et al. 2012; Kijak et al. 2017). The rapid emergence of CTL escape variants suggested a strong and early CTL response with the minor T/F viral strains contributing to rapid and varied profiles of HIV-1 quasispecies evolution during AHI (Kijak et al. 2017). However, our study was limited to acute infection and therefore we cannot precisely predict the contribution of each variant to disease progression.

The selection bottleneck is often more severe in female-to-male transmission than in male-to-female transmission, except when a female donor has a higher viral load, when male recipients have a genital ulcer or inflammation or in male-male receptive anal intercourse (Carlson et al. 2014). In the male-male transmission cases that were studied, the transmission bottleneck was less severe than has been previously shown in both male-female and female-male transmission, likely representing the well-known vulnerability of the thin simple columnar epithelium that lines the rectal mucosal surface as a site of entry (Dezzutti and Hladik 2013; Tebit et al. 2012). The MSM in this study also have higher risk behaviour as evidenced by documented prevalence of inflammatory STI (Masha et al. 2017; Wahome et al. 2017) and multiple partners (Bezemer

et al. 2014; van Griensven and Sanders 2008) coupled with a social healthcare system that is not sensitised to accurate diagnosis and treatment of STI amongst MSM (Dijkstra et al. 2015; Kombo et al. 2017; Sanders et al. 2011), leading to early loss to follow up and reduced uptake of HIV treatment (Hassan et al. 2012) which may result in higher viral loads and the likelihood of higher inoculation dose. Thus, multivariant infections may be a result of both biological and social disadvantages surrounding these most at risk populations.

However, we have not taken into account whether the men in this study practice receptive or insertive anal intercourse, which would modify their risk of infection and therefore likely the transmission bottleneck. These considerations notwithstanding, findings of low multiplicity infection and limited viral evolution preceding peak viremia can be interpreted to suggest a crucial but finite window of potential vulnerability of HIV-1 to vaccine-elicited immune responses, but which is likely less homogenous in MSM.

6.3.2 HIV-1 recombination and subtype composition

The ability of HIV-1 to evade the immune system and antiretroviral therapy depends on genetic diversity within the viral quasispecies and recombination is an important mechanism that helps to generate and maintain this genetic diversity (Archer et al. 2008; Baird et al. 2006; Hu and Temin 1990; Smyth, Davenport, and Mak 2012). The evolutionary rate of HIV-1 can also be elevated by recombination, which is an additional mechanism to mitigate deleterious mutational load. Recombination occurs much more frequently than mutation and encompasses template switching between RNA strands during reverse transcription and can lead to a new recombinant form in case of dual infection with distinct subtypes (Baird et al. 2006; Magiorkinis et al. 2003; Simon-Loriere et al. 2009). The increasing worldwide prevalence of circulating recombinant forms could imply a replication or transmission advantage of some of these strains, but little is known about how recombination rates vary within the HIV-1 genome. It is assumed that some

of these mosaic genomes may have properties that have led to their prevalence, particularly in the case of the circulating recombinant forms (CRFs) (Archer et al. 2008).

In an analysis of HIV-1 recombination in *gag* and *pol* in T cells, recombination was favoured at a number of recombination hot spots, where recombination occurs six times more frequently than at corresponding cold spots (Smyth, Davenport, and Mak 2012; Smyth et al. 2014). However, hotspots and cold spots were spread evenly across the genome and occurred near important features of the HIV-1 genome, although not at sites immediately around protease inhibitor or reverse transcriptase inhibitor drug resistance mutations (Cromer et al. 2016). In a separate study of the complete HIV-1 genome, recombination hot and cold spots differed from those observed in inter-subtype recombinants. However, hotspots within *env* were enriched at the V1, V2 and V5 and helped carry forward resistance-conferring mutations in the diversifying quasispecies while cold spots were enriched within *vif* (Song et al. 2016).

A consensus has not been reached as to whether HIV recombination in infected macrophages occurs in a similar way to when primary CD4 T cells are infected. Cromer *et al* used HIV-1 with silent mutation markers along with next generation sequencing to compare the mutation and the recombination rates of HIV-1 directly in T cells and macrophages (Cromer et al. 2016). T cells had a 30% higher mutation rate than macrophages, but there was a more than four-fold higher recombination rate in macrophages compared to T cells. The difference could not be attributed to different reliance on CXCR4 and CCR5 co-receptors between T cells and macrophages, but the lower rate of strand transfer in T cells may have been associated with increased dNTP concentration in T cells compared to monocyte derived macrophages. Notwithstanding, the pattern of recombination across the HIV-1 genome remained constant between T cells and macrophages despite a difference in overall recombination rate. This indicates that the difference in recombination rates is a general feature of HIV-1 DNA synthesis during macrophage infection (Cromer et al. 2016).

Even though there was enrichment for recombination hotspots in some genes, no gene by itself when sequenced could accurately represent the subtype of the full-length genome. In fact, *pol* subtyping which has been used in most published studies as it also provides information on the prevalence of drug resistance mutations could not identify up to two-thirds of recombinant viruses in our cohort. This is especially important as the description of inter-subtype differences in HIV-1 pathogenesis, virulence and disease progression are often reliant on subtype classification from the sequencing of only one gene and should therefore only be interpreted in the context of that gene.

For instance, the fast disease progression attributed to subtype C compared to subtype A and D is derived from studies of partial sequences of the *pol* or *env* genes but unique recombinant forms of HIV-1 exist and often have unique breakpoints that cannot be inferred from partial sequencing (Amornkul et al. 2013; Kaleebu et al. 2001; Kiguoya et al. 2017; Sanders et al. 2017). However, in this study, faster decline of CD4 T cells in subtype C was only observed if *vif, vpr, rev* and *nef* were subtype C, with subtype A *gag, pol* and *vpu* associated with the fastest CD4 T cell decline. Interestingly, inter-subtype recombination conferred increased protection from CD4 T-cell decline if *tat, vpu, env, rev* and *nef* were recombinants. *Vif* was the only gene where recombination was detrimental. However, this is a small study in which there were very few patients infected with pure subtype C or D, and these findings should be confirmed in larger studies.

