Immunosenescence in HIV Infection

A thesis submitted for the degree of Doctor of Philosophy

Wong Choon Lim Glenn

Wolfson College

Supervisor:

Professor Sarah Rowland-Jones

Nuffield Department of Clinical Medicine
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Good game.

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Abstract

Immunosenescence is the progressive loss of immune tissue that result from (i) functional, (ii) clonal or (iii) systemic changes to the cells or tissue that make up the immune system. While this process occurs gradually during the ageing process, there is overwhelming evidence to implicate HIV infection in driving all three aspects of immunosenescence by inducing high levels of immune activation. In the current literature, there is a dearth of data on the impact of HIV-2 infection or untreated perinatal HIV-1 infection in driving immunosenescence, and the key goal of this thesis is to mend this gap in knowledge.

Both HIV-2 and paediatric HIV-1 infection provide excellent settings for verifying the mechanistic theories that relate to HIV-1-associated immunosenescence. The majority of HIV-2 infected individuals control their infection, which raises the question of how HIV-2 controllers are able to generate robust T-cell responses, when equivalent responses are rare in HIV-1 infected cohorts. Our hypothesis is that the former is an outcome of sustained or more robust homeostasis due to lower immune activation levels. Altogether, we show that primary immune resources are better preserved in HIV-2 than in HIV-1 infection – including more substantial HPC and naïve T-cell populations that correlate well with thymic output. The preservation of primary immune responses during HIV-2 infection is expected to contribute to robust effector functions and a younger phenotype of virus-specific memory T-cells. Accordingly, we show that HIV-2 specific memory T-cells reflect an earlier differentiated phenotype than HIV-1 specific T-cells, are highly polyfunctional and exhibit strong virus suppressive capabilities.

In perinatally HIV-1 infected children – who by virtue of their age represent slow progressors – the presence of a hyperactive thymus may be sufficient to compensate against the early onset of immunosenescence and result in younger immune cell phenotypes. Yet, in measuring leucocyte telomere length and mtDNA content by qPCR, which qualify as biomarkers of senescence, we observed that leucocytes from vertically-infected Zimbabwean children present with shortened telomeres and an increase in mtDNA content, which signals the onset of advanced cellular senescence.

Declaration of Contribution From Other Authors

For the DPhil thesis entitled: Immunosenescence in HIV Infection

For Submission in Trinity Term 2016 by

Wong Choon Lim Glenn (Student No: 543878)

For the thesis entitled: Immunosenescence in HIV Infection, I declare that data from our collaborators, Mathieu Angin[1] and Asier Sáez-Cirión[1], were included and reanalysed in Chapter 4 of the thesis as their data could be examined - both in isolation and juxtaposed with my own data - in parallel to draw valuable conclusions pertaining to a more complete understanding of HIV-2 specific CD8 T-cell responses. The authors have given me the permission to reproduce and reanalyse the data for my thesis. Specifically, the data relates to the HIV-2 suppressive activity of CD8 T-cells and appears in **Section 4.2.3** and **4.2.4**, in Figures **4.6**, **4.7** and **4.9**. In addition, the enumeration of TREC+ cells by qPCR was performed by Appay *et al.*[2], prior to my inclusion into the study; part of this data appears in Chapter 3, Figure **3.3**. Appay & Sauce *et al.*[2] performed the enumeration of primary immune resources and magnitude of CMV responses in uninfected young and old donors, and the data are added in Figures **3.1**, **3.2** and **5.3** to demonstrate the extent of change with age. Finally, Laura Papagno[2] worked with me to set up the tetramer assays used in Figure **4.3**.

Wong Choon Lim Glenn
6 Sep 2016

[1]Institut Pasteur, HIV Unit, Inflammation and Persistence, Paris 75015, France
[2]INSERM U1016, Institut Cochin, Cytokines and Viral Infections Team, Paris 75014, France

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Abbreviations

AIDS – Acquired Immune Deficiency Syndrome
ART – Antiretroviral Therapy
ARV – Antiretroviral
cDNA – Complementary DNA
CA - Capsid
CMV - Cytomegalovirus
DNA – Deoxyribonucleic Acid
EBV – Epstein-Barr Virus
Env - Envelope
dNTPs – Deoxynucleotide Triphosphates
dsDNA – Double Stranded DNA
Gag – Group Specific Antigens
GALT – Gut Associated Lymphoid Tissue
GWAS – Genome Wide Association Study
HIC1 – HIV-1 Controllers
HIC2 – HIV-2 Controllers
HIV-1 – Human Immunodeficiency Virus Type 1
HIV-2 – Human Immunodeficiency Virus Type 2
HLA – Human Leucocyte Antigen
HPC – Hematopoietic Progenitor Cells
ICS – Intracellular Cytokine Staining
IFN – Interferon
IN- Integrase
IRF5 – Interferon Regulatory Factor 5
KIR – Killer-Cell Immunoglobulin-Like Receptor
LTNP – Long Term Non-Progressor
LTR – Long Terminal Repeat
MHC – Major Histocompatability Complex
MMqPCR – Monochrome Multiplex Quantitative Polymerase Chain Reaction
mRNA – Messenger RNA
MTCT – Mother to Child Transmission
NADIs – Non-AIDS Defining Illnesses
NC - Nucleocapsid
Nef – Negative Regulatory Factor
OB – Obliterative Bronchiolitis
ORF – Open Reading Frame
PBMC – Peripheral Blood Mononuclear Cells
PBS – Phosphate Buffered Saline
PCR – Polymerase Chain Reaction
PD-1 – Programmed Cell Death Protein 1
PMTCT – Prevention of Mother to Child Transmission

PCR – Polymerase Chain Reaction
PIC – Pre-integration Complex
Pol - Polymerase
Rev – Regulator of Expression of Viral Proteins
RNA – Ribonucleic Acid
RTE – Recent Thymic Emigrants
SAMHD1 – SAM domain and HD Domain- containing Protein 1
SIV – Simian Immunodeficiency Virus
SNP – Single Nucleotide Polymorphism
Tat – Transactivator of Transcription
TCR – T-cell Receptor
TE – Tris-EDTA
TRIM5α – Tripartite Motif-Containing Protein 5
TNF-α – Tumour Necrosis Factor Alpha
UNAIDS – United Nations Programme on HIV/AIDS
UTR – Untranslated Region
Vif – Viral Infectivity Factor
Vpr – Viral Protein R
Vpx – Viral Protein X
WHO – World Health Organisation

Chapter 1: Literature Review and Introduction

1.1 HIV-1 Origin and Epidemiology

1.1.1 The AIDS Pandemic

Acquired Immunodeficiency Syndrome (AIDS) was first recognized as a disease in 1981, with the emergence of homosexual men who succumbed to unusual opportunistic infections and rare malignancies that signalled a physiological state of immunodeficiency.[1] The causative agent was isolated, subsequently identified as a retrovirus, and is now termed human immunodeficiency virus type 1 (HIV-1). The closest known relative of HIV-1 then was visna, which was found in sheep and is also a prototypic member of the genus Lentivirus.[2] Additional lentiviruses were soon isolated in other primates, and a second causative retrovirus, Human immunodeficiency virus type 2 (HIV-1) was identified in 1986.[3-4] Almost four decades later, what was originally thought to be an isolated disease that affected a minority population of male homosexuals is now recognised as one of the most devastating infectious diseases that have emerged in recent history.

Since its initial discovery, approximately 78 million people have been infected with HIV, from which an estimated 39 million have succumbed to the disease.[5] Nevertheless, we exist in an era of unparalleled advancement in HIV treatment possibilities, which is accompanied by the increased prevalence of targeted public health campaigns – all of which have contributed to significant progress in halting and reversing the spread of AIDS. New HIV infections have fallen in occurrence by 35% since 2000 (58% among

children) and AIDS-related deaths have sharply plummeted by 42% since its peak in 2004.[6] The aversion of approximately 30 million new HIV infections and 8 million AIDS-related deaths is attributed to the concerted global effort to reduce the spread of HIV infection.

In spite of the progress made in AIDS prevention, the sheer number of new infections and people living with HIV ensures that the AIDS pandemic remain a priority in global health policies. According to the World Health Organisation (WHO), 36.9 million people are currently living with HIV and an estimated global prevalence of 0.8% has been estimated in adults aged 15-49 (Figure 1.1).[7] Moreover, 2 million new infections were discovered in just 2014 alone. Sub-Saharan Africa remains the most affected region with a HIV prevalence of almost 5%, and the region accounts for nearly 71% of the population that is infected with HIV worldwide. The latter also shines a harsh light on the gross inequalities of the world – access to treatment remains a problem for HIV-infected individuals, with only 41% and 32% of adults and children with access to treatment respectively. The problem becomes more pronounced for lower income populations, with only a third of the 28.6 million people eligible for anti-retroviral treatment (ART) accessing treatment in low and middle-income countries.[8-9]

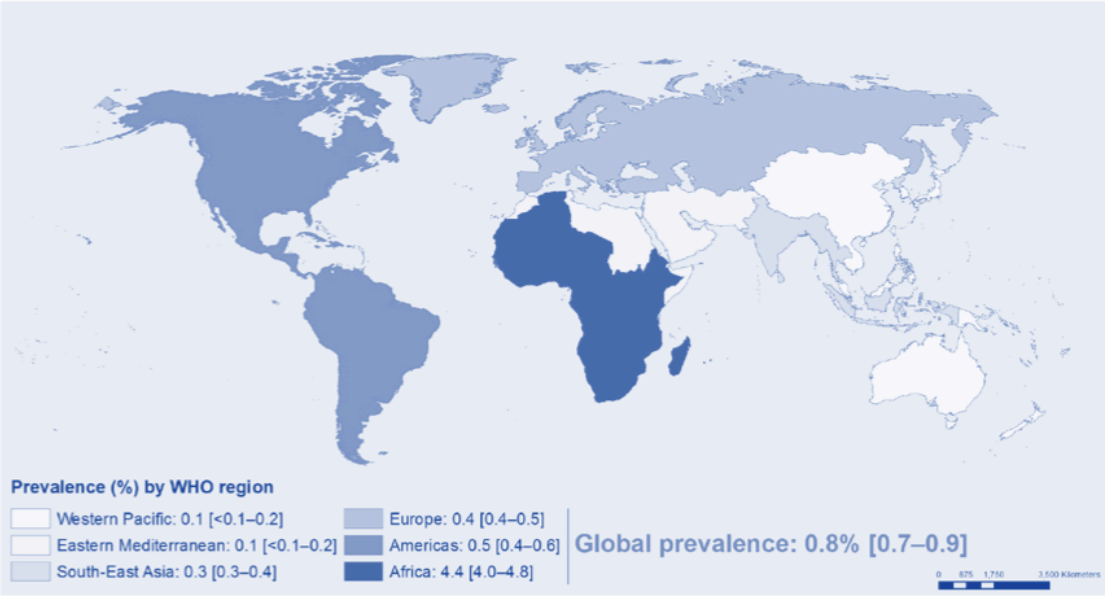

Figure 1.1: Global adult HIV prevalence. An estimated 0.8% of adults aged 15-49 years live with HIV. Sub-Saharan Africa remains the most severely affected (4.4%). (Adapted from[7])

In addition to the gaps in ART coverage across the world, the prevention of AIDS is hindered by the even more fundamental problem of a lack of an effective HIV vaccine or cure. A comprehensive understanding of immune correlates in populations that manifest immune control to HIV-1 and HIV-2 and delayed progression to AIDS is expected to yield critical information to vaccine and therapy design. In this work, we focus on the impact of both viruses on immune exhaustion and senescence, which impacts on the replicative potential and polyfunctionality of T-cells. As the latter is critical to sustaining effective elimination of the virus, we foresee that factors contributing to T-cell senescence are likely to be important in establishing the threshold that marks the progression of AIDS following infection.

1.1.2 The Origin of HIV-1

Both HIV-1 and HIV-2 entered the human population through multiple zoonotic transfers of simian immunodeficiency viruses (SIV) that naturally infected African primates. As a lentivirus, most SIV transmissions occur horizontally, between individuals of the same species; and examples of cross-species transmission and infiltration into the host germline of new species are rare – but can lead to the integration of an endogenous, vertically transmissible part of the genomic loci.[10] Examples include two prosimian endogenous lentiviruses, which independently penetrated the germ-lines of both the grey mouse lemur (pSIVgml) and the fat-tailed dwarf lemur (pSIVfdl) about 4 million years ago.[11-12] Most SIV infections appear to be non-pathogenic;[13] SIVcpz is an exception which leads to AIDS in two distinct subspecies of chimpanzees.[14-15] Of the approximately 40 primate species that are infected with SIV, SIVcpz is of greatest interest to HIV researchers since it bears the closest genetic resemblance to HIV-1 (Figure 1.2).[13]

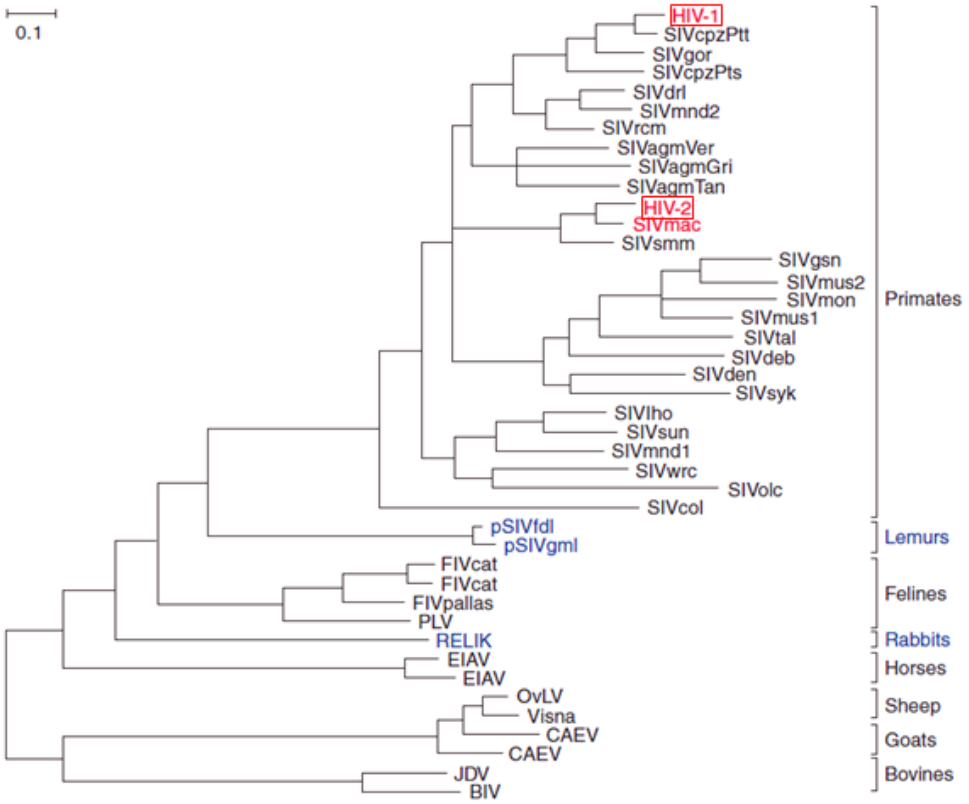

Figure 1.2: Phylogeny of lentiviruses based on Pol sequences. Evolutionary tree constructed from Pol sequences derived from various mammalian lentiviruses using maximum likelihood methods; host species are indicated on the right. Exogenous viruses are depicted in black; HIV-1, HIV-2 and SIVmac appear in red text, with both HIV viruses highlighted within red boxes. Endogenous viruses are displayed in blue. Scale bar represents 0.10 amino acid replacements per site. (Adapted from[13])

Despite its phylogenetic relationship with HIV-1, the perceived lack of a SIVcpz reservoir left the source of HIV-1 contentious. The endangered status of chimpanzees, and the early observation that SIVcpz infections were rarely observed in the wild, fuelled these reservations.[16-17] The first isolates of SIVcpz, for example, were derived from animals housed in primate centres or sanctuaries.[18] The initial doubts have however been addressed by increased opportunities for the non-invasive sampling of wild-living ape populations, which uncovered multiple naturally occurring SIVcpz strains. From studying these strains, it also became clear that all SIVcpz isolates could be separated into either of two subspecies-specific lineages - SIVcpzPtt or SIVcpzPts - which infected the *Pan t. troglodytes* and *Pan t. schweinfurthii* chimpanzee subspecies respectively.[13] Of the two strains, the former is most closely related to the pandemic form of HIV-1.[19-20] In 2006, the additional discovery of SIVgor in wild gorillas has revealed a second potential reservoir of SIV that had contributed to the evolution of HIV-1.[21]

The term HIV-1 does not refer to one virus, but encompasses four distinct lineages which are referred to as groups M, N, O or P – each of which resulted from an independent cross-species transmission event. Group M was the first to be discovered, due to its higher prevalence, and represents the pandemic form of HIV-1 that is responsible for the majority of HIV infections observed worldwide.[13] Group O was identified in 1990; and covers less than 1% of global HIV-1 infections – its observation is restricted to Cameroon, Gabon and countries in its immediate vicinity.[22-23] Group N and P are both exceedingly rare, the former with 13 documented cases and the latter with only two known cases to date.[24-25]

All four HIV-1 groups, as well as SIVgor, cluster with SIVcpzPtt, suggesting that SIV from *Pan t. troglodytes* is the most likely original reservoir of both HIV-1 and SIVgor.[13]

Neither chimpanzees nor gorillas appear to be the natural hosts for SIV, but were infected by cross-species transmission from other primates.[26] SIVcpz, for example, is a complex mosaic virus that has evolved from recombination events between SIVrcm (from red-capped mangabeys) and a virus that more closely resembles SIVs which infect the *Cercopithecus* species.[27] HIV-1 groups M and N are closely related to SIVcpzPtt strains from southern Cameroon and implicates their chimpanzee origin; while existing phylogenetic data point towards a gorilla origin of HIV-1 Group P.[28] Finally, the origin of Group O remains obscure due to its lack of particular closeness to any existing SIV strains – but its clustering with both SIVcpzPtt and SIVgor suggests that either could have led to its existence.[13]

1.1.3 The Origin and Epidemiology of HIV-2

In contrast to the global prevalence of HIV-1, HIV-2 has remained predominantly endemic to West Africa since it was first isolated from AIDS patients in Guinea Bissau and Cape Verde in 1986.[3] In the late 1980s, West African nations - including Guinea-Bissau, the Gambia, Senegal, Cape Verde, Côté d'Ivoire, Mali, Sierra Leone, and Nigeria - all reported a prevalence rate of >1%; this amounted to between 1.5 - 2 million individuals from the region.[29-30] Unlike HIV-1 however, HIV-2 prevalence has experienced a global decline in infection rates; an observation that has largely been attributed to the lower transmissibility of HIV-2.[31-32] HIV-2 has a lower infectivity than HIV-1; perinatal transmission rates of HIV-2 stabilised at 1.2% in a prospective cohort of women in Ivory Coast as compared to 24.7% for HIV-1.[33] In Guinea-Bissau alone, prevalence rates have fallen from 8.3% to 4.7% in the years between 1990 and 2000.[34]

While the absolute number of individuals with HIV-2 remains small in Europe, HIV-2 has spread to multiple European nations, particularly to cities with socio-economic ties to West Africa and Portugal. Portugal exhibits the highest prevalence in Europe, where HIV-2 accounts for approximately 10-13% of all HIV cases, and contributes to 4.5% of HIV progressors.[35] The War of Independence between Portugal and its former colony, Guinea-Bissau, has widely been cited as the reason for the spread of HIV-2 into Portugal, which paved the way for entry into other European cities. In France, where the cohort for our study originates, HIV-2 infection was observed to contribute to approximately 1.8% of the 10,184 newly diagnosed HIV cases between 2003 and 2006, although these cases were largely imported from Senegal and Côté d'Ivoire[36]

HIV-2 originates from a lentivirus that infected a different species of monkey – the sooty mangabey (*Cercocebus atys atys*).[37-38] First proposed in 1989, this speculation was affirmed by demonstrating the close resemblance between sequences of HIV-2 from native West Africans to that of locally circulating SIVsmm strains.[37,39] Unlike SIVcpz, SIVsmm is highly prevalent in captivity and in the wild, and is also non-pathogenic in its native host – suggesting that sooty mangabeys are likely to be the natural reservoir for the virus.[40]

Since its initial discovery, at least eight distinct lineages of HIV-2 have been identified, groups A-H – each originating from an independent transfer event. Group A is the predominant strain that is found throughout western Africa, while group B is localized in Côté d'Ivoire.[41-42] Together, HIV-2 groups A and B account for approximately 1-2 million HIV-2 cases. All other groups were isolated from single cases of HIV-2 infection, with the exception of Group F, which was found in an additional patient.[43-44] Based on *gag* sequences, groups C, G and H are most closely linked to SIVsmm sequences from

Côté d'Ivoire; Group D to SIVsmm strains from Liberia; and groups E and F to an SIVsmm strain from Liberia; the genetic links most likely hint at the origin of zoonosis.[37,39,41,45]

1.1.4 Natural History of HIV-1 Infection

Infection with HIV-1 initiates the progressive depletion of CD4 T-cells, which are the preferred targets of infection and replication.[46] While the timeframe differs between individuals, three distinct phases can often be distinguished – the acute, asymptomatic and symptomatic phase (Figure 1.3a):

1) *Acute or primary infection* – lasting two to eight weeks – is typically characterised by high levels of virus, with viral loads escalating beyond 10^7 copies/ml.[47-48] Clinically, this period may be characterised by fever, lymphadenopathy, pharyngitis and cough.[49-50] With the initiation of adaptive immune responses, particularly virus-specific cytotoxic activity, viral loads decline precipitously.[51-53] During this phase, CD4 T-cell counts decrease dramatically but are restored to a near normal level within 3-4 months of infection.[54] There is a surge in the number of CD8 T-cells, however, which is stabilised but remains elevated until the symptomatic phase.[55]
2) *Asymptomatic phase* – Approximately six months after infection, HIV viral load stabilises at a set point for an indefinite period.[56-57] The viral set point differs greatly between subjects and provides a valuable prognostic marker, where higher viral set points are usually followed by faster rates of CD4 T-cell decline.[58-60] While clinically asymptomatic, a dynamic cycle of virus production and clearance ensues and the daily plasma turnover rate is estimated at 30%-50%.[61-63] Despite

the misleading presentation of stabilised viral load and CD4 T-cell counts, which nevertheless tend to gradually decline, the virus is far from latent and this phase generally heralds the development of symptomatic illness.

3) *Symptomatic phase* – T-cell numbers remain collectively unperturbed in the preceding phases, as elevations in CD8 T-cell counts compensate the decline in CD4 T-cell numbers – this superficial stabilisation of overall T-cell count is described as a period of unselective or indiscriminate T-cell homeostasis.[55] Approximately 18 months prior to the onset of AIDS, homeostatic mechanisms give way to a total collapse in T-cell numbers and AIDS-defining symptoms – including Kaposi's sarcoma, tuberculosis, *Pneumocystis carinii* pneumonia and non-Hodgkin's lymphoma – begin to surface when CD4 T-cell counts fall below 200 cells/ μl.[64-67] Reasons for this failure in T-cell homeostasis continue to emerge but the major mechanisms refer to the progressive immunosenescence and exhaustion of the proliferative capacity of T-cells; as well as the deterioration of the architecture of lymphoid organs.[68-71] This phase is also characterised by progressively increasing HIV viral load.[55-57]

The length of the asymptomatic phase is typically used to determine whether individuals are slow, rapid or intermediate progressors (Figure 1.3).[72-74] The majority of HIV-1 infected individuals (70-80%) experience intermediate disease progression where CD4 T-cell counts gradually decline over 6-10 years with concomitant rise in HIV viral load and eventual manifestation of AIDS-related illnesses. 10-15% are rapid progressors who experience steep CD4 T-cell decline and develop AIDS within the first few years of infection and late progressors (~5%) remain healthy without significant shifts in CD4 T-cell count or viral load over a decade.[74-75]

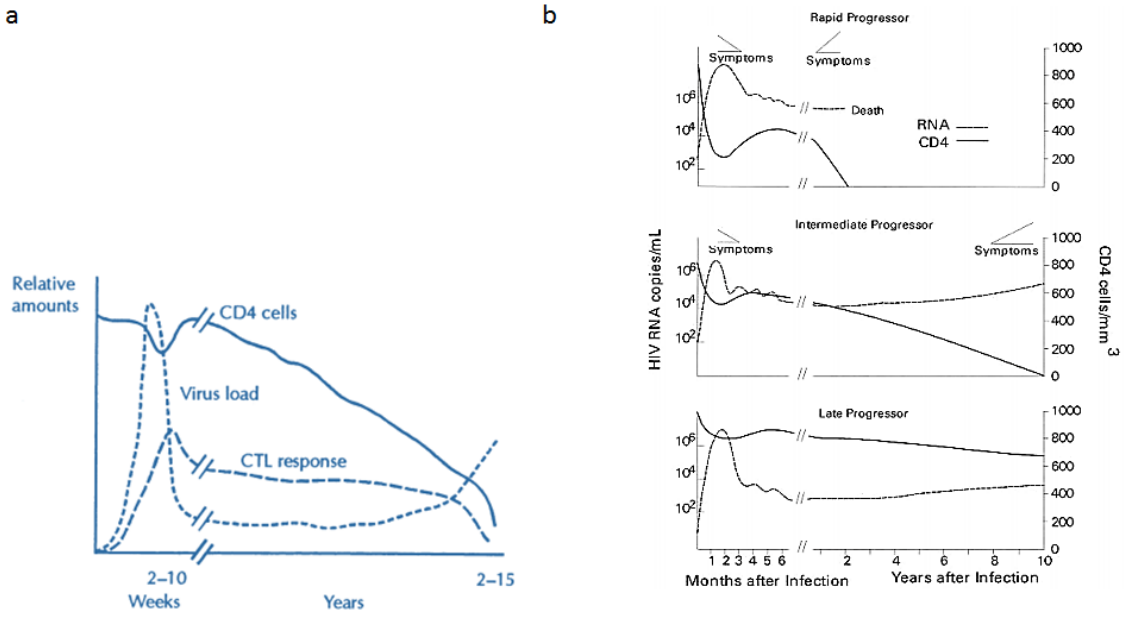

Figure 1.3: HIV disease progression. (**a**) Typical course of HIV-1 infection *in vivo* and (**b**) natural history of HIV-infection in rapid progressors (top), intermediate progressors (middle) and late progressors (bottom). (Adapted from[75,76])

1.1.5 Natural History of HIV-2 Infection

Both HIV-1 and HIV-2 infection can lead to AIDS, but progression to AIDS occurs far less frequently in individuals (15-20%) infected with HIV-2 – those who are infected with the latter typically remain asymptomatic for more than a decade.[77-78] This is a stark contrast to HIV-1 infection, where time of progression to AIDS in treatment-naïve subjects follows a normal distribution with a mean duration of 11 years, for more than 99% of patients in Caucasian cohorts.[79] HIV-2 infection is also characterised by a longer asymptomatic phase and slower disease progression. In a group of Senegalese female commercial sex workers for example, AIDS related mortality was 33% for HIV-1 infected patients as compared to none for HIV-2 infected patients throughout five years of observation from initial seroconversion.[78] In addition, patients with HIV-2 infection

display higher CD4 T-cell counts, lower viral loads and generally lower levels of immune activation.[77,80-81] For recent seroconvertors, plasma viral loads are on average 28-fold lower in HIV-2 infection than HIV-1 infection.[82]

As with HIV-1, HIV-2 viral loads have significant prognostic value – patients with an undetectable viral load have a similar survival probability as uninfected controls, while those with a viral load set point of more than 10 000 copies/ mL had a much higher risk of mortality within two decades of infection (Figure 1.4).[83] However, unlike most HIV-1 controllers, HIV-2 controllers comfortably retain undetectable plasma viral loads for decades and show no significant differences in risk of mortality as compared to uninfected individuals. When HIV-1 and HIV-2 patients are matched by disease stage, HIV-2 patients reveal significantly lower plasma viral loads, while proviral loads remain comparable.[77,84-85] Since the rate of viral shedding hinges on the levels of virus in the plasma, the gap in plasma viral load likely explain the difference in risk of transmissibility between HIV-1 and HIV-2.

Nevertheless, once advanced immunodeficiency becomes apparent, individuals with HIV-2 infection progress at a rate that is clinically indistinguishable from their HIV-1 infected counterparts, with the exception of a lower frequency of patients presenting with Kaposi's sarcoma.[86] In the Gambia, equally high mortality rates were described for both HIV-1 and HIV-2 infected patients with CD4 T-cell count of less than 200 cells/ µl. However, CD4 T-cell counts at the time of mortality remains higher for HIV-2 infection than HIV-1 infection.[87]

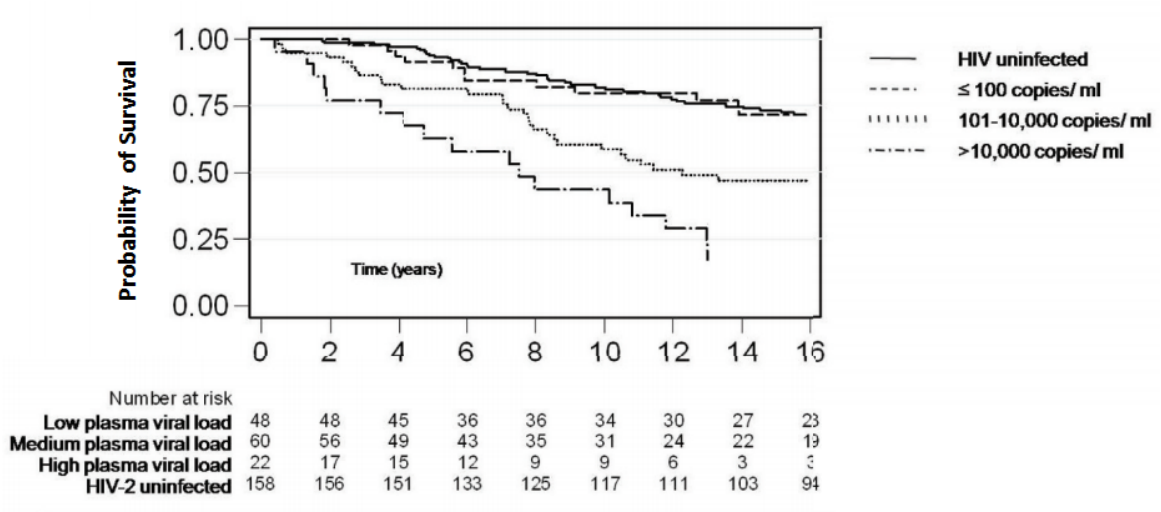

Figure 1.4: Mortality of HIV-2 infection based on viral loads. Kaplan-Meier plots depicting the relative survival probability of HIV-2 patients with viral load of < 100, 101 - 10,000, and >10000 copies/ mL. (Adapted from[83])

1.1.6 Mother to Child Transmission of HIV-1

Mother to child transmission (MTCT) remains a significant source of new HIV-1 infection that continues to drive the present day epidemic, particularly in Sub-Saharan Africa.[88] Globally, approximately 10% of the 34 million of people living with HIV-1 are children; 95% of these children live in Sub-Saharan Africa and 90% of them were perinatally infected.[88-89] While sociological conditions are improving in Sub-Saharan Africa, socioeconomic and sociocultural factors remain a substantial force in driving the paediatric epidemic, which usually result from poverty and unequal opportunities in receiving treatment.

According to UNAIDS, the mean percentage of pregnant women living with HIV in 2015, and accessing PMTCT, is estimated to be 92% in Western and Central Europe, 90% in Eastern and Southern Africa but only 48% in Western and Central Africa.[89] An estimated

1.4 million women living with HIV become pregnant globally and, without access to treatment, there is a 15-45% chance of transmitting the virus to their offspring.[90] MTCT of HIV-1 can occur during three phases of pregnancy: (i) *in utero* (principally during the third trimester), (ii) *peripartum* (through exposure to mucosal fluids during labour) or (iii) *postpartum* (during breastfeeding). *Peripartum* and *postpartum* transmissions are the main source of MTCT, however, the provision of treatment and replacement feeding can drastically reduce transmission rates to 1-2%.[91-92] Hence, the global provision of PMTCT is undoubtedly key to preventing vertical transmission and remains a priority in halting the AIDS epidemic. By upscaling efforts to increase PMTCT exposure, the number of children born annually with HIV was reduced by half in less than decade, from 400 000 in 2009 to 240 000 in 2013.[93]

1.1.7 Natural History of Paediatric HIV Infection

The high rates of HIV transmission in infancy that existed from more than a decade ago is a result of the only recent refinement in knowledge of effective MTCT and late availability of PMTCT with highly effective antiretroviral drugs (ARVs) in developing countries; the result is the presentation of an increasing number of mature children with longstanding HIV infection at healthcare clinics across South Africa today.[94] For context, the percentage of HIV-1 positive pregnant women receiving ARVs in 2007 was 22%, and this figure has since risen to 82% in 2013.[88] Today, HIV-1 prevalence across South Africa ranges between 2-8% for adolescents aged 12–19 years old.[95-98] The appearance of older children with longstanding HIV-1 has allowed for a more detailed understanding of the natural history of paediatric HIV infection.

Paediatric HIV-1 infection is characterised by two distinct modes of disease progression – as the survival probability of vertically-acquired HIV is initially dominated by a very high risk of rapidly progressing disease, with median survival to two years of age clinically observed in more than 50% of cases, there was an initial assumption that survival to older childhood was exceptional.[99] However, more recent epidemiological data indicates that while 20-25% are rapid progressors and die by two years of age, the remaining 75% of HIV-infected adolescents are slow progressors and survive to a median age of 7 years without access to treatment.[100-104] In Sub-Saharan and South Africa, approximately one third of children with vertically transmitted HIV survive to a median age of ten years or longer.[94-95] The rapid progressor group tend to include infants who were infected by intrauterine transmission, while the slow-progressor groups is composed of infants who were infected closer to birth.[105-106]

In slow progressing children, HIV-1 infection continues to be characterised by gradual CD4 T-cell depletion, but children tend to present with higher CD4 T-cell counts than adults when matched for stage of disease progression.[107] Non-progression, characterised by strong cytotoxic responses and high CD4 T-cell counts in the absence of ART, occur at much higher rates in paediatric infection (5-10%) than in adult infections (approximately 0.3%).[100,108-109] Most strikingly, non-progressing HIV-infected children who maintain absolute CD4 T-cell counts within the normal rage of age-matched uninfected children do so despite median viral loads of >30 000 copies/ mL of plasma, while adult controllers typically present with undetectable or extremely low viraemia (Figure 1.5).[110-111]

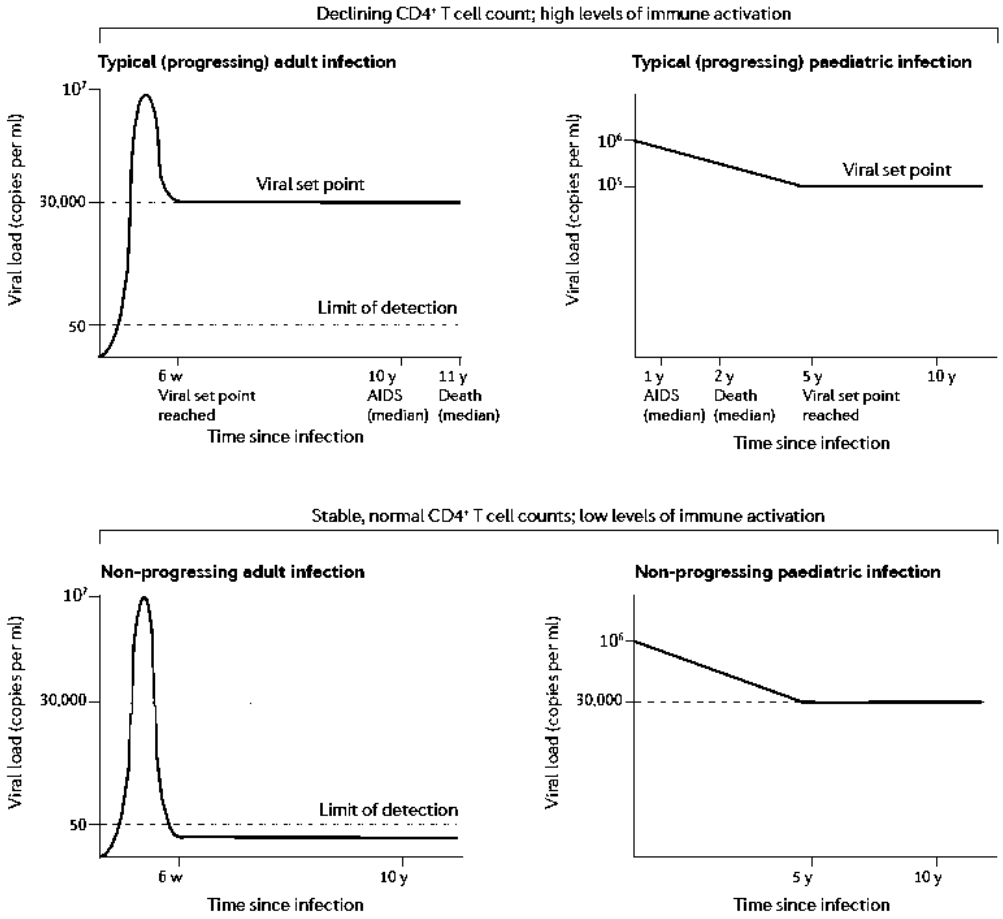

Figure 1.5: Changes in viral load following paediatric and adult HIV infection. Typical, progressing adult and paediatric infection (**top**). In treatment naïve HIV-1 infection of adults, viral set point occurs after 5 weeks and median time to AIDS is 10 years. In ART-naïve paediatric infection, viral set point only occurs after 5 years and median time to AIDS from the viral set point is 1 year. Typical non-progressing adult and paediatric infection (**bottom**). Non-progressing elite controller adults – viral loads tend to be low or undetectable while paediatric elite controllers have a much higher viral set point. (Adapted from[111])

In adult HIV infection, plasma viral loads tend to decrease by between 100 - 1000 fold and settle at a set-point within six weeks of acute infection. The viral set point for paediatric HIV-infection occurs much later (median age of 5 years) and is higher in children than adults.[112] Non-progressing HIV adult and paediatric infection are both characterised by low levels of immune activation but while the former case is achieved through HLA-mediated suppression of viral replication, the mechanisms for lower immune activation in children despite high levels of viraemia are currently unclear.[104] An interesting parallel can be drawn between non-progressing paediatric infection and SIV infection in their natural hosts – such as in the sooty mangabey and African green monkey; both cases present with normal CD4 T-cell counts, low levels of immune activation and high median viral loads of approximately 10^5 RNA copies per ml.[40] Thus, non-progressing paediatric HIV-1 infection may provide windows for the visualisation of a functional cure for AIDS.

1.1.8 Complications of longstanding paediatric HIV infection

Healthcare providers in Sub-Saharan Africa are familiarising themselves with a clinical picture of slow-progressing paediatric HIV-infection that is characterised by pronounced stunting, severe immunosuppression, or chronic complications usually associated with untreated HIV in adults.[113] In Harare, recently diagnosed or undiagnosed vertically acquired HIV remains the major cause of hospital admission and in-hospital deaths among adolescents in the region.[114] While presentation with the common cough is typical and is often met with presumptive treatment for undiagnosed tuberculosis, respiratory illnesses are the most common cause of death among HIV-infected adolescents and these cases usually follow a long medical history that is suggestive of chronic lung disease. While lymphocytic interstitial pneumonitis (LIP) remains the most common cause of lung

disease (30-40%) in younger children,[115] the aetiology of lung disease in older children is less well defined. The detailed screening of HIV-1 infected older children with pulmonary function tests, Doppler echocardiography and chest radiography reveal a pattern of pathogenesis that is compatible with small airway disease, or more precisely identified as obliterative bronchiolitis (OB).[116]

1.2 The HIV-1 and HIV-2 Genome and Structure

1.2.1 HIV-1 and HIV-2 Genome

Both HIV-1 and HIV-2 have two copies of a positive-sense, single-stranded RNA (ssRNA) genome that dimerize at their 5' ends.[117-118] Despite differences in their origins, HIV-1 and HIV-2 display similar genomic architecture, with significant homology at the proteomic level (Figure 1.6).[119] Like most exogenous retroviruses, the HIV-1 and HIV-2 genomes encode the major structural genes – *gag*, *pol* and *env* – which are responsible for building the structural core of the virus within three open reading frames (ORFs).[120] The gene *gag* encodes the Gag polyprotein precursor, which is post-translationally processed to generate the matrix, capsid, nucleocapsid and p6 proteins.[121] The Gag-Pol polyprotein yields protease, reverse transcriptase and integrase, while the *env* gene encodes a 30 amino-acid signal peptide, gp120 and gp41.[122] In terms of the main structural genes, HIV-1 and HIV-2 approach approximately 60% sequence identity in gag and pol, and 40% identity in env respectively.[123]

HIV-1 and HIV-2 are complex retroviruses that encode other regulatory (Tat, Rev) and accessory proteins (Nef, Vif, Vpr, Vpu/Vpx) in its genome; these protein products further regulate and enhance the replicative fitness of the virus. For example, Tat (in two isoforms Tat-1 and Tat-2) acts as a transactivator of the HIV promoter in the long

terminal repeat (LTR) sequence by promoting the elongation of transcription through the polyadenylation signal – leading to the effective generation of full-length transcripts.[124] The accessory proteins generally work to counteract host cellular mechanisms that hinder viral replicability. Among the accessory proteins, Nef has received most interest from HIV researchers due to its key involvement in inhibiting host T-cell responses by promoting the downregulation of CD4 (also downregulated by Vpu) and MHC-I molecules.[125-127] By limiting MHC-I:CD8 interactions, which are essential for CD8 T-cells to mount HIV-specific responses, Nef negates the potent killing capacity of cytotoxic CD8 T-cells. In addition Vpr has also been noted for enhancing HIV infectivity and replication in non-dividing cells, such as macrophages, by facilitating the nuclear localisation of the preintegration complex (PIC).[128]

In addition to differences in the sizes of some ORFs and untranslated regions between the genomes and HIV-1 and HIV-2, one of the more striking differences lies in their differential usage of a specific accessory protein: HIV-1 and HIV-2 uniquely possess *vpu* and *vpx* respectively.[129] This difference remains a fascination, because it is believed to hold clues that explain the difference in virulence and lower transmissibility of HIV-2. Like Vif, which counters host restriction factor APOBEC3G,[130-131] Vpx counteracts SAMHD1, APOBEC3A and IRF5.[132-135] The ability of HIV-2 to infect primary human mononuclear cells, including macrophages and dendritic cells, has been attributed to the antagonism of SAMHD1 that is expressed in those cell types.[132] The latter is a dGTP-stimulated triphosphohydrolase that cleaves dNTPs, and via this mechanism, – depletes the availability of dNTPs for viral cDNA synthesis.[136] Nevertheless, the infectivity of HIV-2 does not appear to be universal amongst myeloid cells, as certain subsets of dendritic cells remain refractory to the virus, leaving the subject controversial.[137] Its HIV-1 counterpart, Vpu, on the other hand, participates in the downregulation of CD4 by

promoting its proteosomal degradation while facilitating the successful release of virions from infected cells.