In an investigation of subtype-specific differences in Gag-protease driven replication capacity using chimeric viruses expressing patient derived Gag-Protease, subtype C Gag-Proteases exhibited substantially lower replication capacities than those expressing subtype B Gag-proteases, regardless of whether a subtype B or C backbone was used (Kiguoya et al. 2017). In East African cohorts, they observed a hierarchy of Gag-Protease-driven replication capacities, i.e. subtypes A/C < D < inter-subtype recombinants, which is consistent with reported inter-

subtype differences in disease progression based on subtyping by *pol*. Chopera *et al* went a step further and used site directed mutagenesis to demonstrate that while some rare polymorphisms within p24 reduced *in vitro* viral replicative capacity in both subtype B and C, downstream compensatory mutations reduced the adverse effect in subtype C but not B (Brockman et al. 2012; Chopera et al. 2012; Kiguoya et al. 2017). Indeed, patient-derived subtype B sequences harbouring the M250I mutation exhibited *in vitro* replicative defects, while those from subtype C did not. The structural implications of the M250I polymorphism were predicted by protein modelling to be greater in subtype B than C, providing a potential explanation for its lower frequency and enhanced replicative defects in subtype B.

6.3.3 Transmitted drug resistance

The numbers of people with drug resistant virus has increased over time largely as the result of growing levels of resistance to NNRTI drugs in sub-Saharan Africa (Gupta et al. 2012). One reason for this is that just one mutation – notably the K103N mutation – causes high-level resistance to the NNRTIs efavirenz and nevirapine and cross-resistance between the two drugs (StanfordTDRM/CPR). Widespread use of combination ART for treatment, prevention of mother to child transmission and pre and post exposure prophylaxis requires continuous monitoring of its efficacy. For instance, single-dose nevirapine (NVP) is quite effective in preventing transmission of the HIV-1 from mother to child; however, many women develop resistance to NVP in this setting (Antunes et al. 2015; Capretti et al. 2016; De Nardo et al. 2016; WHO 2016).

Up to 23% (0.1-23%) of newly diagnosed individuals are infected with HIV-1 variants harbouring transmitted drug resistance mutations (TDRM) (WHO 2016). Some TDRM have been shown that they revert to wild-type, but most mutations have been reported to persist for up to 8 years in the absence of therapy (Pingen et al. 2015; Pingen, Sarrami-Forooshani, et al.

2014; Pingen, Wensing, et al. 2014). Factors associated with increased TDRM include poor treatment adherence, poor absorption in some patients, being a man reporting sex with men, young age, higher CD4 T-cell count, and being a member of a transmission cluster (Levintow et al. 2018). Some evidence also suggests that thymidine analog mutations, caused by the drugs zidovudine and stavudine, may develop more quickly in people with HIV-1 subtype C (Skhosana et al. 2015).

In addition, mutations in the reverse transcriptase (*RT*) and protease genes during antiretroviral therapy can be associated with a reduction in viral infectivity and replicative capacity. Insertion of the resistance-conferring regions into an NL4-3-based molecular background was indeed shown to result in chimeras that displayed a modest but significant reduction in replication capacity compared to the drug-susceptible chimeric viruses (Armstrong, Lee, and Essex 2011; Simon et al. 2003). In a few studies, the presence of transmitted drug resistance mutations was negatively correlated with the viral load and with $CD4^+$ T cell count decay (Leda et al. 2018). However, it is hopeful that in as much as the prevalence of HIV-1 drug mutations continues to rise, there is little evidence of widespread resistance to newer drugs, and therefore many TDR strains are unlikely to influence the activity of currently preferred first-line ART regimens (Rhee et al. 2018). We found drug resistance mutations to non-nucleoside reverse transcriptase inhibitors, integrase inhibitors and protease inhibitors. However, most of the drugs to which these mutations confer resistance to are not used in first line treatment of HIV-1 except nevirapine, efavirenz and dolutegravir, to which only 4 of the patients showed a resistant genotype.

6.3.4 Preadaptation to the newly infected partner's HLA type

Cell-mediated immunity imposes a strong selective pressure on HIV-1 evolution. Recent research has generated clear evidence that the transmitted/founder viruses often contain both adapted and non-adapted CTL epitopes and that immune escape mutations are highly predictable based on host immunogenetic profiles (Carlson et al. 2015; Katoh et al. 2016; Monaco et al. 2016; Monaco, Ende, and Hunter 2017; Moore et al. 2002). The impact on viral control and disease progression of transmitted polymorphisms that are either preadapted to or non-associated with the new host's HLA type has previously been shown. In a cohort of 169 Zambian heterosexual subtype C transmission pairs, almost one-third of possible HLA-linked target sites in the transmitted virus Gag protein were already adapted, and this transmitted preadaptation significantly reduced early immune recognition of epitopes (Monaco et al. 2016).

The degree of adaptation of the transmitted *gag* to the HLA class I (A, B and C) alleles present in the newly infected patients in this study could be estimated from a comparison of the HLA-class I driven polymorphisms present in the transmitted/founder virus to the HLA-class I alleles of the patient. Only mutations that were present in all the sequences obtained per patient were selected, as this suggests that they were transmitted from a pre-adapted virus as opposed to rapidly selected in the new patient. The identified polymorphisms were linked to a wide variety of HLA-A and B alleles, but none was identified within HLA-C. In addition, transmission of polymorphisms was not homogeneous between all the proteins encoded by *gag* with p17 and p24 transmitting a larger proportion of polymorphisms than p2p7p1p6. Preadapted residues were particularly frequent in certain epitopes which may constitute hot spots of immune recognition. Surprisingly, unlike other studies, there was no correlation between the degree of preadaptation and either viral load or the rate of CD4 T cell decline, although specific polymorphisms were shown to be significantly associated with disease progression in this small subset of patients.

Indeed, other groups have shown that transmitted preadapted and non-associated polymorphisms show opposing effects on set-point viral load and the balance between the two is what is significantly associated with higher set-point viral loads (Carlson et al. 2015; Monaco et al. 2016). Overall, the relative ratio of the two classes of polymorphisms was found to be the major determinant of CD4 T-cell decline. Thus, even before an immune response is mounted in the new host, the balance of these opposing factors can significantly influence the outcome of HIV-1 infection.

6.4 Phenotypic characteristics

To study the phenotypic characteristics conferred on the transmitted/founder virus by *gag*, 33 individuals whose founder virus could be unambiguously identified were selected and 48 founder *gag* sequences cloned into a NL4.3 backbone. Twenty-four of these 48 founder variants were derived from single variant infection in 24 patients while 24 were from multivariant infection in 9 patients. Testing three independent clones ensured that the observed phenotypic differences were not due to the confounding effect of backbone mutations that might have arisen during the cloning process and provides an estimate of experimentally induced variation.

6.4.1 Replicative capacity conferred by the transmitted *gag*

RC assays measure the ability of HIV-1 to reproduce and spread *in vitro* in the absence of host immune factors and is among several host cofactors believed to play a role in HIV-1 disease progression. There was a strong positive correlation between the vRC of subtype A Gag-NL4.3 chimeric viruses with both the set point viral load and CD4 T-cell decline, confirming that our replicative capacity assay is physiologically relevant, and that RC conferred by the transmitted/founder virus *gag* is important for disease progression.

Impairment of RC has already been independently associated with a slower rate of CD4 T-cell loss and disease progression in subtype C and B newly infected patients from discordant couple and mother to child transmission cohorts respectively (Adland et al. 2015; Brockman et al. 2010; Claiborne et al. 2015; Kiguoya et al. 2017; Naidoo et al. 2017; Prince et al. 2012). Claiborne *et al* used RC as a measure of HIV-1 fitness to explore its role in events early after infection that set the course for long-term disease progression and untangle viral RC and SPVL, which are themselves partially linked, to reveal the contributions each makes separately to different aspects of the host response (Claiborne et al. 2015). Unlinking the effects of SPVL and vRC probed the already complex effect of HIV-1 characteristics on the quality of the immune response to the virus and revealed evidence that high RC infection was accompanied by generalized immune activation, T-cell exhaustion and higher proviral DNA load in quiescent cells. Claiborne *et al*'s observation that high RC viruses appeared to gain entry into resting cells more efficiently than low RC viruses suggests that the rate or ease of viral entry and replication in different subsets of $CD4^+$ T cells might vary as a function of RC.

In a separate study, persons with a baseline *pol* replication capacity less than the median of the study population had a slower rate of HIV-1 disease progression as assessed by time to $CD4^+$ T-cell count <350 cells/μL blood, rate of viral load increase, treatment initiation, or death (Wright et al. 2011). At baseline, having a *pol* replication capacity below the median was associated with a lower mean viral load and a decreased prevalence of phenotypic evidence of resistance to protease inhibitor. Lower baseline *pol* replication capacity has also been associated with decreased baseline viral loads and decreased rates of subsequent $CD4^+$ T-cell loss in a study of chronically infected haemophiliac patients.

The precise mechanisms by which *gag* modifies virus fitness remain speculative. The viral capsid protein present within the Gag polyprotein precursor is the site of important T-cell epitope targets, and immune responses to Gag can be more important than responses to other

viral proteins in terms of controlling viral replication. The MA protein at the N terminus of the Gag polyprotein precursor directs its intracellular transport to the plasma membrane (Klein, Reed, and Lingappa 2007; Scarlata and Carter 2003), which is the predominant site of virus assembly in most cell types. Mutations affecting residues of the MA protein induce a range of perturbations in the virus assembly process, including a defect in particle release (Freed et al. 1994; Yuan et al. 1993), and the redirection of assembly to the endoplasmic reticulum or other intracellular compartments, in particular multivescicular bodies or late endosomes (Hermida-Matsumoto and Resh 2000; Ono 2009; Ono and Freed 2004; Ono, Orenstein, and Freed 2000; Spearman et al. 1994). Point mutations have been previously shown to influence replicative capacity with Gag residues 483 and 484, located within the Alix-binding motif involved in virus budding being identified as a major contributor to subtype-specific replicative differences (Kiguoya et al. 2017). Indeed, some HLA class I associated polymorphisms did correlate negatively with the Gag-mediated RC in our participant set. It is however important to note that Gag is also important in determining viral susceptibility to intrinsic and IFN-induced antiviral defenses.

The host factors that select for founder viral replicative capacity or allow for propagation of one variant over the other are not well understood. Indeed, founder viruses are rarely the most dominant variant in the donor patient or of the highest replicative capacity (Monaco, Ende, and Hunter 2017; Deymier et al. 2015; Kijak et al. 2017). We therefore compared replicative capacity in cases of multivariant infection to elucidate whether the transmission bottleneck within an individual patient would select for variants of similar replicative capacity traits and observed a wide range of replicative capacity of intra patient founder viruses and no evidence for selection of similar replicative capacity suggesting that any reasonably fit virus could be transmitted. In addition, overrepresentation of some sequences within the patient were not a

result of high replicative capacity of that virus and may have been a result of either a high inoculation dose of that variant, or high fitness of genes outside of *gag.*

Replicative capacity is not a routine clinical monitoring assay. Currently few companies offer a test of HIV-1 replication capacity as part of their combined phenotypic/genotypic HIV-1 drug resistance test. Their RC tests either utilize a single-replication-cycle format to compare the *pol* replication rate of a recombinant pseudotyped virus derived from clinical specimens with that of a reference pseudotyped virus for which the replication capacity is set at 100%, or compares the rate at which HIV-1 taken from blood reproduces and compares this to the median rate at which a number of drug-sensitive (wild-type) strains of HIV-1 reproduce (Gonzalez-Ortega et al. 2011; Kouyos et al. 2011; Sune et al. 2004). In such clinical settings where viral replicative capacity measurements are provided as part of HIV-1 treatment monitoring, patients with a lower RC are also less likely to have any HIV-1 mutations associated with resistance to protease inhibitors, either occurring naturally or as a result of being infected by a person with drug-resistant virus, than those in the higher RC group (Ammaranond and Sanguansittianan 2012; Quinones-Mateu and Arts 2002).