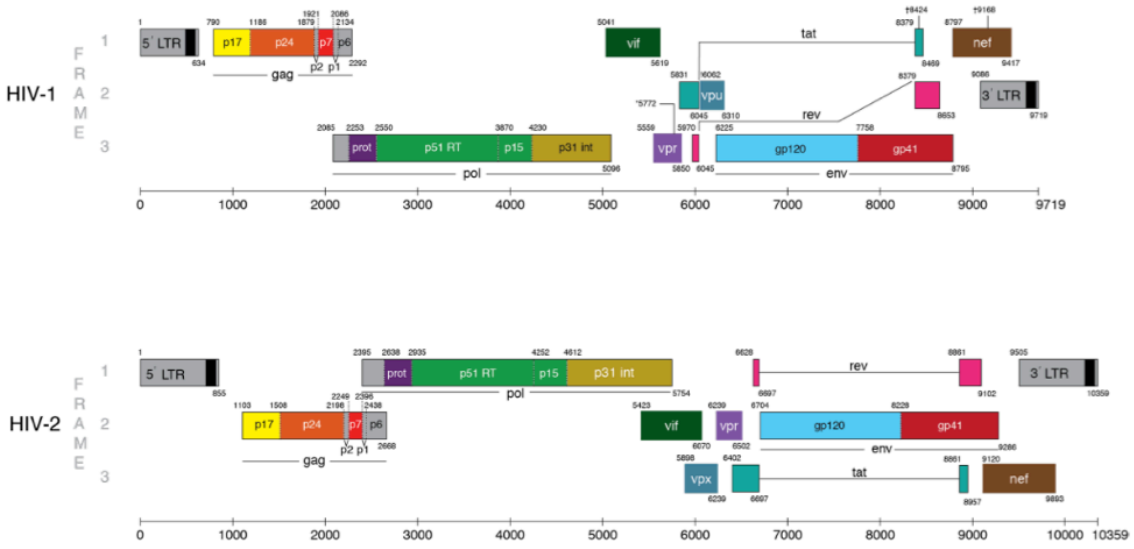

Figure 1.6: The genomes of HIV-1 and HIV-2. ORFs are displayed as rectangles and coloured according to the mature protein products. The location of the ATG start codon relative to the distal end of the 5' LTR is used to map gene positions. (Adapted from[138])

1.2.2 The Structure of HIV-1 and HIV-2

Both HIV-1 and HIV-2 are retroviruses of the genus *Lentivirus* that belongs to the family *Retroviridae*. Typical of retroviruses, mature virions are spherical with a diameter of 1,000–1,500 Å, and possess an envelope that is composed of host-derived phospholipid bilayer (and associated membrane proteins including MHC molecules) and heterotrimeric complexes of the viral glycoproteins gp41 and gp120 (gp105 in the case of HIV-2); the former act as ectodomain while the latter makes up the transmembrane domain.[139-140] The heterotrimeric complexes of gp41 and gp120 form 4 – 35 spikes that stagger themselves across the envelope (Figure 1.7).[141-142]

At the core of the mature virion is the viral RNA genome, and genomic stability is maintained by association with the nucleocapsid (NC) protein p7, which also participates in the packaging of genomic RNA into nascent virions.[143] A conical capsid made up of p24 (CA) – or p26 in HIV-2 – protects the viral genome and critical enzymes involved in viral replication – including reverse transcriptase, integrase and protease.[144] In addition, the accessory proteins Vpr, Vif and Vpu (or Vpx in the case of HIV-2) are contained within the matrix of the capsid. The matrix of the mature virion is also made up of a p17 core which provides overall structural stability by anchoring to the inner surface of the plasma membrane via myristolated residues.[145]

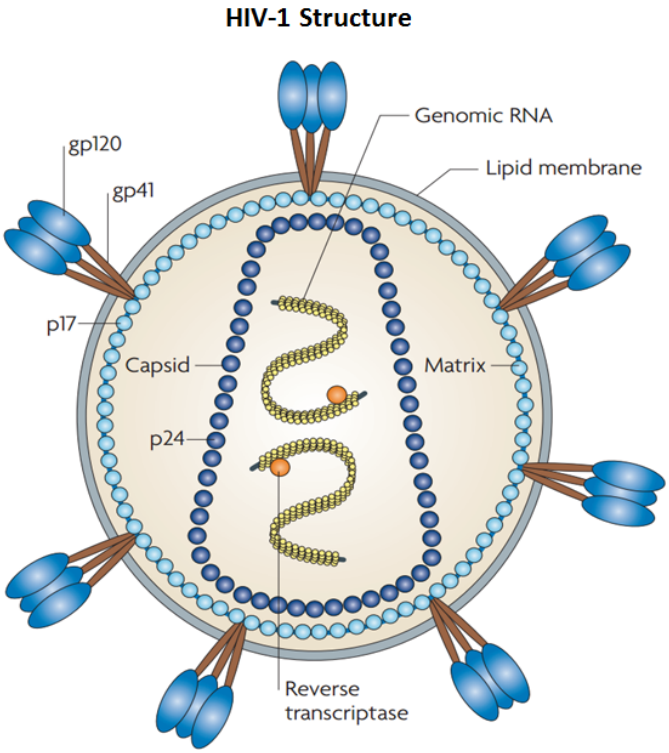

Figure 1.7: The structure of HIV. Schematic diagram of the mature HIV virion**,** which maps the location and arrangement of major HIV structural proteins. (Adapted from[146])

1.2.3 Structure and Function of Gag

The assembly and budding of lentiviral particles are orchestrated by the Gag polyprotein.[147] The final phases of the HIV replication cycle are defined by the transcription of viral genes and export of viral RNAs from the nucleus to the cytoplasm, where viral proteins are synthesised. Gag is first produced as a 55 kDA precursor polyprotein (57 kDA for HIV-2) and seeds the formation of the virus particle.[148] At the time of assembly, a 160 kDA GagPol polyprotein precursor containing the necessary enzymes of replication – protease, reverse transcriptase and integrase – is expressed at approximately 5% of the level of Gag through the clever reprogramming of ribosomal frameshifting events during Gag translation.[149-150]

The precursor protein encompasses the matrix (MA), capsid (CA), nucleocapsid (NC) and p6 domain with two spacer domains, SP1 and SP2, which flank the NC segment (Figure 1.8). The MA domain directs Gag to the plasma membrane and facilitates the fusion of viral *Env* glycoproteins into the nascent virions.[151] During virus assembly, CA drives Gag multimerisation while NC packs RNA genome into virions.[152-153] The p6 domain completes the budding process by recruiting the endosomal sorting apparatus that catalyses the membrane fission step.[154] Finally, the viral protease which is encoded within the GagPol precursor cleaves the Gag precursor to release the mature MA, CA, NC and p6 products – this important step initiates a restructuring process that finalises the assembly of the mature virion.[155] The p6 protein is also involved in the packaging of accessory proteins – Vpr and Vpx/Vpu – into the virus capsid.[156]

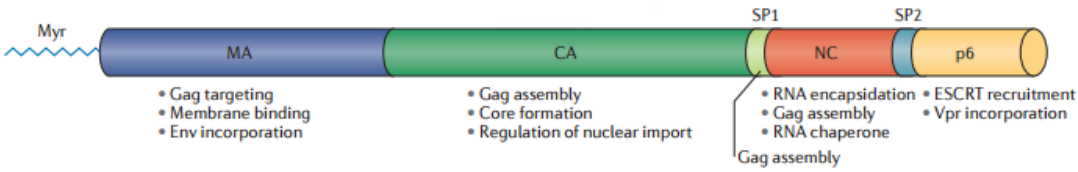

Figure 1.8: Major domains of the Gag precursor polyprotein and their functions. A linear schematic diagram of the Gag precursor polyprotein. Each major domain – including matrix (MA), capsid (CA), nucleocapsid (NC) domains – is individually coloured and their primary functions are indicated below. (Adapted from[121])

HIV-2 gag is similar in organisation and genetic structure to HIV-1 with the exception of a larger precursor product of 57 kDA as opposed to the 55 kDA product of HIV-1.[148] The closeness of the two proteomes is reflected in the ability of HIV-1 and HIV-2 Gag to combine into functional virus particles.[157] The involvement of Gag in virion assembly and release is illustrated in Figure 1.9.

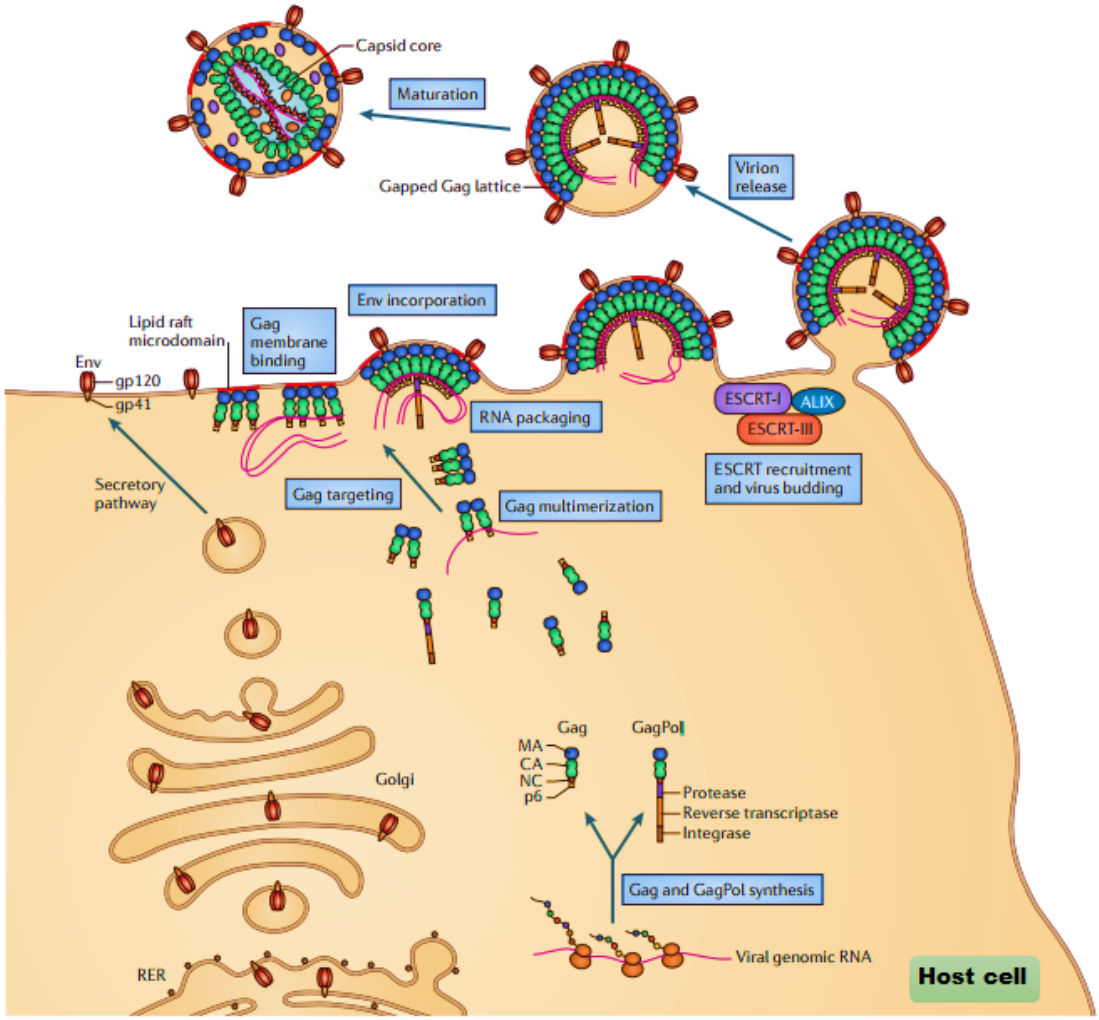

Figure 1.9: The involvement of Gag in HIV assembly. The Gag precursor is synthesised in the cytosol from full-length, single-stranded viral RNA. Gag recruits the viral genomic RNA, multimerises and traffics to the plasma membrane where it is anchored to the lipid bilayer via insertion of its amino-terminal myristate that directly interacts with the phospholipid phosphatidylinositol-(4,5)-biphosphate. The GagPol precursor polyprotein is synthesised by ribosomal frameshift events during translation of the Gag-encoding viral RNA. The proteolytic cleavage of Gag and GagPol poly protein precursors is a necessary step that completes the assembly of mature virions. (Adapted from[121])

1.3 HIV-1 pathogenesis and the Role of Immune Activation

1.3.1 Mechanism for CD4 T-cell decline

The gradual loss of CD4 T-cells in untreated HIV infection and progressive repercussions on both innate and adaptive immunity allow the establishment of AIDS-defining opportunistic infections and malignancies. The mechanisms for CD4 T-cell decline fall within three distinct categories – increased destruction, decreased production and redistribution (Figure 1.10).[158] All three processes interact and contribute synergistically in driving HIV progression.

1) *Increased Destruction*: During acute HIV-1 infection, the lymphoid cells of the gut-associated lymphoid tissue (GALT) is severely compromised as part of the massive CD4 T-cell onslaught and never fully recover – even during effective viral clearance in the asymptomatic phase of HIV-1 infection.[159-161] HIV-1 infection also contributes to profound damage within other peripheral lymphoid tissues – inducing follicular and germinal centre hyperplasia or involution that are apparent in the acute and late phases of infection respectively.[162-163] Since B-cells do not allow for productive HIV-1 replication, the major involvement of B-cell clearance in the manifested hyperplasia suggests that indirect effects of HIV replication and infection are likely to play an important role in HIV pathogenesis.[164] By decimating lymphoid tissues, HIV-1 further interferes with lymphocyte trafficking and redistribution which compromises immunologic integrity.

   During chronic HIV-1 infection, increased destruction of infected and bystander cells also occur through direct cytotoxicity or the initiation of programmed cell death pathways in infected or 'bystander' cells; triggers of apoptosis include

membrane-bound viral or host immune factors. Examples of host mechanisms include Fas/FasL interactions that evolve from the respective, increased expression of Fas and FasL on lymphocytes and innate immune cells upon activation.[165-167] Other studies have revealed that HIV-1 can also mediate CD4 T-cell apoptosis via a Fas-independent mechanism. Recently, it was found that HIV-1 infection contributes to the upregulation of TNF-related apoptosis-inducing ligand (TRAIL) by infected dendritic cells and macrophages, resulting in overall higher levels of TRAIL in plasma;[169-170] this is further accompanied by the increased expression of death receptor 5 (DR5; TRAIL receptor) on infected circulating T-lymphocytes which altogether contributes to the ongoing CD4 T-cell depletion. Furthermore, increased resistance towards apoptosis was observed to be a correlate of HIV-1 immunity and clues to the relevant mechanisms are reflected in the relative expression of host factors that mediate apoptosis. As compared to HIV-1 progressors, the T-cells from long term non-progressors (LTNPs) are more resilient to Fas-mediated apoptosis and exhibit relatively low expression of IFNα, TRAIL and DR5.[171-172]

As for viral factors, several HIV-1 encoded proteins can participate in apoptotic pathways in immune cells – TCR binding of gp120 on CD4 triggers apoptosis, as does gp120 signalling through coreceptors CXCR4 and CCR5.[173-174] The involvement of HIV-1 accessory proteins, Nef, Tat and Vpr, in mediating CD4 T-cell apoptosis have also been extensively studied.[175-181] For example, Nef sensitises infected cells to apoptosis through the upregulation of surface CD95 and CD95L;[182] HIV-1 Nef, in the form of exosomes, is also highly present in the plasma of HIV-1 infected individuals and triggers the apoptotic death of bystander CD4 T-cells through interaction with CXCR4.[181]

Finally, HIV-1 protease is a potent inducer of apoptosis in infected cells and performs this function by either cleaving the inactive procaspase 8 into its active proapoptotic Casp8p41 product; or via a separate mechanism – by cleaving the anti-apoptotic Bcl-2.[183-184] The key involvement of HIV-1 protease in potentiating HIV replication and disease progression is reflected in the subsequent use of protease inhibitors in ART, which successfully resulted in lower viremia and prolonged mortality.[185-187]

2) *Decreased Production*: HIV-1 can infect CD34+ multipotent haematopoietic progenitors and trigger apoptotic pathways, thereby interfering with lymphocyte renewal and maturation by reducing thymic input.[188] Moreover, thymic output is also compromised in HIV-1 patients, either through the direct cytopathicity of HIV-infected thymocyte precursors or by activating apoptosis in uninfected immature thymocytes. The latter ultimately results in tissue atrophy and decreased circulating naïve lymphocytes.[189-191]

3) *Redistribution*: Resting CD4 T-cells, which tend to be refractory to HIV-1 infection, upregulate surface CD62L when exposed to HIV; this directs their trafficking to inflammatory lymph nodes where resting CD4 T-cells aggregate and are further exposed to the milieu of proapoptotic signals that can trigger their demise.[192] CD25+FoxP3+ T regulatory (Treg) cells similarly respond with the upregulation of CD62L when affronted with HIV – leading to an increased migration of Tregs to lymphoid tissues, where they exhibit their characteristic immunosuppressive behaviour.[193-194]

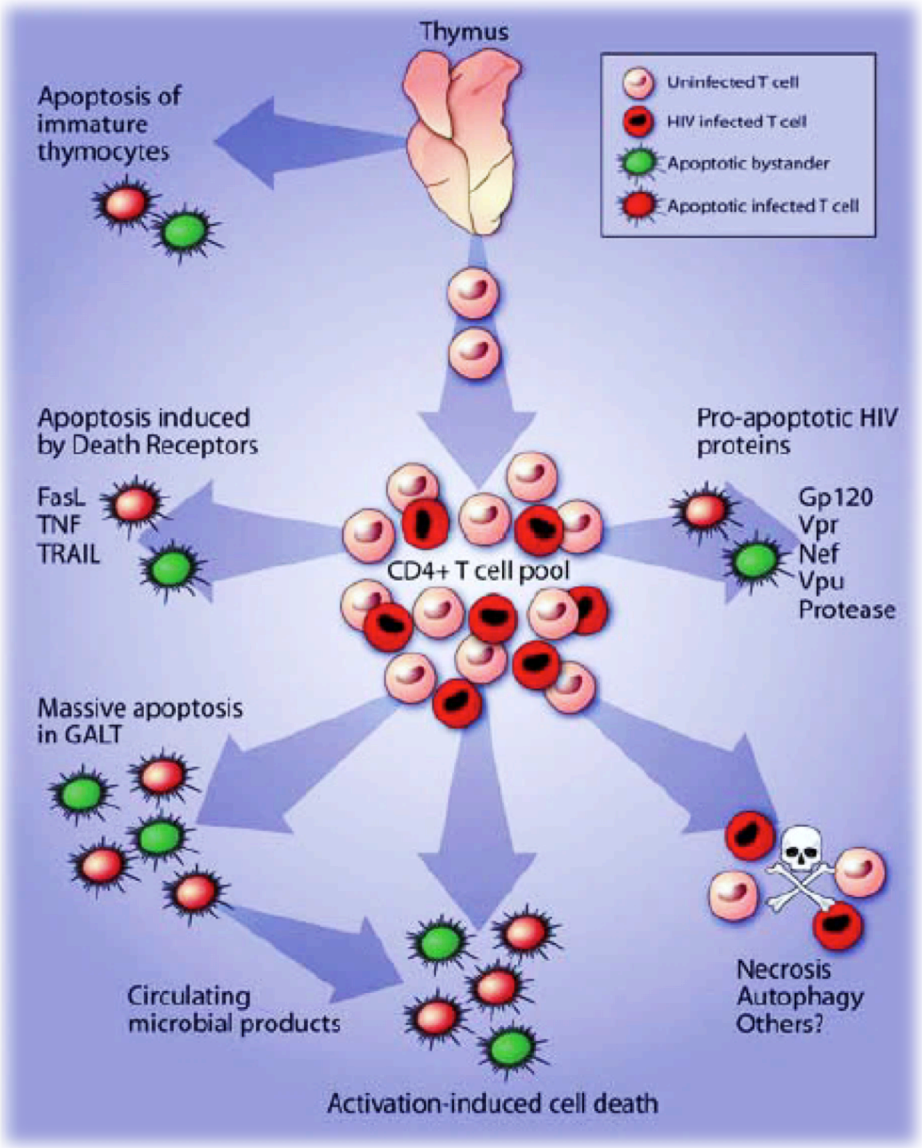

Figure 1.10: Mediators of CD4 T-cell death in untreated HIV-1 infection. Decreased thymopoiesis and excessive apoptosis induced by host (FasL, TNF, TRAIL) and viral factors (gp120, Vpr, Nef, Vpu, Protease) act in concert to orchestrate progressive CD4 T-cell decline. (Adapted from[158])

1.3.2 The Role of Immune Activation in HIV Pathogenesis

The discovery of ongoing HIV-1 replication despite apparently low viral loads during the chronic asymptomatic phases of infection indicated that the virus could by itself, initiate, orchestrate and sustain the immune dysfunction that contribute to AIDS.[195-198] This hypothesis is reinforced by the drastic improvements in AIDS prognosis by the effective use of ARVs which strongly restricted viral replication. However, the mechanistic mode by which continuous viral replication resulted in HIV progression remains poorly defined. A model based purely on CD4 T-cell depletion and failure to maintain a sufficient homeostatic response is necessarily inadequate in explaining disease progression as it fails to incorporate the complexity of dynamic interactions with other immune cells and explain the increased incidence of non-AIDS morbidity associated with HIV-1 infection. The idea that chronic immune activation could play a major role in AIDS pathogenesis was submitted first by Ascher and Sheppard and subsequently by Grossman and colleagues in the late 1980s and early 1990s – the primary involvement of immune activation in driving disease progression has since gained broad recognition.[199-201]

The early proponents of the immune activation model of AIDS pathogenesis showed that the level of CD8 T-cell activation – derived by measuring CD38 and HLA-DR expression – proved a better correlate of disease progression than viral load.[202-205] Since then, inexorable effort have gone into unravelling the relevant mechanisms and we now have a clearer understanding of how immune activation directs the onset of AIDS. Collectively, the data suggest that the prolonged periods of heightened immune activation during HIV-1 infection predisposes immune cells to cell death and intense proliferation cycles, ultimately resulting in the systemic immune exhaustion that manifests in individual cells as premature replicative senescence or compromised renewal potential.[189-191]

At the cellular level, one of the more direct result of immune activation is the skewing of lymphocyte populations towards more activated and differentiated subpopulations with diminished proliferative prowess. The latter can be mediated by (i) promoting lymphocyte turnover, (ii) inducing hyperglobulinaemia through the polyclonal activation of B-cells into terminal differentiation or by (iii) triggering the apoptosis of early differentiated T-cells and B-cells.[206-209] The induction of IFN-α stimulated genes by immunogenic products is a widely cited mechanism where infection with HIV-1 increases the susceptibility of lymphocytes to apoptosis through TNF- and Fas-related pathways.[210-213] In addition to causing quantitative defects in specific lymphocyte compartments, repeated cycles of immune signalling through HIV-1 or microbial signals also result in qualitative functional loss, as observed in the upregulation of inhibitory receptors such as PD-1 and CTLA-4 in lymphocytes.[214-218] The role of PD-1 in imposing lymphocyte anergy is supported by the observation that PD-1 inhibition restores cellular and humoral responses against the persisting virus.[219-220]

On a broader, more systemic level – activated and more highly differentiated lymphocytes tend to express distinct receptor profiles (i.e. increased expression of CXCR3, CCR6 and CD11c and corresponding reduction in CD62L, CCR7, CXCR4 and CXCR5 expression) during the course of HIV-1 infection, resulting in the trafficking of affected lymphocytes to inflammatory extralymphoid tissues rather than lymph nodes.[221-222] Since lymph nodes are instrumental to refining optimal cellular and humoral immune responses, researchers have speculated that the widespread redistribution of activated lymphocytes to inflammatory sites probably dampens the immune response against HIV in chronically viraemic individuals.[223] The overall relevance of immune activation to HIV disease progression is suggested by the gene-expression profiles of lymphocytes during HIV or

SIV infection, where hyperactivated profiles are closely associated with pathogenic outcomes.[224-225]

1.3.3 Sources of Immune Activation in HIV Infection

While the virus itself provides a central source of immune activation, infection with HIV-1 exposes the host to a myriad of stimulatory signals – including microbial products and opportunistic pathogens. Accordingly, immune activation from opportunistic infections can be considered a consequence of the immune dysfunction inflicted by the virus itself. The key players involved in systemic immune hyperactivation are discussed in this section.

1) *HIV and its antigens*: In the most direct sense, HIV drives immune activation of HIV-specific CD4 and CD8 T-cells through immediate TCR signalling. HIV RNA also activates plasmacytoid dendritic cells and other TLR-7 expressing immune cells, such as macrophages, and via this mechanism – promotes the secretion of type I interferon and TNF-α which contribute to and perpetuate bystander activation.[226-227] Although HIV-1 viraemia correlates with markers of T-cell activation,[228] it is not the sole source of immune activation as T-cell hyperactivation is maintained even when HIV viraemia remains undetectable, as is often the case for elite controllers that spontaneously control viral replication.[229]
2) *Microbial Translocation*: GALT has been revealed as the preferential target of HIV-1 replication during HIV-1 infection.[160] During the onset of HIV-infection, mucosal lymphoid tissues provides the virus with its first abundant source of targets for intensive replication, often resulting in the vast clearance of GALT-associated CD4 T-cells before peak viraemia is achieved.[230-231] Observations from

tissue sections and cells isolated from biopsies indicate that the GALT functions as a major reservoir for generating HIV virions throughout all stages of disease progression.[161,232] The significance of GALT in sequestering virus and allowing its persistent replication is highlighted by the presence of HIV virions in the GALT despite several years of ART exposure.

Among CD4 T-cells in the GALT, there is a specific bias towards the depletion of Th17 cells, which display a heightened sensitivity to activation-induced cell death.[233-235] In the gut, Th17 cells actively secrete IL-17 and IL-22, which (i) promote the recruitment of neutrophils that act extensively in the defense against bacterial and fungal infections and (ii) maintain gut epithelial barrier function.[236-237] Through the action of IL-21 and IL-22, Th17 cells preserve mucosal integrity by facilitating epithelial regeneration and supporting the production of tight junction proteins.[237-240] Hence, the drastic loss of Th17 cells during the acute phase of HIV-1 infection results in severe structural and functional impairment to the GALT – ultimately upsetting the fine balance between mutualism and pathogenicity as commensal bacteria are allowed unrestricted access across the gut. Compromise to the mucosal barrier is further potentiated by the exposure of epithelial cells to gp120, which increases mucosal permeability through the disruption of tight junctions.[241-242] The importance of Th17 integrity in the control of HIV pathogenesis can be appreciated by noting the preservation of gut mucosa in LTNPs, who retain healthier Th17 T-cell proportions in the gut and display correspondingly higher Th17 counts in circulation than HIV progressors.[243-244]

When GALT is compromised, an avalanche of immunogenic viral and bacterial products, as well as cytokines, that exponentially drive immune activation are released (Figure 1.11). Stimulatory signals from commensal flora – including

peptidoglycan, lipoteichoic acid, lipopolysaccharide (LPS), flagellin, ribosomal DNA, and unmethylated CpG-containing DNA – [245-246] elicit potent inflammatory responses by activating a plethora of NOD-like receptors (NLRs) and Toll-like receptors (TLRs) which are expressed on multiple immune cell types.[247] Among the range of microbial products that are released from the intestinal mucosa, serum levels of LPS have been shown to associate most closely with lymphocyte activation and HIV progression.[248] While bystander effects contribute significantly to the downstream activation of lymphocytes, the systemic translocation of LPS also has direct consequences on the predisposition of CD4 T-cells to HIV infection and subsequent activation; LPS and other microbial products have also been shown to upregulate CCR5 on CD4 T-cells, thus enlarging the pool of cellular targets for HIV infection and replication.[249-250]

The initial burst of immune hyperactivation from microbial translocation is succeeded by the profound, chronic release of bacterial antigens, as the integrity of the GALT remains compromised and is only very slowly restored during the entire course of infection.[251-253] In addition, serum levels of microbial products remain elevated throughout the chronic phase of pathogenic HIV and SIV infection, leading to speculation that the HIV-induced decimation of the GALT perpetuates systemic immune hyperactivation from the onset of infection to the final stages of AIDS.[254]

3) *Coinfections:* Immunodeficiency resulting from persistent HIV infection has led to increased rates of cytomegalovirus (CMV) and Epstein-Barr virus (EBV) reactivation and replication.[255-262] These are highly prevalent coinfection pathogens that usually remain latent in HIV uninfected subjects. There is substantial evidence to show that the subsequent activation and expansion of

CMV-specific lymphocytes – a situation which manifests more seriously in mature patients – further directs the maturation and accumulation of virus specific lymphocytes with senescent profiles.[263-264]

4) *Homeostatic proliferation:* Although the mechanisms that govern T-cell homeostasis during chronic HIV-1 infection remains poorly understood, the decline in CD4 T-cell populations signal the physiologic release of homeostatic cytokines (IL2, IL7 and IL15) that promote the peripheral expansion and generation of proinflammatory, effector T-cells that further drive bystander activation.[265-267] The increased production of IL15 during HIV infection is also thought to enhance HIV-associated hypergammaglobulinaemia.[268]

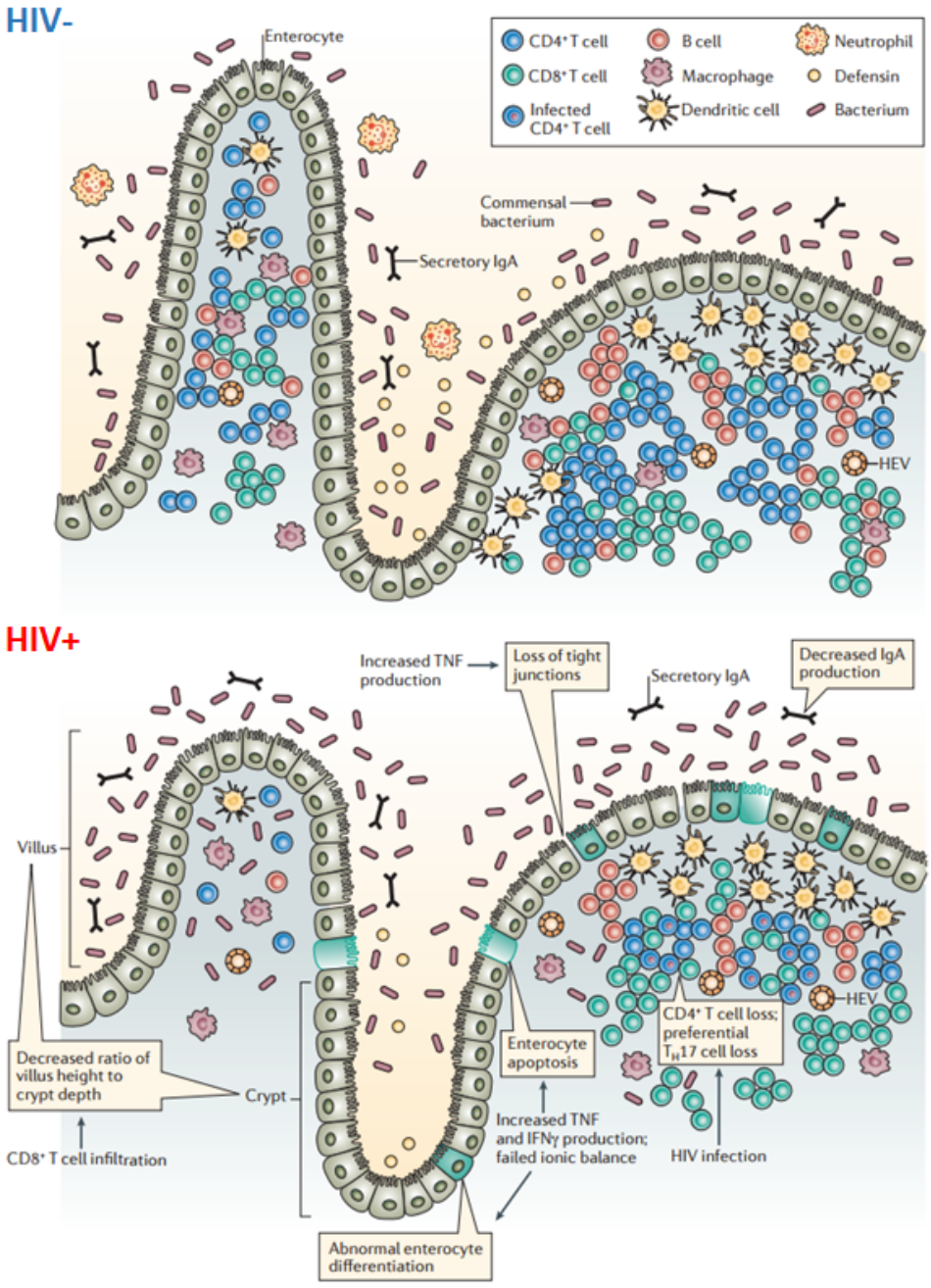

Figure 1.11: Loss of GALT integrity during HIV infection. (**top**) The intestinal epithelium of a healthy individual depicting continuous enterocyte lining that prevents the translocation of commensal bacteria living in the intestinal lumen. Neutrophils (recruited by Th17 cells), defensins and secretory IgA regulate the growth of commensal bacteria. (**bottom**) The intestinal epithelium of an HIV-1 infected individual is discontinuous due to the loss of tight junctions; a reduction in villus height is also typical due to abnormal enterocyte differentiation and apoptosis. HIV-1 infection drives the loss of CD4 T-cells, particularly the Th17 T-cell subset. Lower IgA levels result from B-cell dysfunctions and together with the loss of TH17 cells, permit bacterial overgrowth and translocation across the intestinal barrier. (Adapted from[243])

1.4 HIV-2 Infection – A Model of Functional Cure for HIV-1?

Decades of research have helped to identify correlates of protective HIV immunity that have shown reliable association with disease management and long-term non-progression. Experimental evidence for genetic, immunological and viral factors have been defined in the literature and key examples include the acquisition of strong *Gag*-specific cytotoxic CD4 and CD8 T-cell responses,[269-271] and NK-cell derived IFNγ responses.[272-273] Genetic polymorphisms associated with HIV controllers include the possession of specific HLA-B alleles (eg. HLA-B57, HLA-B27),[274-275] CCR5-δ32 alleles,[276-277] and the co-expression of complementary KIR and HLA-B alleles (eg. KIR3DL1 and HLA-*Bw4*).[278-279] Collectively, the protective features associated with HIV-1 control result in the stifling of viral replication during both acute and chronic phases of infection, which otherwise initiates and sustains damage to the host immune system via mechanisms initiated by immune activation.

This key interest in distinguishing long term non-progressors (LTNPs) from progressors stems from a widely held hypothesis that LTNPs have acquired natural immunity against HIV and that HIV vaccines should ideally replicate the immune responses found in LTNPs. Unfortunately, LTNPS of HIV-1 infection are extremely rare (<2% in most cohorts) and it is in this regard that the paradigm of HIV-2 infection distinguishes itself.[280] HIV-2 infection presents a contrasting scenario where most infected individuals qualify as LTNPS, achieve a normal lifespan, and only a small percentage (15-20%) progress to AIDS.[78,281-282] In Caio, we observed that 37% of HIV-2 donors qualify as elite controllers, with viral loads of < 100 copies/ml maintained over ten years.[281] This large pool of LTNPs in HIV-2 cohorts, and the parallels observed in HIV-1 and HIV-2

immunology poises the latter as an informative, yet relatively underutilised, resource for understanding HIV-1 disease management.

Although HIV-2 is believed to have entered the human population through a different species of SIV-infected monkeys - the sooty mangabey rather than the chimpanzee – both HIV-1 and HIV-2 share 30-60% homology in both sequence and structure.[283] In addition, both viruses infect CD4 T-cells via the same range of coreceptors and a comparable deficit in T-lymphocyte function has been observed in individuals with HIV-1 and HIV-2 infection, when patients with similar CD4 T-cell counts are compared.[284-286] For example, lymphocytes from progressors of HIV-1 and HIV-2 infection display a similar burden of heightened immune activation – in terms of CD38 and Ki67 expression – and exhibit proliferative defects when stimulated with antigens or anti-CD3 antibodies. *In vitro* studies have further established that HIV-1 and HIV-2 display comparable cytopathic potential and replication kinetics in culture.[283] Clinically, HIV-2 patients who develop AIDS progress in a manner and time frame that is indistinguishable from HIV-1 non-controllers.[87,287] It is, therefore, surprising that HIV-2 is controlled by the majority of the infected population despite having demonstrated a strong potential to kill; we believe that understanding how disease progression is evaded in HIV-2 may yield lessons that are applicable to the control of HIV-1.

The relevance of HIV-2 infection - in the context of protective immunity against HIV-1 - is further underscored by data which suggest that preceding HIV-2 infection can limit the rate of disease progression and viral evolution following HIV-1 super-infection.[288-289] In a cohort of 223 participants, a 36-month difference was observed in the time required for dual infected and single HIV-1 infected individuals to progress to AIDS. These findings provide further evidence that overlapping immune responses are triggered during both

HIV-1 and HIV-2 infection, and supports the possibility that HIV-2 infection can prime the immune system to recognize and cope with subsequent HIV-1 exposure.

Currently, studies examining T-cell responses in HIV-2 infection have mainly provided proof of concept to the conclusions derived from studying LTNPs in HIV-1 infection. Our group and others have shown that both CD4 and CD8 T-cells from LTNPs of both HIV-1 and HIV-2 infection mount significantly greater Gag-specific responses and T-cell polyfunctionality than progressors.[281, 290] It is necessary to further our exploration of HIV-2 immunity to obtain perspectives which not only complement, but also supplement the current literature that is applicable to HIV-1 disease management. What remains particularly intriguing is how older people (55-80 years of age) with HIV-2 infection have a similar risk of mortality to uninfected controls, whereas age at infection is one of the strongest risk factors for HIV-disease progression.[282,291] This finding has prompted us to examine whether HIV-2 infected individuals are less prone to the accelerated immunosenescence that accompanies HIV-1 infection in this work.

1.5 The T-cell Response to HIV-2 Infection

1.5.1 Virus Specific CD8 T-cell Responses: HIV-1 versus HIV-2

The first description of HIV-2 specific T-cell responses was made in 1993, in a Gambian cohort of HIV-2 controllers where responses were targeted against an identifiable Gag epitope (1A; TPYDINQML).[292] In this study, HIV-2 Gag-specific responses were detected in over 55% of study participants, much higher than the proportion of Gag-respondents (~25%) that were reported in their previous HIV-1 study.[293] The high frequency of HIV-2 specific CD8 T-cells led the authors to conclude that an increased frequency of HIV-2 specific responses could be responsible for explaining the less pathologic consequences of HIV-2 infections. However, studies in the past two decades have failed to replicate the observation of extremely high frequencies of virus-specific CD8 T-cells in HIV-2 controllers;[294-296] and the overall data are more compatible with the preservation of qualitative rather than quantitative features of CD8 T-cell immunity in HIV-2 control. Unlike the case of HIV-1 infection, HIV-2 controllers demonstrate better T-cell polyfunctionality,[270,297] promiscuity of TCR usage,[298-299] and tendency towards early differentiated phenotypes.[290,299] Within the HIV-2 proteome, Gag appears to be the most immunodominant target of the CD8 T-cell response.[281,296] Accordingly, the bulk of known immunogenic HIV-2 epitopes that are recognised by CD8 T-cells are found within Gag (Table 1.1).[300]

In addition to the direct killing of infected CD4 T-cells by cytotoxic means, soluble factors released from CD8 T-cells could also be involved in the restriction of HIV-2 replication.[301-303] Among the cytokines and chemokines of the CD8 T-cell arsenal, the production of high levels of β-chemokines is consistently reported as a correlate of the contact-independent control of HIV replication; chemokines associated with HIV-2

protection include MIP-1α, MIP-1β and RANTES, which prevent cellular infection by M-tropic virus through direct binding to CCR5 and disrupting membranal interactions between virus and host.[301,303] The relevance of CD8 T-cell-derived soluble factors in limiting HIV-2 replication is reflected in the data from *in vitro* primate studies, which demonstrated that exposure to the supernatant of cultured CD8 T-cells from HIV-2 infected baboons was sufficient to suppress HIV-2 replication in autologous CD4 T-cells.[304] Overall, β-chemokine production in CD8 T-cells is relatively higher in HIV-2 controllers than uninfected subjects and the level of production correlates well with CD4 T-cell count.[301]

Table 1.1: List of known HIV-2 epitopes targeted by HIV-2 specific CD8 T-cells. The position of the indicated epitopes are marked relative to the HXB2 reference genome. Publications related to the listed epitopes can be researched through their Pubmed ID.

Protein	Position	Sequence	HLA restriction	Reference (PubMed ID)
gag	24–32	GGKKKYKMK	B*35	1721107, 10203028
gag	71–80	EIINEEAAEW	A*25	10203028, 8760412
gag	77–85	SLFNTVCIW	A*02	19016530
gag	77–85	SLFNTVCVI		12817012
gag	130–138	PPSGKGGNY	B*35	7584954
gag	173–188	QALSEGCTPYDINQML		17823657
gag	179–190	CTPYDINQMLNC	B*58	Bertoletti, personal communication 1998 via LANL
gag	180–188	TPYDINQML	B*53(01); B*58(01)	19016530, 15832290, 18562522, 7690804, 23558015, 18622680
gag	234–251	SDIAGTTSTVDEQIQWMY		17823657
gag	240–249	TSTVDEQIQW	B*58(01)	23558015
gag	240–249	TSTVEEQIQW	B*57; B*58(01)	12817012, 9499105, 8959245, 15832290, 18622680
gag	249–266	MYRQQNPVPVGNIYRRWI		17823657
gag	254–262	NPVPVGNIY	B*35(01)	7584954, 19016530, 23558015
gag	257–274	PVGNIYRRWIQIGLQKCV		17823657
gag	295–304	SYVDRFYKSL	A*24	18562522
gag	296–313	YVDRFYKSLRAEQTDPAV		17823657
gag	298–306	DRFYKSLRA	B*14	23558015
gag	304–321	LRAEQTDPAVKNWMTQTL		17823657
gag	308–317	QTDPAVKNWM	B*53	18562522
pol	329–337	NPDVILIQY	B*35	7584954
gp160	21–34	LNSWGCAFRQVCHT		21849066
gp160	376–383	TNCRGEFL	Cw*04	7677956, 10203028
gp160	584–592	EKYLQDQAR	B*14	1372650, 10203028
gp160	586–593	YLQDQARL	B*08	1372650, 10203028
nef	75–82	VPLRPMTY	B*35	7584954

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1.5.2 Virus Specific CD4 T-cell Responses: HIV-1 versus HIV-2

HIV-1 specific CD4 T-lymphocytes are preferentially targeted by the virus and depleted from the host repertoire. Conversely, in the majority of HIV-2 infected individuals, the CD4 T-lymphocyte compartment remains preferentially targeted but quantitatively preserved.[137,305] Accordingly, qualitative improvements in their performance are likely to have an exponential impact on disease control.

The virus specific CD4 T-cell response appears to be more robust in HIV-2 infection than in HIV-1 infection. Prospective studies of HIV-2 infection show that the correlates of CD4 T-cell immunity towards HIV-2 are more predictive of disease outcome than CD8 T-cell functions.[296,305-306] The proportion of patients with detectable CD4 T-cell responses against Gag is also higher in HIV-2 donors than in HIV-1 donors, when subjects with equivalent CD4 T-cell counts are compared. HIV-2 specific CD4 T-cells further demonstrate higher proliferative capacity than their HIV-1 specific counterparts, and exhibit greater degrees of polyfunctionality in terms of IFN-γ, TNF-α, MIP-1β, IL-2 and CD107a coexpression.[297]

A characteristic feature of asymptomatic, non-progressive HIV-2 infection is the maintenance of robust CD4 T-lymphocyte help.[296] Furthermore, macaques are less likely to develop early signs of Th dysfunction when infected with HIV-2 than the more pathogenic SIVmac.[307] In summary, the preservation and persistence of both qualitative and quantitative features of CD4 T-lymphocyte function appear to contribute to HIV-2 immunity and may account for much of the sustained viraemic control during the chronic phase of infection.

1.5.3 T-cell responses in HIV-2: Controllers versus Progressors

In HIV-2 infection, an inverse relationship has been demonstrated between CTL responses and proviral load, with stronger cytotoxic T-lymphocyte activity detected in donors with lower viral load.[308-309] Similar to the case for CD4 T-lymphocytes, cytotoxic and IFN-γ responses were more likely to be triggered by HIV-2 Gag, and patients with undetectable viraemia were more competent in mounting virus-specific cytokine responses.[281,290] More specifically, CTLs from non-progressors targeted Gag more frequently, while equivalent responses were absent in the majority of HIV-2 progressors. Ultimately, the establishment of Gag-specific CD8 polyfunctionality reflected a strong inverse relationship to HIV-2 viraemia in infected subjects.

With respect to CD4 T-cells, Type 1 Th (Th1) responses were observed to be more prolific in donors with undetectable HIV-2 RNA than progressors.[310] Furthermore, the magnitude of the CD4 IFNγ response correlated well with proviral load.[306] Both data suggest that the exertion of control by virus-specific CD4 T-cells are important for restricting viral replication and integration in asymptomatic HIV-2 infection.

The observed differences in T-cell functionality between HIV-2 progressors and controllers lead to further questions that have remained relatively unanswered: what accounts for the more efficient T-cell responses that occur in HIV-2 controllers than in progressors? And furthermore, why are T-cell responses more effective against HIV-2 than HIV-1? How are high levels of anti-viral T-cells maintained in controllers who have undetectable plasma viral load for prolonged periods? The conditions for effective HIV-2 control are likely to been seeded during acute HIV-2 infection – which we have little information on – but continue to exert an enduring influence over the control of HIV-2

replication throughout the chronic phase of infection. Studies have also alluded to greater opportunities for the innate control of HIV-2 replication during primary infection, as HIV-2 has shown greater susceptibility to several host restriction factors.[311-312] In this perspective, specific polymorphisms in the genetic loci that code for the relevant host restriction factors (i.e. TRIM5α; APOBEC3f/3G activity) may be more prevalent in the general population. Ultimately, the prompt and sustained clearance of virus would result in lower immune activation throughout the chronic phase of HIV-2 infection.