6.4.2 Is the mechanism of HIV-1 spread between cells important?

As the detrimental effect of vRC remains even in patients of comparable SPVL, it is plausible that this may be a reflection of the mechanism of spread, whereby some viruses may be better at spreading through virological synapses and therefore may not contribute proportionally to the plasma SPVL. There is a large body of data supporting the hypothesis that HIV-1 is indeed inefficient in replicating in mobile CD4 T cells, with cell-cell transmission to cells that would normally be refractory to HIV-1 infection acquiring abortive infection which has then been associated with massive CD4 T cell loss in lymphoid cells through a highly inflammatory mechanism, pyroptosis (Cummins and Badley 2010; Doitsh et al. 2014; Doitsh and Greene

2016; Galloway et al. 2015; Monroe et al. 2014; Munoz-Arias et al. 2015; Sourisseau et al. 2007). In addition, Claiborne *et al* found higher proviral DNA load in resting CD4 T cells of high vRC infection even though proviral DNA loads were similar in activated CD4 T cells (Claiborne et al. 2015), suggesting that high vRC viruses could gain entry to quiescent CD4 T cells as was also shown by Doitsh *et al*'s pyroptosis experiments (Doitsh and Greene 2016). In HIV-1 infection, bystander CD4 T cell death occurs as a result of cell-cell transmission of virions to quiescent cells that are non-permissive to cell-free virus (Doitsh et al. 2010; Doitsh et al. 2014). This is consistent with the observation that high RC viruses destroy CD4 T cells in a manner that is independent of viral load, is accompanied by a heightened inflammatory state and that quiescent cells carry more HIV-1 DNA in patients infected with high RC viruses. It is therefore plausible that high RC viruses are better at inducing virological synapses, which would provide a conduit into quiescent cells that then die by pyroptosis. A possible association between viral RC and cell-cell transmission was therefore investigated.

Even though all donor cells were initially infected at the same MOI, there was a strong correlation between the number of infected target cells and infected donor cells for both cell free and cell-cell transmission. However, the infectivity index defined as the number of new target cell infections resulting from one infected donor cell was higher in cell-cell transmission compared to cell free spread in both CD4 T and Jurkat cells. Replicative capacity did not correlate with infectivity index in cell free infection, but high replicative capacity *gag* conferred a striking increase in cell-cell infectivity on subtype A *gag* on an NL4.3 backbone, but not on subtype C *gag* on MJ4 backbone. This may be because cell free spread of HIV-1 is inefficient or possibly that our replicative capacity assay predominantly measures cell-cell spread not cell free spread. This result may also represent the result of subtype differences in *gag* or the differences in pathogenicity of NL4.3 and MJ4. Nonetheless, within the Kilifi cohort where *gag* sequences were cloned on a NL4.3 backbone that is well known to effectively spread by cell-

cell transmission, the result of high replicative capacity *gag* on cell-cell spread was evident, suggesting an overlap in the mechanisms of cell-cell transmission with those involved in replicative capacity. This was strengthened by observation that the highest expression of pro-apoptotic, autophagy and necrosis markers were observed in high replicative capacity viruses and in high cell-cell but not cell free transmission, further suggesting that the cell death pathways in high replicative capacity infection and cell-cell transmission overlap.

6.5 Which TF characteristics are associated with increased immune activation?

Having identified selected TF characteristics that influenced the rate of CD4 T cell decline and/or viral load, we were able to identify a role for increased cellular activation and aberrant immune dysregulation in patients infected with TF characteristics that were associated with worse outcomes. Multivariant infection, which we have previously suggested may be a result of a less stringent transmission bottleneck, was associated with increased immune activation at acute infection when viral load in that group was also higher, but control of viremia resulted in subsequent reversal of most dysfunction. However, consistent with the reports that events at acute infection disproportionately set the stage for disease trajectory (Braun et al. 2014; Chang et al. 2012; Ling et al. 2007; Selhorst et al. 2017), patients with multivariant infection still lost their CD4 T cells faster that those infected with a single founder virus. Previous studies have shown that in most cases of multivariant infection, the minor variant persists for a long time, often rapidly replacing the major variant as a result of a rapid immune response to the major variants and itself then collapsing as it starts to be targeted by CTL responses (Henn et al. 2012; Kijak et al. 2017). This might explain the burst of CD8 TTE and B cell differentiation that was observed at 6-9 months post infection but did not persist to chronic infection.

Increased CD8 T cell activation and a high proinflammatory milieu had already been identified as a link between high vRC and the rate of CD4 T-cell decline in subtype C infection (Claiborne

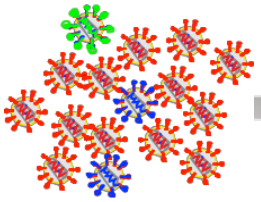
et al. 2015). Similarly, there was increased CD8 T cell activation and exhaustion that persisted at all time points measured with differences still evident after 2 years of infection, suggesting that this activation may not be driven by antigenic load only, and that other pathogenic mechanisms associated with replicative capacity may be involved. This is further supported by the observation that there was increased differentiation in all the cellular lineages that were characterized including B cells, NK cells and monocytes, suggesting that patients infected with high RC are unable to resolve inflammation and immune activation. Increase in activated cells would result in both an increase in activated CD4 T cells that are susceptible to HIV-1 infection, and increased killing of infected CD4 T cells by the activated effector cells which may explain the rapid depletion of CD4 T cells in high RC infection. As these patients are unable to resolve inflammation, continued immune activation eventually led to exhaustion, senescence and anergy.

In both multivariant infection and high vRC infection, innate immune cells including NK cells and monocytes and adaptive cells including T cells and B cells were perturbed. This raises the possibility that the increased inflammatory state observed was not only driven by responses to a few specific epitopes that may be present in sequences from high TF infection. It is more likely that the immune perturbance may have been a result of a combination of other mechanisms such as differences in: i) CD4 T cell target subsets including increased ease of entry into quiescent cells by high RC TF as has been previously reported, ii) efficiency in CD4 T cell entry steps consistent with our observation of increased cell-cell but not cell free spread of high vRC viruses, iii) an inflammatory mode of cell death that is consistent with pyroptosis, and iv) increased sensing by innate immunity.

Changes in immune activation as a result of enrichment for APOBEC mutations were mostly observed in cells involved in adaptive immunity with monocytes and NK cells appearing similar regardless of APOBEC activity. This is unsurprising as APOBEC is known to cause mutations

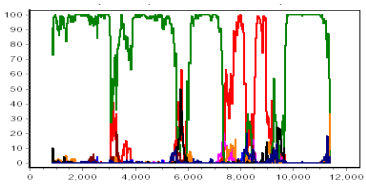
that increase viral diversity and can therefore enhance or reduce recognition of epitopes by either T cells or B cells (Casartelli et al. 2010; Kim et al. 2014; Monajemi et al. 2014; Norman et al. 2011; Tsuji-Kawahara et al. 2010). Figure 6.1 summarizes the founder viruses that were investigated in this thesis and their associated influences on HIV-1 pathogenesis while figure 6.2 summarises potential mechanisms by which the virus characteristics identified in this thesis may ultimately lead to the depletion of $CD4^+$ T cells.