1.6 Immune Activation in HIV-2 Infection

1.6.1 Lower Levels of Immune Activation in HIV-2 Infection

The rates of clinical progression and CD4 T-cell decline during HIV-2 infection are significantly slower than observed in HIV-1 infection, and this has been attributed to a low or undetectable viral set point.[82,283] Despite better outcomes, elevated plasma viral load in HIV-2 infection is still predictive of disease progression.[313] However, viral load measurements are not particularly informative since the majority of infected individuals control viral replication to undetectable levels. Instead, the level of immune activation – which has been shown to be independently predictive of disease outcome in HIV-2 infection – may provide a more accessible and useful indicator of prognosis.[314] The concentration of β2-microglobulin (β2m) in the plasma of infected individuals, for example, has been shown to be predictive of survival and associate with slower CD4 T-cell decline and disease severity.[284,315]

As in HIV-1 infection, the degree of immune activation in HIV-2 infection is elevated relative to uninfected subjects, albeit to lower levels than described for HIV-1 infection. HIV-2 infected individuals present with increased concentrations of soluble markers of

immune activation, such as β2m and sCD14, and higher frequencies of activated T-cells.[285,315-316] When infected subjects are matched by disease stage, HIV-2 infected individuals manifest with similar degrees of qualitative and quantitative change in T-cell activation as HIV-1 progressors – suggesting that immune activation could be an equally important driver of disease progression in HIV-2 infection.[284-286] This argument is further supported by the correlation between immune activation and plasma viral load in viraemic HIV-2 infected donors.[284-285] Despite the relationship between immune activation and disease progression in symptomatic HIV-2 infection, the general outlook of HIV-2 infection – compatible with the milder disease course – is less apparent immune activation as compared to HIV-1.[317-318] Since immunosenescence – largely driven by immune activation – is a key driver of HIV pathogenesis, we propose that both the virus-specific and total T-cell phenotype of HIV-2 controllers are less likely to reflect an image of advanced cellular senescence.

1.6.2 Reasons for Lower Immune Activation in HIV-2 Infection

Due to declining rates of new HIV-2 infections and a lack of information on HIV-2 seroconversion dates, there is a complete void of information on acute HIV-2 infection. Nevertheless, studies of chronic infection have yielded insight on the possible mechanisms that may account for the overall lower immune activation levels in HIV-2 infection and the core findings are described in this section.

1) *Innate Immunity:* The restriction of SAMHD1 by Vpx in HIV-2 infection permits greater infectivity of myeloid cells in HIV-1 infection.[132,319-320] While the outcome of improved HIV-2 immunity may seem paradoxical, the increased penetrance of HIV-2 in innate immune cells may cater for additional antigen presentation routes

that promote the stimulation of more efficient innate immune responses or downstream adaptive T-cell responses. Nevertheless, Duvall *et al.* showed a lack of permissibility of blood-derived mDCs and pDCs to HIV-2 infection in culture, which suggests that the infectivity of monocytes and macrophages may be sufficient in restricting viral replication or that alternative protective mechanisms may have a more significant contribution.[137] Next, the increased susceptibility of the HIV-2 capsid to TRIM5α recognition could potentially lead to more efficient proteosomal processing of HIV-2 proteins, and thus, promote antigen presentation.[311] While TRIM5α restriction is less potent in HIV-1 infection– the molecule efficiently mediates the destabilisation of capsid structure during HIV-2 infection and promotes the occurrence of abortive reverse transcription events and innate immune responses.[321-324] In summary, unlike the picture in HIV-1 infection, the enhanced innate mechanisms could result in the timely clearance of HIV-2 virions during primary HIV-2 infection, thereby preventing the enormous release of antigen load that could otherwise evolve from GALT compromise. Indeed the extent of microbial translocation – measured by LPS quantification – was observed to be lower in asymptomatic HIV-2 subjects than viraemic subjects, and related to severity of disease in the latter.[325]

2) *Adaptive Immunity:* Unlike the case in HIV-1 infection, HIV-2 Nef interacts with CD3-TCR complexes and results in both the downregulation of the latter and the inhibition of apoptosis.[326] Regardless of disease progression status, the extent to which Nef could downregulate CD3-TCR complexes correlated with the level of immune activation in HIV-2 infected subjects but did not relate to disease outcome.[327] However, in viraemic individuals, the potency of HIV-2 Nef-mediated downregulation of CD3-TCR and CD28 related to higher CD4 T-cell

counts and lower expression of exhaustion markers – including PD-1, CTLA4, Fas, and FasL.[328] Indeed, the expression levels of PD-1 and its ligand on T-cells – associated with higher levels of immune activation and CD4 T-cell decline in HIV-2 infected progressors.[329] Therefore, HIV-2 Nef may have an important contribution in controlling HIV-2 viremia by suppressing T-cell exhaustion and death. Furthermore, by relieving T-cells of the high expression of inhibitory molecules such as PD1 and CTLA4, HIV-2 Nef may allow for more effective cytotoxic killing of infected cells by CD8 T-cells. Other viral factors that may contribute to reduced T-cell activation in HIV-2 include the envelope protein gp105, which supresses T-cell activation via a monocyte-dependent mechanism.[330]

Overall, the generalised state of lower immune activation in HIV-2 infection probably results from a combination of all the factors listed above, which participate in effective viraemic control, and delay or prevent the early onset of lymphocyte exhaustion and senescence that so often accompanies chronic HIV-1 infection. The rapid clearance of HIV-2 also results in reduced levels of host- and viral-derived immune signals, altogether minimising the possibility of both bystander and virus-specific activation.

1.7 Immunosenescence in HIV Infection

1.7.1 Ageing, Senescence and Immune Senescence

The intersection between ageing and HIV/AIDS is a rapidly growing field. While improvements in the use of combinatorial ART have been instrumental in prolonging the lifespan of people with HIV, the treatment regime has not quite bridged the gap in mortality between HIV-1 infected and uninfected individuals – even in countries that access excellent heathcare.[331-334] Moreover, illnesses frequently associated with the elderly tend to manifest prematurely in HIV patients.[335] Some of the age-related morbidity are aggravated by the treatment itself, but the premature setting of an ageing cellular phenotype is worrying and consistently manifests itself even in untreated HIV infection.[335-336] Consequently, considerable research has gone into unravelling the mechanisms involved in the presentation of non-AIDS, but age-related morbidity – now widely referred to as non-AIDS defining illness or NADIS – with the aim of designing appropriate therapeutic interventions that will further improve the health of the millions living with HIV.

Ageing is the progressive, time-dependent loss of tissue and organ function.[337] The antagonistic pleiotropy theory has been useful in our understanding of the ageing process and proposes that natural selection favours genetic programmes that bolster reproductive fitness early in life but offers less insurance against health problems beyond our reproductive period.[338] The regulators of cellular senescence represent a set of genes that apply to this theory: molecules that are part of a potent anticancer program which removes neoplastic, promiscuous, or hyperactive cells from tissues by intrinsic or extrinsic mechanisms – but play a role in age related pathologies.[339-343] For example, the mechanisms involved in cell cycle arrest and apoptosis in progenitor cells; and their

unidirectional, irreversible maturation programmes have been fine-tuned at the expense of tissue-repair capacity. The latter could act as an obstacle to recovery from sustained tissue damage, particularly when the latter is a result of modern infections or increased life expectancy, where selection pressure for enhanced survival may have only just begun to act. The problem of cellular senescence is twofold because it has become apparent that our organ systems have evolved the capacity to cope with senescent cells to a limited extent, and beyond a certain threshold, senescent phenotypes predominate due to ineffective clearance – resulting in a gradual ageing program that can be accelerated under extranormal circumstances.[263,344-346]

The immune system is a misnomer in this case as it is far from invulnerable to the ageing process and is recipient to a dynamic plethora of circumstances relating to new and existing infections. Immune exhaustion occurs at three different levels – (i) functional, (ii) clonal and (iii) systemic and HIV infection has demonstrated the ability to interfere with each of these stages.[347-352] Firstly, aged immune cells display functional deficits associated with the limited proliferative capacity or the expression of molecular patterns that down-modulate its activity.[350] Secondly, antigen-specific B- and T-cell clones are driven towards a state of replicative senescence upon persistent activation, resulting in the gradual loss of specific T-cell populations that control recurrent or ongoing infections.[349-352] Thirdly, systemic changes in the composition of immune cells occur during ageing, specifically, age is associated with the loss of lymphocyte renewal capacity – either through reduced lymphopoiesis, thymic involution, increased populations of Treg cells in the periphery and lymph nodes, or a combination of these events;[191,353-357] Treg cells have been observed to become more functionally potent with age.[358-360] As immune activation is a key driver of immunosenescence, all three processes are brought to premature fruition during chronic infection; the effects of persistent immune activation become particularly

evident when the infection persists for decades – as in the case of non-progressive or treated HIV infection.

Functional defects mainly result from the accumulation of unrepaired or uncorrected mutations or alterations in macromolecules that eventually lead to aberrant cellular responses and functions.[361-364] Several molecular mechanisms are involved in these changes, including DNA damage, oxidative stress, reduced macromolecular biosynthesis, degradation and turnover.[340,365] Key among these are the contributions of telomere attrition and mitochondrial dysfunction. The former has been causally linked to apoptosis, growth arrest and tumorigenesis that result from chromosomal instability and the latter is directly involved in determining cellular life and death.[366-369] The most common signalling pathway utilised by each of these stressors is the activation of the DNA damage response mechanism - where ATM or ATR kinases block cell-cycle progression through stabilisation of p53 and transcriptional activation of p21, the cyclin-dependent kinase (Cdk) inhibitor.[370-372] Primary damage to cellular macromolecules can accumulate when repair mechanisms fail to keep up with the rate of damage, or when the rate of damage is accelerated.[361]

Clonal and systemic changes that are characteristic of immune senescence tend to be perpetuated by homeostatic responses which are initiated to compensate primary changes in cell production, renewal and death. These changes are particularly pronounced in lymphocyte populations where thymic involution and the consequently marked reduction in T-cell production trigger peripheral expansion via cytokine-dependent mechanisms involving IL-7, IL-2 and IL-15.[266,268,373-375] A secondary impetus is related to repeated stimulation of lymphocytes with antigens, resulting in multiple cycles of proliferation and apoptotic triggers, as well as advancement towards more differentiated phenotypes.[349-352]

Unlike pathogens that vanish after acute infection, antigens from viruses that establish latent persistent infections – such as CMV, EBV and HIV – establish an evolving equilibrium that eventually collapses from repeated stimulation of the lymphocyte pool, ultimately resulting in the inflation of memory T-cells numbers (Figure 1.12).

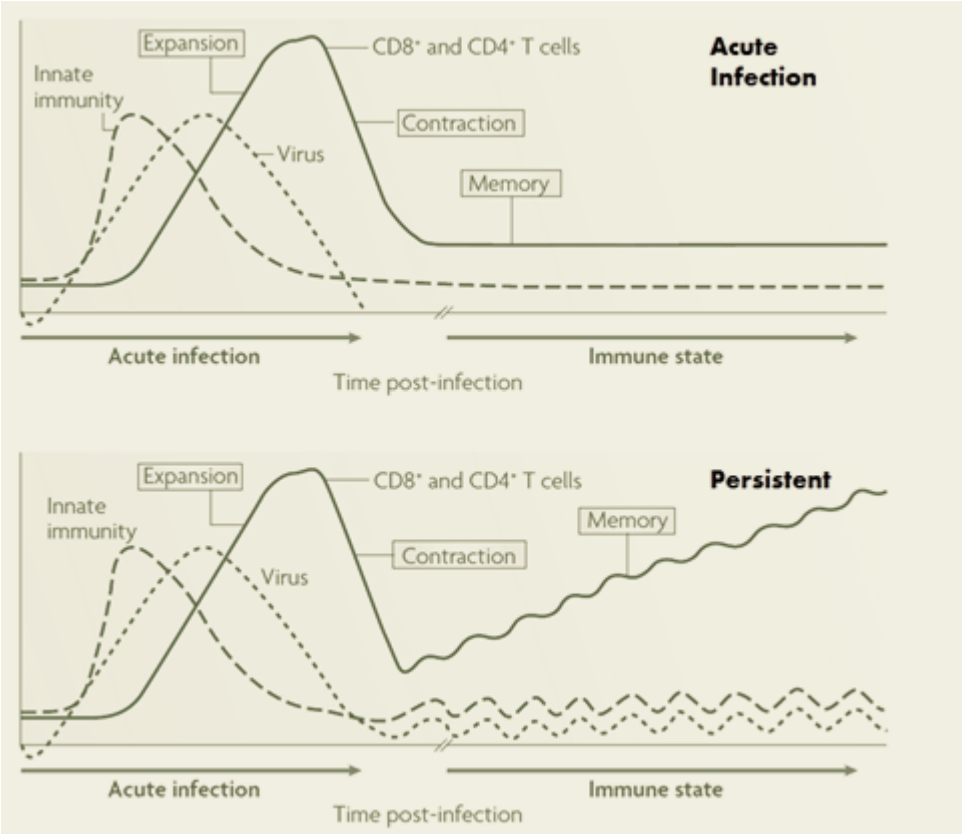

Figure 1.12: Memory inflation during persistent and chronic infection. The immune response to virus that establish acute (**top**) or persistent (**bottom**) infection. In both cases, the kinetics and natural history of viral replication are similar during acute infection, but latent infections result in repeated reactivation cycles through antigen stimulation, this results in the proliferation of long-lived memory T-cells that eventually accumulate. (Adapted from[377])

Thymic involution, which refers to the progressive shrinkage of the thymus with age and the consequent reduction of naïve T-cell output from the thymus, contributes to systemic changes in the composition of T-cells with age and is apparent in humans from as early as 1 year of age.[376-377] The mechanisms involved remain poorly understood, partly because thymocytes and thymic stromal cells are closely linked, and their interdependence makes it difficult to dissect their individual contribution in maintaining thymic integrity.[377-379] A few external processes have been observed to contribute to this process – including reduced thymic input from progenitor cells (HPCs) and defects in the commitment of HPCs to the lymphoid lineage.[380-384] Structural loss, on the other hand, result from impairment to the thymic stromal components that are regulated by soluble factors such as sex hormones.[385] Since the rate of production of new thymic immigrants (RTEs) is highly related to its overall cellularity – even late in senescence – the progressive decline in naïve T-cell production applies homeostatic pressure for peripheral maintenance of the naïve T-cell pool through much of adulthood.[386-387]

Through ageing, or damage from viral infections, the reduced thymic output eventually results in lower TCR diversity. In elderly subjects, the few RTEs that reach the periphery demonstrate diminished survival capacity, resulting in a smaller naïve T-cell compartment.[386] This precious pool of naïve T-cell pool is further compromised in the periphery by premature death, or advanced recruitment into the memory T-cell pool.[377] As there is overlap in the use of survival and maintenance cytokines by both naïve and memory T-cell pools,[387-391] the latter actively compete for the same survival resources and invariably fare better due to their larger representation. The resulting lymphopaenia triggers an excessive production of homeostatic cytokines, ultimately driving the clonal expansion of a pool of naïve T-cells with limited TCR diversity [266,392] Finally, the rapid proliferation of naïve T-cells activates a protocol of systemic memory differentiation

stimuli, which is supported by the compensatory cytokine milieu (Figure 1.13).[393] Altogether, the processes of reduced naïve T-cell production, increased memory turnover and accumulation of memory cells from lifelong encounters with pathogens synergise to limit TCR diversity in old age. The same observations are manifested in HIV infection, where HIV infection accelerates the loss of naïve T-cells and decrease thymic output.[191,263,] During HIV infection, the chronic activation of virus-specific and bystander cells further create the necessary conditions for premature memory inflation and loss of TCR diversity. The clinical consequence of memory inflation and poor TCR diversity is an increased susceptibility to new or opportunistic pathogens and a reduced efficacy of vaccination programmes in insuring against infection – observations that are highly prevalent in the elderly.[394-396] The association between increased TCR diversity and usage with better disease outcome in HIV infection argues in favour of the value of TCR diversity in HIV infection.[298-299] Moreover, poor vaccine responses have been observed in both adult and perinatal HIV-1 infection, and this is suggestive of the severity of the loss of TCR diversity with chronic HIV infection.[397-398]

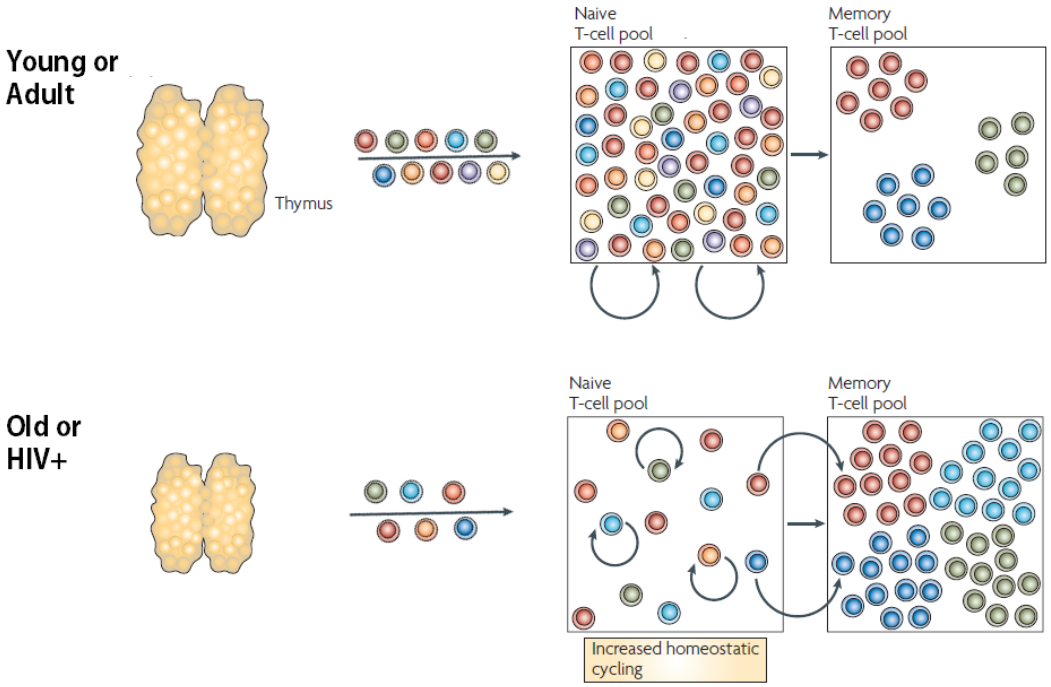

Figure 1.13: Systemic changes in T-cell Senescence during HIV infection. (**top**) T-cell homeostasis in the young or in middle-aged adults. (**bottom**) In the old or HIV positive group, thymic involution or loss of thymic cellularity result in the depletion of RTEs or naïve T-cells. The resulting lymphopenia signals intense homeostatic cycling through IL-7 and to a lesser extent, IL-2 and IL-15. The increased proliferation of a small pool of naïve T-cells and their subsequent differentiation lead to memory inflation, resulting in large pool of memory T-cells with limited TCR diversity. (Adapted from[377])

1.7.2 Telomere Length and Replicative Senescence

In eukaryotic cells, the preservation of linear chromosomes is hindered by two chromosomal end-related problems:

(i) Because the ends of linear eukaryotic chromosomes cannot be distinguished from broken DNA ends that require repair, chromosomal ends are prone to trigger DNA end-joining and recombinational events, which compromise their genetic integrity.

(ii) As DNA polymerase facilitates nucleotide addition only in the 5' to 3' direction when DNA replicates, the enzyme is unable to anneal the gap which remains after the departure of the 5' most RNA primer.[368, 399]

These problems, if left unchecked, could result in the progressive shortening of linear chromosomal ends, but this is prevented by the enzyme telomerase via its telomerase-containing RNA component and reverse transcriptase activity.[368] By providing docking sites for telomeric proteins, telomerase recruits adaptors to chromosomal ends, which helps to establish cellular distinction between chromosomal ends and broken DNA.[400-401] In addition, telomerase synthesises multiple tandem repeats of TTAGG at the 5' primer end,[402-403] which compensate for the gradual loss of genetic information during DNA replication (Fig 1.14).

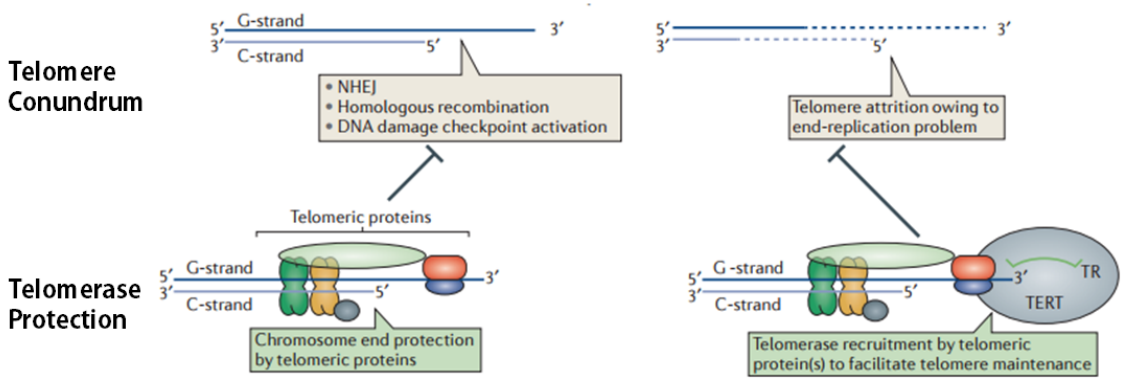

Figure 1.14: Chromosomal end protection via telomerase action. (**left panel**) Unprotected, protein-free DNA may be a target for deleterious DNA end-joining and recombination events. By binding the ends of unprotected DNA, telomeric proteins protect against these events. (**right**) DNA sequences are also lost as a result of incomplete replication by DNA polymerase. Telomerase is recruited by various telomeric proteins, and the resulting complex enzymatically extends chromosomal ends (Adapted from[368])

Although telomerase is highly active in the germ line and stem cells, its activity is drastically diminished in somatic cells. Hence, the telomeric ends of chromosomes in somatic cells have been found to progressively shorten with each cellular division, a phenomenon that becomes dramatically apparent during ageing.[404] The telomeric shortening of linear chromosomal ends is also often accompanied by loss of replicative capacity, a condition described as replicative senescence.[368]

While commonly associated with ageing, replicative senescence can be prematurely accelerated by genetic, immunologic, viral and environmental factors that can further exert their effects on specific cellular subsets. For example, chronic antigenic stimulation by antigen or inflammatory cytokines stimulates the intense proliferation of antigen-specific lymphocytes, to a point where they become markedly less able to proliferate but retain their ability for cytokine secretion.[405] While T- and B- lymphocytes transiently up-regulate telomerase to preserve chromosomal integrity and replicative capacity during

immune activation, this function is lost after repeated encounters with antigen.[349-350] This process results in the accumulation of lymphocytes with critically short telomeres and proliferative defects. The latter are particularly pronounced during chronic HIV-1 infection which leads to persistent activation of lymphocytes. While there is a lack of consensus on a definitive phenotype for senescent T-cells, the respective loss and acquisition of CD28 and CD57 expression have consistently identified senescent T-lymphocytes with a long replicative history, and which reveal shortened telomeres and proliferative impairment.[406]

1.7.3 Mitochondria and the Regulation of T-cell Responses

The free radical theory of ageing is nearing its 60th anniversary and represents one of the most popular and enduring theories of ageing.[407-408] The latter proposes a central role of oxidative stress in driving cellular senescence. At the seat of this theory is the mitochondrion, which is the principle source of intracellular reactive oxygen species (ROS). ROS are toxic byproducts of the aerobic respiration apparatus and result in oxidative damage to important cellular macromolecules – that regulate cell cycling, replicative fitness and apoptosis – when they accumulate. The theory is based on several key observations that emerge with age: (i) enhanced ROS production and decreased mitochondrial function, (ii) accumulation of mitochondrial DNA (mtDNA) mutations, (iii) decline in the activity of ROS-scavenging enzymes with age and (iv) accumulated oxidative damage to cellular proteins, lipids and DNA.[408-411] Since cellular life and death directly hinges on the ability of the mitochondria to supply its energy requirements, mitochondrial dysfunction can have an immediate impact on cellular longevity. In addition, mitochondria participate in signalling events that trigger physiological changes which are important for cellular differentiation, and loss of mitochondrial integrity during

ageing is expected to translate into aberrant cellular behaviour – which on a large scale, leads to loss of tissue function.[412] In this section, we discuss the involvement of mitochondria in initiating and sustaining important immune functions, and provide clues to how immune disorders can result from loss of mitochondrial function through age or immune activation.

The mitochondria are key regulators of innate and adaptive immunity.[413] The induction and maintenance of immune functions are energy-intensive and homeostatic adjustments in mitochondrial behaviour are required to meet the ATP demands of activated cells. The antigen-specific signals that activate T-cell responses and their differentiation into memory cells also trigger mitochondrial biogenesis, highlighting the essential coupling of immune and mitochondrial activity in the execution of immune activity.[414-416] Upon TCR stimulation, naïve T-cells rapidly transition from a quiescent catabolic state, which focus on nutrient assimilation for ATP generation, to a robust anabolic state where nutrients are utilised to generate macromolecules that are required for proliferation.[415] The latter process is sustained by a large increase in glucose consumption that is dependent on ROS signalling.[417]

In terms of T-cell differentiation, mitochondria not only drive the establishment of immune cell phenotype, but energetically support the maintenance of cellular phenotype and their specific functions.[414,418] In addition to supplying energy, mitochondria also participate in signalling pathways that alter cellular phenotype during differentiation. While a traditional view of CD4 T-cell differentiation into specific subsets – such as Th1, Th17 and Th22 cells – have focused on the upregulation of specific transcription factors for driving phenotype (i.e. T-bet and GATA3 for Th1 and Th2 respectively), emerging data indicate that the differentiation process also requires specific metabolic profiles that

are regulated through the mitochondrion.[413] For example, Treg development requires a cellular environment of increased oxidative phosphorylation and decreased glycolytic flux, as compared to Th17 cells.[418]

Finally, at the systemic level, the mitochondrion is involved in maintaining the relative proportions of specific T-cell subsets in circulation. First, the thymic education of T-cells relies on the initiation of mitochondrial apoptosis to execute clonal deletion of autoreactive T-cells.[419-420] Secondly, when an infection is resolved, the mitochondria play a central role in establishing CD8 T-cell memory clearance through apoptosis, where a few long-lived CD8 memory T-cells are preferentially selected for survival. In this phase of infection, effector T-cells (Teff) are deleted while central memory cells (Tcm) are retained. While the molecular mechanisms remain unclear, specific metabolic pathways are initiated in each of the two cell types to favour proliferation and subsequent apoptosis in the former and prolonged survival in the latter.[414,421-422] One clue into how the latter is regulated further hints at the interdependence between immune function and mitochondrial metabolism, and refers to the upregulation of CD36 on Tcm cells; the increased expression of CD36 is essential for fatty-acid uptake, which promotes *de novo* lipogenesis and lysosomal storage of lipids by the mitochondrion.[422] Since fatty-acid oxidation generates much more energy than glucose oxidation, the increased lipid uptake of Tcm cells, relative to Teff cells, is compatible with the catabolic requirements of Tm cells for prolonged survival.

Although the signalling pathways involved in the selection of metabolic profiles require much study and clarification, enough evidence exists to show that the mitochondria regulate T-cell homeostasis at the functional, clonal and systemic level. The key role of the mitochondrion in managing the many facets of T-cell behaviour suggest that deficits

in mitochondrial function through excessive oxidative damage that accumulate with age or chronic immune activation may have severe consequences on immune function, and the patterns of T-cell senescence observed during ageing or chronic viral infections can, in part, be explained by loss of mitochondrial integrity.

1.7.4 HIV-1 Infection: Mitochondrial Dysregulation and Oxidative Injury

The long-term residence of HIV and other chronic infections can result in excess oxidative damage to cells or tissues, either through direct molecular interference or through immune activation as a secondary stressor. Oxidative stress occurs when the production of unwanted ROS and nitrogen molecules outcompetes the levels of antioxidant protection, and the current evidence suggest that HIV infection can contribute to excessive oxidative stress.

While much of the focus on mitochondrial impairment during HIV-1 infection has focused on the impact of nucleoside reverse transcriptase inhibitors (NRTIs), recent evidence substantiates claims that HIV-1 can directly interfere with mitochondrial biology.[337] HIV imposes oxidative injury as a vital mechanism to improve its replicability.[423-425] Clinically, infection with HIV-1 decreases intracellular and systemic glutathione (GSH) levels.[426-427] The latter protects cellular and tissue components from oxidative damage and is also required by CD8 and NK T-cells for viral clearance. While a reduction in antioxidant defense is a converse pathway of inflicting oxidative stress through excessive oxidative input, it yields the same functional results.[428-429]

HIV-1 is able to exert oxidative stress directly with its molecular machinery. HIV-1 gene products such as Tat decrease cellular GSH and mitochondrial SOD2 levels.[430] Loss of SOD2 in mitochondria contributes to increased mitochondrial superoxide production. In animal studies, the tissue-specific transgenic targeting of HIV Tat expression results in mitochondrial damage and mtDNA depletion in the heart, ultimately increasing the incidence of heart failure.[431] Molecularly, mtDNA loss reduces the supply of macromolecules for the proper functioning of the electron transport chain, which results in additional electron leak and superoxide production.[337, 432]

Furthermore, HIV-1 modulates mitochondrial-pathways to differentially manipulate apoptosis as a mechanism to improve its survival and replicability. HIV proteins – including Tat, Nef, Vpr and Protease – have demonstrated the ability to trigger apoptosis in infected and bystander cells through the mitochondrial-dependent pathway, thereby interfering with the efficacy and quantity of immune cells that participate in its clearance.[433] HIV-1 infection of memory CD4 T-cells can also alter T-cell homeostasis by conferring apoptosis resistance in infected cells through the respective downregulation and upregulation of procaspase 8 and anti-apoptotic Bcl2. In this manner, HIV-1 is able to upkeep a reservoir of latently infected cells while contributing to part of the memory inflation observed during HIV-1 infection.[434-435]

Finally, in addition to direct molecular interference, excessive immune activation as a result of HIV-1 infection can drive mitochondrial dysfunction. As discussed earlier, the activation and maintenance of T-cell responses is energetically demanding, and mitochondrial activity and replication is enhanced during immune activation to cope with the high metabolic requirements.[414,418,436] Next, activated phagocytotic and cytotoxic cells also upregulate the production and release of ROS as part of their mode of action, further

contributing to the amount of sustained oxidative damage in chronic infection.[437-438] Therefore, it is not surprising that many studies have drawn a parallel between immune activation and oxidative damage in chronic HIV-1 infection.[436,438-441]

1.7.5 Advanced T-cell senescence during HIV-1 infection

Even though combinatorial ART has resulted in the dramatic decline of AIDS-associated morbidity and mortality, the treatment does not fully restore health and life expectancy of treated HIV-1 subjects remain ≥10 years shorter than the uninfected population. [331-334] ART recipients are at a higher risk for age-associated diseases and mortality and this has resulted in a large consensus that HIV-1 is involved in accelerating immune senescence. Several factors have been linked to the excess morbidity of HIV-1 infected subjects, including chronic immune activation and ART-related toxicity.[337,442] In spite of the latter, the data collectively indicate that infection with HIV is, by itself, sufficient to contribute to the age-related health impairment, since both HIV disease and T-cell activation strongly relate to thymic dysfunction, shortened telomeres and reduced T-cell regenerative potential, even in the absence of treatment.[191,443-445] Overall, both treated and untreated HIV-infected individuals exhibit phenotypes which are compatible with premature immune ageing, such as low naïve to memory T-cell ratios, a less diverse T-cell repertoire and reduced responsiveness to vaccination.[191,263,397-398]

The ART-independent effects of HIV-infection on T-cell senescence are believed to be largely driven by excessive immune activation. HIV infection is associated with high levels of inflammation as defined by the levels of soluble factors including IL-1β, IL-6 and TNFα.[438-439,446-447] Since the elevation of inflammatory cytokines is in part alleviated by ART, HIV replication itself is thought to make a significant contribution to the

hyperinflammatory state. However, some molecules remain elevated despite the effective suppression of HIV replication in the presence or absence of therapy, which suggest the involvement of indirect effects of HIV infection – including co-pathogen load, microbial translocation and irreversible fibrosis of the thymus and lymphoid architecture. Indeed, biomarkers that indicate ongoing microbial translocation, such as sCD14 and LPS, remain high throughout chronic infection, even in the presence of ARVs.[254,448]

The sustained high levels of immune activation result in the residence of a disproportionately large number of exhausted and senescent T-cells in the periphery, whose numbers are further associated with poor clinical outcomes.[449-451] Phenotypically, as compared to CMV and EBV infection, CD8 T-cells from HIV-1 infected donors express low levels of CD28 and are more significantly skewed towards the high expression of both CD57 and CD27 – thus indicating advanced cellular differentiation and senescence.[206,452] Reminiscent of T-cells in aged individuals, HIV-specific CD8 T-cells in HIV-1 infected individuals also reveal shortened telomeres, which associate with their inability to proliferate. In adults and infants, the proportion of CD57+ CD8 T-cells continues to increase during the course of infection and disease progression.[107-109]

Since antigen specific memory T-cells must maintain replicative potential in order to sustain the effective suppression of viral replication, the replicative impairment which results from the premature "ageing" of the CD8 T-cell compartment is likely to be an important determinant in establishing the threshold that leads to the final stages of AIDS, where viral titres increase exponentially and mortality and morbidity ensues. In support of this hypothesis, CD8 T-cells from untreated LTNPS exhibit enhanced telomerase activity and display longer telomeres than progressors.[445,455-456] In addition, this finding was associated with preserved CD8 T-cell polyfunctionality and cytotoxicity. Pertinently, the

use of telomerase activators on CD8 lymphocytes from HIV-1 infected individuals seems to replicate this setting by enhancing proliferation, cytokine/chemokine production and anti-viral cytotoxicity through the preservation of telomere length.[456]

While a wealth of information on HIV-1 induced immunosenescence can be found in the literature, including comparisons with CMV and EBV infection, the subject of immune ageing has remain experimentally unexplored in the context of HIV-2 infection. Recent studies have revealed that despite great differences in viral loads, progressors across both HIV-1 and HIV-2 cohorts express comparable levels of lymphocyte activation markers - HLA-DR, CD38 and Ki67 – when matched for CD4 T-cell counts.[284,286] As immune activation is regarded as the main driver of lymphocyte senescence, it is surprising that HIV-2 progressors maintain much lower viral loads than their HIV-1 counterparts, while exhibiting similar signs of immune activation. This suggests either that immune activation and disease progression are uncoupled in HIV-2 infection, or that compensatory mechanisms, such as the input of lymphoid progenitors or naïve T-cells, are sufficient to offset the presumed functional deficits that are attributed to chronic immune activation. A detailed understanding of how parameters relating to lymphocyte senescence and replenishment align during HIV-2 infection may therefore, yield critical information on how viraemia can be more effectively controlled during progressive HIV-1 and HIV-2 infection.

1.7.6 Meeting the Debt of Senescence – HIV-1 versus HIV-2?

An intricate balance of processes and cellular signals maintains T-cell homeostasis in humans. During HIV-1 infection, continuous antigenic stimulation drives senescence and terminal differentiation in T-cells, which are replenished by the differentiation and proliferation of the naïve T-cell pool.[335,457] The size of the naïve T-cell pool is in turn balanced by the generation of haematopoietic progenitor cells (HPCs), thymic output, and IL7 availability in the serum.[56,258] In the context of HIV-1 infection, disease progression was found to be more closely associated with the loss of naïve CD4 and CD8 T-cells in circulation than with CD57 expression.[263] This suggests that the former may be more relevant in directing disease outcome in HIV-1 infection.

Since the continuous depletion of naïve lymphocytes during chronic HIV infection is not only a function of direct infection by HIV, the maintenance of healthy naïve T-cell numbers relies not only on the effective suppression of viral replication, but also on the reduction of excessive immune activation so that favourable conditions can be created for the renewal of the naïve T-cell pool. Therefore, knowledge of the mechanisms involved in limiting T-cell renewal during HIV infection could provide critical information on how therapeutic interventions can be refined to further bridge the gap in life expectancy between HIV-1 infected and uninfected populations. Moreover, since HIV-2 infection is generally associated with lower levels of viraemia and immune activation, [77,80-81] a comprehensive study detailing and comparing the ability of HIV-1 and HIV-2 infected individuals to maintain T-cell homeostasis will be useful in verifying the immune activation model of T-cell senescence and disease progression.

Although CD4 T-cell decline has largely been the focus of HIV-1 infection, HIV-1 disease progression is actually characterised by general lymphopenia.[263,457] Both CD4 and

CD8 T-cell counts are drastically reduced during chronic infection and the naïve T-cell compartment is preferentially depleted. Although less is known about the fate of other lymphocyte populations during HIV-1 infection, the latter is also characterised by the marked underrepresentation of naïve B-cell and natural killer (NK) cell populations in the periphery.[459-460] Collectively, these deficits suggest a collective impairment in the regeneration of lymphocytes during HIV infection and indicate that the dysfunction in T-cell homeostasis may stem from an earlier origin of lymphocyte development.

While HIV is able to infect thymocytes and contribute to reduced thymopoiesis and loss of thymic architecture, the loss of naïve lymphocyte populations can be further traced to a more upstream origin of lymphocyte development. The percentage and absolute counts of $LIN^{-}CD34^{+}$ haematopoietic progenitor cells (HPCs) and lymphoid progenitors have been observed to decline with naïve and total CD4 T-cell populations during HIV-1 infection, suggesting that haematopoiesis is deficient during HIV infection.[191] Furthermore, HPCs from HIV-1 infected subjects are less predisposed towards lymphoid differentiation. Nevertheless, the lack of correlation between HIV-1 plasma load and HPCs in circulation, and the observation that primary HIV-1 infection contributed only to a modest difference in HPC numbers, led the authors to conclude that HIV-1 is unlikely to have a direct influence on HPC frequency, even though HIV-1 can directly infect HPCs. Indeed, the authors demonstrated that the loss of HPCs was more closely related to markers of immune activation, and that the plasma levels of chemokines that suppressed HPC proliferation (such as IP10 and MIG) were higher in HIV-1 progressors. Due to the lower levels of T-cell activation in HIV-2,[77,80-81] we predict that HPCs in HIV-2 infected individuals may be better preserved than in HIV-1 infection, thus supporting the renewal of the naïve T-cell pool.

In terms of naïve T-cell production, a recent study presented a case of enhanced thymic activity during HIV-2 infection, including higher levels of RTEs via the enumeration of TREC+ T-cells.[461] Together with a separate study which showed that HIV-2 has the capacity to infect but not replicate efficiently in thymocytes,[462] the data collectively suggest that thymic output may be sufficient to maintain T-cell homeostasis in HIV-2 infected individuals. The latter provides a partial explanation for the differential outcomes of HIV-1 and HIV-2 infection – particularly shedding light why age or time of seroconversion – key predictors of HIV-1 disease outcome – do not affect HIV-2 progression. [282]

Even though the conclusions may seem paradoxical, a belief in the benefit of chronic stimulation with low level of stimuli is shared by the hygiene hypothesis or in more clinically relevant settings, in the effectiveness of low dose immunotherapy.[463-466] Since thymic output is adjusted as a homeostatic response to fewer naïve T-cells in the periphery, the small increments in immune activity during HIV-2 infection may stimulate thymopoiesis in a sustainable manner that promotes viral clearance. This argument is further supported by two recent reports, which observed enhanced thymic output in two independent cohorts of perinatal HIV-1 infection.[467-468] Both studies focused on vertically infected children (median age of 13) and reported higher RTEs in the periphery as compared to age-matched, uninfected controls. As vertically-infected children represent a minority group of paediatric controllers who, because of their age, are less susceptible to thymic dysfunction and involution than HIV-1 infected adults, their enhanced capacity for thymopoiesis argue in favour of a case where chronic viral infection is not immediately pathologic as long as homeostatic mechanisms are able to keep up with the changes to the immune system.

1.7.7 Double the Trouble: CMV and HIV Coinfections

In the study of immunosenescence, it is important to also consider the contribution of highly prevalent co-pathogens, such as CMV, that establish latent infection, particularly if they themselves have been implicated in driving senescence. CMV is a herpesvirus of the *Betaherpesvirinae* subfamily and has a universally high prevalence; adult prevalence rates of over 90% have been consistently reported in developing countries, and in developed countries, the seroprevalence rate is approximately between 50-60%.[469-470] A survey conducted in the United States between the years 1988 and 1994 revealed an overall prevalence rate of approximately 59% for individuals beyond the age of six.[471] CMV infection is usually acquired early in life, particularly in the developing world, but progresses to latency and lifelong persistence after primary infection. The detection of active CMV replication – either from reactivation or new infection – in adulthood is usually an indicator of immune dysfunction, and occurs more frequently in the elderly, or in those who receive immunosuppressive drug therapy or suffer from AIDS.[263,472-475]

Equalling the prevalence of CMV in elderly populations, CMV prevalence tends to occur at 75 – 90% in the HIV-1 infected population.[471,476-479] Prior to the ART era, CMV end-organ disease was a major cause of morbidity and mortality in HIV-1 coinfections, and manifested as retinitis, encephalitis, colitis or pneumonitis.[480-481] While the current evidence converge towards a higher risk of CMV reactivation during HIV-1 co-infection,[255-258,261-262] the relevant mechanisms require further clarification. Nevertheless, the similar prevalence of CMV infection among the elderly and HIV-1 infected populations sharpens the parallel between HIV and immunosenescence.

For similar reasons as HIV-1, CMV has been implicated in driving immunosenescence because of its association with an ageing T-cell phenotype during both acute and chronic phases of infection – i.e. the accumulation of terminally differentiated T-cells and memory inflation.[206,255,263,290,452,484] However, since the consensus argues for the requirement of chronic and high antigen stimulation for the induction of immunosenescence,[485-486] CMV infection is unlikely to equal the threat of HIV-1 in mediating T-cell senescence for most of the infected population, since viral loads tend to remain low or undetectable until approximately 70 years of age or during pregnancy.[487] Furthermore, in perinatal survival cases of coinfection with HIV, the premature immune system retains the ability to eliminate CMV viral load within the first two years of birth. However, the resurgence of CMV viral load and the presence of concomitant strong CMV T-cell responses during coinfection with either HIV-1 or HIV-2 suggest that its contribution to immunosenescence should not be overlooked in the different settings of HIV coinfections. [206,255,263,290,452,484,488]

The phenotype of T-cell differentiation and senescence that is driven by CMV infection is quite distinct from the footprint of EBV and both HIV viruses – and this observation applies to both CMV-specific T-cells and T-cells of other specificities. In general, CMV tends to drive T-cells further along the continuum of T-cell differentiation than either HIV-1 or HIV-2.[206,290,452] Specifically, CMV tends to drive T-cells towards terminal differentiation (CD28-CD27-CD57+CD45RA+), while HIV-1 and HIV-2 drives T-cell differentiation towards more intermediate phenotypes that express varying levels of CD28 and CD57. Next, CMV infection is also associated with T-cells that have shortened telomeres.[489] Like HIV-1, CMV infection independently drives memory inflation and increases the proportion of cells that express inhibitory PD-1 and CTLA-4, thereby adding to the pool of functionally anergic memory cells.[490-491] Furthermore, CMV is one

of the most immunodominant pathogens and it has been demonstrated that 50% of the CMV-specific response can be directed towards a single epitope.[485,492] The clonal expansion of the latter can occur in response to the homeostatic signals that evolve from the loss of naïve T-cells during HIV-1 infection and lead a greater loss of TCR diversity. In summary, CMV is likely to have a significant role in driving immunosenescence under exceptional circumstances, such as during coinfection with HIV (Figure 1.15), and the study of HIV-directed immunosenescence should certainly consider the contribution of CMV in driving T-cell phenotype and pathology.

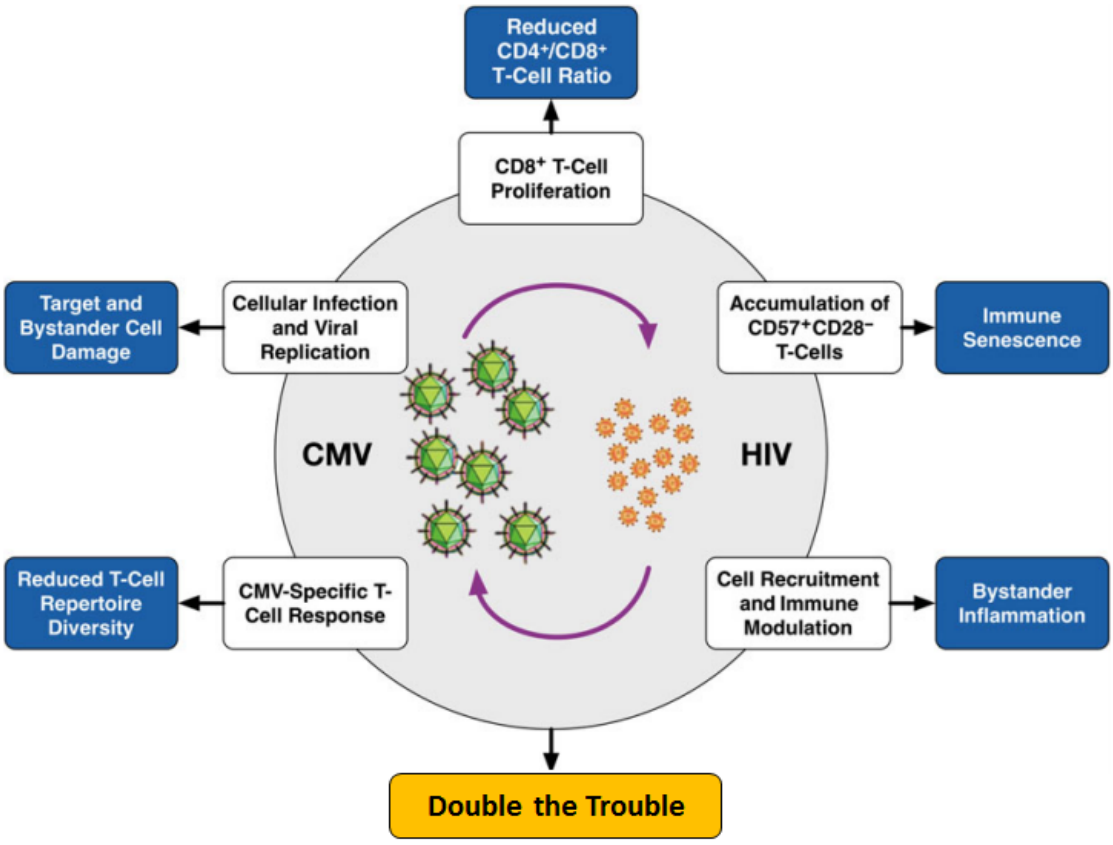

Figure 1.15: Coinfection with CMV leads to aggravated cellular senescence. HIV infection induces CD4 T-cell loss and replicative dysfunction, leading to poor CD8 viral suppressive outcomes and permitting more CMV replication. Infection with both viruses contribute to aggravated inflammatory responses and immune senescence (Adapted from[488])

1.8 Concluding Remarks and Thesis Aims

Overall, immunosenescence or immune senescence – used interchangeably throughout this thesis – is taken to mean the progressive loss of immune tissue that result from (i) functional, (ii) clonal or (iii) systemic changes to the cells or tissue that make up the immune system. While this process occurs gradually during the ageing process, there is overwhelming evidence to implicate the involvement of HIV-1 infection in driving all three aspects of immunosenescence by inducing high levels of immune activation (Figure 1.16). Altogether, relatively little work has been invested into understanding the impact of HIV-2 infection and untreated perinatal HIV-1 infection in driving immunosenescence, and the key goal of this thesis is to mend this gap in knowledge. In achieving the latter, we hope to verify the conclusions made from studying the effects of HIV-1 infection, and inform the design of therapeutics for treating different HIV infections.

Both HIV-2 and paediatric HIV-1 infection provide excellent settings for verifying the mechanistic theories that relate to HIV-1-associated immunosenescence. The majority of HIV-2 infected individuals control their infection, which raises the question of how they are able to generate robust T-cell responses, when equivalent responses are rare in HIV-1 infected cohorts.[290,297,305] Our hypothesis is that the former is an outcome of sustained or robust homeostasis that is subject to less interference from systemic immune activation. During HIV-1 infection, the persistently high levels of immune activation have been shown to be an important driver of the immune ageing phenotype. Since HIV-2 is associated with low levels of immune activation,[77,80-81] the infection is expected to reflect better outcomes in T-cell homeostasis, which may then support high levels of T-cell viral suppressive activity. Alternatively, in perinatally HIV-1 infected children – who by virtue of their age represent slow progressors – the presence of a hyperactive thymus may be

sufficient to compensate against the early onset of immunosenescence and result in younger immune cell phenotypes.[353,467-468] These queries are systematically addressed in the subsequent chapters, with Chapters 3 - 5 focused entirely on HIV-2 infection and Chapter 6 on perinatal HIV-1 infection.

Chapters 3 – 5 represent the most comprehensive work done on T-cell senescence in the HIV-2 context, and a bulk of the data that specifically pertain to CD8 T-cells have been published in the *Journal of Immunology* (Appendix).[494] These chapters employ the extensive use of flow cytometry to compare the T-cell phenotype between asymptomatic HIV-2 controllers and HIV-1 controllers. Chapter 3 is more concerned about aspects of T-cell homeostasis and maintenance of the naïve T-cell pool, while Chapter 4 addresses the functional aspects of T-cell activity. Altogether, we show that primary immune resources are better preserved in HIV-2 than in HIV-1 infection – including more substantial HPC and naïve T-cell populations that correlate well with thymic output. The preservation of primary immune responses during HIV-2 infection is expected to contribute to robust effector functions and a younger phenotype of virus-specific memory T-cells. Accordingly, we show that HIV-2 specific memory T-cells reflect an earlier differentiated phenotype than HIV-1 specific T-cells, are highly polyfunctional and exhibit strong virus suppressive capabilities. Chapter 5 is a short chapter that examines the potential role of CMV as a co-pathogen in driving senescence within the HIV-2 context, and raises questions on whether prolonged infection with HIV-2 promotes CMV reactivation.

In perinatally HIV-1 infected children – who by virtue of their age represent slow progressors – the presence of a hyperactive thymus may be sufficient to compensate against the early onset of immunosenescence and result in younger immune cell phenotypes. While initial plans for a similar study of cellular senescence was planned to

examine the impact of perinatal HIV-1 infection on immune senescence, the loss of cellular samples during shipment precluded this possibility. Instead, we focus on more molecular aspects of leukocyte senescence in Chapter 6. By quantifying the telomere length and mtDNA content – biomarkers of cellular senescence – of leucocytes from vertically-infected children, we observed that leucocytes from vertically-infected Zimbabwean children nevertheless possessed shorter telomeres and higher mtDNA content than uninfected children; which signal the onset of advanced immunosenescence despite the presumed possession of a hyperactive thymus.[467-468]

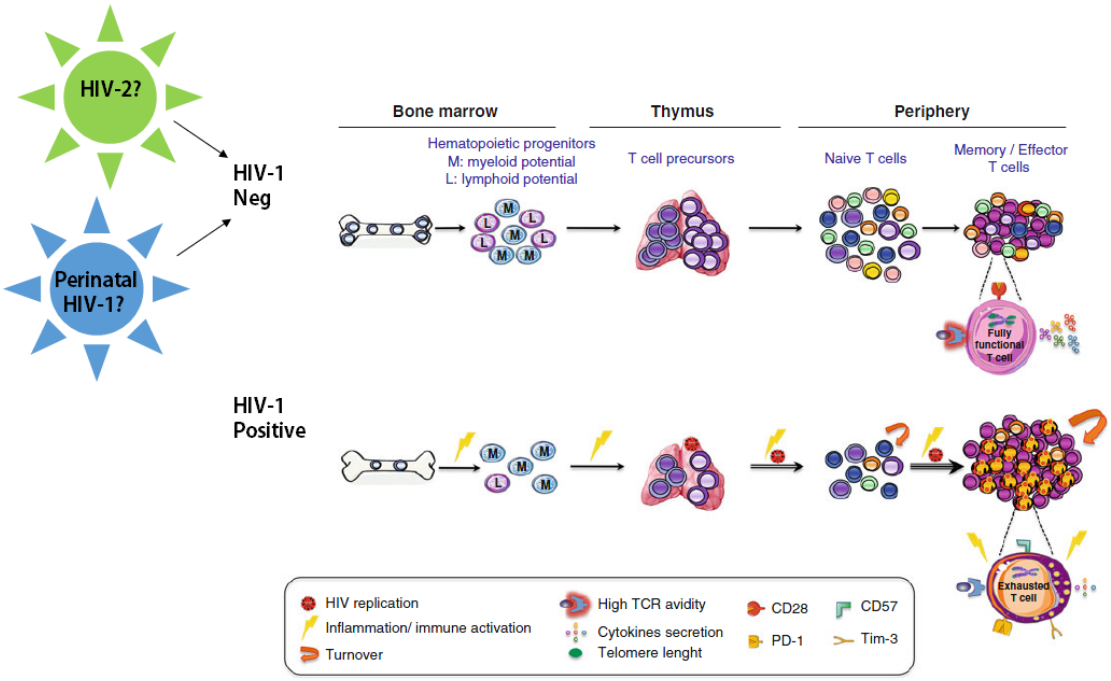

Figure 1.16: The role of HIV-1 in driving T-cell senescence. Persistent HIV replication and the associated chronic immune activation contributes to the collapse of T-cell homeostasis, ultimately leading to functional, clonal and systemic changes in T-cell phenotype. HIV-2 and untreated perinatal HIV-1 infections are hypothesised to reflect better outcomes in T-cell homeostasis because of lower immune activation and stronger thymic output respectively; we expect that parameters associated with T-cell homeostasis may be more compatible with observations from the HIV-1 negative group. (Adapted from[347])

Chapter 2: Materials and Methods

2.1 For Data in Chapters 3-5, 7 and Appendices

2.1.1 Study Population

A national, multicentre cohort was established to study the natural history of HIV-2 infection in adult patients living in France and under the care of local hospitals under the aegis of the ANRS in May 1994 (ANRS Study CO5 coordinated Action 7, Principal Investigator: S Matheron). The main objective of establishing this cohort was to describe the natural history of the infection and the associated clinical predictors of progression; this cohort also allowed the formation of a database to facilitate the collection of biological samples from HIV-2 donors in France. The study protocol incorporates a systematic collection of socio-demographic, clinical, virological and treatment parameters. By the end of June 2009, 749 patients were included in the study and data or samples from at least one follow-up visit were available for 727 patients.

The majority of donors were found to be native to West Africa (80%) and were infected heterosexually (82%) by Group A virus (83%). The median CD4 T–cell count at baseline was 472 cells/ µl, which made up 28% of PBMCs. A quarter of the patients had been receiving antiretroviral treatment prior to their inclusion to this study. Viral loads were measured by a quantitative PCR (qPCR) method with a detection threshold of 100 copies/ml of blood; and viral load was detected in 63% of patients. The median follow-up period was 50 months, and within this period, treatment was initiated for 487 patients who were previously naive to antiretroviral therapy at baseline. Of the 727 patients who

had at least one follow up visit, 386 remained naïve to antiretroviral therapy and 339 were clinically asymptomatic during their last visit.

The PBMCs from 19 of the 386 treatment naïve HIV-2 infected donors were made available to our research group for further analysis. The HIV-2 controllers (HIC2) in Chapters 3-5 are therefore, a subgroup of the ANRS CO5 VIH-2 cohort who were recruited into the ANRS IMMUNOVIR 2 study, which analyses patients with non-progressive infection. All patients in this study reflected characteristics of HIV controllers and were asymptomatic, treatment naïve individuals who met the inclusion criteria for this study. They were (i) infected and remained asymptomatic for at least 5 years; (ii) had a CD4 T cell count of > 400 cells/µl and (iii) a viral load of < 400 RNA copies/ml. HIC2 were compared to three groups of HIV-1-infected individuals: HIV-1 controllers (HIC1) from the ANRS CO21 Codex cohort who met the same inclusion and exclusion criteria as HIV-2 controllers; as well as two groups of HIV-1 viraemic individuals (one with CD4 T cell count below 200 cells/ml and the other with CD4 T cell count above 500 cells/ml) from the Pitié Salpêtrière Hospital (France). PBMCs from uninfected healthy adults were obtained from the French Blood Bank (Etablissement Français du Sang). A summary of the clinical attributes of the patients included in this study are displayed in Tables 3.1 and 3.2 of Chapter 3. All participants gave their written informed consent. This study was approved by the Comité de Protection des Personnes of Ile de France XI.

2.1.2 Blood Separation and Cryopreservation of Samples

Blood samples were collected in 10ml EDTA-coated tubes (BD Biosciences: 368589) at baseline and layered on 15 ml of Lymphoprep (Axis-shield: 114547) in Leucosep Tubes (Greiner Bio-One: 227289). The suspension was subsequently centrifuged at 400*g* for 30

minutes without the application of brakes. Plasma were collected without disturbing the interface, while PBMCs in the plasma-density gradient medium interface were transferred into a separate 50ml Falcon Tube (BD Biosciences: 352070). PBMCs were washed in 1x Phosphate Buffered Saline (PBS) that was added to make up a total volume of 50 ml and spun at 300 x *g* for 10 minutes. The supernatant were discarded and cells were aliquoted in batches of no more than 10^7 cells/ ml of freezing medium [Fetal Bovine Serum (FBS) containing 10% dimethylsulphoxide (DMS)] in a Nunc Cryotube (Sigma-Aldrich: V7634). Cells were kept at -80°C overnight before transferring into a liquid nitrogen tank for long-term storage until use.

2.1.3 Flow cytometry and reagents

All flow cytometry data were collected with an LSR Fortessa (BD Biosciences). Electronic compensation was conducted using antibody capture CompBeads (BD Biosciences: 552843) that were separately stained with individual monoclonal antibodies. Data analysis were performed using FlowJo v9.4.2 or DIVA (BD Biosciences) software. All fluorochrome-conjugated antibodies were titrated to determine the optimal concentration for staining. Fluorescence minus one (FMO) controls – where cells were stained with every antibody in the panel except for the specific antibody being tested – guided the setting of gates for each fluorochrome-conjugated antibody.

All experiments involving PBMCs were performed on previously cryopreserved cells that were thawed according to the following procedure. The cryotube containing the sample of interest was placed in a 37°C water bath; prior to complete thawing, cells were transferred into a 10 ml falcon tube containing 9 ml of pre-warmed (37°C), complete RPMI medium [cRPMI (RPMI-1640 medium with L-glutamine, 10% sterile heat-inactivated FBS and 1%

streptomycin/penicillin)]; the sample was centrifuged at 1500 rpm (Beckman GS-6) for 5 minutes and washed once with FACS buffer (1% FCS, 0.1% NaN3, 2mM EDTA in PBS) before subsequent staining.

Tetramer Phenotyping

Pre-conjugated HLA-B*5301:peptide, biotin and β2 microglobulin monomers were generated and provided by Yanchun Peng from the Weatherhall Institute of Molecular Medicine (WIMM). Immunodominant HIV-2 epitopes were identified by Aleksandra Leligdowicz after screening the IFNγ response of PBMCs from HLA typed HIV-2 infected donors in *ex vivo* Enzyme-linked Immunospot (ELISpot) Assays.[281] From these assays, the optimal epitope sequences and peptide lengths were identified.[290] Monomeric complexes (50 μg) of HLA-B*5301 p26 TPYDINQML (TL9) and p26 DRFYKSLRA (DA9) were reconstituted into tetramers by adding Extravidin-PE (Sigma: E-4011) at 49 μl/50μg of monomers and the solution was incubated overnight at 4°C in the dark. PBMCs were stained with pre-titrated concentrations of PE-conjugated tetramers (1 μg/ 10^7 PBMCs) for 15 minutes at 37°C before adding the appropriate panel of antibodies (Table 2.1). Cells were washed once with FACS Buffer and fixed with 2% paraformaldehyde before they were analysed with the LSR Fortessa (BD Biosciences).

Immunomagnetic sorting of $CD34^+$ cells

HPCs were purified by immunomagnetic sorting using MACS technology, adhering strictly to the provider's recommendations and instructions (Miltenyi Biotech). The purity of enriched populations was > 90% as determined by flow cytometry.

T cell, B cell, and CD34+ HPC Phenotyping

For T-cell, B-cell and CD34+ HPC phenotyping, cells were stained with the appropriate panel of antibodies for 20 minutes in the dark at room temperature (Table 2.1). After incubating with antibodies, cells were washed in FACS buffer by spinning at 1500 rpm (Beckman GS-6) and fixed with 2% paraformaldehyde before analysis. The gating strategy for analysing the phenotype of CD34+ HPCs and T-cells is show in Figure 2.1 and Figure 2.2.

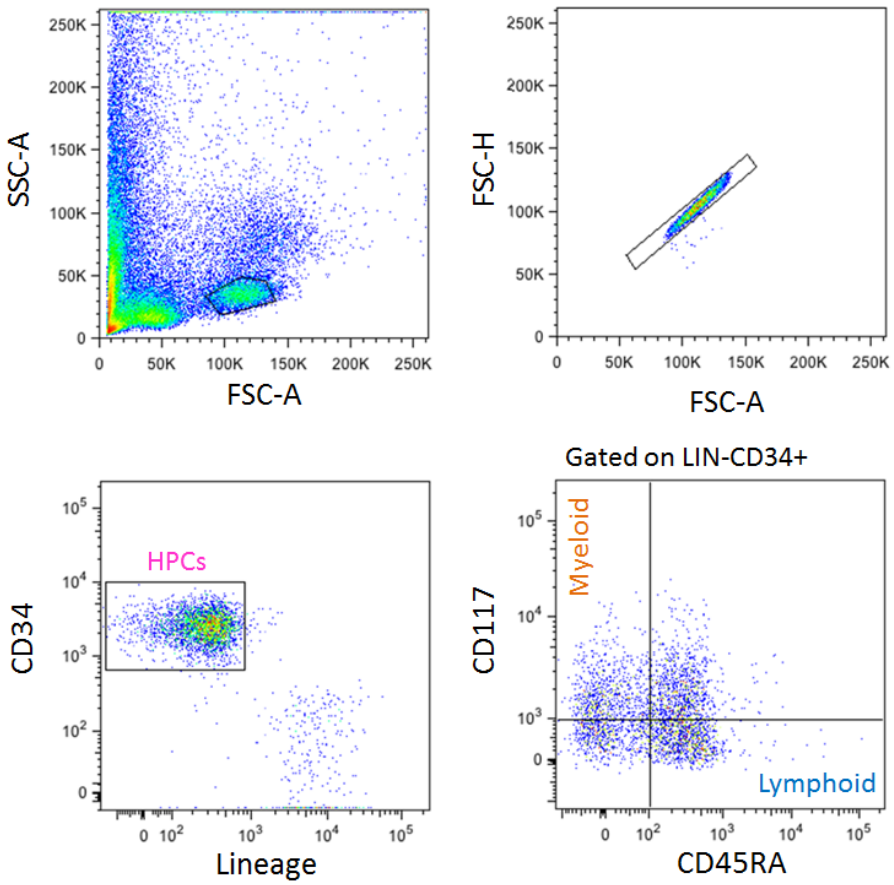

Figure 2.1: Representative Gating for HPC Analysis. Cells were first gated to identify lymphocyte populations, then gated to identify singlets. HPCs were identified as Lin- (CD3-CD14-CD19-CD20-CD56-) CD34+; the latter were divided into CD45RA+CD117- Lymphoid or CD45RA-CD117+ Myeloid HPCs.

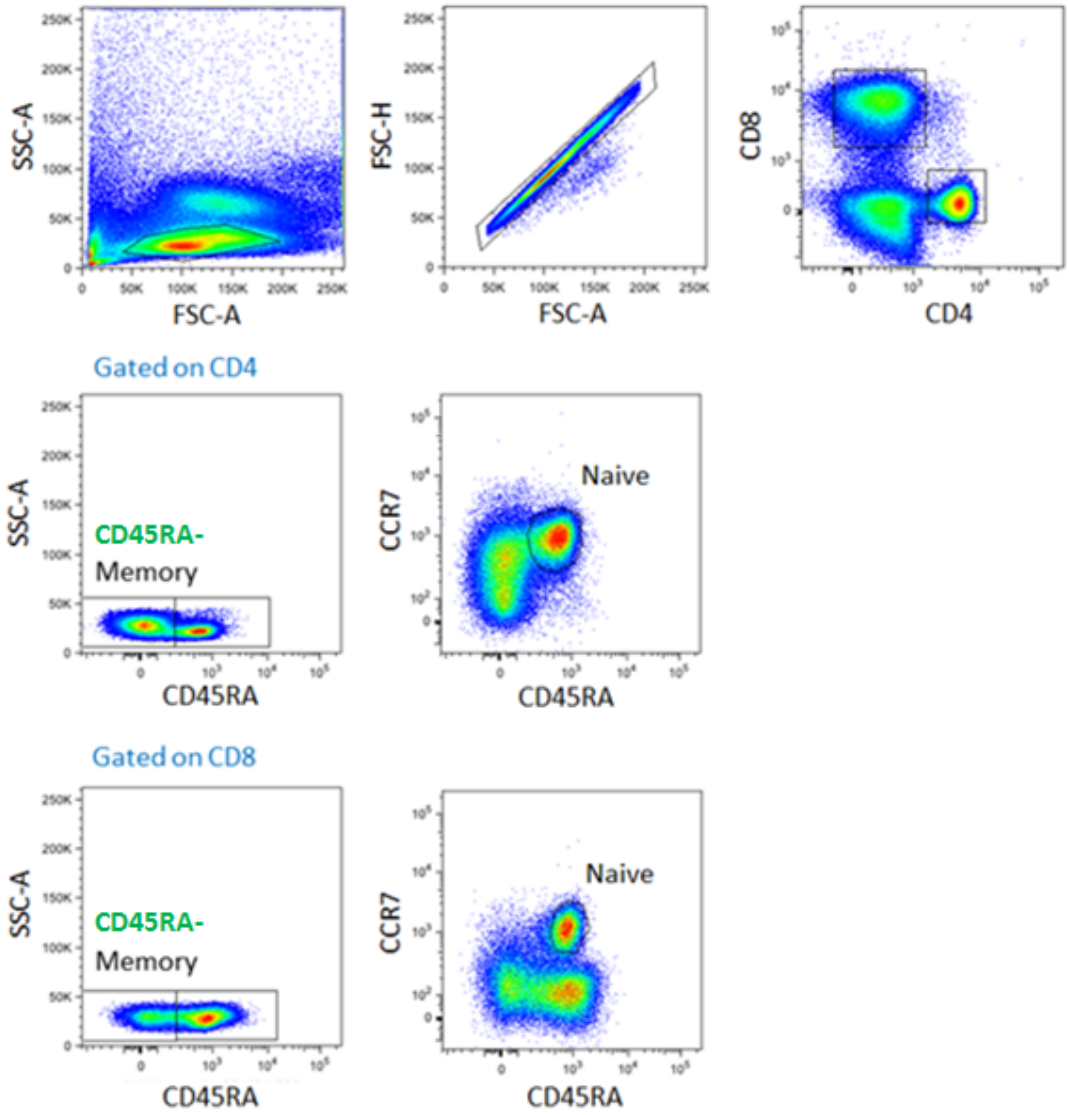

Figure 2.2: Representative Gating for T-cell Analysis. Cells were first gated to identify lymphocyte populations, then gated to identify singlets, followed by the identification of CD4 and CD8 populations. Naïve and Memory T-cells were identified by separately gating for CCR7+CD45RA+ and CD45RA- respectively. The exclusion of CD45RA+ cells in the classification of memory T-cells omits a population of terminally-differentiated memory CCR7-CD27-CD28-CD45RA+ TEMRA cells, which are a population of CD57+ T-cells as they were not consistently observed in high proportions in all HIC2 donors and were absent in the CD4 T-cell compartment of the majority of HIC2 but remains a caveat in further CD57+ analysis of memory T-cells.

Intracellular Cytokine Staining

Four complete HIV-2 subtype A genome sequences from West Africa were available on the Alamos National Laboratory HIV Database. A consensus sequence was generated from these four sequences, and HIV-2 Gag overlapping peptides which spanned the entire Gag proteome were designed using the Los Alamos National Library program PeptGen (Available at: http://hiv-web.lanl.gov). These peptides overlapped by 10 amino acids, were 15 to 19-mer in length and were synthesized by the PEPscreen service from Sigma-Genosys (0.5 – 2 mg of each peptide, 70% purity). Peptides were dissolved in DMSO (Sigma-Aldrich) at 100 mg/ml and diluted with RPMI to a final concentration of 20mg/ ml before being individually filtered. The peptides were stored at - 80 ºC before use. As a high DMSO concentration is cytotoxic, the final DMSO concentration in the respective peptide pools were kept below < 0.04%.

Purified PBMCs were thawed and rested overnight at 37ºC in cRPMI media; viability was then examined by trypan blue exclusion (≥ 95% viable). For stimulation, cells were incubated in the presence of 15 mer overlapping peptides covering the entire HIV-2 p26 (1µg/ml) or clade B HIV-1 Gag (2 µg/ml) protein. For CMV responses, overlapping peptides covering the entire pp65 and IE1 protein were provided by Victor Appay.[263] Peptide stimulated cells were incubated in the presence of anti-CD107α antibodies for 1 hour at 37 °C in a 5% CO2 incubator, followed by an additional 5 hour incubation with secretion inhibitors Monensin (2.5 µg/mL; Sigma-Aldrich) and Brefeldin A (5µg/mL; Sigma-Aldrich: B7651). The BD Cytofix/Cytoperm™ Kit (BD Biosciences: 554655) was used for permeabilisation and fixation of the cells prior to staining for intracellular markers. Cells were first stained with directly conjugated antibodies that targeted specific surface markers (CD3, CD4 and CD8) before the addition of the intracellular cytokine

antibody cocktail; both steps were preceded by a single wash step. For each patient, negative controls were included and signified the incubation of PBMCs in the absence of peptides; a positive control, where cells were activated with *Staphylococcal* enterotoxin B (Sigma-Aldrich: S4881) was also included for each donor. In the calculation of cytokine responses, the magnitude of responses from the negative control were regarded as background and subtracted from the stimulated response prior to further analysis.

Table 2.1: List of Antibodies used for Flow Cytometry

Antibody Panel	Antibody	Supplier	Catalogue
HPC	LIN-FITC	BD	340546
	CD34-PE	BD	345802
	CD45RA-V450	BD	560362
	CD117-PECy7	Beckman	IM3698
	CD38-APC	BD	345807
	CD4-APCCy7	BD	560158
T-cell	CD57-PB	Biolegend	322316
	CD4-HV500	BD	560768
	CD8-BV650	Biolegend	301041
	KI67-FITC	BD	556026
	HLADR-PerCPCy5.5	BD	339216
	CD45RA-ECD	Beckman	IM2711U
	CCR7-PECy7	BD	557648
	CD38-APC	BD	345807
	CD27-A700	Biolegend	302814
	CD31-APCH7	Biolegend	303119
Tetramer	CD8-APCCy7	BD	557834
	CD27-AF700	Biolegend	302814
	CD28-PECF594	BD	562296
	CD45RA-V450	BD	560363
	CCR7-PECy7	BD	557648
	CD127-BV650	Biolegend	351325
	GranzymeB-V450	Thermo Fischer	GRB17
	CD3-BV605	Biolegend	317321
	EOMES	eBioscience	64-4877-41
	TBET-APC	Biolegend	644813
ICS	CD4-HV500	BD	560768
	CD3-ECD	Beckman	IM2705U
	CD8-APCCy7	BD	557834
	MIP1β-FITC	R&D Systems	IC271F
	TNF-PECy7	BD	557647
	IL2-APC	Miltenyi	130091644
	IFNγ-A700	BD	557995
	CD40L-PE	BD	555700
	CD107-Vioblue	Miltenyi	130095520
B-cell	CD19-V450	BD	560353

	IgD-FITC	Beckman	735993
	CD27-PE	BD	555441
	CD38-PECy7	Beckman	A54189
	CD21-ApC	BD	559867
	IgM-A700	EXBIO	A7320C100
Flow-FISH	CD8-APC	Biolegend	344721
	CD57-PB	Biolegend	322315
	CD27-QDot605	Thermo Fischer	Q10065
CD4 Subsets	CD3-APCCy7	BD	340190
	CD4-A700	Biolegend	300526
	CD45RO-E650NC	eBioscience	95-0457
	CD62L-E605NC	eBioscience	93-0629
	CCR6-PerCPCy5.5	Biolegend	353405
	CXCR5-FITC	Biolegend	356913
	CXCR3-PECy7	Biolegend	353719
	CRTh2-APC	Biolegend	350109
	CCR4-V450	BD	561123

2.1.4 In vitro HIV-2 suppression assay

In order to measure the ability of CD8 T-cells to suppress the HIV-2 infection of autologous CD4 T-cells *in vitro*, Angin *et al.* adapted their previously published HIV-1 suppression assay.[495] PBMCs were isolated from peripheral blood by density centrifugation. CD4 and CD8 T-cells were then respectively isolated by positive and negative magnetic-bead sorting (STEMCELL Technologies). CD4 T-cells were activated in cRPMI supplemented with phytohemagglutinin (1 µg/ml) and interleukin-2 (IL-2) (100 IU/ml) for 3 days. Simultaneously, CD8 T-cells were cultured in non-supplemented culture media. Activated CD4 T-cells were infected with HIV-2 SBL strain[496] using a spinoculation protocol[497] and cultured alone or with autologous CD8 T-cells at a 1:1 ratio for 14 days in IL-2 (100 IU/ml) supplemented cRPMI.

SBL is a subtype A HIV-2 virus and accounts for the majority of HIV-2 infections in Guinea Bissau and Europe; it bears close genetic similarity to the prototype HIV-2 virus strain, ROD.[498] The amount of p26 production in culture supernatants was measured every 3-4 days with an enzyme-linked immunosorbent assay (Gentaur: 78902); adhering strictly to the manufacturer's instructions. Overall CD8 T-cell suppressive activity was determined by using the peak of viral replication as a reference point and calculating the log decrease in p26 production after culturing superinfected CD4 T-cells with autologous CD8 T-cells (log[p26 in CD4 T-cell culture/p26 in CD8:CD4 1:1 co-cultures]).

2.1.5 TREC Assay

The TREC assay was performed by Appay *et al.*, and the data were included in Figure 3.3. Thymic function was estimated by the quantification of signal joint T-cell reception excision circles (sjTREC) as previously described.[499] PBMC lysates were subjected to multiplex polymerase chain reaction (PCR) for 22 cycles using sjTREC and CD3γ chain outer primer pairs. Each amplicon set were quantified with the LightCycler (Roche Diagnostics), performed on $1/100^{th}$ of the initial PCR, in independent experiments, but on the same serially diluted standard curve. This highly sensitive, nested quantitative PCR assay allows the detection of one copy of sjTREC in 10^5 cells. The sjTREC value for each donor were averaged from triplicate experiments.

2.1.6 Flow-FISH Measurement of Telomere Length

Previously frozen PBMCs were used for the determination of telomere length by Flow-FISH with the Telomere PNA Kit/ FITC (Dako: K5327). Samples were first stained for T-cell surface markers, CD8-APC, CD27-QDot605, CD57-PB and CD45RO-QDot605,

for 20 minutes at room temperature before subsequent fixation with 12.5 mM BS^3 (Thermo Scientific: 21580). The reaction was quenched by adding 9 µl of 1M Tris Buffer, pH 7.5. Thereafter, samples were washed with cold FACS buffer; 100 µl each of the hybridization buffer and FITC-conjugated telomere-specific PNA probes were added and the mixture was heated to 80 °C for 10 mins with a heating block. Samples were subsequently left to incubate overnight at room temperature and in the absence of light. After overnight incubation, samples were incubated with 100 µl of the washing buffer for 10 minutes at 40 °C before performing two wash cycles. Cells were resuspended in 60 µl of FACS buffer. Data were collected with the ImageStream100 (Amnis Corporation) and analysed with the Ideas Application v5.0 software.

2.1.7 Statistical analysis

Statistical analyses were performed using Prism software (GraphPad). The specific test that was used in each experiment is indicated in the figure legend. *P* values >0.05 were considered not significant.

2.2 For Data in Chapter 6

2.2.1 Study Population

The natural history of untreated vertically-acquired HIV is, for most, initially characterised by rapid progression (20 - 25%) and mortality before two years of age. However, in sub-Saharan Africa, approximately 35% survive to a median age of ten years without prior treatment.[94-95] In order to identify, recruit and initiate treatment for HIV-infected children, Rashida *et al.* offered routine provider-initiated testing and counselling (PITC) to children aged 6 – 16 at the main primary healthcare clinics (PHC) from six high density suburbs in southwest Harare, for over a two year period. HIV prevalence in adolescents aged 12 - 19 fell between 2-8% in communities across sub-Saharan Africa[95-98] and a prevalence of 5.3% for children aged 6 - 15 was observed in this study.[116]

When children tested HIV positive at the PHCs, their guardians or parents were approached for informed consent so their wards could be included in our clinical cohort, where information relating to growth and lung function were closely monitored. In addition, blood was collected from these children every 6 months prior to ART, and quarterly upon initiation of ART, for immunological studies.

The inclusion criteria for participation in the study were:

- Between 6-15 years of age.
- Test HIV-positive following PITC at PHCs
- Willingness to receive HIV care at the PHCs
- Residence in suburb where the PHC is located
- Informed consent by parent and assent from child

The exclusion criteria for participation were:

- Age <6 years or >16 years.
- Residence outside the suburb where PHC is located
- Previous tested HIV positive and receiving treatment

In preparation for future immunological studies, we requested for the recruitment of 200 age-matched children who tested HIV negative as controls, and obtained blood samples after consent and assent were respectively granted by the guardians and children involved. For telomere length measurement, we used DNA that was extracted from whole blood; both PBMC separation and DNA extraction were performed by laboratory technicians whom we personally trained at the Biomedical Research and Training Institute (BRTI). This study has been approved by the Medical Research Council of Zimbabwe (AP122/2013).

2.2.2 Collection of Blood and PBMC Isolation

Blood samples were collected in 10ml EDTA-coated tubes (BD Biosciences: 368589) and transported in batches to the BRTI. All centrifugation steps were performed with a Beckman GS-6 centrifuge. Blood samples were spun at 1500 rpm for 5 minutes. Plasma were collected and stored at -80°C. An equal volume of RPMI was added to the volume of blood after removing the plasma. Blood was carefully layered on Ficol at a ratio of 2:1; and spun at 400g for 25 minutes without the application of brakes. PBMCs in the plasma-density gradient medium interface were collected and transferred into a separate 50ml Falcon Tube (BD Biosciences: 352070). Cells were washed with RPMI and spun at 300 *g* for 10 minutes. The supernatant were discarded and cells were resuspended in no more than 10^7 cells/ ml of freezing medium [Fetal Bovine Serum (FBS) containing 10% dimethylsulphoxide (DMS)] in a Nunc Cryotube (Sigma-Aldrich: V7634). Cells were

kept at -80°C overnight before they were transferred into a liquid nitrogen tank for long-term storage until use.

2.2.3 DNA Extraction from whole Blood

Lysing of RBCs

2 – 5 ml of blood were transferred into a 50 ml Falcon tube and vortexed vigorously before adding TE 20-5 (Tris 20 mM-EDTA 5mM) pH 8.9 buffer to a total volume of 40 ml. The solution was mixed by inverting the tube 4 – 5 times and allowed to stand on ice for 15 minutes. The sample was then centrifuged at 3500 rpm for 15 minute. After removing the supernatant, the white pellet was dislodged and washed again with TE buffer. This wash step is repeated until the pellet appeared white or slightly pink in colour.

Lysing of WBCs and Protein Digestion

2 ml of TE 20-5 buffer was added to the pellet, followed by the subsequent addition of 100 µl of 20% SDS (final concentration of 1%) for protein denaturation. The suspension was mixed by inverting the tube 4 – 5 times.

20 µl of proteinase-K was added and the resulting solution was inverted 4 – 5 times before placing the sample tube in a shaking water bath and incubating overnight incubation at 42 °C. Samples were allowed to cool to room temperature after overnight incubation. 2.5 ml of 7.5M ammonium acetate were added and the sample was mixed gently by inverting the tube several times. 10 ml of ice-cold absolute ethanol was added and again, the tube was inverted to precipitate the DNA-ammonium salt complex. The sample was subsequently centrifuged at 5000 rpm for 30 minutes. The supernatant was

removed and 4 ml of sterile TE 20mM–EDTA 5mM-Nacl 0.2M, pH 8.0 buffer was added to the sample before vortexing briefly for 5 – 10 seconds. The DNA suspension was dissolved by placing the tube in shaking water bath at 52 °C. Once dissolved, the sample was allowed to cool on ice before we precipitated the DNA with 8 ml of ice-cold ethanol; this mixture was centrifuged at 5000 rpm for 30 minutes and the supernatant was removed completely. The DNA pellet was allowed to air dry and finally 150 µl of TE 20-1 buffer was added to resuspend the DNA; the sample was placed overnight in a water bath at 58 °C. Samples were spun briefly at 1000 rpm after overnight incubation and stored in a 1.5 ml Eppendorf tube after being labelled with the study number and the date of sample collection. DNA concentration was quantified by Nanodrop and the samples were stored at 4 °C. All DNA samples had 260/280 ratios of approximately 1.85.

2.2.4 Monochromatic Multiplex qPCR (MMqPCR)

Methods

The multiplex assay that we used was modified from a previously published protocol,[500] and these modifications have now been published by our collaborator, Helen Côté.[501] For each PCR reaction, 8 µl of master mix was added to 2 µl of DNA that was diluted to approximately 20 ng/ µl. The mixture was added in duplicates to a 96 well LightCycler 480 plate (Roche: 04729692001). The final concentration of reagents in the master mix was 1x LightCycler Faststart SYBR Green I Master (Roche: 03003230001), 1.2 mM EDTA (Sigma-Aldrich), and 0.9 µM of each of the four MMqPCR primers (Table 2.2). Prior to PCR, the plates were centrifuged at 1500*g* for 2 minutes at room temperature. The qPCR was performed on a LightCycler 480 using the following thermal cycling and temperature profile (Figure 2.3).

The rate of temperature increment was fixed at 2.2°C /s for all steps except during the melting curve analysis. The protocol begins with a 95 °C enzyme activation (hot-start) incubation for 15 minutes, followed by two cycles of sequential 94 °C and 49 °C incubation for 15 seconds each. Thereafter, the amplification step was repeated 40 times with the following program: 94 °C for 15 seconds, 62 °C for 10 seconds, 74 °C for 15 seconds, 84 °C for 10 seconds and 88°C for 15 seconds, with signal acquisitions at the end of the 74°C and 88 °C steps. For mtDNA amplification, the two cycles of 94 °C and 49 °C prior to the amplification step were omitted (Figure 2.3). After PCR cycling, a melting curve program was initiated where samples were heated at 95 °C incubation for 1 minute, allowed to cool to 45°C before being heated again to 95°C at a rate of 0.11 °C/ s. In the last heating step, fluorescence signals were repeatedly acquired at temperature intervals of 0.2 °C

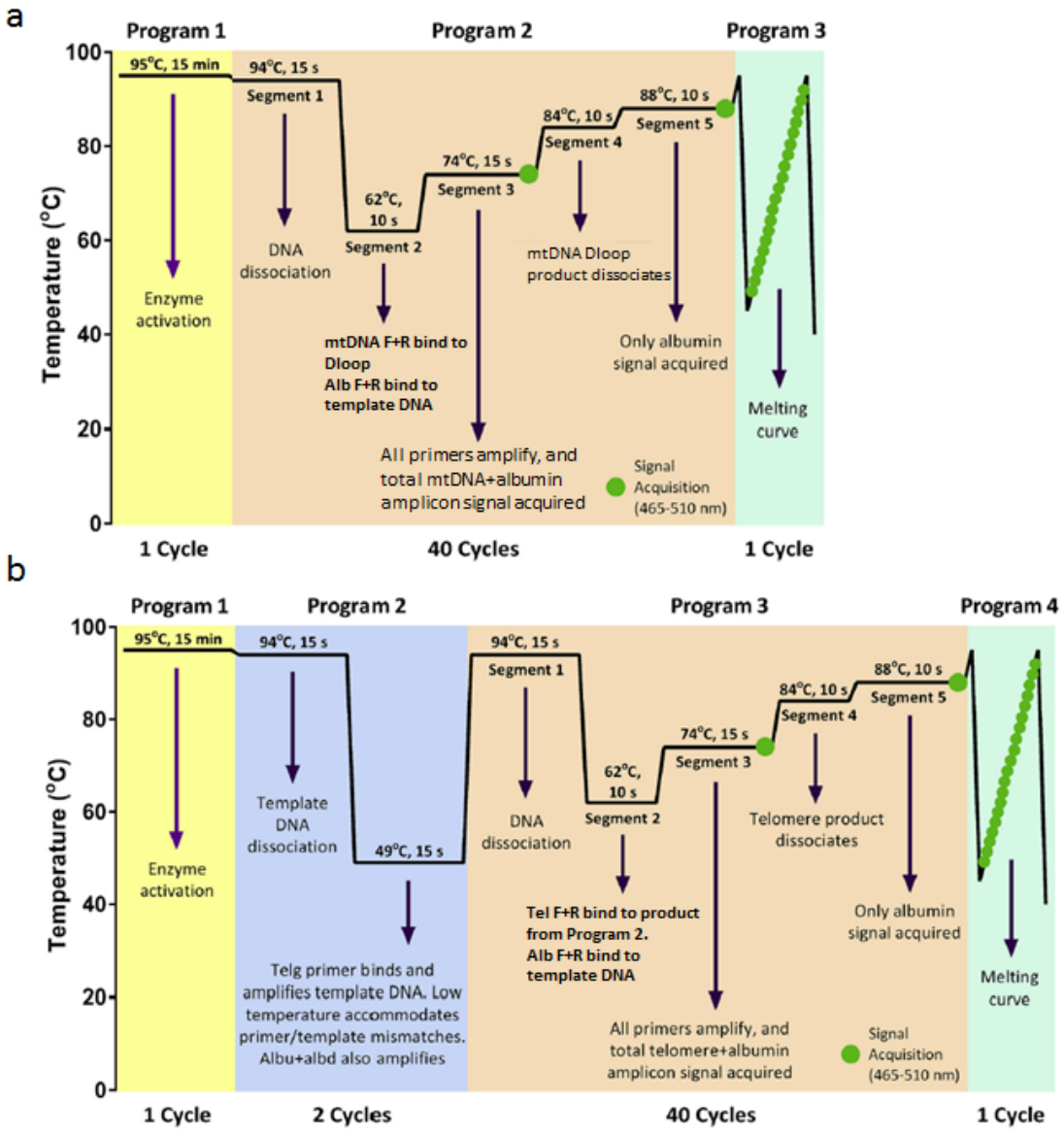

Figure 2.3: qPCR Cycling Profile for Telomere and mtDNA Measurement. Schematic plot of the monochromatic multiplex real-time quantitative PCR cycling profile for mtDNA (**a**) and telomere (**b**) copy number enumeration. The green circles are used to mark segments were signal is acquired. (Adapted from[501])

Data were acquired with the LightCycler 480 software version 1.5.1.62 SP2 (Roche). Each run included samples for standard curve generation as well as two negative and two positive internal controls (ICs). Samples that were used in the standard curve were added to the centre of the plate to avoid plate edge effects. The data were exported as text format and analysed with Microsoft Excel in order to separate the two sets of acquisition data (74 °C and 88 °C). The data were then converted into grid format by using the LC480 Conversion Software Version 2.0, that latter was developed by the Heart Failure Research Centre in Amsterdam. Baseline corrections and C_T values were subsequently calculated using the LinRegPCR version 2012.1 software.[502] The latter establishes a baseline and aligns the exponential phase of the fluorescence curve for all samples within a common window of linearity. This software determines the C_T as the cycle immediately before the upper limit of the window of linearity, to ensure that the fluorescence threshold is reached during exponential amplification. The PCR efficiency for each acquisition was calculated from the standard curve generated by each plate of samples.

Primer and Primer Design

Table 2.2 Primers used in MMqPCR

Primer	Nucleotide Sequence
Albumin F	5'-**CGGCGGCGGGCGGCGCGGGCTGGGCGG**AAATGCTGCACAGAATCCTTG-3'
Albumin R	5'-**GCCCGGCCCGCCGCGCCCGTCCCGCCG**GAAAAGCATGGTCGCCTGTT-3'
Tel F	5'-**ACACTAA**GGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3'
Tel R	5'-**TGTTAGG**TATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'
mtDNA F	5'- **ACGCTCGACA**CACAGCACTTAAACACATCTCTGC -3'
mtDNA R	5'-**GCTCAGGTCATAC**AGTATGGGAGTGGGAGGGGAAA-3'

Telomeric DNA Amplification for T/S Ratio Determination

Relative average telomere length can be measured by qPCR using primers that hybridize the telomeric hexamer repeats, since the number of binding sites for the primers increase with telomere length. These primers can prime at multiple locations along the tandem repeats of telomeric DNA but are inherently problematic as a series of irregularly-sized products can be generated, thus preventing the acquisition of a consistent fluorescence readout with the SYBR Green I (Roche: 03003230001) during the assay. The melting temperature of these products could melt at high enough temperatures to overlap the melting curve of the single copy gene amplicon and are, therefore, unsuitable for use in MMqPCR assays. In order to circumvent this problem, the Tel primers listed above were designed to generate a short, fixed length product (Figure 2.4). Only Tel F is able to prime DNA synthesis along chromosomal telomeric sequences, as a mismatched base at the 3' terminal of Tel R prevents active elongation. However, Tel R is able to hybridise with multiple segments of the Tel F extension product and exactly one configuration of these hybridisation sites allow active elongation, thereby enabling the generation of a single, fixed-length product (79 bp).