Multiple founder variants



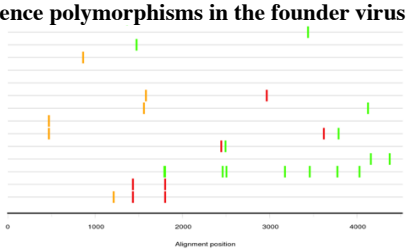
- Occurs more frequently in MSM
- High viral load in the first month of infection
- Faster CD4 T cell depletion
- One T/F was often over-represented/dominant, although not necessarily having the fastest replicating *gag*
- No selection for T/F of similar replicative capacity
- Increased immune activation at acute infection

Inter-subtype recombination



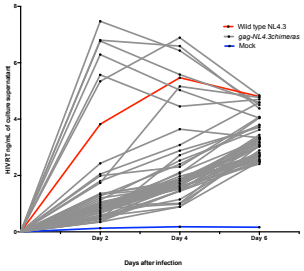
- Partial subtyping is inaccurate
- Recombination breakpoints were enriched *in gag*, *pol*, *tat* and *env*
- Recombination was beneficial to the patient when it occurred in *env*, *tat*, *vpu*, *rev* and *nef*

Sequence polymorphisms in the founder virus genome



- **HLA associated polymorphisms** were enriched in p17 and p24. Polymorphisms in p17 were associated with HLA-A and those in p24 associated with HLA-B
- **APOBEC induced hypermutation** was associated with increased activation and exhaustion of of CD4 and CD8 T cells, and increased expression of CD86 on B cells.
- **Transmitted drug resistance** mutations associated with higher set point viral load but not CD4 T cell depletion. 4/38 patients received founder variants that were resistant to the first line treatment drugs nevirapine, efavirenz and dolutegravir

High replicative capacity conferred by *gag*



- Faster depletion of CD4 T cells
- Higher viral loads
- HLA associated polymorphisms that modulate replicative capacity were enriched in p17
- Higher cell-cell but not cell free transmission
- Increased caspase 1 in quiescent cells
- Increased expression of RNA associated with cell death pathways
- Disruption of immune homeostasis throughout infection

Figure 6.1: HIV-1 founder virus characteristics that influence pathogenesis. Factors that influenced disease outcome include 1) A less stringent transmission bottleneck as evidenced by multivariant infection, 2) HIV-1 subtype per gene 3) Sequence polymorphisms including HLA associated mutations, APOBEC induced hypermutation and transmitted drug resistance mutations and 4) replicative capacity conferred by the transmitted *gag*.

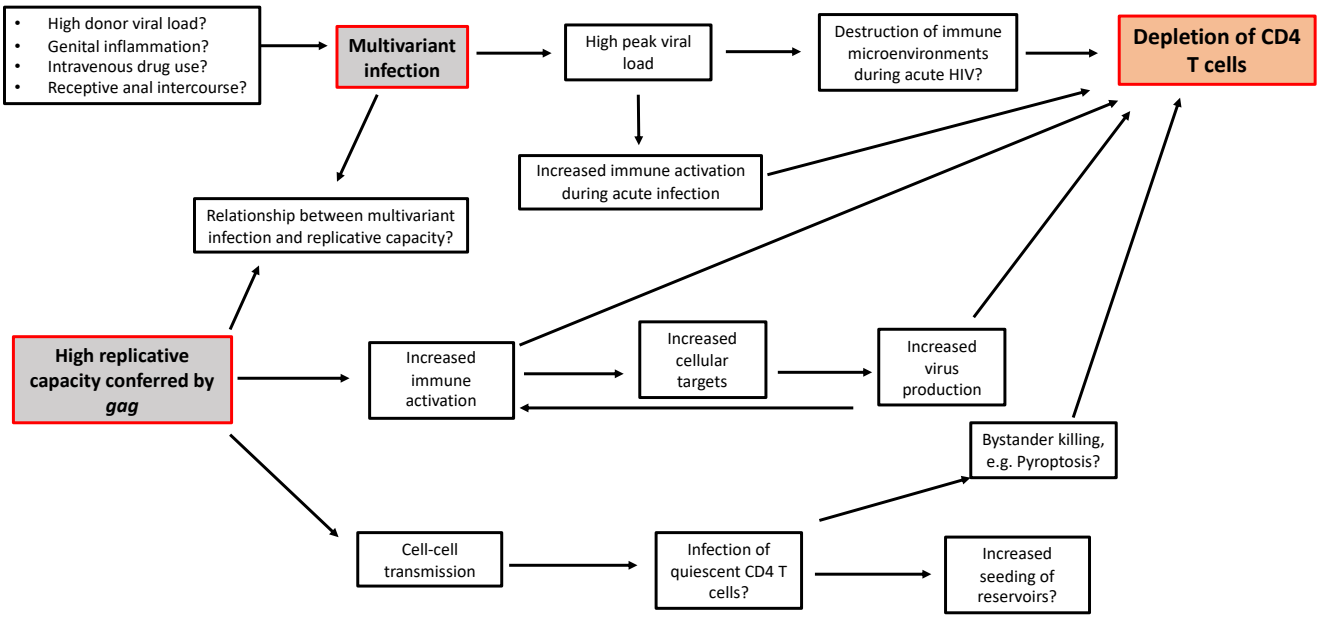


Figure 6.2: Hypothesis for the depletion of $CD4^+$ T cells. Multivariant infection is associated with high peak viral load which may lead to the depletion of $CD4^+$ T cells through increased immune activation during acute HIV-1 infection and increased destruction of immune microenvironments. Multivariant infection is likely a result of a less stringent transmission bottleneck which may result from a high inoculation dose or increased permissiveness as a result of genital inflammation, intravenous drug use or receptive anal intercourse. On the other hand, TFv whose gag confers increased *in vitro* replicative capacity was associated with increased immune activation which may increase cellular targets and increased virus production

ultimately leading to depletion of both HIV infected and uninfected $CD4^+$ T cells. The ability of high replicative capacity viruses to preferentially spread by cell-cell transmission may potentially increase the proviral DNA load in resting $CD4^+$ T cells and therefore contribute to establishment and maintenance of the latent reservoir.