Relative TL was collected as a ratio of the quantity of telomeric DNA (T) normalized to the copy of the single-copy nuclear gene, albumin (S), yielding the T/S ratio. The T/S ratios were calibrated with a coefficient that was based on samples (n = 35) that were assayed using both MMqPCR and Flow-FISH, such that leukocyte T/S ratio were comparable to the mean lymphocyte telomere length in kb as determined by Flow-FISH.[503] The standard curve was generated by serial dilution (1:2) of whole blood pooled from 24 healthy individuals, ranging from approximately 0.16 – 21.00 ng/µl of DNA across eight standard samples, ultimately creating a 128-fold linear range ($R^2 > 0.99$) standard curve. The Hi-IC consisted of pooled WB DNA extracts, and the Lo-IC included DNA from cultured cells with long telomeres (K562).

Albumin Primers

Albumin primers were designed to yield an amplicon that would melt at a much higher temperature than the telomere amplicon. Fluorescent signals from the albumin primers can then be acquired during Segment 5 of Program 3 (Figure 2.3b), where the temperature is high enough to completely melt the telomere amplicon but also low enough to keep the albumin amplicon double-stranded and hybridised with SYBR Green I. The albumin amplicon has a size of 98 bp.

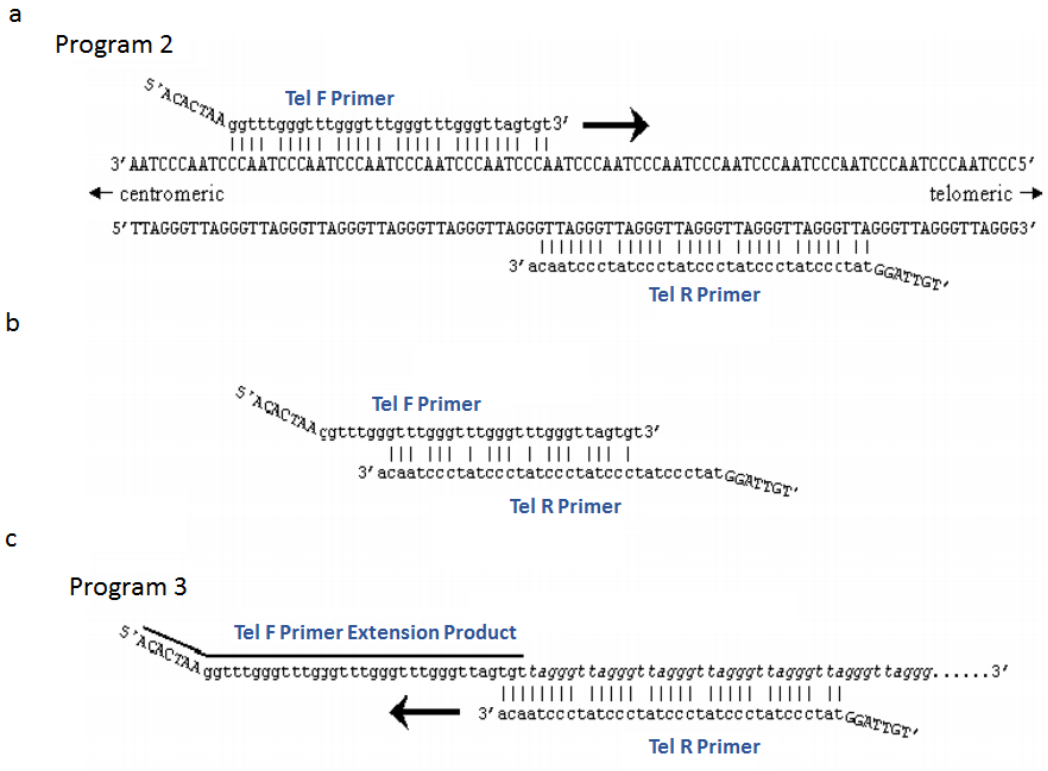

Figure 2.4: Mismatch Strategy of Tel Primer Design. (**a**) During Program 2 (Figure 2.3), Tel F amplify products from telomeric DNA, while synthesis at Tel R is inhibited by a base pair mismatch at the 3' end. (**b**) Tel F and Tel R may only hybridize perfectly along a complementary 3 bp overlap, and this configuration is not stable enough to enable primer dimer formation. (**c**) During Program 3, Tel R can hybridize along many points of the Tel F Primer Extension Product, but can only prime DNA synthesis in the configuration shown, as other results create a mismatch at the Tel R 3' end. The overbar of the Tel F Primer Extension Product marks the primer sequence itself; the capitalised sequence prevents the 3' ends of the telomere PCR product from priming DNA synthesis in the middle of other copies of the telomere PCR product. (Adapted from[500])

mtDNA Primers for estimation of mtDNA oxidative damage

The mtDNA control region or Dloop regulates mtDNA replication and transcription;[504-505] it refers to a segment of non-coding DNA that contains the origin of replication and the origin of transcription for both strands of DNA. The Dloop accumulates mutations at a higher frequency than any other segment of mtDNA under oxidative stress;[505] it is a hot spot for polymorphisms associated with ageing as well as life-threatening heart and lung diseases.[506-511] Because of these associations, the Dloop region has been extensively utilised to estimate oxidative damage in mitochondria or by approximation, cells and tissues.[510-513]

Based on the mitochondrial reference sequence from Mitomap, the D-loop sequence begins at position 577 of the 5' end and ends at position 594.[514] The two Mito primers were designed to amplify a 149 bp fragment (M) that is located slightly upstream of the mtDNA Dloop region as it contained fewer polymorphisms but was essential for mtDNA replication [between nucleotide regions 325–348 (Mito F) and 452–474 (Mito R)]. mtDNA content was recorded as a ratio of the quantity of mtDNA (M) normalized to the copy of the single-copy nuclear gene, albumin (S), yielding the M/S or mtDNA ratio. The standard curve on each plate was generated by serial dilution (1:5) of two plasmid DNA sequences obtained from Helene Côté, which collectively encompasses the albumin and D-loop regions at a 1:50 ratio. Standards ranged from 5,075,625 to 325 copies of albumin, and from 237 952 683 to 15 229 copies of D-loop, creating a standard curve with a 15,625-fold linear range ($R^2 > 0.99$).

2.2.5 Quality Control of T/S and M/S Data

Each run included two internal controls, a long-telomere or high mtDNA (Hi-IC) and short-telomere or low mtDNA (Lo-IC) control. The negative control consisted of master mix with Buffer AE added. For the telomere essay: The Lo-IC consisted of pooled WB DNA extracts, and the Hi-IC included DNA extracted from cultured cells with long telomeres (K562). For the mtDNA essay: The Lo-IC consisted of a 1:4 dilution of pooled volunteer whole blood DNA and the Hi-IC utilised an extract from cultured SKBR3 cells. The negative control contained Buffer AE in place of template DNA.

The T/S or M/S ratios of both internal controls, PCR efficiencies, and percentage difference between duplicates were logged for each run. The data from each run was accepted if four out of the following five conditions were met: (i) the negative control is negative or at least 3 C_T below the lowest standard; (ii) the T/S ratio of both internal controls are within 2 SD of logged values from previous runs performed under the same conditions; (iii) the PCR efficiency for both acquisition data sets fell within 90-100% (i.e. between 1.8 – 2.0 fold amplification per cycle); (iv) the difference between the two PCR efficiencies were < 2.5%; (v) The mean absolute difference in T/S or M/S ratio among duplicates were less than < 10% for all 40 samples. Data were only accepted if they fell within the linear range of the standard curve.

2.2.6 Statistical analysis

Statistical analyses were performed using Prism software (GraphPad). The specific test that was used for each experiment is indicated in the figure legend. *P* values >0.05 were considered not significant.

Chapter 3: Preservation of Lymphopoietic Potential in HIV-2 Infected Controllers

3.1 Introduction

In the absence of an effective vaccine that prevents the acquisition of HIV-1, the long term suppression of viral replication in infected individuals remains a major objective to prevent further disease progression and dissemination. Although progression to AIDS can now be significantly delayed by ART – the establishment of natural HIV control, which is observed in HIV-1 controllers (HIC1) and rare patients who gain independence from ART after early initiation, remains a priority.[317,516] A significant portion of the HIV literature, therefore, seeks to identify immune correlates of natural HIV control in an attempt to decipher the immunological mechanisms that underlie effective control of HIV-1 replication in the absence of ART. With information of the latter, the immune system can be modulated by therapeutic approaches to mimic the immune parameters of HIV-1 controllers, in the hope of arriving at a functional cure of HIV-1 infection.

As HIV-1 controllers remain exceedingly rare, HIV-2 infection provides an ideal model to identify immune correlates that are associated with natural HIV control as the majority of infected individuals are disease controllers (HIC2) that remain asymptomatic and present with undetectable viral load.[78,280-282] Although HIV-2 shares the same modes of transmission and intracellular mechanisms of replication as HIV-1, it provides a unique model of attenuated infection by a human immunodeficiency virus.[315,318,516] Compared to HIV-1 infection, HIV-2 infection is predominantly characterised by a slower decline of CD4 T-cells and lower levels of immune activation.[82,283,317-318] Nevertheless, HIV-2

infected patients who progress to AIDS manifest similar clinical manifestations that match the severity of those infected with HIV-1.

In the present work, we focus on the potential role of cellular immunity in preserving low HIV-2 replication. Although CD8 T cell mediated immunity is involved in the control of HIV-1 replication at the early stages of acute infection, its effectiveness is often lost in the later stages of chronic infection.[517] Moreover, chronically HIV-1 infected individuals present with substantially decreased thymic output, fewer naïve T-cells, B-cells and NK T-cells, as well as an increased likelihood of the presentation of activation and senescent markers on memory T-cells.[189-191,263,457,459-460] The latter process, which is reminiscent of immune aging, probably impacts on the regenerative capacity of the HIV-specific CD8 memory T-cell pool and the adaptability of the T-cell compartment, thus affecting their long-term ability to control viral replication and counteract the emergence of escape variants respectively.

Unlike HIV-1 infection, the description of potent and polyfunctional Gag-specific CD4 and CD8 T-cells in a large majority of HIV-2 donors led us to reason that their continuous renewal is likely to be essential for maintaining long-term immune efficacy against HIV-2.[290,296-297,305] In an earlier study, Sauce et al. described reduced naïve T-cell numbers and haematopoietic stem cells (HPCs) in HIV-1 infected individuals; the severity of these symptoms worsened with disease progression and could be reversed by ART.[191] Moreover, it was discovered that HIV-1 elite controllers lacked these characteristics and retained a healthy population of memory T-cell precursors, suggesting that the retention of these cells is probably important for HIV control. As immune activation was discovered to be a primary driver of these symptoms,[202,205,255] we suspected that HIV-2 infected individuals would be less susceptible to the loss of primary immune resources. In

the context of HIV-2 infection, the preservation of HPCs and naïve T-cells in a majority of HIC2 would help to explain how most HIV-2 infected individuals sustain HIV-2 suppression during the chronic phase of infection, while most HIV-1 infected patients lacked this capacity and ultimately progress to AIDS.[290,297,305,518-520] We embarked on the fine characterisation of donors from the French ANRS CO5 HIV-2 cohort who qualify as HIC2, with particular focus on the potential capacity of these individuals to maintain healthy numbers of naïve and memory T-cell precursors. We describe for the first time that HIV-2 infected patients retain a robust lymphopoietic capacity and T cell production capacity, suggesting the absence of a marked premature immunosenescent phenotype.

3.2 Results

3.2.1 Cohort Characteristics: ANRS CO5 Cohort

A very small proportion of the ANRS CO4 French HIV cohort (n = 46880) was found to be either long-term nonprogressors (LTNPS) or HIV-1 controllers (HIC1). In the absence of treatment, the former group of patients (0.42%) remained asymptomatic for decades and maintain high CD4 cell counts while the latter group (0.22%) spontaneously controlled HIV replication.[521] As HIV-2 was considered to be a model for spontaneous HIV control, due to the high frequencies of HIV-2 controllers, the ANRS CO5 cohort was established in 1994 to broaden the database of HIV controllers for the study of immune correlates of natural HIV control in France.

The CO5 cohort is a national prospective study initiated in 111 clinical centres in France, with inclusion criteria set to recruit HIV-2 infected individuals of at least 18 years of age who have lived in France for at least 1 year and have given informed consent concerning their participation. LTNPS were defined as individuals with asymptomatic HIV infection for at least 8 years, and nadir CD4 T-cell count > 500 cells/µl; while HIV-2 controllers remained asymptomatic for at least five years and had plasma HIV-2 viral load of less than 500 copies/ml. The cohort includes 749 patients, with 6.1% and 9.1% of donors presenting as LTNPs and controllers respectively.[312]

From the CO5 cohort, we were randomly assigned 19 HIV-2 positive individuals with controlled viraemia in the absence of ART (Table 3.1), which we studied and compared them to uninfected (from the French Blood Bank) or HIV-1 infected donors (Table 3.2). The frequency of female patients in the HIV-2 group was 60% (n=12) and the median age

was 49 years (Interquartile rage (IQR): 42-52). At inclusion, median time since diagnosis was 13.6 years (IQR: 9.7-19.3) and median CD4 T-cell count was 893 cells/µl of blood (IQR: 707-1170 cells/µl). Out of these donors, two individuals had detectable, albeit low, HIV-2 viral load (54 and 117 RNA copies/ml). Most donors originated from West African countries (n=16), with a small minority born in France (n=3) and Colombia (n=1). Overall, this subgroup of donors exhibit characteristics that are compatible with the description of HIV-2 controllers (HIC2). They were (i) diagnosed at least 5 years ago and had remained clinically asymptomatic throughout the period of diagnosis; (ii) had a CD4 T cell count of > 400 cells/µl and (iii) a viral load of < 400 RNA copies/ml. HIC2 were compared with HIV-1 controllers (HIC1) from the ANRS CO21 Codex cohort who met the same inclusion and exclusion criteria as HIV-2 controllers;[522] as well as two groups of HIV-1 viraemic individuals (one with CD4 T cell count below 200 cells/ ml and the other with CD4 T cell count above 500 cells/ml) that were recruited from the Pitié Salpêtrière Hospital (France).

Regarding HLA expression, the most common allele, C*04, was carried by nine individuals (45%, five were homozygotes). Overall, 11 individuals (55%) bore homozygote alleles, suggesting a genetic background of limited diversity, and possibly arriving from bottleneck populations. The HIV-1 protective B*57 and B*27 alleles were found in three donors.[523-525] The contribution of HLA genotype to HIV-2 disease progression remains unclear, although alleles associated with susceptibility, such as the HLA-B*35 and B*1503 alleles, were found in eight of our donors.[526-527]

Table 3.1 Clinical attributes of HIV-2 infected patients

ID	Gender	Age (years)	CD4 count (cells/µl)	Time since diagnosis (years)	Viral load (copies/mL)	HLA A	HLA B	HLA C	Country of Birth
012-006	M	44	707	20.5	<40	03/23	35/53	04/04	Ivory Coast
012-009	F	54	1,118	27.3	<100	03/74	14/15	07/08	Guinea Conakry
012-045	F	43	858	22.7	<40	1/33	15/35	04/14	France
012-073	M	52	891	11.3	<100	02/03	49/57	07/18	Ivory Coast
012-084	F	50	1,776	8.8	<40	68/68	07/52	-	Ghana
012-088	M	70	502	7.4	117	02/02	27/53	02/04	Senegal
012-101	F	34	1,212	12.5	<40	03/26	58/58	03/07	The Gambia
013-035	F	48	859	9.2	<40	34/34	15/53	02/04	Guinea Conakry
013-037	M	59	1,300	8.8	<40	01/29	44/57	06/16	Colombia
013-049	F	52	604	25.6	<40	02/23	15/52	02/16	Guinea Conakry
019-010	F	40	1,170	15.4	-	33/68	53/53	04/04	Ivory Coast
023-008	F	41	-	5.0	<40	34/34	7/53	04/07	Ivory Coast
028-012	F	28	827	10.2	<40	-	18/78	05/16	Guinea Conakry
028-016	F	49	895	11.3	<40	02/68	15/51	14/16	Ivory Coast
036-018	F	39	1,036	12.7	<100	03/03	35/53	04/04	Ivory Coast
036-019	M	53	1,228	17.6	<40	23/23	07/14	07/08	Guinea Bissau
045-007	F	50	-	16.2	40	33/33	53/53	04/04	Ivory Coast
051-007	M	48	399	14.4	<40	02/29	39/44	07/16	France
075-001	M	52	413	18.2	54	24/25	35/44	05/12	France
082-005	M	57	1,090	23.0	<100	23/34	53/53	04/04	Ivory Coast

Table 3.2 Clinical attributes of HIV-1 infected patients

Group	Gender (%female)	Age (years)	CD4 count (cells/µl)	Viral load (copies/mL)	Number
HIV1-negative	51%	39 [33-47]	860 [610-1,110]	NA	43
HIV1+ CD4<200	30%	40 [32-48]	82 [42-136]	127,330 [3,040-543,000]	22
HIV1+ CD4>500	11%	39 [31-48]	690 [560-910]	16,540 [800-6,300]	15
HIV1+ controllers	53%	48 [42-51]	751 [519-953]	<40 [<40-57]	13
HIV2+ controllers	60%	49 [42-52]	893 [707-1,170]	<40 [<40-88.5]	19

Values are expressed as: median [interquartile range]

3.2.2 HIV-2 controllers show preserved CD4 and CD8 production capacities

Exhaustion of lymphopoiesis is a major correlate of disease progression in HIV-1 infection. Previous analyses by Sauce *et al.* highlighted a marked decline of primary immune resources in HIV-1 progressors, including CD34+ haematopoietic progenitor cells (HPC) and naïve CD4 and CD8 T-cell numbers;[191] Sauce et al. also measured the number of these cells in uninfected young (18-24 years) and old (75-96 years) donors, and the data are included in this thesis to demonstrate the extent of cellular loss during ageing. These alterations, which are the hallmark features of advanced immune aging,[347] were not observed in HIC1. We therefore performed similar analyses in HIC2, directly analysing the frequency and phenotype of $CD34^+$ haematopoietic progenitor cells (Figure 3.1C). The frequency of circulating HPCs ($CD34^+Lin^-$) in HIC2 was high compared to levels found in untreated HIV-1 infected patients with low CD4 T-cell counts, and equivalent to those observed in HIC1 (Figure 3.1A). In a similar manner, HIC2 displayed a high proportion of lymphoid precursor progenitor cells that were identified by the $CD117^-$ $CD45RA^+$ phenotype among $CD34^+$ cells (Figure 3.1B). Moreover, we found high levels of naïve lymphocytes within the CD4 and CD8 T-cell compartments of these individuals (Figure 3.2A and B). Our results demonstrate the maintenance of effective lymphopoiesis in HIV-2 infection**,** which is likely to be linked to a capacity that sustains the long-term production of naïve CD4 and CD8 T-cells.

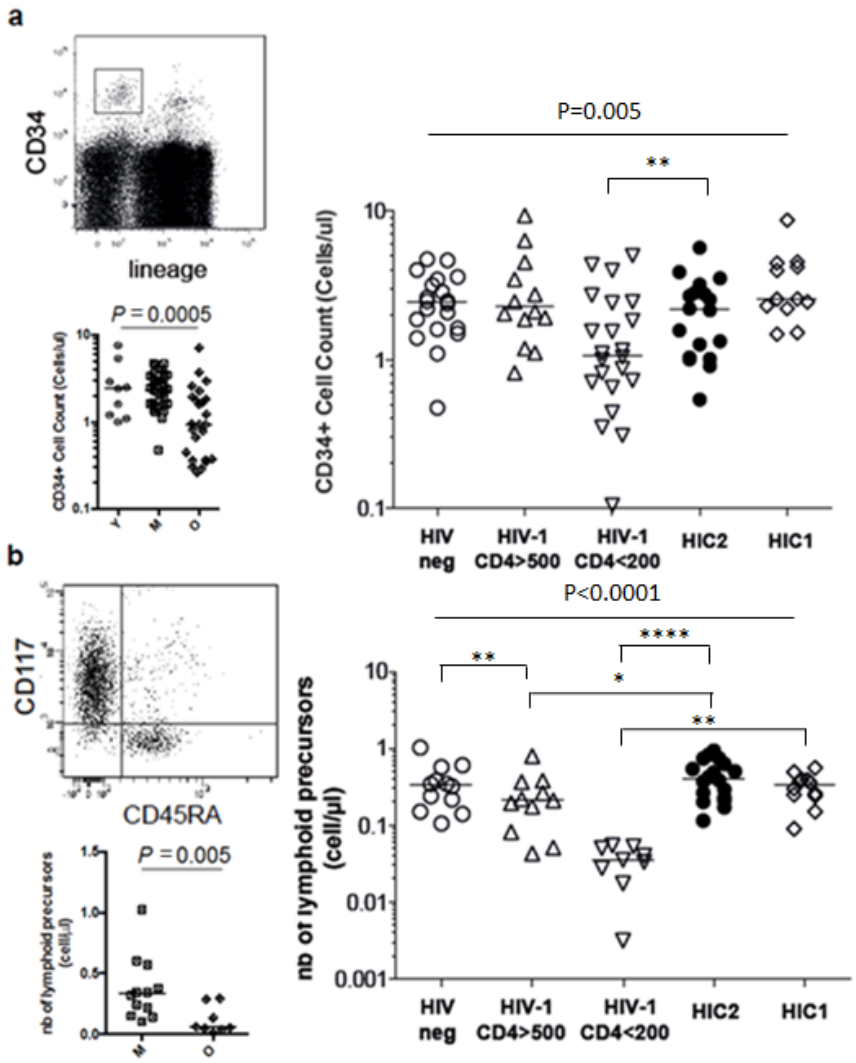

Figure 3.1. Lymphopoietic capacity is preserved in HIC2. (**a**) Representative plots of CD34 and lineage staining to identify HPC within total PBMCs from a HIV-2 infected patient (left panel) and reanalysed data from Sauce *et. al.* showing absolute counts of $CD34^+$ Lin^- cells in (lower left panel) young (Y:18-24 years old (n=9)), middle-aged (M:25-55 years old (n=20)) and elderly persons (O:75-96 years old (n=8)); and (right panel) middle aged healthy adults (HIV neg (n=43)), treatment naïve viraemic HIV-1 infected patients with high (HIV-1 CD4>500 CD4 T-cells/µl (n=22)) or low (HIV-1 CD4<200 CD4 T-cells/µl (n=15)) CD4 T cell count, HIV-1 controllers (HIC1 (n=13)) and HIV-2 controllers (HIC2 (n=19)). (**b**) Representative example of CD117 and CD45RA staining on $CD34^+$ enriched cells to identify circulating lymphoid precursors in a HIV-2 infected patient (lower left panel) and absolute counts of $CD117^-$ $CD45RA^+$ $CD34^+$ cells in (lower left panel) middle aged (n=12) and elderly persons (n=8) and (right panel) middle aged healthy adults (HIV neg), treatment naïve viraemic HIV-1 infected patients with high (HIV-1 CD4>500 CD4 T-cells/µl) or low (HIV-1 CD4<200 CD4 T-cells/µl) CD4 T cell count, HIV-1 controllers (HIC1) and HIV-2 controllers (HIC2). The Kruskal-Wallis and Dunn's Test were used to perform multiple comparisons that included all groups of data. All significant differences are shown. Bars indicate the median.

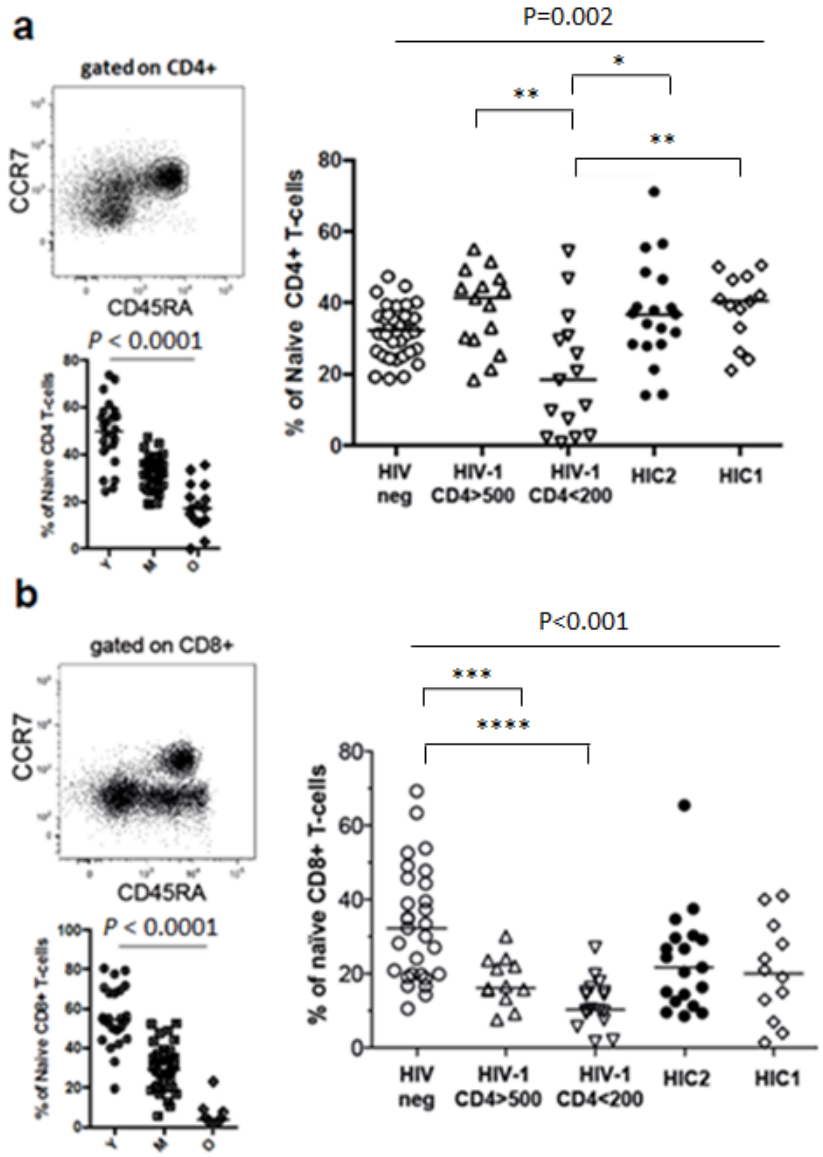

Figure 3.2. Healthy Naïve CD4 and CD8 T-cell populations in HIC2. Representative example of CCR7 and CD45RA staining on CD4 T-cells (**a**) and CD8 T-cells (**b**) to identify naïve cells in a HIV-2 infected patient (left panel) and frequency (right panel) of naïve cells in middle aged healthy adults (HIV neg), treatment naïve viraemic HIV-1 infected patients with high (HIV-1 CD4>500 CD4 T-cells/µl (n=22)) or low (HIV-1 CD4<200 CD4 T-cells/µl (n=15)) CD4 T cell count, HIV-1 controllers (HIC1 (n=13)) and HIV-2 controllers (HIC2 (n=19)). The decline of naïve CD4 and CD8 T-cell percentages in uninfected individuals with age is reflected in the bottom left panels of (**a**) and (**b**) respectively (reanalysed data from Sauce *et. al.*), where groups of young (Y:18-24 years old (n=24)), middle-aged (M:25-55 years old (n=44)) and elderly (O:75-96 years old (n=23) individuals are shown. The Kruskal-Wallis and Dunn's Test were used to perform multiple comparisons that included all groups of data. All significant differences are shown. Bars indicate the median.

In HIV-1 infection, the progressive decrease in naïve T-cell proportions in HIV-infected adults probably results from the antigen-driven maturation of these cells in the absence of adequate T-cell renewal capacity due to the declining thymic output of new cells.[189-190, 528] It was previously reported that thymic function is preserved in HIV-2-infected individuals with low viral load and that HIV-2 replication in thymocytes is impaired;[461-462,529] the combination of both these factors are likely to contribute to the maintenance of high naïve T-cell counts in HIV-2 infected patients and we were keen to determine whether the proportion of naïve lymphocytes in HIC2 was a reflection of a productive thymus. Appay *et al.* measured signal joint T-cell receptor (sjTCR) excision circles (TREC) levels in total PBMCs, and we used these data as a measure of thymic function in analysing HIC2. Naïve CD4 and CD8 T-cell counts directly correlated with the amount of sjTREC/ml (Figure 3.3b), suggesting that the maintenance of these cells was related to sustained thymic activity. The close correlation between naïve CD8 and naïve CD4 T-cell counts in blood that was taken from the same individuals also corroborates our conclusion (Figure 3.3a), since this equilibrium is often lost as we age or become infected with HIV-1.[189,335]

We also estimated the percentage of recent thymic emigrants in peripheral blood by studying CD31 expression, which primarily relates to the thymic history of naïve CD4 T-cells.[530] In line with the TREC data, a strong correlation was observed between the frequency of recent thymic emigrants ($CD31^+$ Naïve CD4 T-cells) and naïve CD8 and CD4 T-cell counts (Figure 3.3b and c). Most importantly, thymic output was also positively correlated to the percentage of CD34+ HPCs, and the ratio of lymphoid to myeloid progenitors, indicating its relationship and dependence on sustained lymphopoiesis (Fig 3.3d). Taken together, these data support the maintenance of a robust lymphopoietic capacity and thymic output in HIC2, which contributes to the preservation of the naïve CD4 and CD8 T-cell compartments. The latter is central to the mounting of

de novo responses, as recently shown in elderly subjects and old vaccinated primates;[531-532] and is critical for the sustenance of effective T-cell responses against retroviruses that demonstrate high evolutionary rates, such as HIV-1 and HIV-2.[533-537]

3.2.3 Lymphocytes from HIC2 appear less senescent than HIV-1 infected subjects

We subsequently analysed for differences in the memory lymphocyte compartments of HIC2 versus their HIV-1 infected counterparts. While the percentages of memory CD8 T-cells in HIC2 were higher than uninfected controls, they were less sizeable than HIC1 or HIV-1 infected individuals with higher CD4 T-cell counts (Figure 3.4b), suggesting a state of reduced antigen-induced maturation or inducement of T-cell homeostasis to drive the maturation of naïve CD8 T-cells in HIC2 as compared to HIV-1 infected subjects. The number of memory CD4 T-cells were comparable between HIC2 and uninfected subjects, but were much higher in HIC2 than in HIC1 and viraemic HIV-1 individuals with high and low CD4 T-cell counts (Figure 3.4c); this data suggest that memory CD4 T-cells are better preserved in HIC2 than in the different settings of HIV-1 infection.

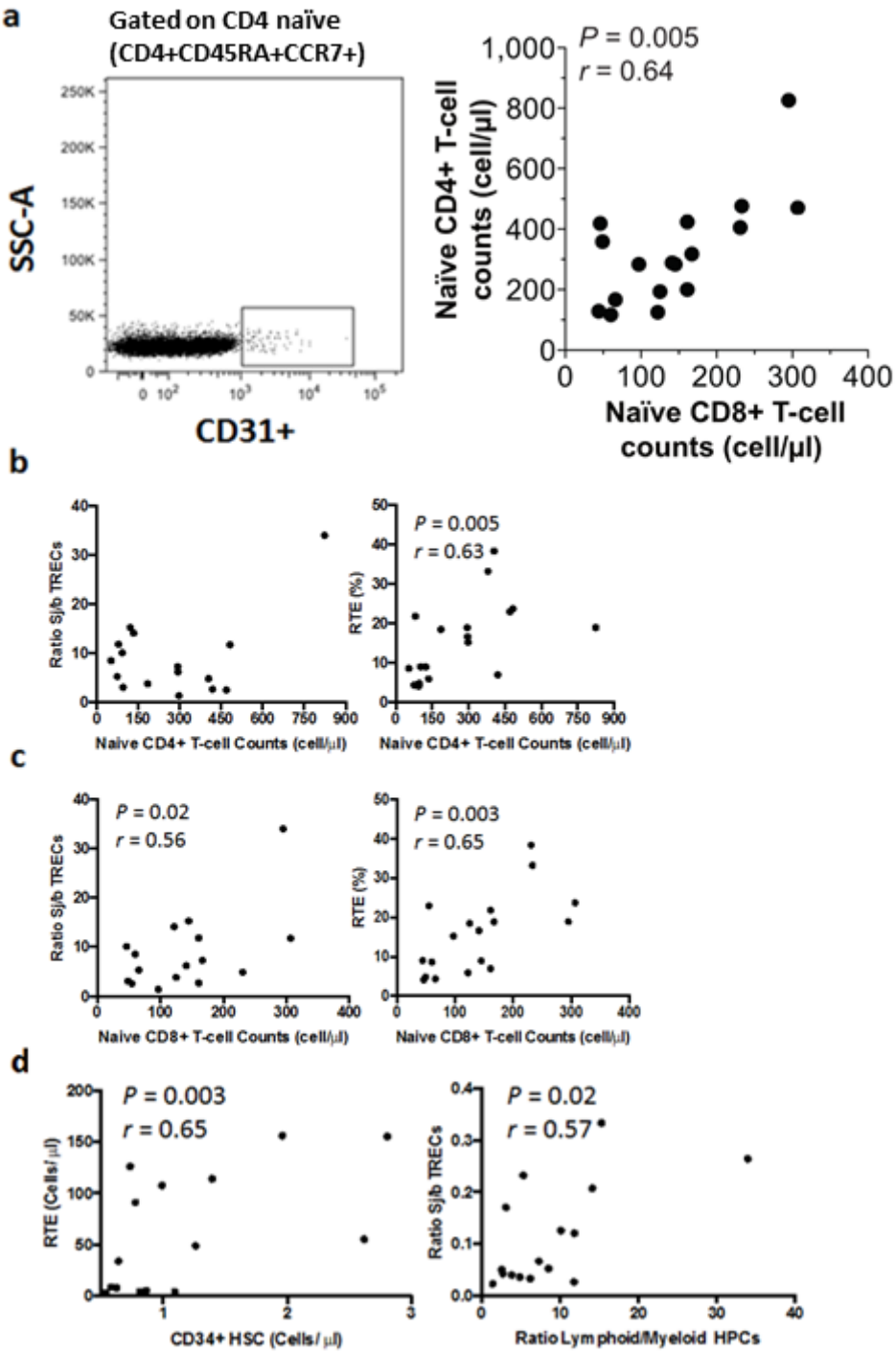

Figure 3.3. Correlation between thymic function and Naïve T-cells. (**a**) Representative example of CD31+ naïve CD4 T-cells (left panel); and correlation between naïve CD4 and CD8 T-cells (right panel). (**b**) Correlations between thymic output (left panels) and naïve CD4 (**b**) and CD8 (**c**) T-cell counts in HIC2. (middle and right panels) Correlations between the percentages and counts of recent thymic emigrants and naïve CD4 (**b**) and CD8 (**c**) T-cells in HIV-2 controllers. (**d**) Correlations between thymic output and the number of circulating CD34+ HSCs (left) and ratio of lymphoid to myeloid progenitors (right). The Spearman‘s rank test was used to determine correlations.

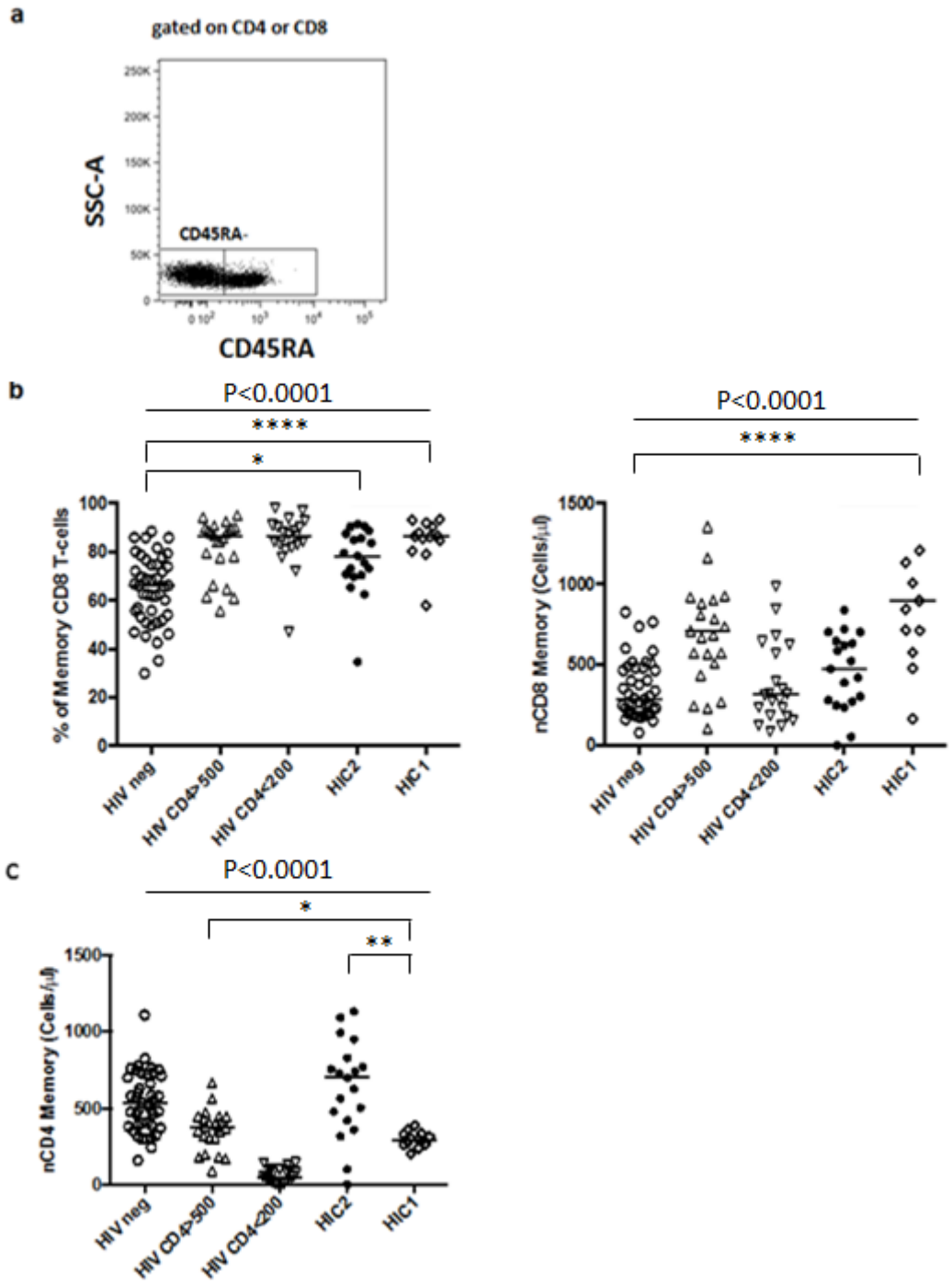

Figure 3.4. Comparison of memory T-cell compartments. Representative example of CD45RA- memory CD4 and CD8 T-cells (**a**) and frequency (left panel) and number (right panel) of memory CD8 T-cells. (**b**) Memory CD4 T-cells in HIC2 versus HIV-1 infected and uninfected donors. (**c**) The graphs include T-cells from middle-aged healthy adults (HIV neg), treatment naïve viraemic HIV-1 infected patients with high (HIV-1 CD4>500 CD4 T-cells/µl) or low (HIV-1 CD4<200 CD4 T-cells/µl), HIV-1 controllers (HIC1) and HIV-2 controllers (HIC2). The Kruskal-Wallis and Dunn's Test were used to perform multiple comparisons that included all groups of data. All significant differences are shown. Bars indicate the median.

More importantly, we were interested in the quality of memory T-cells in HIC2 donors. The high expression of CD57 in T-lymphocytes is generally associated with repeated antigen encounters, leading to loss of proliferative capacity and polyfunctionality; and these cells are observed in high frequencies during HIV-1 or CMV infections.[255,405,452,454,538-539] The numbers of CD57+ memory CD8 T-cells were comparable between HIC2 and uninfected donors, and lower in HIC2 than viraemic HIV-1 infected donors, suggesting a less senescent profile of CD8 memory T-cells in HIV-2 versus HIV-1 infected donors (Figure 3.5b). Nevertheless, the percentage of CD57+ expressing memory CD8 T-cells in HIC2 showed close correlation with activation antigens such as CD38 and HLA-DR (Figure 3.6c), but not with the proliferation marker, Ki67. These observations suggest that the level of systemic immune activation remains coupled to the generation of terminally differentiated CD8 T-cells in HIC2.[540-541] The percentages and numbers of CD57+ memory CD4 T-cells in HIC2 appeared higher than HIC1 and HIV uninfected donors, although these differences were not statistically significant (Figure 3.5a). Surprisingly, the number of CD57+ CD4 memory T-cells in HIC2 were correlated with CD4 T-cell counts in HIC2 (Figure 3.7b), suggesting that the increased presence of terminally differentiated CD4 T-cells may be related to the increased survivability of CD4 T-cells in non-progressing HIC2.

For the majority of HIV-1 infected donors, there is a strong inverse relationship between systemic immune activation and CD4 T-cell levels in HIC1 donors.[229] Since HIV-2 infection is associated with lower levels of immune activation,[80-81,284,317-318,529] we reasoned that HIC2 may be exempt from exhibiting a similar phenotype. Similar to what Leligdowicz *et al.* observed in a Caio cohort, the expression of activation markers HLA-DR and CD38 on both CD4 and CD8 T-cells did not relate to CD4 T-cell counts in HIC2.

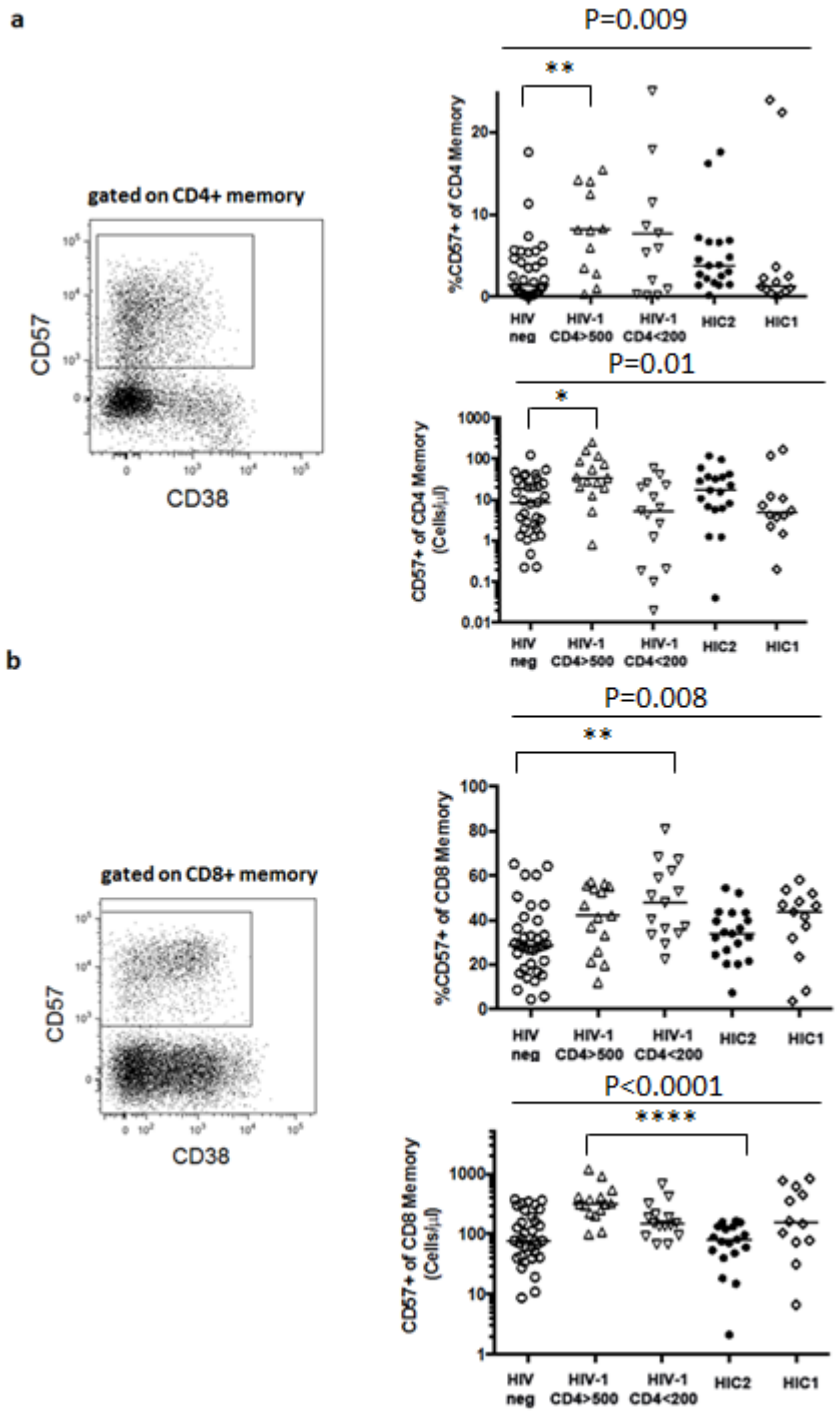

Figure 3.5. CD57 expression in HIC2 versus HIV-1 infected donors. (left panels) Representative staining to identify CD57+ memory CD4 (**a**) and CD8 T-cells (**b**); (right panels) frequency and number of CD57+ memory CD4 (**a**) and CD8 T-cells (**b**) in middle aged healthy adults (HIV neg), treatment naïve viraemic HIV-1 infected patients with high (HIV-1 CD4>500 CD4 T-cells/µl (n=22)) or low (HIV-1 CD4<200 CD4 T-cells/µl (n=15)) CD4 T cell count, HIV-1 controllers (HIC1 (n=13)) and HIV-2 controllers (HIC2 (n=19)). The Kruskal-Wallis and Dunn's Test were used to perform multiple comparisons that included all groups of data. Bars indicate the median.