6.6 Limitations of the study

The present study used cross-sectional data and cannot account for possible selection bias with regard to the influence of potential confounders such as STDs or risk behaviour in the study population. A substantial proportion of the subjects had limited behavioural information available for analysis therefore no firm conclusions could be drawn regarding particular risk factors and the likelihood of single versus multiple virus transmission. However, it is now well known that acute infection fuels heterosexual spread of HIV-1 (Pilcher et al. 2004). On average, individuals are hyper infectious beginning before the onset of the acute retroviral syndrome and continuing for ~6 weeks thereafter (Chakraborty et al. 2001; Gray et al. 2001; Pilcher et al. 2004). Thus, studying this phase of HIV-1 infection is still relevant.

The experimental approach taken in this study also presents with some limitations. The use of a subtype B proviral backbone for subtype A *gag* could result in suboptimal pairing between the *gag* genes derived from acutely infected patients and the subtype B NL4.3 infectious molecular clone backbone. Secondly, we did not take into account interactions between *gag* and the other genes in the autologous virus. However, a majority of the analysed constructs produced infectious progeny virus. As different HIV-1 infectious molecular clones become more widely available, it will be important to further validate this system by cloning in these HIV-1 clade A *gag* genes into other backbones in order to ensure that there is a minimal bias introduced due to backbone incompatibilities. One such study was by Kiguoya *et al* where they

demonstrated that subtype differences in replicative capacity that they observed in subtype B and C derived *gag-pro* chimeras cloned on to NL4.3 backbone persisted even when they cloned a few of the *gag* sequenced on to a subtype C backbone (Kiguoya et al. 2017). Likewise, similarity between the replicative capacity of *gag* chimeras on a lab adapted backbone and the RC of the entire patient derived IMC have previously been demonstrated (Brockman et al. 2012; Chopera et al. 2012; Kiguoya et al. 2017; Claiborne et al. 2015).

Thirdly, a cell line rather than primary cells was used to measure replication. Therefore, care must be taken when interpreting the results as cell lines do not always accurately replicate the primary cells. The CEM-based GXR25 cell line used for these replication studies is one of the few established T cell lines able to support entry and replication of CCR5-tropic strains of HIV-1.GXR25 cells resemble primary $CD4^+$ T cells morphologically and possess several receptors associated with primary CD4 T cells that are important for HIV permissiveness such as CD4, CCR5 and CXCR4. However, However, in order to support efficient replication of CCR5-tropic strains, the GXR25 cells must be propagated for no less than 4 months prior to infection. Properly passaged cultures can support replication even after passaging for up to 1 year. Careful monitoring of CCR5-tropic replication throughout passaging is essential for successful experiments. Additionally, the parent CEM cell line from which the GXR25 cell line was derived exhibits high levels of cyclophilin A, up to 2 to 4-fold higher expression than the Jurkat cell line. Due to high levels of cyclophilin A, the replication defect normally associated with the canonical HLA-B*57 associated escape mutation, T242N, which is attributed to a decreased ability of capsid to bind cyclophilin A, cannot be easily detected in this particular cell line. Thus the CEM-based GXR25 cell line is not ideal for studying replication defects associated with mutations in the HIV-1 capsid cyclophilin-binding loop (Claiborne, Prince, and Hunter 2014).

6.7 How can this study inform HIV-1 intervention strategies?

Our study raises the concern that current subtyping of HIV-1 that is often based on partial gene sequencing that has informed most of our knowledge on HIV-1 subtype differences in pathogenesis, epidemiology, and has therefore to some extent also driven vaccine design strategies may be inaccurate. In addition to accurately accounting for genetic differences between HIV-1 subtypes, the future design of CTL-based vaccines will also need to account for differential effects of host-driven viral evolution and HLA-preadaptation on viral fitness.

Successful antiretroviral treatment of HIV-1-infected individuals reduces viremia to undetectable levels, restores $CD4^+$ T-cell counts, reconstitutes the immune system to some degree, and significantly prolongs life (Rutstein et al. 2017). Despite this, combination ART does not fully restore immune function, does not eradicate viral reservoirs and viral rebound back to baseline levels occurs upon treatment interruption (Aiuti and Mezzaroma 2006; Eberhard et al. 2016; Burton et al. 2005). Strategies aimed at mitigating persistent immune activation and eradicating the latent viral reservoir will contribute immensely toward improving the quality of life of HIV-1-infected individuals and will help to curb the epidemic. Research work presented in my thesis suggests that this may involve strategies that sieve out high RC founder viruses and tighten the stringency of the transmission bottleneck to prevent multivariant infection.

A better understanding of the mechanisms driving HIV-1 induced immunological abnormalities and the processes by which they ultimately cause disease is crucial for unveiling avenues for pursuing these more advanced therapeutic interventions. Having identified the near full-length TF genomes from patients with faster disease progression and inability to resolve immune activation, future work to identify a viral genotype and phenotype that is associated with

bystander CD4 T-cell death will allow examination into the precise HIV-1 genes involved and will provide novel insights into HIV-1 pathogenesis. It will also open up such genes as candidates for a prophylactic vaccine that would be capable of arresting the decline of CD4 T cells at acute HIV-1 infection.

Furthermore, the sequences and chimeric viruses that I have generated provide a useful resource in the field of immunogen design, for their utility in designing transmitted/founder virus peptide sets and for use in functional viral inhibition assays to probe effective immune responses against transmitted/founder viruses. Eventually, the ultimate aim is to identify specific mechanisms associated with viral RC that are involved in CD4 T-cell death, and that can be harnessed to inform novel design approaches/strategies and assessments of effective prophylactic HIV-1 vaccine and/or lifelong functional cure.

6.8 Future work

The development of future HIV-1 vaccines will require strategies to increase breadth of immune responses to counteract the challenge of HIV-1 genetic diversity. One of the strategies that has been used in to increase breadth in vaccine development is the use of multiple immunogens either in combination or using a prime-boost regimen. Infection with multiple variants provides a natural experiment through inoculation with more than one distinct HIV-1 variant. Future investigation into breadth of T cell and antibody responses in the context of multivariant infection would therefore help to inform on the degree to which use of more than one immunogen can help to achieve broadly adaptive immune responses, and whether breadth of responses will correlate with number of founder variants.