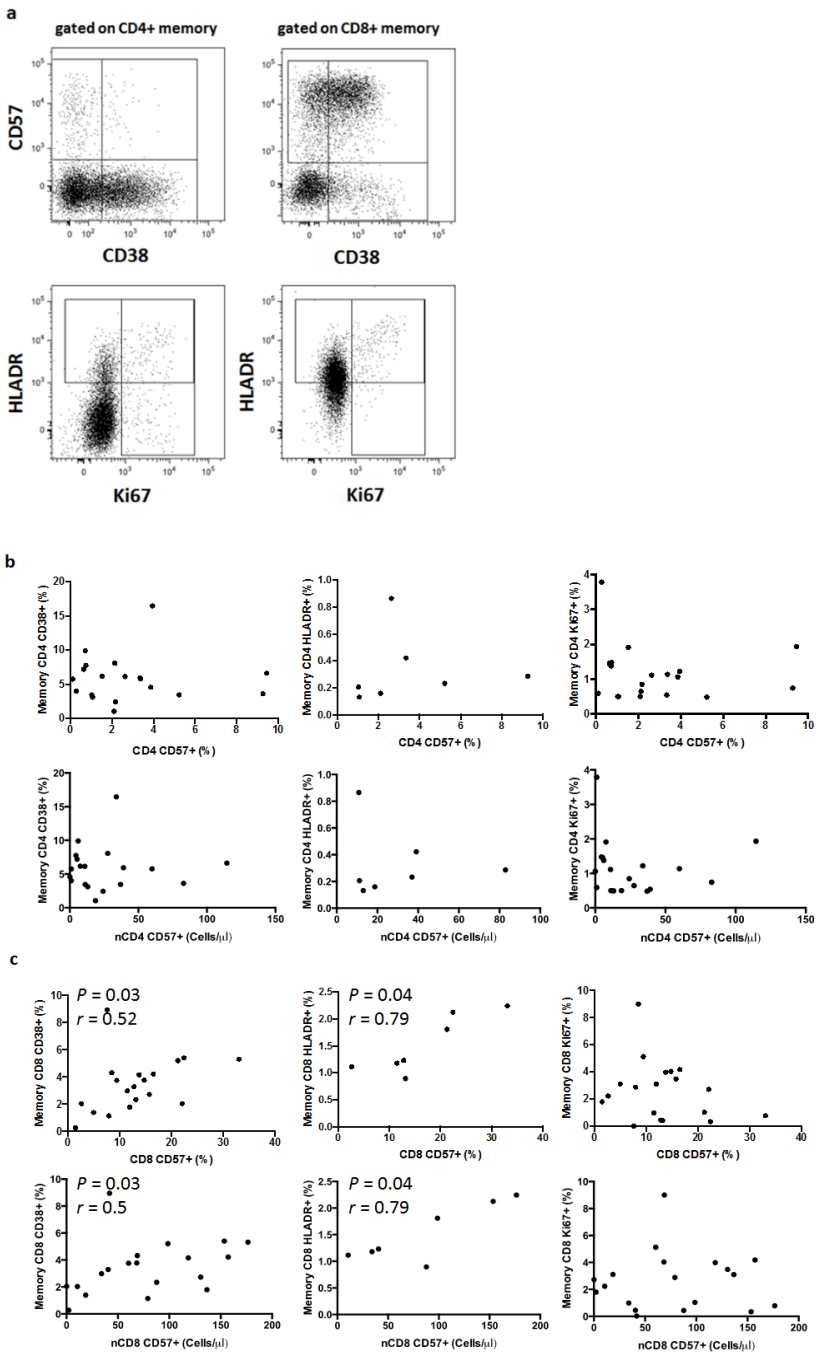

Figure 3.6. Correlation between CD57 expression and expression of activation markers CD38 and HLA-DR in memory CD8 T-cells. (**a**) Representative staining to identify CD38+ (top) and CD57+ (bottom) memory T-cells in (left) CD4 and (right) CD8 T-cells; (**b**) Correlation between the percentages (top) and numbers (bottom) of CD57+ CD4 T-cells with the percentages of CD38+ cells (left), HLA-DR+ cells (middle) and Ki67+ memory CD4 T-cells (right). (**c**) Correlation between the percentages (top) and numbers (bottom) of CD57+ CD8 T-cells with percentages of CD38+ cells (left), HLA-DR+ cells (middle) and Ki67+ memory CD8 T-cells (right). The staining of PBMCs with anti-HLADR antibodies was only possible for eight of the nineteen HIC2 subjects.The Spearman‘s rank test was used to determine correlations.

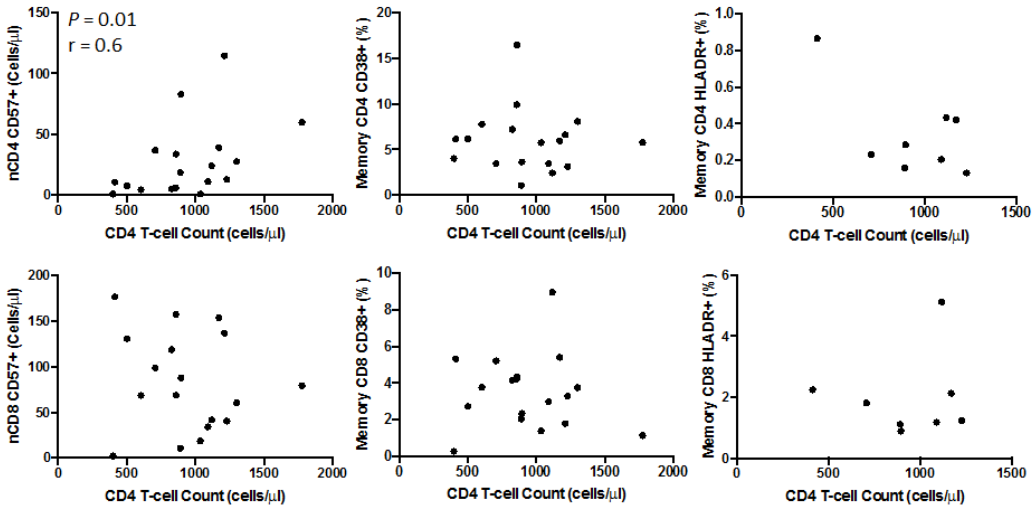

Figure 3.7. Correlation between CD4 T-cell counts and the expression of T-cell senescent and activation markers. Correlation between CD4 T-cell counts and the expression of senescence and activation markers in CD4 (top) and CD8 (bottom) T-cells. The relationship with the number of CD57+ cells (left), and the percentages of CD38+ (middle) and HLA-DR+ (right) cells are shown. The staining of PBMCs with anti-HLADR antibodies was only possible for eight of the nineteen HIC2 subjects..The Spearman‘s rank test was used to determine correlations.

3.3 Discussion

Disease progression in HIV-1 is dependent on the rate of CD4 decline, and on the rate at which HIV-specific CD8 T-cells are able to sustain the cellular immune response over time;[517,542] both processes are directly influenced by the host ability to renew lymphocytes that are depleted, either from direct HIV infiltration or in the performance of their cytotoxic functions. In HIV-1 infection, both the *de novo* production of new lymphocytes, and the peripheral homeostatic division of existing lymphocytes are critical for naïve T-cell renewal;[543-545] it was, therefore, not surprising that HIV-1 infected individuals who fared better possessed a robust capacity to sustain both the production of haematopoietic progenitors and stronger thymic output.[191] Since the onset of immunosenescence is also a result of the decreased production of primary immune cells, the ability to quantitatively and qualitatively study attributes of HPCs directly from blood, facilitates the study of its relevance in settings of chronic infections with pathogens that establish high latency, as the latter are extremely adept at driving memory inflation.[377] The characterisation of HPCs, therefore, allows us to study immunosenescence with greater breadth and depth, especially since the conventional and preferred approach has been to focus on the study of "senescent" T-cells that are $CD28^{-}CD57^{+}$ and reveal diminished functionality and proliferative capacity. [538-539]

Our study recapitulates the loss of HPC numbers, and more specifically, the loss of HPCs that are primed for lymphoid differentiation with age and HIV-1 disease progression. However, the investigation of these building blocks of innate and adaptive immunity has remained hitherto unchartered with respect to HIV-2 infection. This study, together with the assessment of CD8 T-cell effector functions that will be discussed in the subsequent chapter, represent a most comprehensive T-cell study in the context of HIV-2 infection.

Although the immune dynamics responsible for the slow progression of HIV-2 infection has remained puzzling for years, we are gaining momentum in piecing together the precise picture of events that contribute to this largely non-progressive infection with a HIV virus. At equivalent plasma viral loads, proviral DNA loads are very similar between HIV-1 and HIV-2 infection.[306,515] When left unchecked, the survival of HIV-2 infected patients is strongly related to both CD4 T-cell counts and plasma viral load.[83] However, in contrast to HIV-1, HIV-2 is often controlled to undetectable plasma viral loads in the absence of cART, which most likely involves a complex immune-related mechanism. We demonstrate here that the competent preservation of lymphopoietic and naïve T-cell output, despite having survived a median age of 13.6 years since infection (albeit with controlled viral replication), may be pertinent to the control of a strain of virus that if allowed to survive, is able to demolish CD4 T-cell immunity in a manner and time frame that resembles its devastating HIV-1 counterpart.[87,287] Owing to the maintenance of a strong lymphopoietic capacity, there is a greater potential for HIC2 to sustain T-cell mediated immune responses than HIV-1 infected subjects, therefore allowing the former to suppress viraemia over decades of chronic and latent infection without exhibiting AIDS symptoms.

The requirement and importance of a sustained virus-specific response in long-standing HIV-2 infection may seem trivial, and paradoxical for an infection that is characterised by controlled viremia in most of the infected population; but the high proviral loads – equivalent to HIV-1 – observed during HIV-2 infection presents a potential risk of re-emergence.[306,515] Evidence from HIV-1 infection and their respective controllers, suggests that HIV-1 is able to reemerge from a latent infection within resting memory CD4 T-cells, which provide a stable reservoir for the integrated proviral genome to exist for decades.[546-549] Considering the substantial proviral load that exists during HIV-2 infection

and the simultaneous preservation of high CD4 T-cell numbers, it seems reasonable to expect a significant risk of HIV-2 reactivation during prolonged infection. However, the lack of phylogenetic separation between proviral HIV-2 sequences across decades of asymptomatic HIV-2 infection suggests that this might be unlikely throughout most of adulthood.[80,537] The fear and possibility of reemergence during chronic HIV-1 infection, even when viral RNA has been reduced to undetectable levels, has nevertheless necessitated a requirement of lifelong ART.[550] That an equivalent requirement may be redundant in the majority of HIC2 cases hints at the natural existence and sustenance of adaptive immune responses that remain capable of suppressing HIV-2 replication – this argument is supported by the durability of HIV-2 specific responses that is observed across the majority of HIC2 cases.[290,297,299,305] As with latent CMV infection, we cannot exclude the possibility of a higher risk of reemergence during the later phases of life, and believe that HIC2 should be continuously monitored as they age.

We also believe that HIV-2 may be continuously replicating in lymphoid tissue despite the observation of undetectable plasma viral loads, as continuous antigen stimulation is required for the persistence of HIV-2 specific T-cells, which have been shown to occur at higher frequencies in HIV-2 infection than in HIV-1 infection.[305] Long-lived antigen-specific memory CD4 and CD8 T-cells require different stimuli for their survival; while antigen availability has been shown to be obligatory for the retention of memory CD4 T-cells, the subsistence of specific memory CD8 T-cells clones in the periphery appears to be more reliant on the secretion of homeostatic cytokines, such as IL-15 and IL-7.[375,551-555] Nevertheless, the preserved functionality and cytotoxic capability of CD8 T-cells rely on the continuous presence of antigen.[556-562] Hence, the preservation of highly effective HIV-2 specific CD4 and CD8 T-cells across decades of seemingly asymptomatic infection is likely to be fueled by repeated exposures to antigen, albeit undetectable by

current laboratory standards. The description of clear correlations between Gag-specific T-cell responses and viraemia, even in prolonged asymptomatic HIV-2 infection, further supports their relevance in preventing the salient re-emergence of HIV-2 virus.[281,290,306]

Before probing into aspects of T-cell functionality, we were keen to apply the conventional approach of studying CD57 expression on T-cells from HIC2, as a substantial amount of research exists to support their relevance and implication on T-cell functionality. [452,454,538-539] In addition, only a very small minority of existing HIV-2 studies have attempted to study the relationship between the systemic expression of CD57 on T-cells and immune activation or disease progression, although several have demonstrated a relationship between immune activation and HIV-2 disease progression.[285-286,317,529] We observed that the proportion and number of senescent CD57+ CD8 T-cells were lower in HIC2 than their HIV-1 infected counterparts (particularly those with CD4 T-cell counts <200 cells/μl) and comparable to uninfected controls; suggesting that memory CD8 T-cells from HIC2 possess a 'younger' phenotype, even when compared with HIC1. Nevertheless, the visibly larger memory CD8 T-cell compartment in HIC2, relative to uninfected individuals, and the observed correlation between the expression of CD57 and T-cell activation markers on memory CD8 T-cells, suggest that even in clinically asymptomatic HIV-2 infection, the systemic levels of immune activation is high enough to influence T-cell homeostasis.[202,224,449] Unfortunately, strict limitations in sample availability prevent the broader and deeper study of immune activation and its implications in HIC2, although earlier studies have clearly demonstrated higher CD4 and CD8 T-cell activation in HIC2 than in uninfected controls.[285]

The few studies that have examined CD57 expression on T-cells were performed by Duvall *et. al.*, where sizeable populations of CD57+ HIV-2 specific CD4 and CD8 T-cells

were described, despite the fact that a majority of HIV-2 specific CD4 T-cells did not express CD57.[297,305] However, Duvall *et al.* also reported that the HIV-2 specific CD4 T-cells that *did* express CD57 were more polyfunctional.[297] In line with their conclusions, we observed that the majority of memory CD4 T-cells in HIC2 were CD28+CD57-, but observed higher percentages of CD57+ memory CD4 T-cells than uninfected controls and HIC1 even though these observations did not translate to differences in absolute numbers. Most notably, we observed a positive relationship between the number of CD57+ memory CD4 T-cells and the CD4 T-cell counts of HIC2. Since HIV-2 specific CD4 T-cells appear to abstain from a CD57+ phenotype,[305] it is reasonable to conclude that the bulk of CD57+ memory CD4 T-cells in HIC2 are unlikely to be specific for HIV-2, and we believe that the strongest argument for the relationship between the numbers of CD57+ memory CD4 T-cells and the CD4 T-cell count is the better overall survival of CD4 T-cells, which is either necessary or reflective of durable viraemic control over HIV-2. Nevertheless, HIV-2 specific CD4 T-cells will benefit from the generalised resilience of the CD4 T-cell compartment and, in turn, participate more effectively in viral clearance.

The possession of durable memory CD4 T-cells in HIV-2 infected subjects could be related to a resistance towards apoptosis that was conferred during primary infection and retained throughout the chronic phase of infection. Indeed, it has been shown that CD4 T-cells from HIV-2 infected individuals are less prone to apoptosis during the chronic phase of infection and that this resistance to apoptosis strongly associated with non-progressing disease.[317] Our observation of higher numbers of memory CD4 T-cells in HIC2 than all other HIV-1 infected groups, including HIC1, is consistent with this argument. The resilience of memory CD4 T-cells in HIV-2 infected individuals may be related to the activity of Nef, which is likely to be most significant during acute infection, but could still play a role during chronic infection because of active viral replication. Indeed the ability

of Nef to downregulate TCR:CD3 remains functionally important in viraemic HIV-2 infected subjects – as the potency of Nef-mediated downmodulation of TCR:CD3 remained positively correlated with CD4 T-cell counts in these subjects.[328] By downregulating the molecules that directly respond to antigenic stimulation, Nef effectively renders CD4 T-cells refractory to the functional impairment caused by persistent and repeated antigen stimulation. In fact, HIV-2 Nef also suppresses the induction of Fas, Fas-L, PD-1 and CTLA4 expression and in this context, renders CD4 T-cells less susceptible to apoptosis that may be induced by antigen-specific or bystander activation. The expression of PD1 and CTLA4, further permits effective cytotoxic killing of HIV-2 infected cells by CD8 T-cells.[217] The imprint of Nef activity on CD4 T-cells during primary infection could be stable over time or sustained by the low levels of HIV-2 replication during asymptomatic infection; such an argument would explain the specific uncoupling of CD57 expression with activation markers in memory CD4 T-cells but not memory CD8 T-cells in our group of HIC2.

Overall, we demonstrate that asymptomatic HIV-2 infection is supported by healthy populations of memory T-cell precursors, including naïve T-cells and haematopoietic progenitors. We show that these properties of T-cell renewal are likely to be linked to preserved thymic function – an observation that is consistently described in other studies.[461,529] We also demonstrate that in general, memory CD8 T-cells display a 'younger' phenotype in HIC2 than in HIV-1 infection with respect to CD57 expression – comparable to the levels of expression observed in uninfected individuals despite evidence of heightened systemic immune activation. Since immune activation is a key driver of disease progression in HIV-1 infection, this elevation presumably falls below the threshold required to trigger and snowball the progression of AIDS in the majority of HIV-2 cases, since most infected individuals identify as HIC2. The absence of samples

from HIV-2 progressors in our cohort is a major limitation that prevents the conduct of a more comprehensive study to explore the relevance of our findings to a broader scope of HIV-2 infection that spans various degrees of HIV-2 progression, and a future study capable of the latter is a necessary complement to this work.

Chapter 4: Potent T-cell Responses and Viral Suppression in HIC2

4.1 Introduction

In this chapter, we focus more specifically on the contribution of cellular functionality in the control of HIV-2 infection. Viral, genetic, and host immune responses probably synergise to establish HIV-1 control in HIC1;[269-279] and with respect to host immune responses, the frequency and magnitude of Gag-specific CD8 T-cell responses is most often singled out as a major contributor to spontaneous HIV-1 control during both acute and chronic phases of HIV-1 infection.[269-271,564] Out of the 6 major HIV-1 proteins (Tat, Rev, Nef, Gag, Pol and Env), Gag-specific responses are the first to emerge following HIV-1 infection, and these early responders – Gag-specific CD8 T-cells – were able to effectively eliminate infected cells.[565] In addition, the frequency and magnitude of Gag-specific responses are the strongest predictor of survival past infancy. In cases of vertical infection, strong correlations between Gag-specific T-cells and viral load, as well as subsequent survival, emerge from as early as the first 3 months of life.[566]

Earlier studies on T-cell responses in HIV-2 patients have indicated that Gag-specific CD8 T-cells are a major correlate of HIV-2 control. In fact, this relationship could be localised to a single Gag epitope and be detected at a lower threshold among HIV-2 infected subjects than HIV-1 infected subjects, as the mapping of CD8 T-cell responses to multiple HIV-1 Gag epitopes are required to demonstrate a similar strength of association in the latter group.[271,281,564] Accordingly, we were keen to determine whether protective HIV-2 specific T-cell functionality was preserved in our cohort of HIC2, and to devise a

method to reasonably compare the phenotype and suppressive capacity of HIV-2 specific T-cells with HIV-1 specific T-cells, given the lack of HIV-2 progressors in our cohort.

While less attention has been given to CD4 T-cells, emerging evidence suggests that virus specific CD4 T-cells may be more relevant to disease control in HIV-2 than in HIV-1, as prospective studies that include HIV-1 controllers, indicate that the high quality of the CD4 T-cell response in HIC1 are more likely to be an outcome, rather than a determinant, of effective HIV-1 control.[281,567-569] The higher frequencies of Gag-specific CD4 T-cells during HIV-2 infection, relative to HIV-1 infection, is a common trope in the HIV literature, and this observation is significantly associated with better prognosis and prevails even when donors are matched for CD4 counts.[296,305] In comparisons where differences in the frequencies of Gag-specific CD4 T-cells are less apparent, CD4 T-cells from HIC2 reveal higher proliferative prowess and polyfunctionality than HIC1.[297,306,570-571] Moreover, Foxall *et al.* demonstrated an inverse relationship between the magnitude of the HIV-2 specific CD4 T-cell response and proviral load; an observation which strongly reflects their importance in establishing viraemic control during acute infection.[306] Taken together, the combined preservation and potency of CD4 and CD8 T-cell functions during HIV-2 infection, are more likely to result in an effective synergy that sustains viral suppression than cases of HIV-1 infection; and the better preservation of CD4 T-cells in the former is likely to be indispensable in driving the disparate outcomes of disease progression that have been described for each of the two viruses.[296,305,317]

Given the importance of HIV specific T-cell responses in establishing viraemic control over both HIV-1 and HIV-2, we reasoned that the persistent renewal of lymphocytes that was demonstrated in Chapter 3 would show more relevance to asymptomatic phenotype of HIC2 if they also demonstrated strong effector functions. From the French ANRS CO5

HIV-2 cohort, we describe strong Gag p26-specific cytokine responses from CD4 and CD8 T-cells; as well as strong CD8 T-cell mediated suppression of HIV-2 infection of autologous CD4 T-cells *ex vivo*. The magnitude of these T-cell responses correlated well with the CD4 T-cell counts of our donors, suggesting that these factors are important correlates of HIV immunity.

4.2 Results

4.2.1 HIC2 maintain robust T-cells cytokine responses

Figure 4.1 shows the gating strategy for the detection of specific cytokines through intracellular cytokine staining. In order to assess the overall magnitude of HIV-2 specific T-cells in HIC2, we performed intracellular IFNγ staining on T-cells from PBMCs that were stimulated with 15-mer overlapping peptides that spanned the entire proteome of the HIV-2 p26 protein; 16 (84.2%) and 17 (89.5%) of the 19 donors generated IFNγ+ CD4 T-cell and CD8 T-cell responses respectively (Figure 4.2a). Among p26 responders, an ostensible proportion of TNFα, MIP1β and IL2 producing CD4 and CD8 T-cells were also observed, with the expression of CD40L and CD107 mostly confined to CD4 and CD8 T-cells respectively.

The expression of IL2 by CD4 and CD8 T-cells were however, less frequently observed than the expression of other cytokines, including IFNγ and TNFα (Figure 4.2a). Among p26 responders, the frequencies of IFNγ+ CD8 T-cells were higher than observed for CD4 T-cells, although these differences were not statistically significant when we compared their absolute numbers (Figure 4.2b). Nevertheless, the median frequency of Gag-specific CD4 T-cells were approximately one magnitude lower in this cohort than reported in previous HIV-2 studies,[297,306] which suggest that the consensus sequence used in generating the overlapping HIV-2 Gag peptides may not include the immunodominant MHC-II epitopes that are pertinent in this cohort. Among responders that mounted both CD4 and CD8 T-cell responses to p26, a clear relationship was observed between the number of IFNγ secreting CD4 and CD8 T-cells, suggesting that their survival might be intimately linked (Figure 4.2c). Overall, our data parallels the observations from other

studies in showing that HIC2 retain robust virus specific responses despite the appearance of asymptomatic infection and undetectable plasma viraemia.

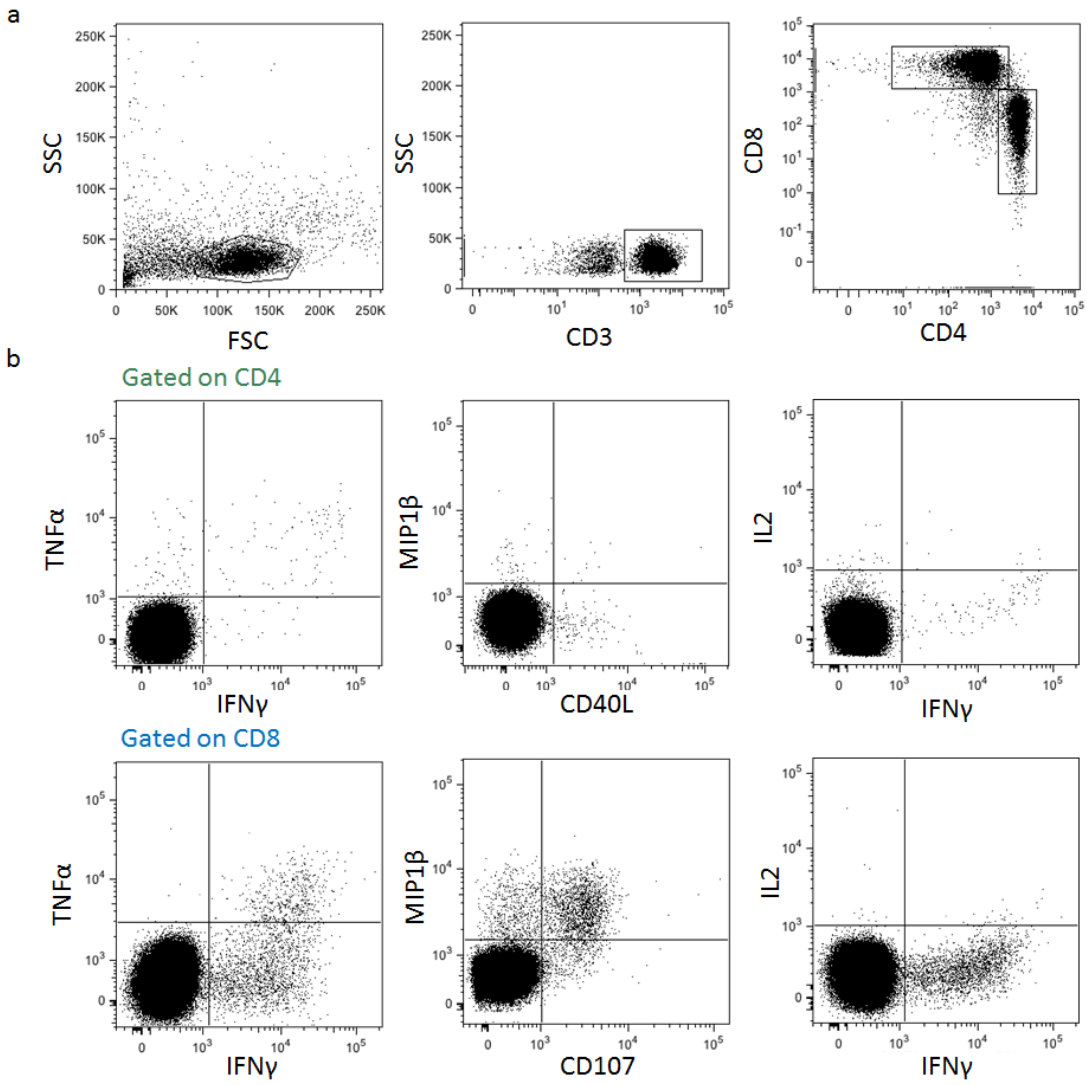

Figure 4.1: HIV-2 Gag-specific responses in HIC2. (**a**) Representative staining and gating strategy for determining the percentage of cytokine secreting CD4 and CD8 T-cells. After gating for singlets (not shown) and lymphocytes and CD3 positivity, cells were gated on CD4 and CD8 expression. (**b**) Within CD4 and CD8 T-cells, the gating of IFNγ, TNFα, CD107, CD40L, MIP1β and IL2 expressing cells are demarcated.

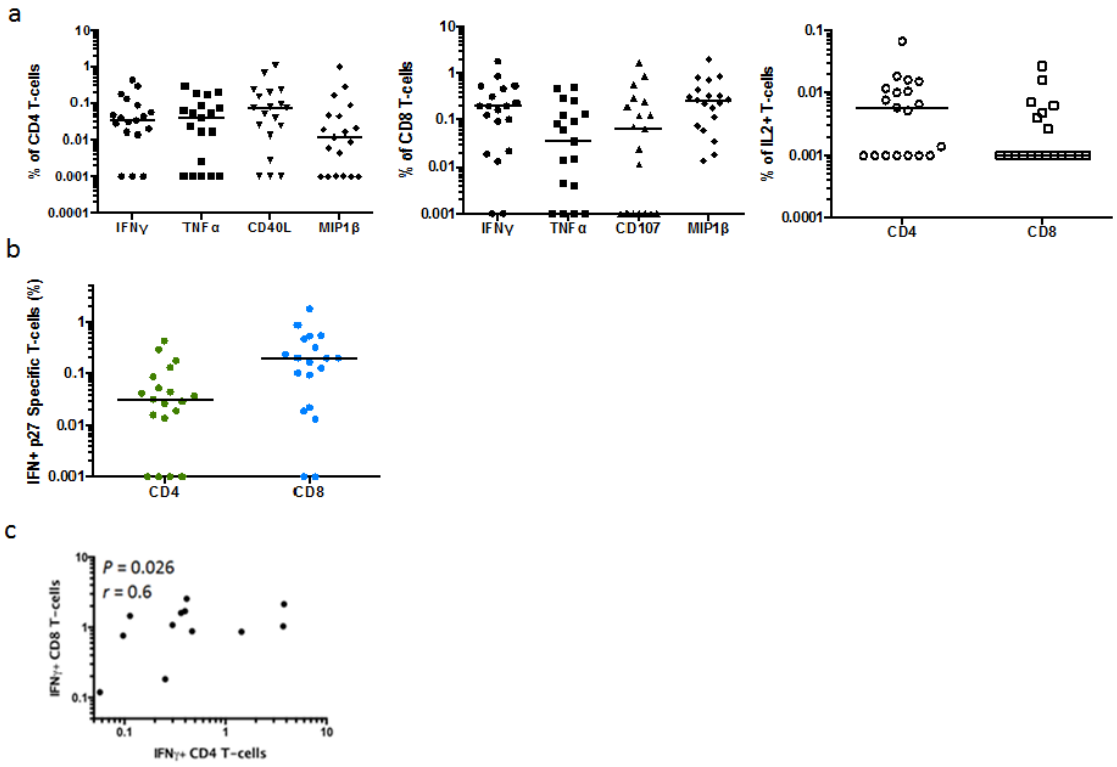

Figure 4.2: Gag-specific CD4 and CD8 T-cells in HIC2. (**a**) Percentages of cytokine secreting CD4 (left) and CD8 T-cells (middle) upon 6h stimulation with 15-mer peptides spanning the entire p26 proteome. Percentages of IL2+ p26 specific CD4 and CD8 T-cells (right). (**b**) A comparison between the percentages of IFNγ+ CD4 and CD8 T-cells. (**c**) Positive correlation between the numbers (cells/µl) of IFNγ+ CD8 and CD4 T-cells. The Spearman's rank test was used to determine correlations. Values equivalent or lower than zero were assigned the value 0.001. Bars indicate the median.

4.2.2 Phenotype of p26-specific CD8 T-cells

In viraemic HIV-2 individuals, the IFNγ response of Gag-specific CD8 T-cells was found to correlate inversely with viraemia, suggesting that they may play an important role in controlling HIV-2 infection. Therefore, we further committed to phenotyping CD8 T-cells that were specific for p26. The magnitude of p26-specific CD8 T-cell responses detected in most patients were comparable to those observed in HIC1 (Figure 4.3a), although the comparison remains unbalanced as different antigens are required for the stimulation of HIV-1-specific responses. These cells exhibited other effector functions, including the expression of TNFα, MIP-1β and CD107 (Figure 4.3b), highlighting a polyfunctional profile that has been previously described.[281,290,298]

Next, we proceeded by using recombinant tetrameric MHC class I-peptide complexes to more precisely capture the memory differentiation phenotype of p26-specific CD8 T-cells (Figure 4.4a). We analysed in particular, CD8 T-cells that were restricted by HLA-B*5301, as B*53 was the most common allele among our donors (Table 3.1). We were able to generate HLA-B*5301 tetramers with the immunodominant p26 epitope TPYDINQML (TL9);[292,572] All of the seven HLA-B*53 patients tested positive for TL9-specific CD8 T-cells. We also detected HLA-B*1401 restricted, DA9-specific CD8 T-cells in two out of two HLA-B*14 patients.

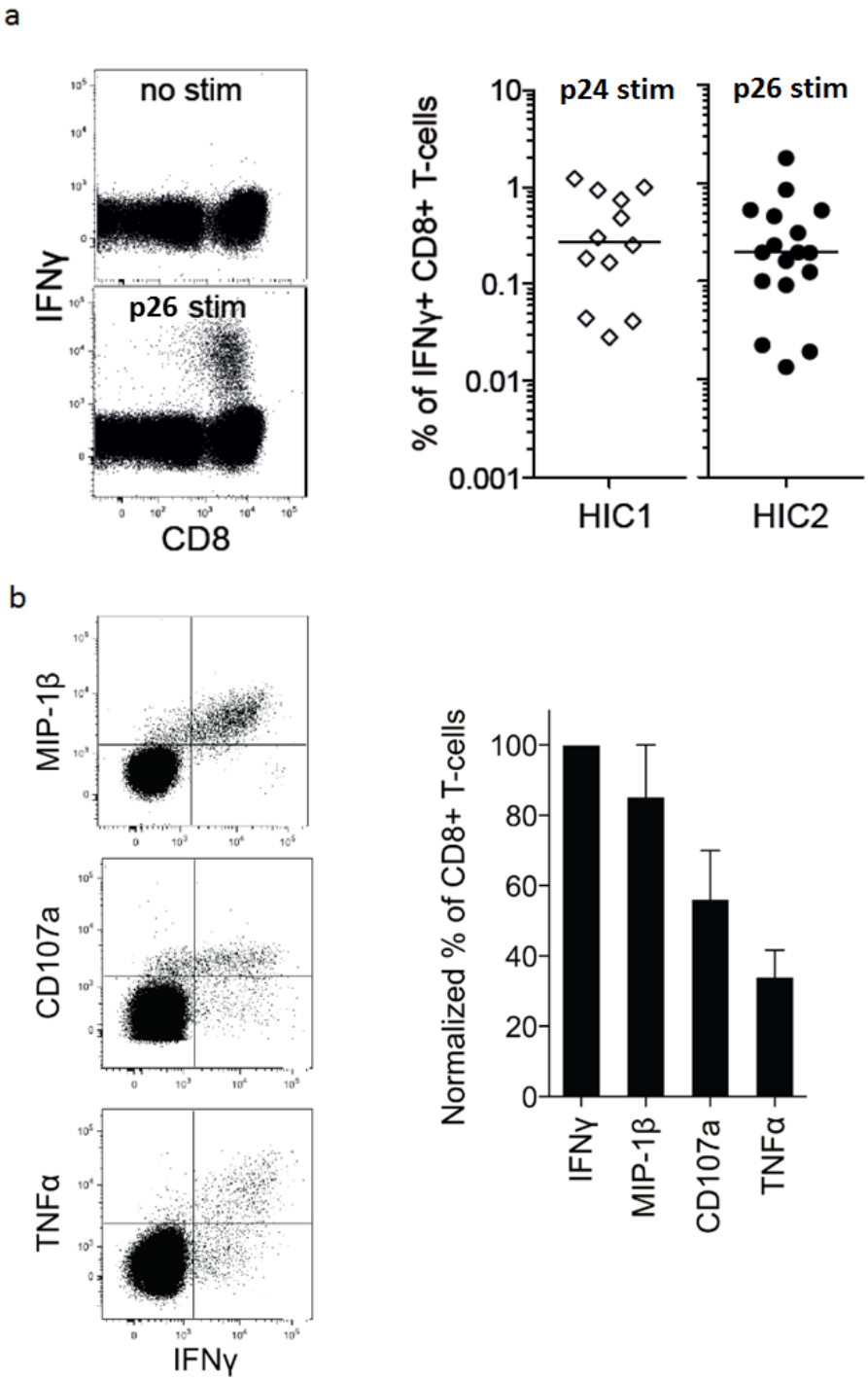

Figure 4.3. Polyfunctional cytokine response by CD8 T-cells. (**a**) Representative example of IFNγ secretion in CD8 T-cells from a HIV-2 infected patient unstimulated or upon stimulation with p26 overlapping peptides (left panel) and frequencies of $IFN\gamma^+$ CD8 T-cells in HIV-1 controllers (HIC1) and HIV-2 controllers (HIC2) upon stimulation with HIV-1 and HIV-2 peptides respectively (right panel). (**b**) Representative examples of IFNγ, CD107a, TNFα and MIP-1β secretion in CD8 T cells from a HIV-2 infected patient upon stimulation with p26 overlapping peptides (left panel). Co-expression of intracellular MIP-1 β, CD107a and TNFα by IFNγ+ CD8 T-cells (right panel).

The differentiation phenotype of HIV-2-specific CD8 T-cells was subsequently assessed by the surface expression of CD27, CD28, CCR7, CD45RA and CD127 (Figure 4.4b), and compared to the phenotype of HLA-B*2705 restricted, KK10-specific CD8 T-cells from HIC1, the latter selected as the prototype of p24-specific effector cells in HIV-1 infection.[573-574] Overall, both groups of HIV-specific CD8 T-cells displayed similar phenotypes, being mainly $CD27^{+}CCR7^{-}CD127^{-}$, with slight but noticeable differences. HIV-2-specific CD8 T-cells trended towards higher CD45RA expression and the expression of CD28 was significantly higher on HIV-2-specific CD8 T-cells (Figure 4.5a); in line with previous observations of HLA-B*3501 restricted p26 NPVPVGNIY (NY9)-specific CD8 T-cells. Together, the data indicate that HIV-2-specific CD8 T-cells are in general less differentiated, displaying an even younger phenotype than highly effective p24-specific CD8 T-cells from HIC1. Accordingly, HIV-2 specific CD8 T-cells are likely to retain proliferative potential, as previously suggested.

Nevertheless, despite their relatively early memory differentiation state, HIV-2-specific CD8 T-cells express high levels of the transcription factors T-BET and EOMES (4.5b), which regulate memory differentiation and the acquisition of effector functions.[575] In line with this observation, HIV-2-specific CD8 T-cells also express high intracellular levels of Granzyme B that were comparable to the levels expressed by HIC1 (Figure 4.5a). Taken together, our data support the presence of robust Gag-specific CD8 T-cell responses in HIC2, which are perpetuated by cells that exhibit an early differentiation phenotype but possessed strong effector potential.

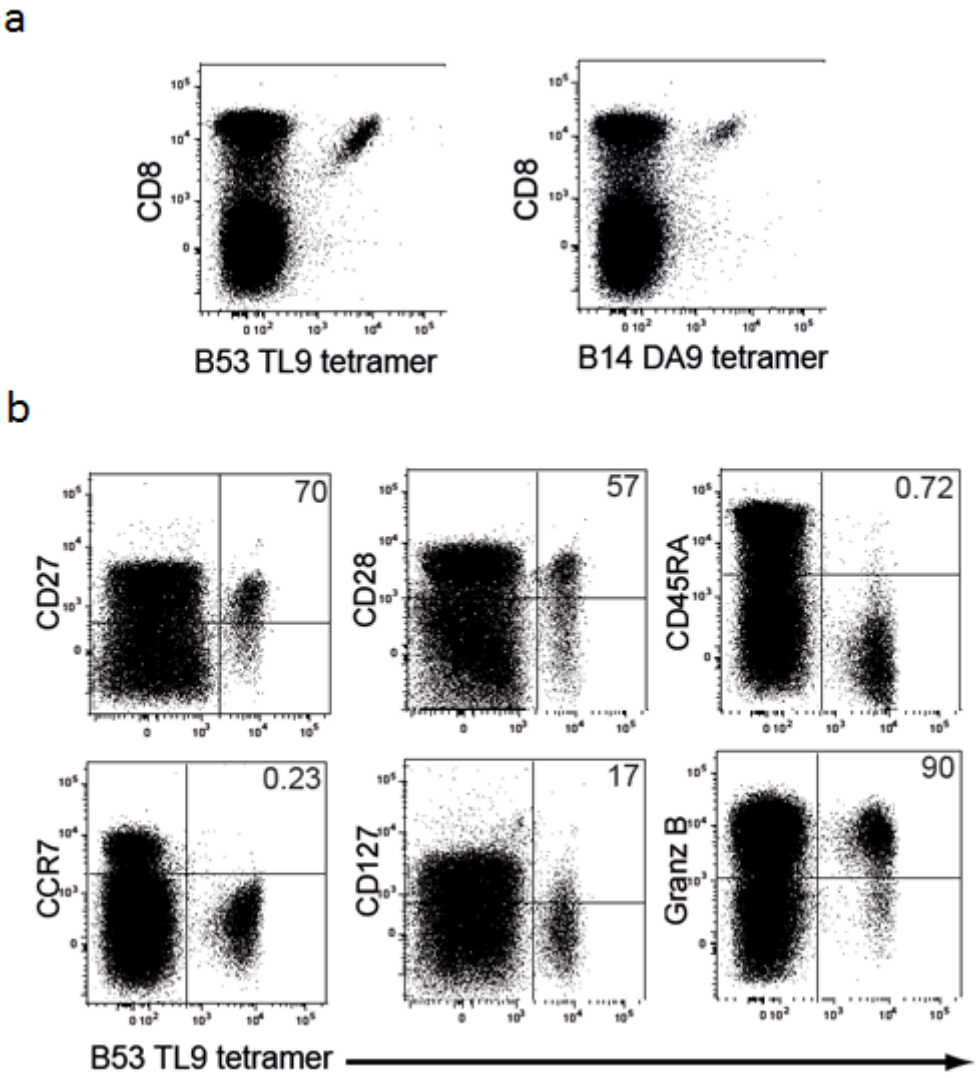

Figure 4.4. Characterisation of p26-specific CD8 T-cells in HIV-2 infected individuals by MHC I tetramers. (**a**) Representative staining of HLA-B*5301 restricted p26 TPYDINQML (TL9) (left panel) or HLA-B*1401 restricted p26 DRFYKSLRA (DA9) (right panel) specific CD8 T-cells from HIV-2 infected patients. (**b)** Representative staining profile for the expression of the cell surface markers CD27, CD28, CD45RA, CCR7, CD127 and intracellular Granzyme B in TL9-specific CD8 T-cells.

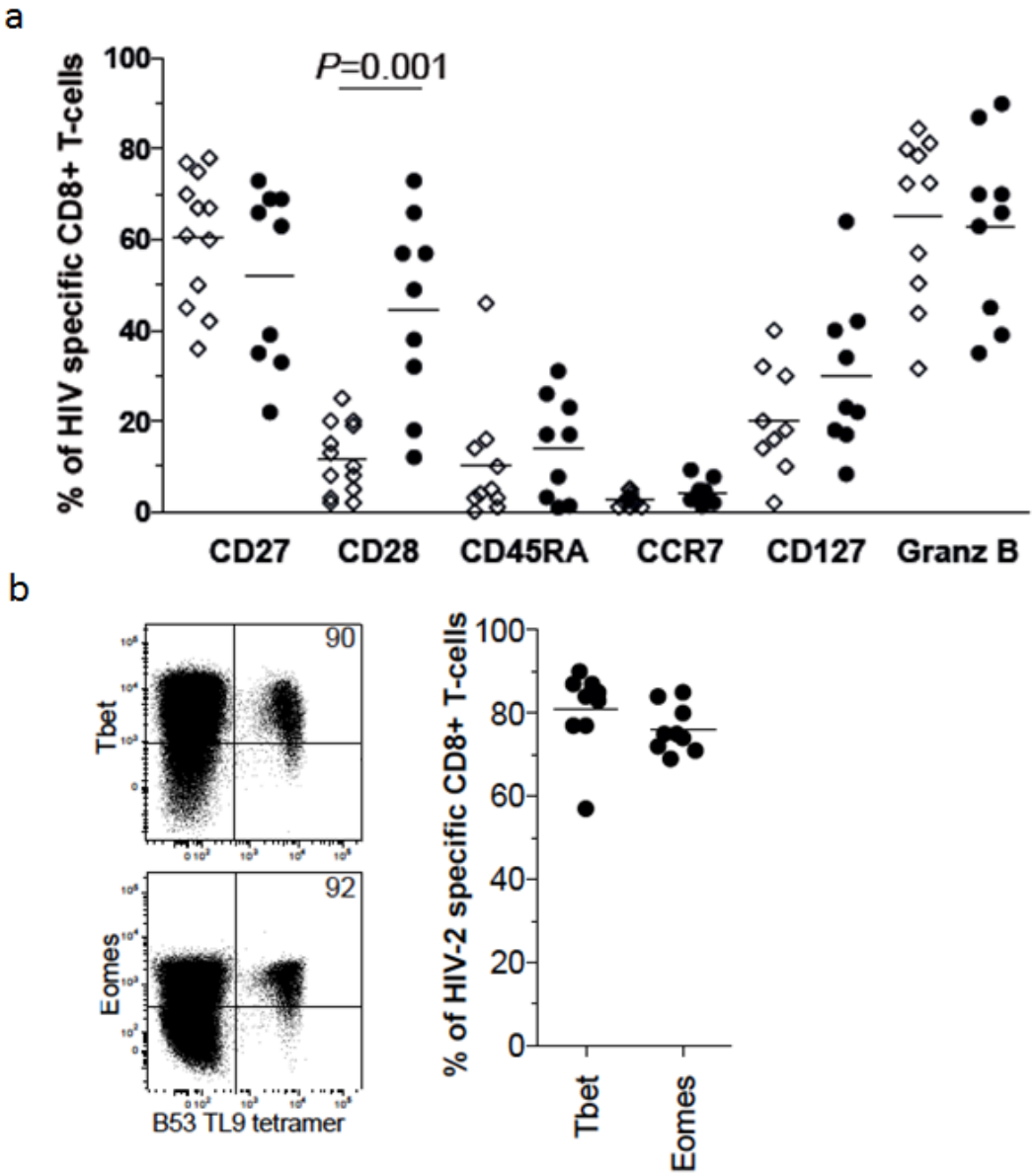

Figure 4.5. p26 specific CD8 T-cells are less differentiated but retain strong effector potential. (**a**) Comparative expression of the cell surface markers CD27, CD28, CD45RA, CCR7, CD127 and intracellular Granzyme B between B27 KK10-specific CD8 T-cells from HIV-1 infected patients (open diamonds (n=12)) and B53 TL9 or B14 DA9-specific CD8 T-cells from HIV-2 infected patients (full circles (n=9)). The Mann-Whitney test was used for comparing groups. Bars indicate the median. (**b**) Representative staining for the intracellular expression of the transcription factors T-bet and Eomes in B53-TL9 specific CD8 T-cells (left panel). Expression of the transcription factors T-bet and Eomes in B53 TL9 or B14 DA9 specific CD8 T-cells from HIV-2 infected patients (right panel).