In addition, in as much as replicative capacity is a predictor of disease progression, the mechanisms underlying high replicative capacity are not well understood. Viruses that exhibit high replicative capacity may have increased efficiency of target cell entry but may also be

better at any of the steps involved in the HIV-1 life cycle. The data presented in this thesis suggests that high replicative capacity may be a result of efficient spread by cell-cell mechanisms as opposed to cell free spread. However, few studies have been made of cell-cell transmission in primary HIV-1 isolated from patients whose disease outcome is known. In addition, it is not known whether HIV-1 subtypes differ in their ability to spread through virological synapses, or whether founder variants from elite controllers differ from those of fast progressors in their mechanism of cell entry and therefore ability to deplete $CD4^+$ T cells and cause inflammation. Furthermore, it is not known whether increased efficiency of cell-cell spread is a feature of transmitted/founder variants, compared to the variants that are not transmitted from the chronic donor. Several studies have demonstrated that resting cells that would normally be refractory to infection with cell free HIV-1 can gain virus through cell-cell spread. It is therefore possible that founder variants that can spread efficiently through cell-cell mechanisms may be associated with higher proviral DNA load. Future work should interrogate the role of HIV-1 spread through virological synapses in HIV-1 pathogenesis is therefore needed.

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Appendix 1: Optimization of the PCR protocol

Appendix 1.1: Rationale

A subset of samples could not be amplified using the standard near length-genome or half genome method that had been successfully used for most samples. This section outlines modifications to the protocol that enables successful amplification.

Appendix 1.2: RNA extraction step

Modifications to this step were aimed at increasing the quantity and quality of RNA extracted in samples where no genomes were successfully amplified, and in samples where the RNA yield and quality as measured on the nanodrop was low. Modifications were as follows: RNA was extracted from 420 µl of plasma instead of 140 µl. All steps were carried out exactly as described in the material and methods except that: 1) the buffer volumes were increased three fold including the carrier RNA/AVL buffer, 2) due to increased volume, six rounds of spinning were required in order to pass all the plasma/Carrier RNA/AVL buffer/Ethanol solution through the QIAamp columns, and 3) final elution was done in 100 µl Buffer AVE.

Following extraction, reverse transcription to cDNA was carried out on the same day to avoid RNA degradation by freeze-thaw cycles.

Appendix 1.3: cDNA synthesis step

Occasionally, some RNA extracted from some samples contained the genes closest to the 3-prime end from where cDNA synthesis had been initiated but not genes towards the 5-prime end, suggesting that unsuccessful amplification may have been a result of incomplete cDNA synthesis or shredding or degradation of RNA. Thus, modifications to this step were aimed at

increasing the likelihood of generating RNA that was long enough to amplify two 5kb half genomes that overlapped by the *vif* gene, which is located at the middle of the HIV-1 genome.

Reagent concentrations and cycling conditions were not modified. However, the following adaptation was made. Half genome cDNA was generated instead of near full-length genome cDNA. This was by using the 3 prime primer 1.3'3' PlCb primer to generate cDNA from the 3-prime half of the genome and using the primer Vif1 that would contain the *vif, pol* and *gag* genes (Figure S1). This resulted in increased efficiency of amplifying the 5-prime half genome.

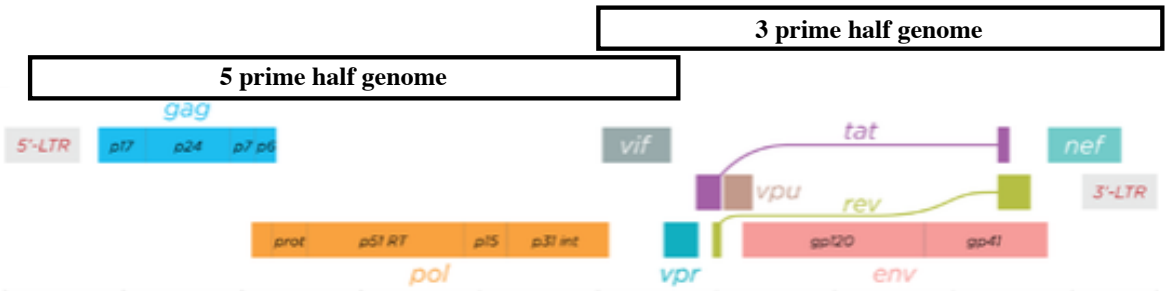

Figure S1: HIV-1 Genome map showing coverage of the half genome amplicons.

Two overlapping half genomes were amplified spanning from the U5 region of the 5' long terminal repeat region to the 3' long terminal repeat and overlapping by the complete *vif* gene.

Appendix 1.4: Single genome amplification step

There was a subset of samples whereby the gag, pol, env and vif genes could be amplified individually, but 5kb half genome amplification was unsuccessful. In these patients, we hypothesised possible mismatches between the half genome primers and the cDNA template, which would result in suboptimal primer binding and subsequently unsuccessful amplification. Therefore, modifications to this step were aimed at optimising primer binding and increasing specificity in an initial PCR reaction which would be followed by a second nested PCR reaction

with increased primer binding efficiency but reduced specificity but using PCR product from the first round PCR which was highly sensitive. A touch down protocol was optimised to achieve this. Figure S2 shows 5kb PCR product from 8 different patients.

PCR reactions were performed in 1x Q5 Reaction Buffer, 1x Q5 High GC Enhancer, 0.35mM of each dNTP, 0.5µM of primers and 0.02 U/µl of Q5 Hot Star High-Fidelity DNA Polymerase (New England Biolabs, USA) in a total reaction volume of 25µl.

Cycling conditions for the first round PCR reaction of the 3 prime half genome were 98°C for 30s, followed by 10 cycles of 98°C for 10s, 72^{O}C – 30s (Decrease the annealing temperature by 1.5 °C in every cycle for 9 cycles) and 72°C for 4.5minutes, followed by 30 cycles of 98°C for 10s, 62^{O}C for 30s and 72°C for 4 minutes with a final extension at 72°C for 10min. Cycling conditions for the second round nested PCR reaction of the 3 prime half genome were 98°C for 30s, followed by 10 cycles of 98°C for 10s, 72^{O}C – 30s (Decrease the annealing temperature by 1.5 °C in every cycle for 9 cycles) and 72°C for 4.5min, followed by 30 cycles of 98°C for 10s, 64^{O}C for 30s and 72°C for 4 min with a final extension at 72°C for 10min.

Cycling conditions for the first round PCR reaction of the 5 prime half genome were 98°C for 30s, followed by 10 cycles of 98°C for 10s, 72^{O}C – 30s (Decrease the annealing temperature by 1.5 °C in every cycle for 9 cycles) and 72°C for 4.5min, followed by 30 cycles of 98°C for 10s and 72°C for 4 minutes and 30 seconds with a final extension at 72°C for 10min. Cycling conditions for the second round nested PCR reaction of the 5 prime half genome were 98°C for 30s, followed by 10 cycles of 98°C for 10s, 72^{O}C – 30s (Decrease the annealing temperature by 1.5°C in every cycle for 9 cycles) and 72°C for 4min, followed by 30 cycles of 98°C for 10s, 66^{O}C for 30s and 72°C for 4 min with a final extension at 72°C for 10min.

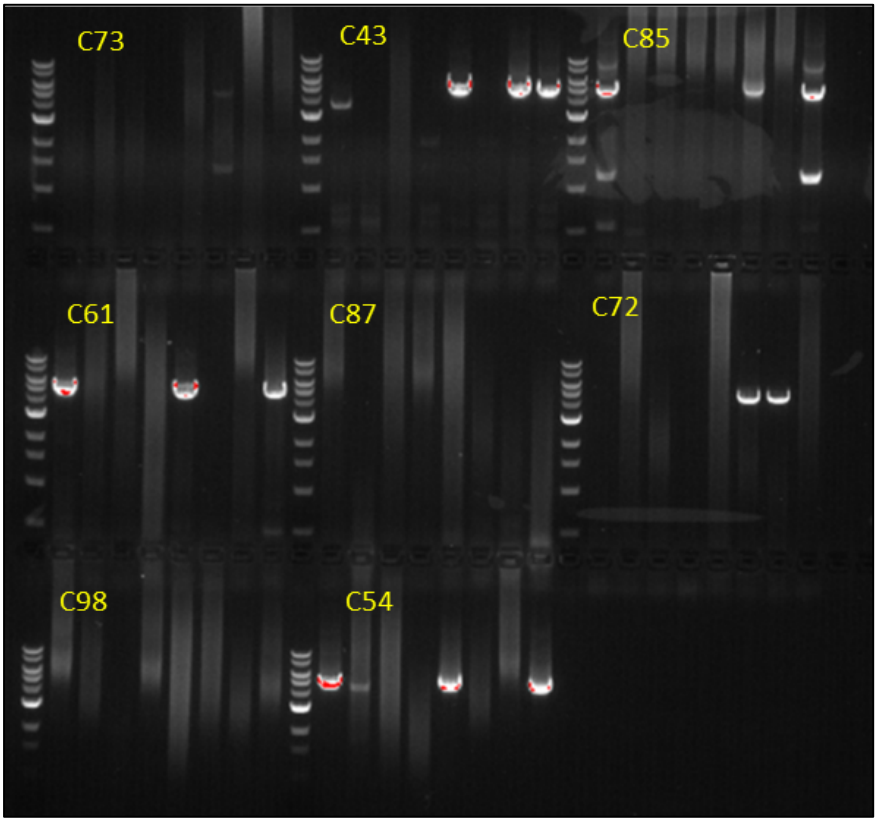

Figure S2: Agarose gel showing eight replicates of 5' half genomes from eight different patient samples. The bright bands are 5 kb half genomes.

Barcoding PCR to prepare amplicon library for PACBIO sequencing

Positive amplicons would need to be pooled together in libraries containing up to 100 unique amplicons for PACBIO sequencing. To enable deconvolution of unique sequences and identification of each patient's sequences, amplicons were barcoded by amplification with the PCR primers that had been used to generate the amplicons, but which had been modified by the addition of a 20 nucleotide DNA barcode. Barcoding was done on the first-round single genome amplicons as described in the materials and methods but was not always successful. In such cases, the following modifications were made to the basic protocol: 1) Second round nested

PCR product was used as template instead of the first round amplicon, 2) PCR product was cleaned using the Wizard ® SV Gel & PCR Clean-up System (Promega) before being used as template and 3) DNA concentration was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Scientific) and 0.5 – 1.0 ng of this DNA used as template. Figure S3 shows barcoded half genome amplicons on an agarose gel.

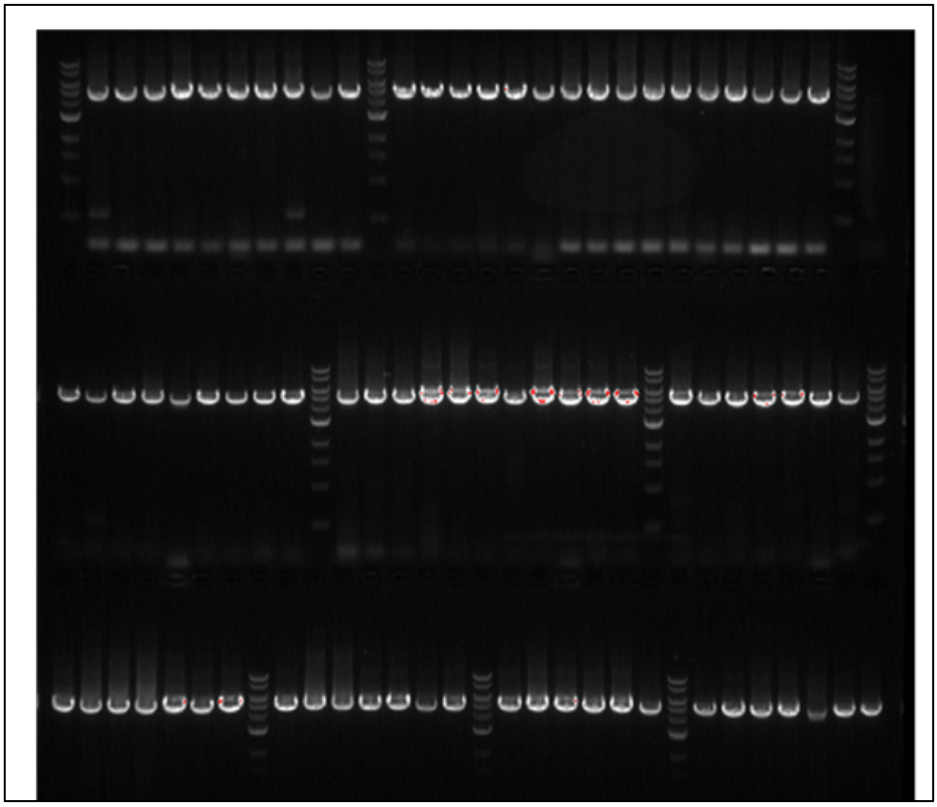

Figure S3: Agarose gel 5kb half genome amplicons that have been barcoded before library preparation for PACBIO sequencing.