4.2.3 Unstimulated CD8 T-cells from HIV-2 controllers suppress HIV-2 replication

In order to examine whether the strong phenotypic potential of HIV-2 specific cells translated into an effective capacity to suppress HIV-2 infection. Our collaborators Angin *et al.*, from the Pasteur Institute, performed virus suppression assays using T-cells from the same HIC2 donors. HIV-1 suppression assays have provided the most robust method to distinguish between the functional capacities of CD8 T-cells in HIV-1 controllers and non-controllers; with HIV suppression assays, the group previously showed that HIC1 possessed HIV-specific CD8 T-cells with a robust capacity to suppress infection by killing HIV-infected autologous CD4 T-cells. The group successfully adapted this approach to the study of HIV-2 by using an SBL virus strain and using supernatant p26 concentration as a read-out for determining the efficacy of viral suppression.

Angin *et al.* found that CD8 T-cells from HIC2 have a strong capacity *ex vivo* to suppress HIV-2 infection of autologous CD4 T-cells (Figure 4.6a), with a median reduction in p26 production of 2.7 logs [IQR: 1.3-3.3]) (Figure 4.6b). The viral suppression capacity shown by CD8 T-cells from HIC2 was remarkably high, comparable to the levels observed with CD8 T-cells from HIC1 donors. Similar to what they observed in HIV-1 controllers, the capacity of CD8 T-cells from HIV-2 controllers to suppress HIV-2 replication was relatively stable over time (Figure 4.6c). There was, however, some heterogeneity in HIV-2 suppressive capacity among our donors, with log p26 reduction ranging from 0.02 to 3.7 (Figure 4.6b). With respect to disease progression, the CD8 T-cells from two patients with the steepest decline in CD4 T-cell counts in the 3 years preceding this study (92.5 and 215.7 cells/mm3/year) showed no *ex vivo* capacity to suppress HIV-2 infection in our assay (Figure 4.7a).

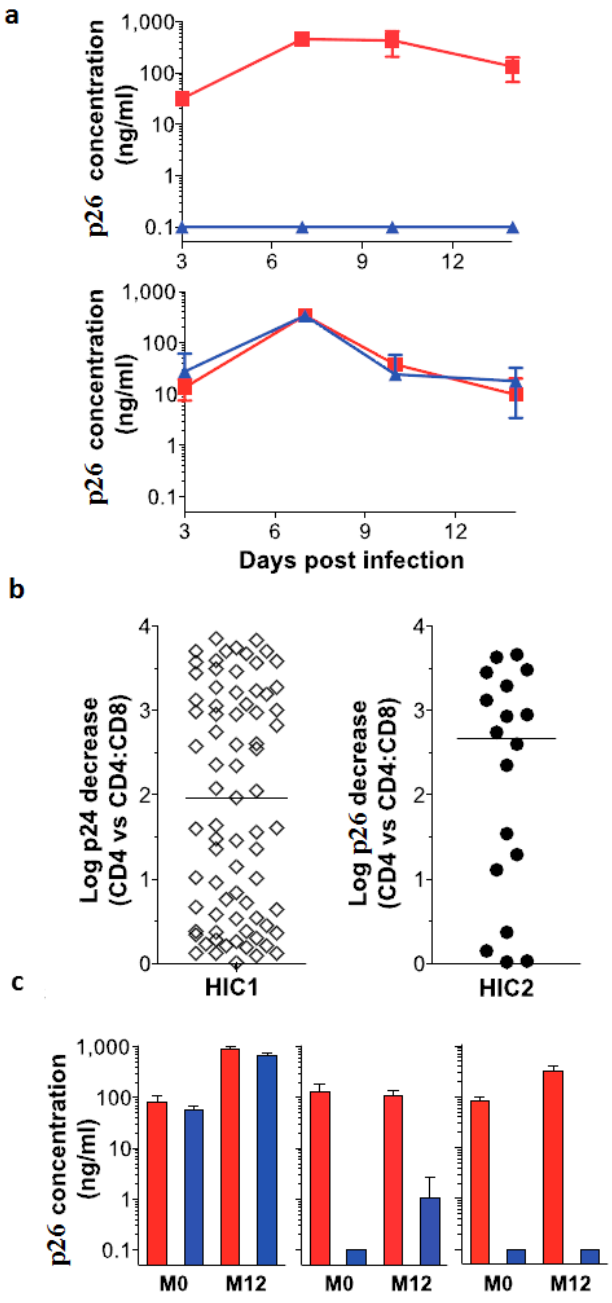

Figure 4.6. *Ex vivo* viral suppressive capacity of HIV-2 infected individuals CD8 T-cells. (**a**) Data from Angin et. al. showing examples of p26 concentration in the supernatant of CD4 T-cells isolated from an HIV-2 controller (top) or an healthy donor (bottom), alone (red) or co-cultured with autologous CD8 T-cells (blue) at different days post infection. Error bars indicate the mean and standard deviation. (**b**) Log p26 decrease (p26 concentration in supernatant of infected CD4 T-cells alone divided by p26 concentration in supernatant of infected CD4 T-cells co-cultured in presence of autologous CD8 T-cells) in HIV-1 (open diamonds) or HIV-2 controllers (black circles). (**c**) p26 concentration in supernatant of CD4 T-cells isolated from three different HIV-2-infected individuals, alone (red columns) or co-cultured with autologous CD8 T-cells (blue columns) at time of inclusion (Month 0, M0) or 12 months later (M12). Error bars indicate the mean and standard deviation. Bars indicate the median

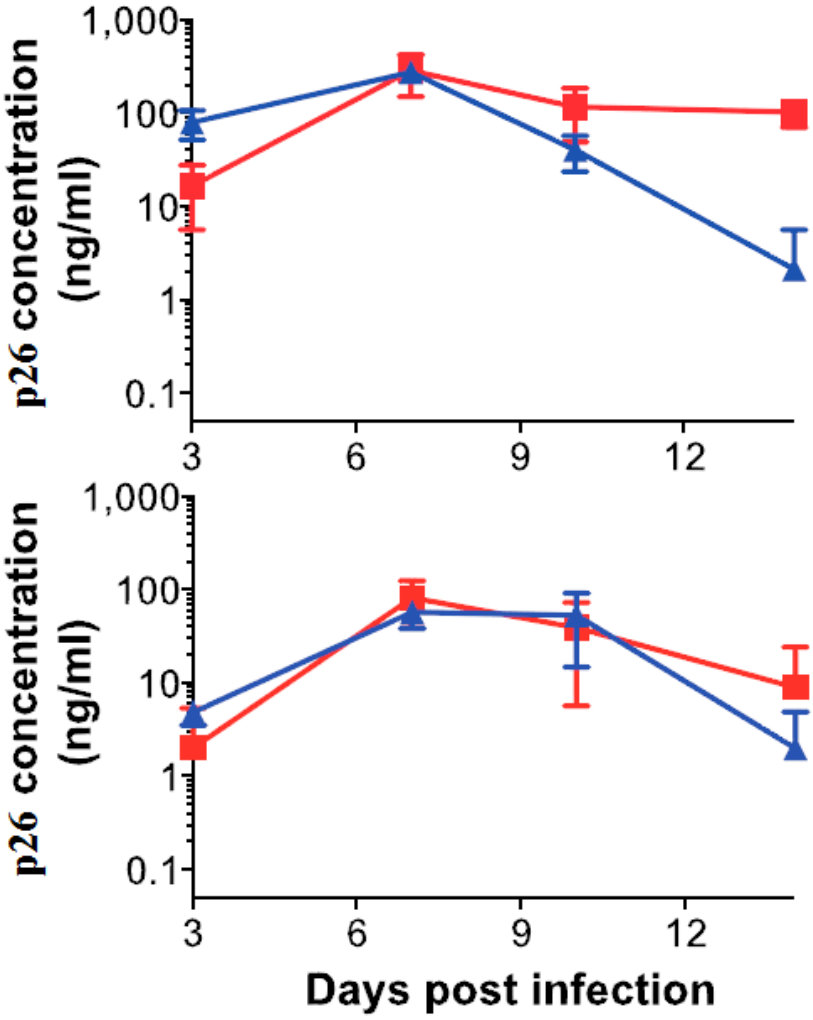

Figure 4.7. HIV-2 viral suppressive activity is absent in donors with steep CD4 T-cell decline. Data from Angin et. al. showing p26 concentrations in the supernatant of CD4 T-cell cultures from HIV-2 infected individuals with rapidly decreasing CD4 T-cell counts (92.5 cells/mm3/year (left) and 215.7 cells/mm3/year (right)), alone (red) or co-cultured with autologous CD8 T-cells (blue) at different days post infection. Error bars indicate the mean and standard deviation of measurments from the same subject.

4.2.4 HIV-2 Gag-specific Responses Relate to Markers of Disease Progression

In HIV-1 infection, a small subset of treatment-naïve patients experience eventual CD4 T-cell decline despite years of undetectable viral replication and are referred to as "elite controller progressors".[191] The lack of prospective HIV-2 studies has not allowed the distillation of a similar population in HIV-2 infection, but does not preclude its existence. Accordingly, we attempted to look for correlations between the strong suppressive activity and cytokine response of HIV-2 specific CD8 T-cells and CD4 T-cell counts from donors.

Although CD4 T-cell counts from our HIC2 cohort do not differ from uninfected controls (Figure 4.8b), we observe clear relationships between the magnitudes of the cytokine response of HIC2 donors with their CD4 T-cell count (Figure 4.8a). Specifically, the number of IFNγ+ and IFNγ+TNFα+ CD4 and CD8 T-cells were positively correlated to CD4 T-cell count. Likewise, a positive correlation between the CD4 T-cell counts of our donors and the capacity of CD8 T-cells to mediate HIV-2 suppression *ex vivo* was discovered; the latter also appeared to be more related to the p26-specific CD4 T-cell – rather than CD8 T-cell – response (Figure 4.8b and 4.9). Overall, the data suggest that the potency of HIV-2 specific T-cell activity in HIC2, specifically, the potency of cytotoxic and cytokine secreting potential, dictate their potential for disease management with the outcome of preserving CD4 T-cell counts. Nevertheless, our current data is insufficient in allowing us to comment on whether avid Gag-specific CD4 T-cell responses are a contribution or consequence to effective HIV-2 CD8 T-cell suppression, but their consistent association with spontaneous HIV-2 control suggests more of the former.[297,305-306]

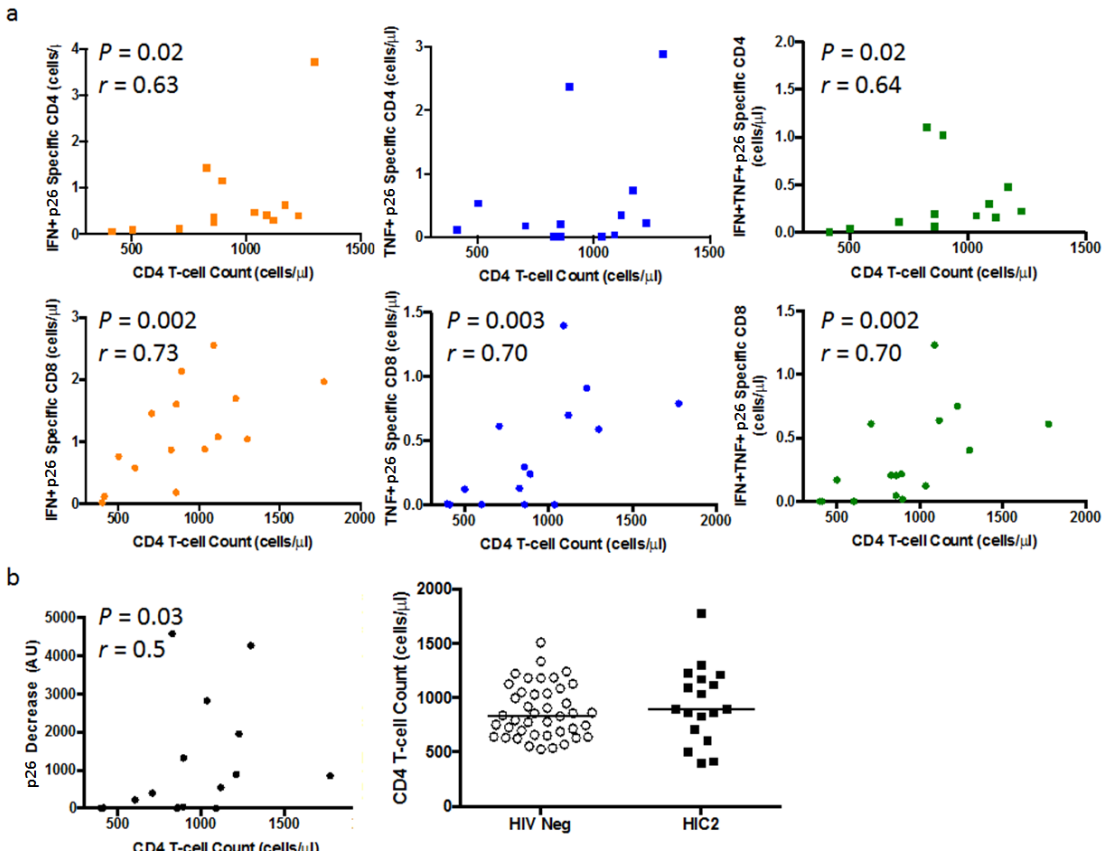

Figure 4.8. Correlation between HIV-2 specific responses and CD4 T-cell count. (**a**) Correlations between the number of p26-specific CD4 (top) and CD8 (bottom) cytokine secreting cells with CD4 T-cell count. The numbers of IFNγ+ (left), TNFα+ (middle) and IFNγ+ TNFα (right) CD4 and CD8 T-cells are shown. (**b**) (left) Correlations between CD4 T-cell counts (cells/mm3) and log p26 decrease (p26 concentration of supernatant from cultures of infected CD4 T-cells alone divided by p26 concentration of supernatant from infected CD4 T-cells co-cultured with autologous CD8 T-cells) in HIV-2-infected individuals. (right) CD4 T-cell counts from HIV negative and HIC2 populations. The Spearman's rank test was used to determine correlations. Bars indicate the median.

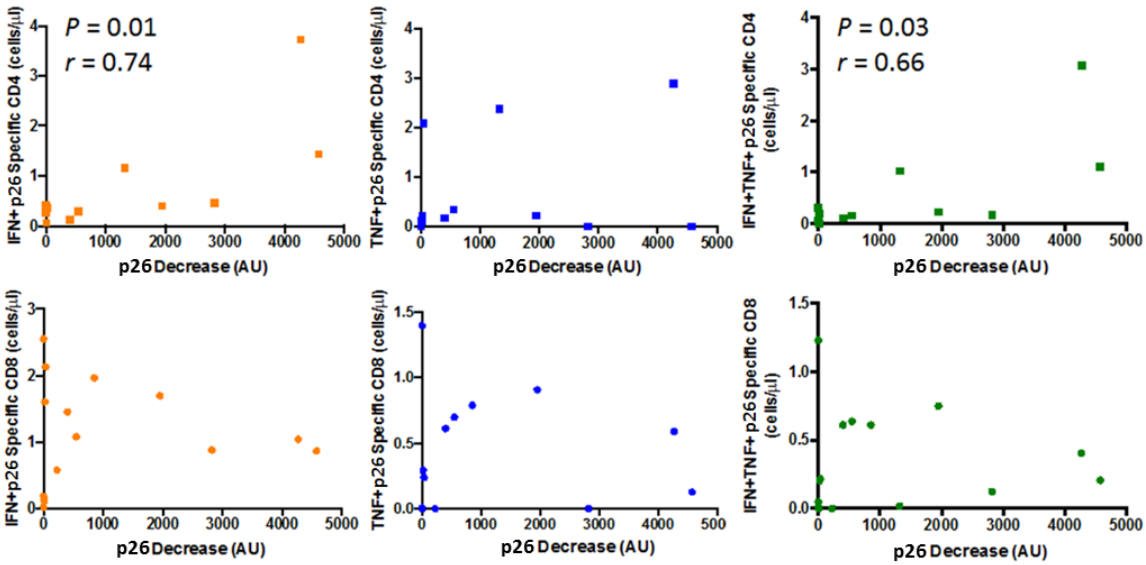

Figure 4.9. Correlation between HIV-2 specific CD8 T-cell cytotoxic capacity and Gag-specific T-cell responses. (**a)** Correlations between p26 decrease and the numbers of cytokine secreting p26-specific CD4 (top) and CD8 (bottom) T-cells. The numbers of IFNγ+ (left), TNFα+ (middle) and IFNγ+ TNFα (right) of CD4 and CD8 T-cells are shown. The Spearman's rank test was used to determine correlations.

4.3 Discussion

The strong lymphopoietic and thymic potential in HIC2, and the renewal of naïve CD4 and CD8 T-cells would be superfluous if unaccompanied by strong HIV-2 specific effector functions. We demonstrate in this chapter that HIV-2-specific T-cells from HIC2 harbour particularly potent effector functions that relate to an exceptional capacity to suppress viral replication in autologous CD4 T-cells. The strong cytokine response from HIV-2 Gag-specific CD8 T-cells in HIC2 parallels the levels observed in HIC1, and reflects a polyfunctionality that relates to better clinical outcome.

Reminiscent of the overall younger phenotype of memory CD8 T-cells in terms of CD57 expression, HIV-2 p26-specific are $CD27^{+}CCR7^{-}CD127^{-}$ and exhibit high expression of CD28 and to a lesser extent, CD45RA. Indeed, $CCR^{-}CD27^{+}CD28^{+}$ CD8 T-cells are known to express low levels of CD57 and PD-1.[406,576] CD28 and CD27 are costimulatory receptors that regulate T-cell activation and act in the further generation of antigen-primed cells. The combination of CD27 and CD28 is particularly useful in identifying different stages of T-cell differentiation as subsequent loss of expression appears to be irreversible. [406,576] Accordingly, CD8 T-cells can be separated into 'early', 'late' or 'intermediate' memory cells in a linear sequential pattern characterised by the initial loss of CD28 and subsequent loss of CD27 expression (i.e. CD28+CD27+, CD28-CD27+ and CD28-CD27- cells respectively). HIV-2 specific CD8 T-cells, comparable to those from HIC1, tended to be enriched in the intermediate CD28-CD27+ phenotype. Hence, the higher expression of CD28 in HIV-2 specific CD8 T-cells suggests a less mature phenotype. Thushan de Silva *et. al* arrived at a similar conclusion, but the team only examined the expression of CD45RO and CD27 on HIV-2 specific CD8 T-cells in juxtaposition with CMV- and EBV-specific CD8 T-cells and not in relation to HIV-1

infection.[290] This study of the ANRS CO5 cohort therefore, provides dimensional perspective to his earlier argument.

While CD45RA can be associated with terminal differentiation, this relationship is not definitive. On the contrary, CD45RA re-expression is associated with cell quiescence and is a characteristic of long-lived effector memory cells.[206,406] In HIV-1 infected patients, CD45RA expression was restricted to a rare population of resting T-cells that functionally resemble memory cells in terms of retaining proliferative capacity and exhibiting strong IFNγ and TNFα responses to antigenic stimulation.[577] This rare subset of HIV-specific memory cells appears only during acute infection when viral replication is inhibited or after viral containment by cART. The high prevalence of CD45RA expressing HIV-2 Gag-specific CD8 T-cells in HIC2 may be instrumental in controlling HIV-2 viremia and the archetypical expression of CD27, CD28 and CD45RA on HIV-2 Gag-specific CD8 T-cells is, therefore, a significant departure from the pattern that is predominantly observed during HIV-1 infection.[206,452] Armed with potent effector functions, the predominant preservation of these early-differentiated HIV-2 specific memory CD8 T-cells during chronic infection may represent the difference between uncontrolled viral replication and viral control.

Although overall, strong CD8 T cell-mediated HIV-2 suppression is observed in HIC2 patients, some heterogeneity, as in HIC1, is observed in these patients.[519,522-523] The absence of or low CD8 T cell-mediated HIV-2 suppression in four patients may be related to several factors. First, we cannot exclude the possibility that the capacity for CD8 T cell suppression remained undetected due to the absence of immunodominant epitopes that were not expressed by the HIV-2 SBL virus strain used in our experiments. However, Saez-Cirion *et. al.* did demonstrate that the lack of suppressive CD8 T-cell activity in

HIV-1 controllers could not be reversed even in the presence of autologous virus.[519] Secondly, the absence of CD8 T-cell suppressive activity may result from the stringent resolution of viremia, resulting in the contraction of the HIV-2 specific CD8 T cell response to a limited pool of high quality memory T-cells, whose quantity fell below the detection threshold of the assay.[548,578-579] Furthermore, we were only able to analyse CD8 T cell responses in peripheral blood, which precludes the observation of robust responses that were previously observed in infected tissues from HIV-1 elite controllers.[580] Alternatively, innate or humoral responses, or even infection with unfit virus, could have a preponderant role in controlling viremia in these patients.[581] Finally, HIC2 that demonstrated low CD8 T-cell suppressive activity could have been sampled shortly before undergoing clinical progression.

With respect to CD4 T-cells, the data presented in this chapter supports the rhetoric proposed by several of our predecessors, that the HIV-2 specific CD4 T-cell response is pertinent to viral control. The potency of the HIV-2 specific CD4 T-cell response is inversely related to proviral load, and donors with stronger cytokine responses exhibit better clinical outcome.[305-306] As in the case of HIV-1, HIV-2 specific CD4 T-cells remain preferentially targeted during HIV-2 infection but are quantitatively preserved – implying that qualitative improvements or preservation of function – despite infection with virus – could have a significant impact on viral control.[137,305] Virus-specific CD4 T-cells contribute to viral clearance through their plethora of helper functions that augment and stimulate CD8 T-cell and B-cell responses; they have also demonstrated cytotoxic capabilities and participate in the production of effector cytokines.[582-584] In this study, we reveal close associations between the frequency of Gag-specific CD4 T-cells with both CD8 T-cell suppressive activity and CD4 T-cell counts.

An earlier study by Duvall *et. al.* showed that highly polyfunctional Gag-specific CD4 T-cells expressing multiple cytokines were enriched in asymptomatic HIV-2 controllers.[297] In addition, the pattern of cytokine expression shifted with their maturation status – the majority (~80%) of Gag-specific CD4 T-cells belonged to an intermediate CD57-CD27+CD45RO+ subset, but early differentiated CD4 T-cells expressing IL2 and terminally differentiated but polyfunctional CD57+ CD4 T-cell expressing high levels of M1P-1β and CD107 were also found. Due to limited sample availability, we were unable to commit to the deeper characterisation of CD4 T-cells in HIC2 from the ANRS CO5 cohort. However, HIV-2 specific CD4 T-cells from our donors were observed to be highly polyfunctional and coexpressed high levels of TNFα, MIP1β and CD40L in addition to IFNγ. The limited expression of IL2 and CD107a by Gag-specific CD4 T-cells in our cohort suggests that they were chiefly at an intermediate stage of differentiation, and that HIV-specific T-cells bearing this phenotype are likely to be most relevant to HIV-2 control. The paucity of IFNγ+IL2+ CD4 T-cells in this study, which Duvall *et. al.* claims to be the distinguishing factor in HIV-2 infection, is likely to be related to the duration of HIV-2 infection.[297,305] While the mean age of asymptomatic donors in their study was 36 years of age, those in our cohort averaged 48 years of age

Antigen-specific CD4 and CD8 T-cells are both key players of the adaptive immune system, and their interdependence on each other suggests that interventions which improve and preserve the functions of both groups of lymphocytes are likely to be necessary for the management of HIV-1 infection. The intensity of prolonged HIV-1 replication contributes to a process of premature immune aging that exerts a toll and weakens cellular immunity in infected individuals.[335] HIV-2 specific CD4 and CD8 T-cells are able to effectively suppress viral replication in HIC2 and this prevents chronic immune activation, which delays further deficit to the lymphopoietic system, altogether

allowing the sustenance of an effective adaptive immune response that establishes long-term control over the virus. This equilibrium between HIV-2 and host immunity may be referred to as a virtuous cycle, in contrast to the vicious cycle in HIV-1 infection, where notwithstanding exceptional HIC1 cases, poorly controlled viral replication results in elevated chronic immune activation. In many ways, the immune correlates of asymptomatic HIV-2 infection described in this study suggests desirable endpoints for treating chronic HIV-1 infection – the neutralization of excessive immune activation and preservation of lymphopoietic and thymic output.

A complete picture explaining the benign virtuous cycle of HIV-2 infection remains unresolved; whilst many epidemiological studies have found an association between specific HLA class I alleles and HIV-1 disease outcome, i.e. rapid or delayed progression to AIDS,[585] the contribution of specific HLA alleles in HIV-2 to HIV-2 control is still unclear. Evidence exists to show that HLA-B*35 and B*150 alleles are associated with HIV-2 disease progression[526,586] – but several individuals in our study bore HLA-B*35 allele, yet controlled their infection. Similarly, in the context of HIV-1 infection, HLA class I homozygosity has been shown to be associated with rapid progression;[527,587] but the majority of HIC2 in this study were homozygotes. Interestingly, no protective HLA alleles have been identified in association with better clinical outcome in HIV-2 infection. It was suggested by Yindom *et. al.* that this might be linked to the fact that HIV-2 infection is easier to control than HIV-1,[526] and the relaxed requirements for HIV-2 control may not have allowed for the distillation of immunodominant epitopes that are restricted by specific HLA molecules. Moreover, unlike the partial control exerted by CD8 T cells during HIV-1 infection,[588] the stringent CD8 T-cell control that persist during chronic HIV-2 infection could limit the emergence of escape variants, and

therefore abrogate the requirement for HLA Class I molecules that possess sufficient flexibility to adapt to new variants.

While still hypothetical at this stage, a greater sensitivity of HIV-2 to intracellular restriction factors may also play a crucial role in constraining the initial replication of HIV-2 in CD4 T-cells. It has been shown that HIV-2 is more susceptible to restriction by tripartite motif protein isoform 5 alpha (TRIM5a) than HIV-1.[311] Subsequently, it was found that tetherin interacts differently with both HIV viruses, depending on whether the interaction is mediated by Vpu or Env, as in the case of HIV-1 and HIV-2 infection respectively.[589-590] The differential usage of protein adaptors might translate to the different sensitivities of HIV-1 and HIV-2 to tetherin control at the cell membrane. While cART-naïve HIV-2 infected patients from the ANRS CO5 HIV-2 Cohort have demonstrated high levels of APOBEC3F/G editing activity on proviral DNA in a previous study, immunovirological parameters were not found to be associated with the latter.[312]

Finally, it has been proposed that by countering SAMHD1, the HIV-2/SIVsmm-specific Vpx enhances the sensing of HIV-2 by dendritic cells, which may induce potent CD8 T-cell responses by providing additional routes for antigen presentation.[132,591-592] This argument remains highly debatable, as several groups have reported that dendritic cells are refractory to HIV-2 infection, which could in turn, restrict HIV-2 replication.[137,593] Overall, an enhanced sensitivity of HIV-2 to host restriction factors may be crucial in limiting its initial replication at the acute stage – a protective measure which prevents excessive damage to the host immune system and provides the necessary window of opportunity for the subsequent induction and enactment of an effective T-cell response, ultimately establishing and maintaining control over the virus for several decades. Once activated, this timely bottleneck would prevent the expenditure and subsequent

exhaustion of primary immune resources, as well as the consumption of HIV-specific T-cells – overall halting the development of the hyperinflammatory state that has become synonymous with HIV-1 infection.[202,223,335] Combined together, the synergy between innate and adaptive factors may provide the necessary groundwork for establishing a virtuous cycle of HIV-2 infection, which exemplifies the requirement of a holistic mechanism for natural control over the virus.

Chapter 5: CMV – A Partner in Crime?

5.1 Introduction

Immune activation is often cited as the main driver of HIV pathogenesis and disease progression. An obvious source of activation during HIV infection is the immune response against HIV itself, which drives the expansion and turnover of high numbers of HIV specific T-cells. However, CMV seropositivity has also been observed to accompany HIV-1 infection in a large proportion of HIV-1 cases (60 – 99%) and is by itself, a significant source of immune activation and immunosenescence even in the presence of ART.[476-477,452,484,594-596] In a HIV-1 cohort in Paris, CMV seroprevalence rates rose to 97.6% by the time recruitment ended in 2011.

In the pre-ART era, these interactions between HIV-1 and CMV required clinicians to respond with far greater immediacy, manifesting in a range of comorbidities – including retinitis, encephalitis, colitis or pneumonitis – in addition to being a significant risk factor in driving mortality.[480-481] Although interest in this area of research began to decline, partly due to the substantial elimination of the development of CMV end-organ disease by ART, a fast-growing body of evidence suggests that the reprieve afforded by ART is only temporary, as coinfected individuals began to present with a range of non-AIDS related morbidities that are usually more prevalent in the elderly (i.e. cardiovascular and cerebrovascular disease, liver disease, atherosclerosis, osteoporosis, cognitive impairment and frailty).[598-602]

Even though perinatal coinfections appear to be more deadly in driving mortality,[596,603-604] studies on adult cohorts continue to show an accelerated rate of HIV progression and risk

of mortality despite treatment – presenting a strong case even after adjusting for HIV viral load or CD4 T-cell count.[599,606-610] In a large cohort study of 6111 HIV-1 infected individuals, the increased risk of mortality was shown not to be associated with HIV or CMV end-organ disease but a pattern of pathology that reveals the salient interplay between systematic inflammation and cellular/immune senescence – including the higher incidence of non-AIDS defining cancers, cardio- and cerebro-vascular diseases, and atherosclerosis;[599] there is a wide consensus that the progression of atherosclerotic lesions involves a chronic T-cell mediated inflammatory response.[611-612] Altogether, the data suggest that for those living with HIV, coinfection with CMV remains a significant threat in reconstituting health, to an extent that warrants the modification or improvement of current treatment regimes.[599]

In the context of HIV-1:CMV coinfection, the independent association of CMV with cellular and immune senescence and the more recent pathological phenotypes associated with HIV-1:CMV coinfections have prompted an investigation into the contribution of CMV in driving cellular and/or immune dysregulation and dysfunction – which is more immediately relevant to this work. Apart from driving late differentiated T-cell phenotypes, [290,452,484] the increased levels of immune activation from CMV co-infection also contributes directly to HIV-1 pathogenesis – increasing the incidence of activation-induced cell death through Fas-related mechanisms that act to further contribute to the depletion of lymphocytes.[596,613-614] Moreover, CMV may also activate latent proviral HIV with its molecular repertoire: the IE-2 gene can augment HIV-1 gene expression within the same cell or through CMV antigens that induce the peripheral release of inflammatory cytokines.[615-617] Furthermore, CMV can facilitate the entry of HIV into CD4 T-cells *via* the transcription of its US28 gene, which encodes a chemokine receptor that substitutes for CCR5 in allowing HIV entry into CD4 T-cells.[618] Finally, signals derived from CMV

antigens promote the upregulation of CCR5 by CD4 central memory T-cells, ultimately providing additional routes for cellular infiltration by HIV.[619] The latter mechanisms are likely to apply to HIV-2, which has demonstrated significant overlap with HIV1 in its coreceptor usage.[620-622]

On its own, the establishment of avid CMV responses in infected individuals was associated with a significant reduction of both naïve and total CD4 T-cells in HIV-uninfected young adults, who also present with compromised T-cell renewal capacity.[623-629] The combined effects of CMV and HIV-mediated immune dysfunction are likely to accelerate the breakdown of T-cell homeostasis and synergize in the induction of HIV-associated immunosenescence – as Appay *et. al.* demonstrated in a previous study. In a cohort of HIV-1 infected Parisians, his team found that strong CMV responders presented with particularly low naïve CD4 and CD8 T-cell numbers and the accumulation of CD57+ senescent T-cells – a difference that was more obvious in patients receiving ART.[263] Accordingly, the additional loss of naïve T-cells and CMV-mediated memory inflation may provide a plausible explanation for the acceleration of HIV disease progression that occur in the presence of high CMV T-cell responses and seroprevalence.[594-596]

For coinfected and treatment-naïve controllers, a clear relationship between the avidity of CMV responses and naïve or total T-cell counts was not observed – we rationalised that any association might be masked by the more dominant HIV-1 contribution to immune activation or the overall lower activation status of T-cells in HIV-1 controllers. Accordingly, we hypothesise similar outcomes for HIV-2 and CMV co-infection: the preservation of T-cell functionality and the relatively milder shifts in immune activation status are likely to constitute an immune system that is still capable of managing the

innocuous balance between CMV pathogenesis and immunocompetence, [80-81] denying CMV the premature opportunity – often observed in HIV-1 infection – to direct a shift towards more senescent T-cell phenotypes. We describe lower CMV-specific CD4 and CD8 T-cell responses in HIC2 than HIV-1 infected individuals, and show that these responses do not show clear associations with markers of T-cell activation and senescence in our study.

5.2 Results

5.2.1 CMV-specific responses in HIC2

In order to quantify the total CMV-specific response, we measured the magnitude of cytokine producing T-cells after the stimulation of donor PBMCs with both pp65 and IE1 – principle targets of the CMV-specific T-cell response – overlapping peptides.[263,493] A representative example of the CMV-specific T-cell response of HIC2 is depicted in Figure 5.1. Due to the lack of acces to plasma samples for members of this cohort, we were unable to produce serology data, but the presence of CMV-specific responses in the majority of HIC2 suggests that most of them had been CMV positive or exposed. Of the 19 HIC-2 donors, 12 (63%) and 18 (95%) individuals exhibited pp65-specific CD4 and CD8 T-cell responses respectively; while 10 (52%) and 15 (79%) individuals mounted IE1-specific CD4 and CD8 T-cell responses respectively. CMV-specific CD4 T-cells that were primed with this method expressed IFNγ, TNFα, CD107, IL-2 and MIP1β; while CD8 T-cells expressed only IFNγ, TNFα, CD107 and MIP1β (Figure 5.2a). The magnitude of the pp65-specific CD4 and CD8 T-cell responses were comparable to those observed after HIV-2 Gag stimulation, while IE1-specific CD4 and CD8 T-cell responses were less frequently observed (Figure 5.2b).

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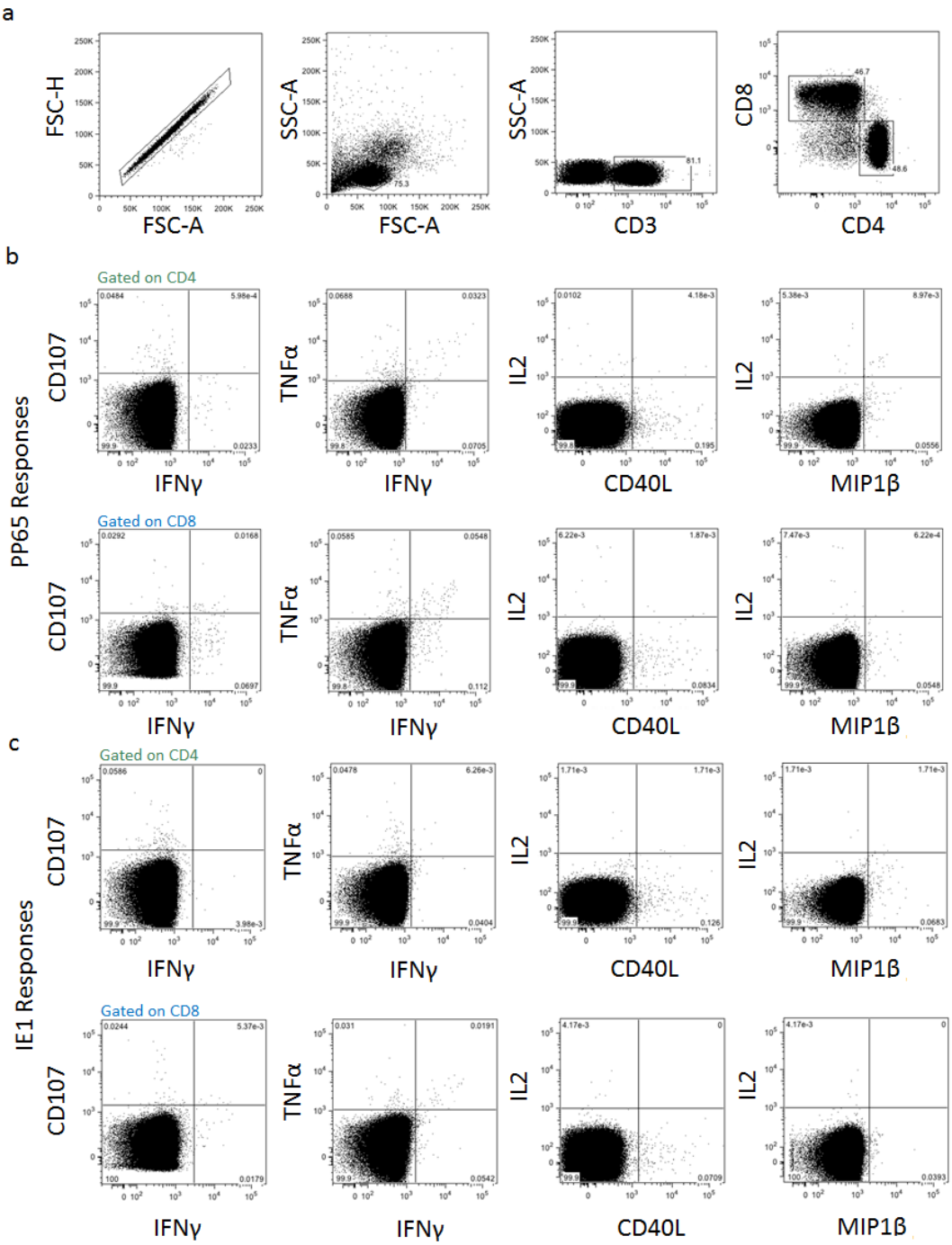

Figure 5.1: HIV-2 Gag-specific responses in HIC2. (**a**) Representative staining and gating strategy for determining the percentage of cytokine secreting CD4 and CD8 T-cells. After gating for singlets, lymphocytes, and CD3 positivity, cells were gated on CD4 and CD8 expression. Within CD4 and CD8 T-cells, the gating of IFNγ, TNFα, CD107, CD40L, MIP1β and IL2 expressing cells are indicated for (**b**) PP65-specific and (**c**) IE1-specific CD4 and CD8 T-cell responses.

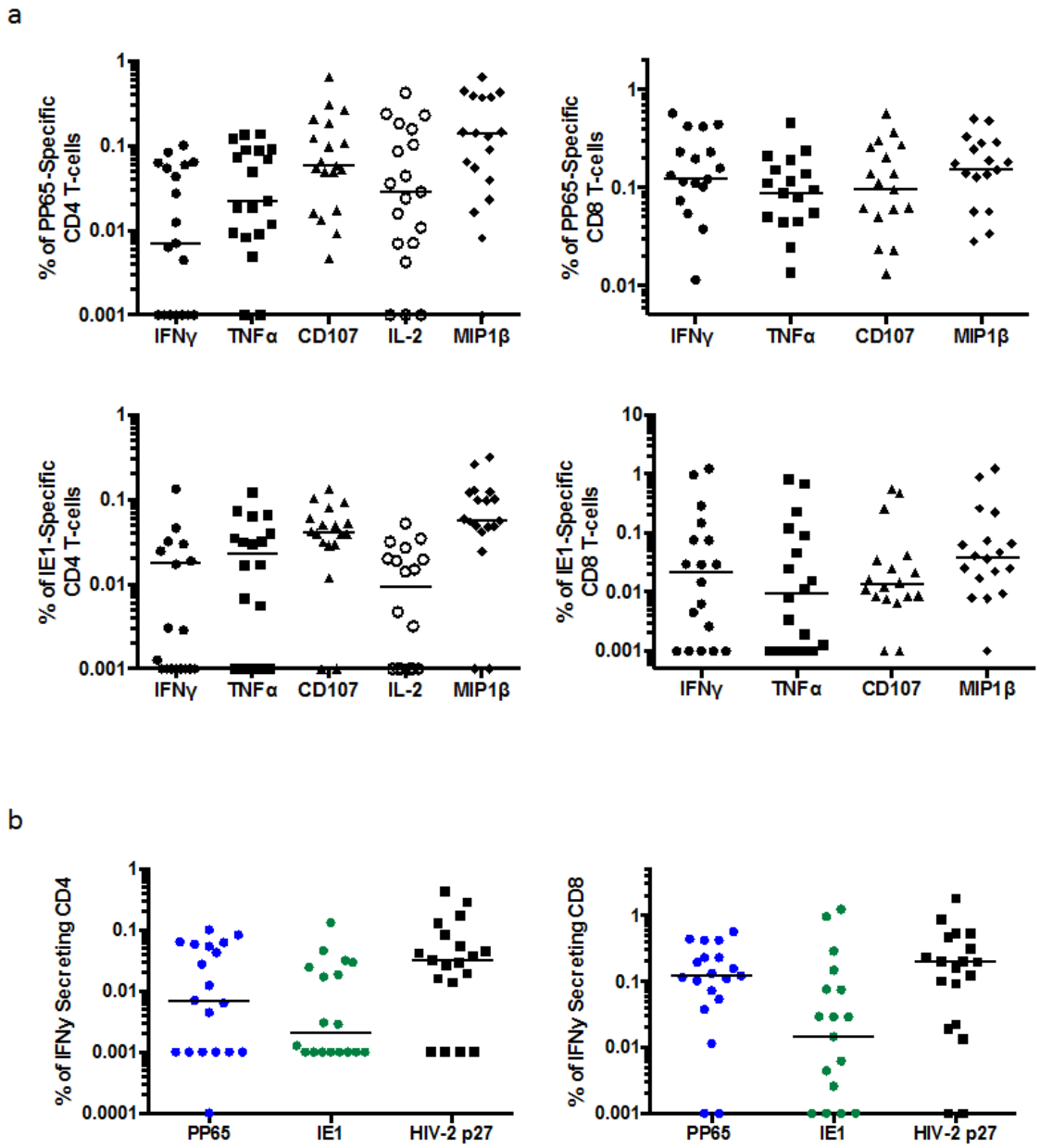

Figure 5.2: PP65 and IE1-specific CD4 and CD8 T-cells in HIC2. (**a**) Percentages of cytokine secreting CD4 (left) and CD8 T-cells (middle)from 19 HIC2 donors upon 6h stimulation with 15 mer peptides spanning the entire PP65 (top) or IE1 (bottom) proteome. (**b**) A comparison between the percentages of IFNγ+ CD4 (left) or CD8 (right) T-cells after stimulation with PP65, IE1 and HIV-2 p26. Values equivalent or lower than zero were assigned the value 0.001. Bars indicate the median.

5.2.2 Lower CMV-specific responses in HIC2 than HIV-1

Appay *et. al.* have shown that the accruement of T-cell responses against CMV infection is associated with age (data shown in Figure 5.3a) and HIV-1 progression, and was closely related to a reduction in naïve CD4 T-cell counts and elevated CD57 expression, even in treated HIV-1 infected middle-aged individuals.[263] The resurgence of CMV-specific T-cell responses with age and HIV-1 status is attributed to a loss of immune competence and the consequential increase in antigen load; and the gradual expansion of memory T-cell populations over time further contributes to this phenomenon.[263,377,472-475,487]

We hypothesised that the magnitude of CMV-specific responses in HIC2 is likely to be reflective of their relatively preserved immune functionality as opposed to the phenotype observed in chronic HIV-1 infection,[191] and be more compatible with the lower frequencies associated with HIV uninfected, middle-aged donors. Indeed, the magnitude of pp65- and IE1- specific CD8 T-cell responses in HIC2 donors aligns closely with this hypothesis, matching the frequencies observed in the HIV negative group but were lower than the levels observed in HIC1 (Figure 5.3b). Fewer CMV-specific CD4 T-cell responses were also detected in HIC2, with the exception that they were also considerably lower than the frequencies observed in the HIV negative group, at least with respect to PP65-specific responses. These observations suggest that unlike the case with HIV-1 infection, infection with HIV-2 triggeres less profound CMV-specific expansions.

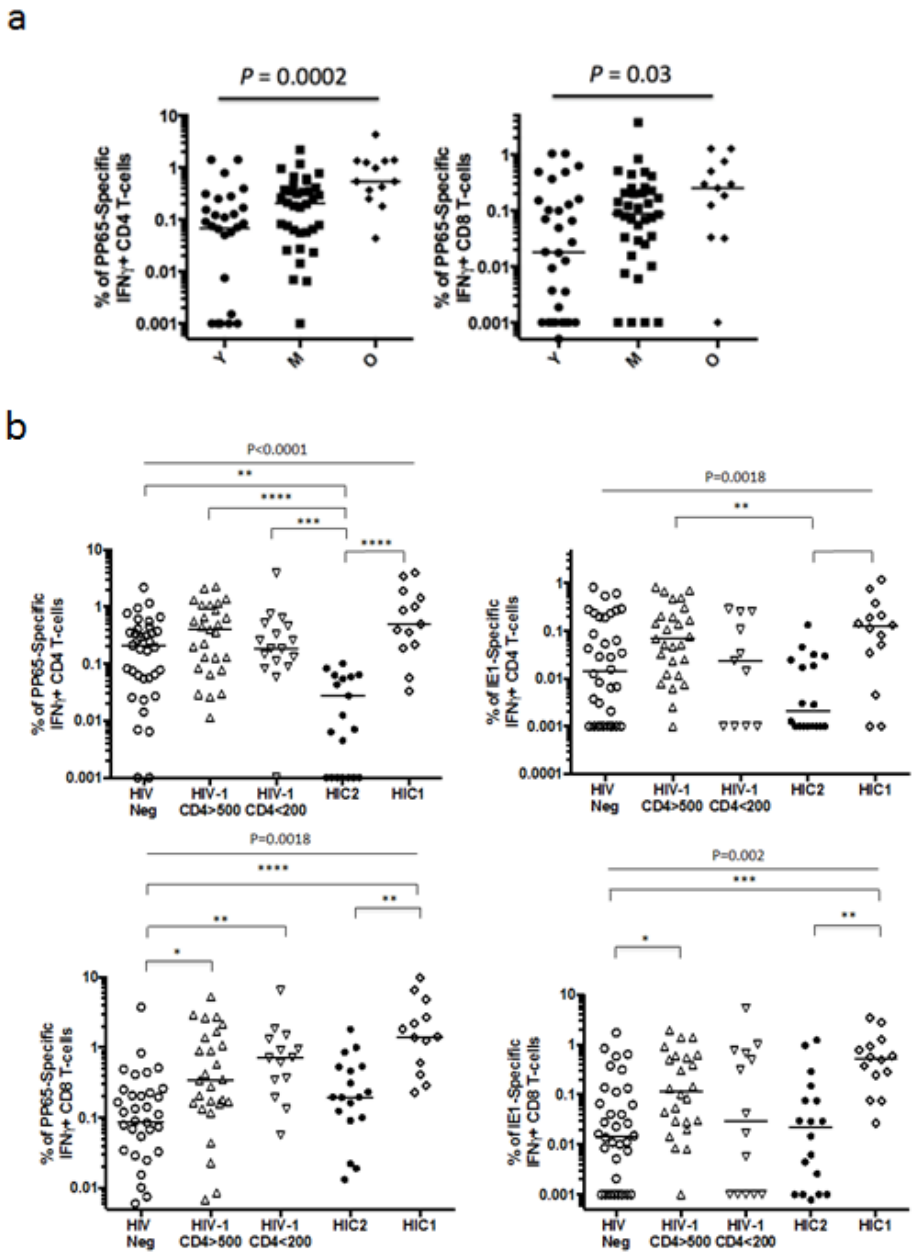

Figure 5.3: Comparison of PP65 and IE1-specific CD4 and CD8 T-cells in HIC2 and HIV-1 infected donors. (**a**) Data from Sauce et. al. comparing the percentages of PP65-specific IFNγ+ CD4 (left) or CD8 (right) T-cells in HIV-2 negative donors that were separated into different age groups; data obtained using PBMCs from the French blood bank (Y:18-24 years old (n=34)), middle-aged (M:25-55 years old (n=36)) and elderly persons (O:75-96 years old (n=21)). (**b**) Percentages of IFNγ secreting CD4 (top) and CD8 T-cells (bottom) in middle aged healthy adults (HIV neg (n=36)), treatment naïve viraemic HIV-1 infected patients with high (HIV-1 CD4>500 CD4 T-cells/µl (n=27)) or low (HIV-1 CD4<200 CD4 T-cells/µl (n=11), HIV-1 controllers (HIC1 (n=14)) and HIV-2 controllers (HIC2 (n=19)) upon 6h stimulation with 15 mer peptides spanning the entire PP65 (left) or IE1 (right) proteome. The Kruskal-Wallis and Dunn's Test were used to perform multiple comparisons that included all groups of data. All significant differences are shown. Bars indicate the median.

5.2.3 HIV-2 and CMV responses influence CD4 T-cell homeostasis

Next, we wanted to ascertain whether the magnitude of HIV-2 or CMV-specific responses in HIC2 could contribute to T-cell memory inflation in HIC2. Unlike the case of HIV-1 infected adults, we did not observe a relationship between the pp65-specific CD4 T-cell response and the percentage and number of naïve CD4 T-cell counts (Figure 5.4).[263] However, we observed strong negative correlations between the magnitude of both p26- and pp65- specific CD8 T-cell responses with the percentages of circulating naïve CD4 T-cells, which was not observed for naïve CD8 T-cells (Figure 5.5). Moreover, the magnitude of p26-specific CD8 T-cell responses was also closely related to the numbers of memory CD4 T-cells, but not memory CD8 T-cells (Figure 5.4 & 5.5). Nevertheless, by analysing the expression of CD57 on T-cells, we observed that the potency of HIV-2 or CMV-specific T-cell responses did not relate to the terminal differentiation of either CD4 or CD8 T-cells from HIC2 (Figure 5.7).

5.2.4 HIV-2 and CMV responses do not relate to markers of senescence

Several studies have reported relationships between HIV-1 and CMV specific cytokine responses to cellular markers of activation and senescence.[306,630] Most notably, Foxall *et. al.* demonstrated a positive correlation between the IFNγ+IL2+ Gag-specific CD4 T-cell response and the expression of HLA-DR within total CD38+CD4 T-cells. Due to the paucity of IFNγ+IL2+ p26-specific CD4 T-cells, and because we only had HLA-DR staining for eight samples, we were unable to perform an identical comparison. Instead, we looked for correlations between the percentages of IFNγ+ CMV or HIV-2 specific cells and the expression of HLA-DR. We found a slight correlation between the magnitude of pp65- and IE1- specific CD4 responses (not statistically significant) and the

percentage of HLA-DR+ CD4 T-cells, which was not observed for p26-specific CD4 T-cell response (Figure 5.6).

In addition, we compared the magnitude of the virus-specific IFNγ response of CD4 and CD8 T-cells to the respective expression of CD38, and Ki67. Generally, we found no correlation between the HIV-2 and CMV-specific CD4 and CD8 T-cell responses to the expression level of T-cell activation markers, with the exception of a negative correlation between the p26-specific CD4 T-cell response and the percentages of Ki67+ CD4 T-cells (Figure 5.7). Altogether, the data suggest that in HIC2, the magnitude of HIV-2 specific responses may be more influential in driving systemic T-cell activation and differentiation than CMV-specific responses, with a specific bias towards the alteration of CD4 T-cell phenotype; but the magnitude of CMV-specific responses may, beyond a threshold level of immune activation, be informative of dysregulated T-cell homeostasis.

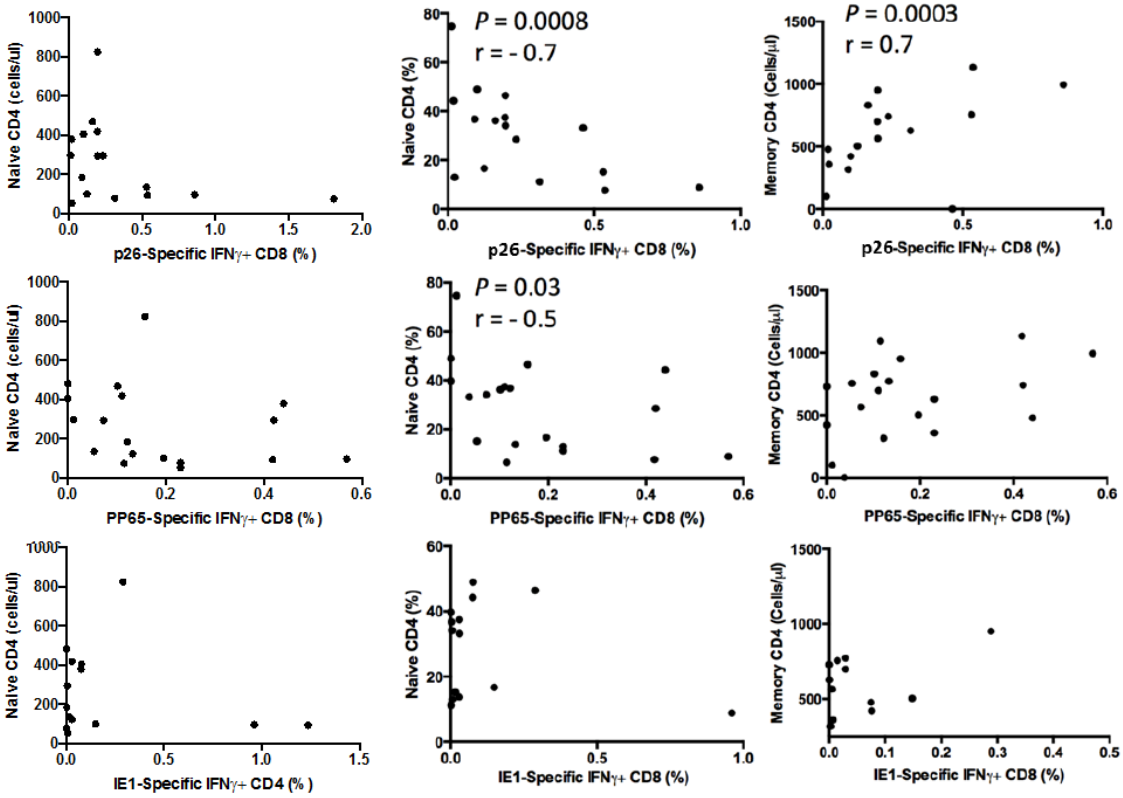

Figure 5.4: Correlation between HIV-2 and CMV responses with Naïve and Memory CD4 T-cell numbers. Correlations between the percentages of p26 (top); PP65 (middle) and IE1 (bottom) -specific, IFNγ producing CD4 (left) and CD8 (right panels) T-cells, and the percentages (left) and numbers (middle) of naïve CD4 T-cells as well as the numbers of memory CD4 T-cells (right). The Spearman‘s rank test was used to determine correlations.

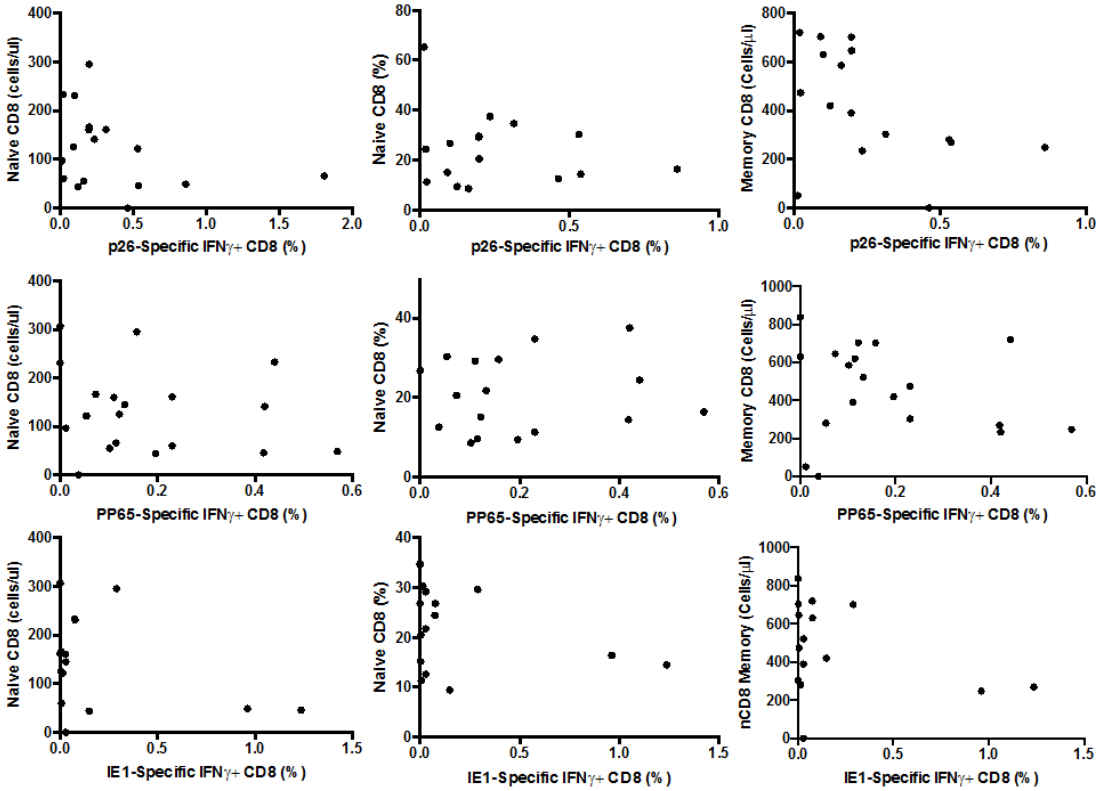

Figure 5.5: Relationship between HIV-2 and CMV responses with Naïve and Memory CD8 T-cell numbers. Relationships between the percentages of p26 (top) and PP65 (bottom) -specific, IFNγ producing CD8 T-cells, and the percentages (left) and numbers (middle) of naïve CD8 T-cells as well as the numbers of memory CD8 T-cells (right). The Spearman‘s rank test was used to determine correlations.

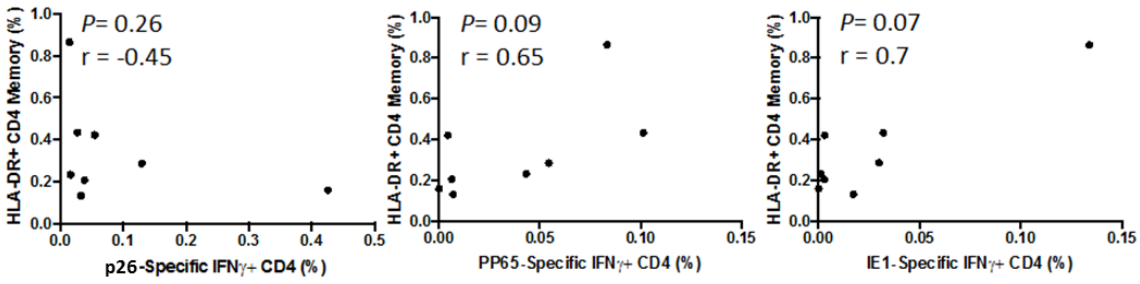

Figure 5.6: Correlation between HIV-2 and CMV responses with HLADR expression. Correlations between the percentages of p26 (left); PP65 (middle) and IE1 (right) -specific, IFNγ producing CD4 T-cells, and the expression of HLADR within CD38+ memory CD4 T-cells. The staining of PBMCs with anti-HLADR antibodies was only possible for eight of the nineteen HIC2 subjects The Spearman‘s rank test was used to determine correlations.

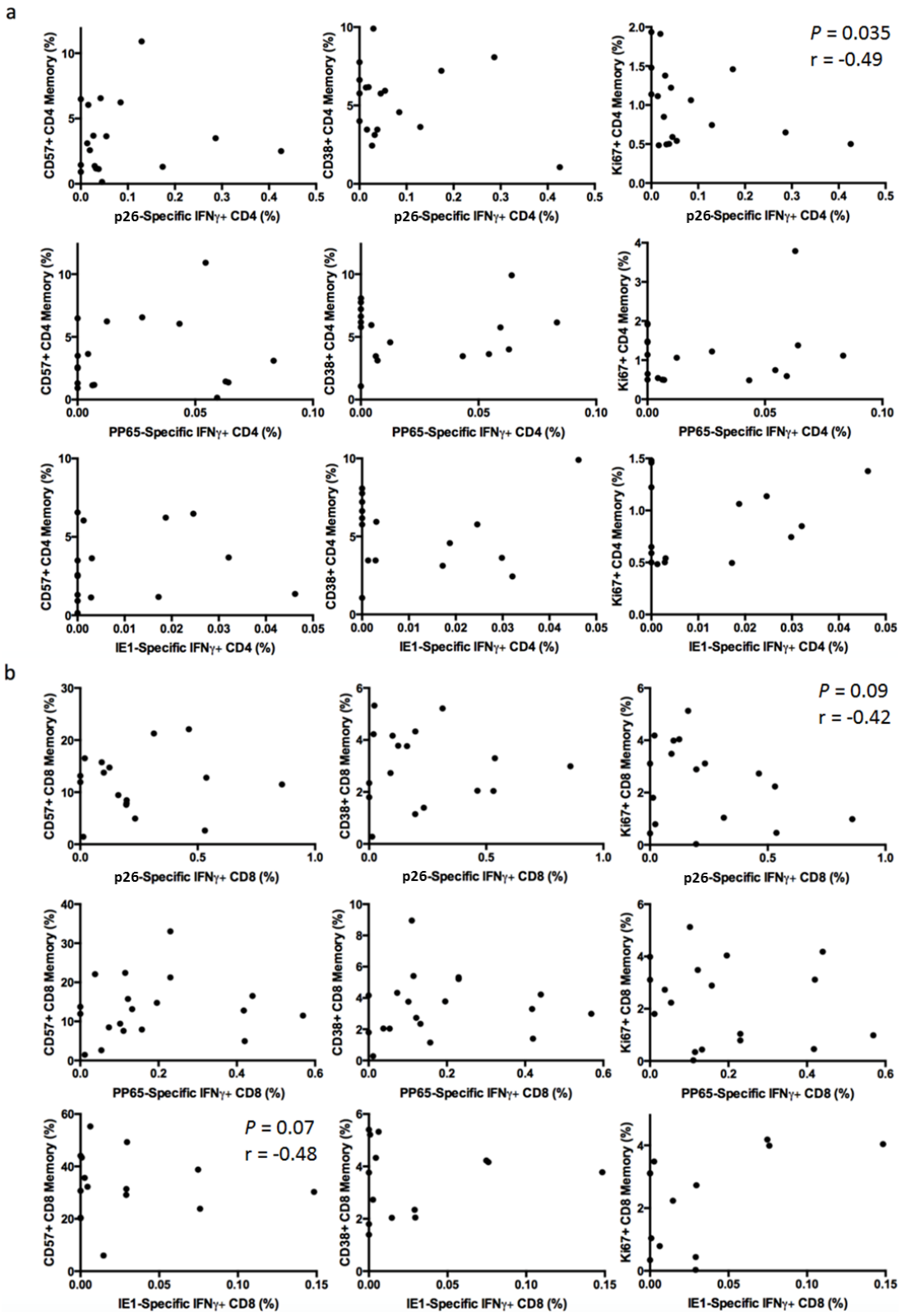

Figure 5.7. Correlation between HIV-2 and CMV responses to CD57, CD39 and KI67 expression. Correlations between the percentages of p26 (top); PP65 (middle) and IE1 (bottom) -specific, IFNγ producing CD4 (a) and CD8 (b) T-cells, to the respective expression of CD57 (left), CD38 (middle) and Ki67 (right). The Spearman‘s rank test was used to determine correlations.

5.3 Discussion

The prevalence of CMV infection differs between geographic regions and affects 60-90% of the population worldwide;[484,594-596] its manifestation is also dependent on socio-economic status, being particularly high in developing countries.[632-633] In West Africa, seropositivity hovers between 70 - 90%, and rises in accordance with age.[633] As expected from the West African background of our donors, 95% of HIC2 displayed CD8 T-cell responses towards either pp65 or IE1. While initial investigations suggested that immunosuppression was the leading cause of CMV reactivation – a perception inspired by its association with chronic HIV infection – more recent evidence indicates a key role of immune activation in promoting CMV reactivation.[263,600,634] The observation of strong CMV responses in HIV-1 infected individuals can then be rationalised as a product of the heightened immune activation which accompanies chronic HIV infection.

Regardless of whether immunosuppression or immune activation is the main culprit in driving CMV responses during HIV infection, both are considerably less pronounced during HIV-2 infection,[82,283,315-316] which made it unsurprising that the magnitude of pp65 and IE1 responses in HIC2 fell considerably below the levels observed in HIV-1 infected donors. Nevertheless, the observation that CD4 T-cell responses towards PP65 ($P<0.0001$) or IE1 ($P = 0.07$) occur less frequently in HIC2 than HIV-negative donors remains curious, particularly because the CD8 T-cell responses in HIC2 closely match those of the HIV negative group. We believe that the specific systemic effect of HIV-2 infection on the CD4 T-cell subset could be due to several reasons. Again, the ability of HIV-2 (but not HIV-1) Nef to downmodulate TCR signaling and induce an anergic profile in CD4 T-cells could be relevant in this context – rendering CD4 T-cells less responsive to antigenic stimulation.[326-329] Alternatively, the low frequency of CMV-specific CD4 T-cells may be

outcompeted by a more diverse repertoire of circulating memory CD4 T-cells, which may be skewed towards the recognition of other pathogens, such as HIV-2. Finally, the idea that other CMV proteins, such as US3 or UL55, may be more immunodominant in driving CMV-specific CD4 T-cells responses in HIC2 remains possible.[635]

In terms of systemic immune activation, we found that the magnitude of HIV-2 p26-specific CD8 T-cell responses was more closely related to the percentages and numbers of naïve and memory CD4 T-cells than CMV pp65- or IE1-specific CD8 T-cell responses. In addition, the magnitude of the HIV-2 p26-specific CD8 T-cell response did not seem to be related to the systemic maturation of naïve CD8 T-cells. We believe that this phenomenon could be related to the continuous presence of HIV-2 antigen in the periphery, possibly due to the persistent replication of HIV-2 in lymphoid tissues, which may continue to drive the expansion of p26-specific CD8 T-cells; and activate the systemic migration of naïve CD4 T-cells to germinal centres in lymphoid tissues, where they present antigens to support the affinity maturation of B-cells.[679] The observation that pp65-specific CD8 T-cell responses are also related, albeit to a lesser extent, to the decline in the percentages of naïve CD4 T-cells suggest that systemic bystander activation, resulting from HIV-2 activity in lymphoid reservoirs, may also be involved in simultaneously stimulating the peripheral maturation of naïve CD4 T-cells and expansion of CMV specific CD8 T-cells.[493] A previous study on the ANRS CO5 cohort – where our HIC2 donors were recruited from – demonstrated substantial levels of sCD14 in circulation,[316] A separate study showed that untreated individuals suffering from both chronic HIV-1 and HIV-2 infection revealed similar levels of sCD14 and LPS in their plasma;[325] and that the levels of LPS in plasma correlated with the CD4 T-cell counts of both HIV-1 and HIV-2 infected donors. Altogether, the literature suggests that GALT

remains compromised during chronic HIV-2 infection, which may be due to active HIV-2 replication, and serve as a persistent source of bystander activation.

Although we generally observed a lack of association between HIV-2 and CMV T-cell responses with the expression of T-cell markers of senescence and activation, we observed two striking trends – even within our relatively small group of HIC2. Firstly, while p26-specific CD8 and CD4 T-cell responses did not correlate well with systemic T-cell activation, our data are suggestive of an inverse relationship between the magnitude of HIV-2 specific CD4 and CD8 T-cell responses and the percentages of actively proliferating cells – an influence that was more profound for memory CD4 T-cells than memory CD8 T-cells. Together with the association between the magnitudes of the HIV-2 specific CD8 T-cell response and the slight memory inflation of CD4 T-cells, these data further indicate that prolonged infection with HIV-2 could eventually contribute to the destabilisation of T-cell homeostasis and the systemic impairment of T-cell proliferation. The relationship that Foxall *et. al.* observed between homologous Gag-specific CD4 T-cell responses and systemic CD4 T-cell activation corroborates this viewpoint, although we did not observe an identical relationship in our study. Secondly, our data reveal a slight correlation between systemic CD4 T-cell activation and the magnitude of CMV-specific T-cell responses, which suggests that the slight increment in systemic immune activation during asymptomatic HIV-2 infection could be sufficient to trigger CMV reactivation, or at least, the peripheral bystander expansion of CMV specific CD4 T-cells – which may contribute significantly to pathology as HIC2 age.

There are several possible explanations for the discordant conclusions revealed by our data and those described by Foxall et. al.;[306] the genetic predisposition or infection history of a predominantly Caucasian cohort could contribute to differences in the susceptibility

of CD4 T-cells to immune activation caused by HIV2. Secondly, and more importantly, although both cohorts qualify as HIC2, our group of mostly West African natives had a mean CD4 T-cell count (938 cells/µl) that was approximately 36% higher than the cohort studied by Foxall *et. al.* (686 cells/µl), which suggests that the HIV-2 infected individuals in the latter study could be developing signs of HIV progression. Therefore, strong relationships between the magnitude of the HIV-2 specific CD4 T-cell response and systemic T-cell activation could indeed be an early indicator of progression towards AIDS.

Although the juxtaposition of both our data and those revealed by Foxall *et. al* hints at a possible negative impact of prolonged HIV-2 infection on T-cell senescence, we have not arrived at a point in HIV-2 research where we can make the assumption that any irregularity in the immune profiles of HIC2 is anything more than unusual, particularly since most HIV-2 studies are cross-sectional in nature. In fact, a longitudinal study showed that for the majority of HIV-2 infected adults, HIV-2 infection had no effect on survival over a nine year study period. Moreover, their data indicate that age is not a risk factor in determining the mortality of HIV-2 infected individuals.[282] However, at the end of the nine year period, approximately 90% of their donors were still under 54 years of age – this falls within the median age range of participants described in most cross-sectional HIV-2 studies which also feature a high percentage of asymptomatic controllers (>60%).[291,290,296,305-306] While this might be difficult to achieve, considering the fast declining prevalence of HIV-2 infections within the past decade,[31-32] additional prospective or cross-sectional studies of HIV-2 infected mature individuals, particularly beyond the age of 60, could yield divergent outcomes in the age-dependent risk of HIV-2 mortality. The latter is probable considering that CMV viral loads – a pathogen which similarly remains latent for most of adulthood – only begin to escalate in HIV uninfected

or healthy individuals after the age of 70.[487] The clinical implications of CMV reactivation are manifold, and includes an increased resistance to vaccination, increased mortality after transplantation; and a higher incidence of heart and lung disease, diabetes, cancer and autoimmunity.[598-602] Overall, it is not difficult to envisage a similar pathology in older, HIV-2 infected individuals.

Chapter 6: Molecular Senescence in Vertically-Infected Children

6 .1 Introduction

Unlike the situation in HIV-2 infection, spontaneous control of HIV-1 viremia is restricted to a small proportion of privileged individuals that are referred to as HIV-1 controllers.[72-75,522] The overrepresentation of specific MHC class I alleles – such as HLA-B27 and HLAB-57 – among controllers indicate that viraemic control can be mediated, at least in part, by HIV-1 specific CD8 T-cells.[274-275] Yet, disease progression typically occurs in the presence of a strong, broad repertoire of polyclonal HIV-1 specific CD4 and CD8 T-cell populations that recognize autologous virus. This paradox has now been attributed to the accumulation of a defective functional phenotype of HIV-1 specific T-cells after repeated antigen exposure – often referred to as 'senescent' T-cells.[368,405,456] Senescent T-cells demonstrate a preserved capacity for IFNγ secretion but diminished proliferative responses to antigens;[349-351] their inability to proliferate is linked to the possession of shortened telomeres, which implicates the processes of cellular exhaustion and accelerated immune senescence as key drivers to this phenomenon.

Current evidence now demonstrates that HIV-1 induced T-cell senescence is not only directed at HIV-1 specific T-cells, but affects lymphocytes globally – thus contributing to a generalised state of immune suppression.[191,347] The universal impact of chronic HIV-1 infection on T-cell senescence is a product of heightened immune activation, which is largely contributed by increased microbial translocation from damage to the gastrointestinal tract.[159-161] The latter results in the persistent release of microbial products,

which chronically stimulate both innate and adaptive immune cells through antigen-specific and bystander activation.[248,254,448] Telomere length is now a widely prescribed measure of leucocyte senescence.[367,399,402,445,501] By studying telomere attrition, independent groups have shown that both HIV-1 infected children and adults display markedly shorter telomeres and that HIV-1 controllers demonstrate longer telomeres and enhanced telomerase activity.[455,503,637-638] More strikingly, telomere shortening is evident in HIV-1 infected infants even from as early as two years of age.[638-639]

Studies on adolescent cohorts remain exceedingly rare even though HIV-1 infected adolescents provide a fascinating backdrop for studying advanced immune ageing due to the possession of an intact or hyperactive thymus that may potentially offset premature immunosenescence.[467-468] Our collaborators, Côté *et. al.*, have previously demonstrated ostensible telomere shortening in leucocytes from a group of viraemic HIV-1 infected Canadian adolescents accessing ART.[638] An investigation into the effect of vertical HIV-1 transmission on telomere shortening in ART-naive older children is to our knowledge, unprecedented. Here, we show that untreated vertically infected Zimbabwean children display shortened telomeres vis-à-vis age matched HIV-negative adolescents, and that telomere length is positively associated with CD4 T-cell counts in infected children.

While telomeres are the guardians of genomic integrity, mitochondria hold the key to cellular life and death by meeting energy demands and regulating apoptosis. Alongside ideas of telomere shortening and replicative senescence, the mitochondria takes centre stage in the most important theory on mammalian ageing that has dominated the ageing discourse of the last century – the free radical theory of ageing.[407-408] While our knowledge of the ageing process is far from complete, most biogerontologists agree with the consensus that aging begins with molecular damage that culminates in cellular, tissue

and finally organ dysfunction.[412] The free radical theory of ageing argues that intracellular reactive oxygen species which are metabolic residuals of mitochondrial respiration induce oxidative damage on mitochondrial DNA (mtDNA) and important cellular macromolecules – ultimately driving age-associated tissue damage.[408-411]

The principal contribution of chronic HIV infection to mitochondrial ageing have been traditionally thought to result from the inhibition of mtDNA polymerase γ – the main enzyme responsible for mtDNA replication – by nucleoside reverse transcriptase inhibitors (NRTIs).[640] Subsequent studies have revealed other mechanisms where ART could interfere with mitochondrial stability; for example, azidothymidine (AZT) has been found to inhibit mitochondrial adenylate kinase, promote oxidative stress and directly interfere with the electron transport chain.[641-642] However, two studies on antiretroviral-naïve HIV-infected adults showed a significant reduction of mtDNA content in PBMCs from HIV positive individuals as opposed to their HIV negative counterparts.[436,643] In both studies, mtDNA content was positively correlated with CD4 T-cell count and inversely related to HIV viral load – suggesting that infection with the virus itself could interfere with mitochondria biogenesis. While the molecular mechanisms for HIV-directed changes to mitochondrial biogenesis are currently unclear, microarray studies highlighted the downregulation of mitochondrial genes in PBMCs from treatment naïve but HIV positive individuals.[436]

Studies on the effect of HIV infection on the mitochondrial health of children are exceedingly rare, and the few that exist describe mtDNA depletion in children with access to antiretroviral treatment or who were HIV-exposed but uninfected.[638,644-645] This study attempts to bridge the gap in knowledge by comparing the mtDNA content of untreated HIV-infected children with uninfected age-matched controls. We describe a contrasting

phenomenon of elevated mtDNA levels in PBMCs from untreated HIV infected children that is particularly pertinent in children with critically low CD4 T-cell counts.

In addition to a risk of HIV-related opportunistic infections, the late diagnosis of HIV-1 infection in African children has also contributed to the discovery of chronic conditions that do not manifest in either infants or adults. In ZENITH, it was found that nearly 50% of older HIV-infected children past 11 years of age presented with chronic lung disease (CLD), and constrictive obliterative bronchiolitis (OB) was identified as the probable underlying cause.[116] Since strong, independent, associations exist between a vast array of heart and lung pathologies and abnormalities in mtDNA and leucocyte telomere length (LTL),[506-511,646-648] we sought to determine whether mtDNA or LTL measurements might be useful in predicting systemic disease in HIV-1 infected children. In our study, we observed no differences in the LTL or mtDNA content of blood from HIV-infected children with lung disease and those of other HIV+ children in the cohort.

6.2 Results

6.2.1 Cohort Recruitment and Characteristics: ZENITH Cohort

The Zenith Cohort was recruited from the main primary healthcare clinics (PHCs) from six high density suburbs in southwest Harare. The cohort includes treatment naïve, HIV+ children who were between the ages of 6 - 15 and were willing to receive HIV care at the clinic; HIV-1 viral load data are currently unavailable for this cohort. The mode of transmission was determined to be perinatal based on the following reasons: (i) no sexual debut or history of blood transfusion; (ii) known maternal HIV infection or maternal/sibling death due to HIV infection and (iii) no history of infection with other common sexually transmitted viruses. Moreover, children in our cohort also presented with stunting and delayed puberty; and reported the occurrence of frequent infection events during childhood.[113,116] The clinical attributes of ZENITH participants are summarized in Table 6.1. The mean and median CD4 T-cell counts of HIV-1+ children were 443 cells/µl and 367 cells/µl (IQR: 218-570) respectively. The age difference between HIV- and HIV+ children was not found to be statistically significant.

The FEV_1/FVC ratio is often clinically used to diagnose children with obstructive pulmonary disorders.[648-650] For this study, the clinicians provided the baseline FEV1/FVC ratio z-score for each child if the data were available, and donors with a z-score <-1.64 were considered to be suffering from obstructive lung disease. CD4 T-cell counts were found to be lower for HIV+ children with lung disease (320.6 cells/ µl) than those without (460.9 cells/ µl) – suggesting that the onset of lung disease may be indicative, or at least a result of HIV progression (Figure 6.1).

Table 6.1 Clinical attributes of ZENITH participants

Group	Gender (% female)	Age (years)	CD4 count (cells/µl)	Lung Disease (%)
HIV+ (n=385)	51%	10.7 (6 – 15)	439 (2-2045)	11.6
HIV- (n=98)	59%	10.3 6 - 16	-	-

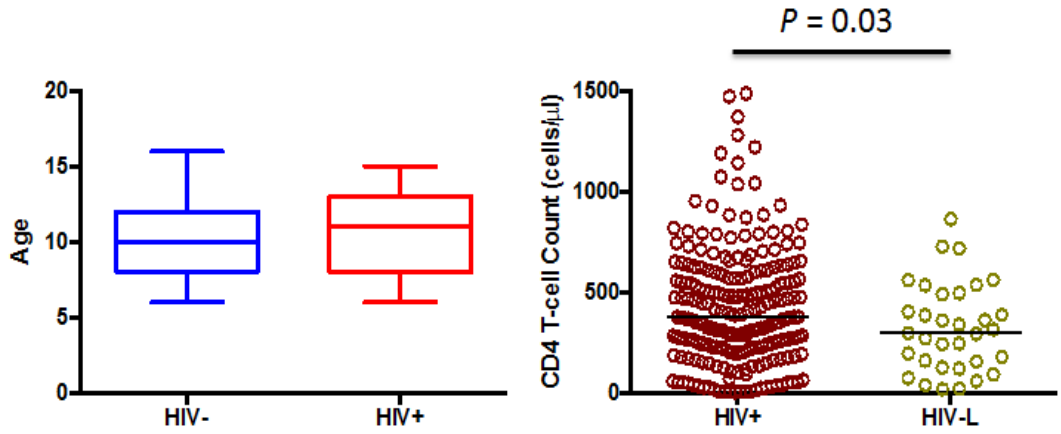

Figure 6.1: HIV+ Children with Lung Disease have Lower CD4 T-cell counts. (left) Box graphs representing the age range of HIV- and HIV+ children, the middle bar represents the median. (right) CD4 T-cell counts of HIV+ children and HIV+ children with lung disease; the difference in CD4 T-cell counts between both groups were found to be statistically significant. The unpaired T-test was used for comparing between groups. Bars indicate the median.

6.2.2 Changes in LTL and mtDNA Content of blood from HIV+ Children

The LTL and mtDNA content of leucocytes that were extracted from the whole blood of 379 children (278 cases and 101 controls) were quantified by real-time PCR and expressed as a ratio of the quantity of telomeric (T) or mtDNA (M) sequences and the copy number of the single copy nuclear gene (S) – referred to as the T/S or mtDNA ratio. In order to study the impact of untreated HIV-infection on the telomere length and mtDNA content of circulating leucocytes, we first compared the telomere length and mtDNA content of PBMCs from infected and uninfected children. Leucocytes from children with vertically-transmitted HIV were found to possess lower LTL ratio (9.8 kb/cell) (Figure 6.1a) and higher mtDNA content (43.3 copies/cell) (Figure 6.1b) than uninfected children (T/S: 10.9 kb/cell; M/S: 33.1 copies/cell) (Fig 6.1a).

6.2.3 Differences in LTL and mtDNA were related to CD4 T-cell counts

Next, we were keen to establish whether changes in LTL or mtDNA ratio were indicative of disease progression in untreated HIV-1 infected children. We observed that vertically-infected children with critically low CD4 T-cell counts (<200 cells/µl) had the shortest LTLs (8.95 kb/cell); significantly lower than those with CD4 T-cell counts <500 cells/µl (10.31 kb/cell) and those who were HIV- (10.9 kb/cell/cell) (Figure 6.2a). Moreover, a positive correlation was discovered between LTL and CD4 T-cell count (Figure 6.2c) – indicating that the severity of LTL shortening was related to disease progression.

Next, the leucocyte mtDNA content of HIV+ Zimbabwean children progressively increased with decreasing CD4 T-cell counts, with HIV+ children with CD4 T-cell counts > 500 presenting with 38.9 copies per cell and those with CD4 T-cell counts < 200 presenting with 46.8 copies per cell; this data suggest that changes to the rate of mitochondrial biogenesis might be related to disease severity (Fig 6.2b). However, the correlation between CD4 T-cell count and mtDNA content was not found to be statistically significant.

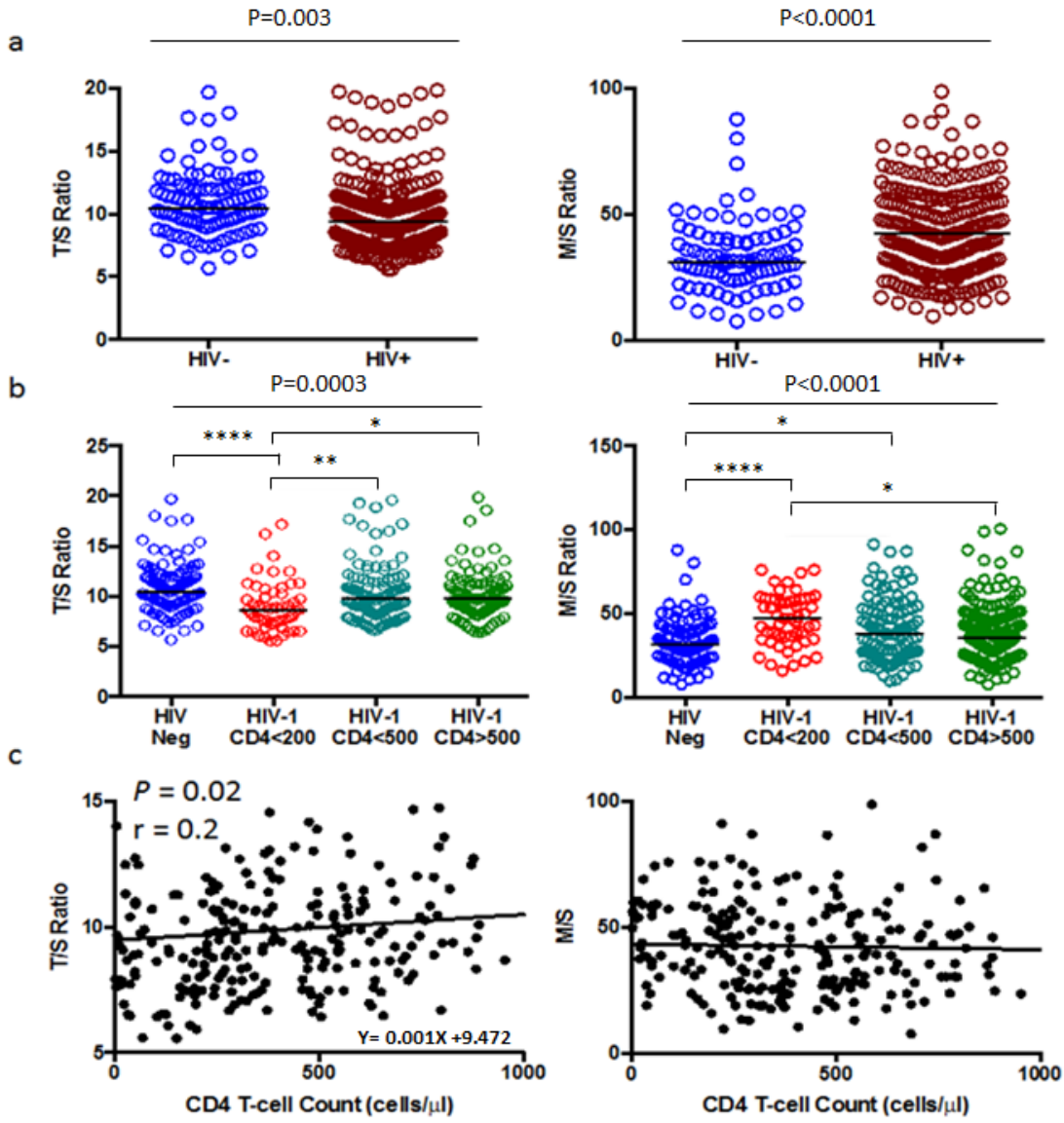

Figure 6.2: LTL and mtDNA content grouped by disease status. (**a**) T/S ratio of HIV- versus HIV+ children (left) and M/S ratio of HIV- versus HIV-1+ children (right) (**b**) T/S (left) and M/S (right) ratio of Zimbabwean children grouped by disease status and CD4 T-cell counts (HIV neg (n=99); CD4 < 200: CD4 T-cell counts < 200 cells/μl (n=54); CD4 < 500: between 200-500 cells/μl (n=110); and CD4 > 500: CD4 T-cell counts >500 (n=95)) (**c**) Correlation between T/S (left) or M/S (right) ratio and CD4 T-cell counts. The unpaired T-test was used for comparing between two groups. The one-way ANOVA with Tukey's multiple comparisons test was used for comparing multiple groups. All statistically significant differences are shown in the figure. The Spearman test was used for determining correlation. Bars indicate the median.

6.2.4 LTL and mtDNA according to Lung Disease Status

Using spirometric data from HIV positive children, we identified children with definitive, obstructive lung disease as subjects whose spirometric z score fell below -1.64. Children who were clinically diagnosed with lung disease presented with the lowest mean (9.6 kb/cell) telomere length and a downward trend was observed when comparing the T/S ratio of HIV-positive children with and without lung disease, but the difference was not statistically significant (Fig 6.1a). While various groups have independently reported a relationship between HIV infection and chronic obstructive pulmonary disease (COPD) on mtDNA depletion, there are no documented mtDNA studies on HIV-associated lung disease.[643-644,652] We found no difference in the mtDNA content of leucocytes from HIV-infected children with or without lung disease, although the mtDNA content of leucocytes from HIV+ children with lung disease (45.1 copies/cell) was much higher than those from HIV- children (33.1 copies/cell) (Fig 7.2).

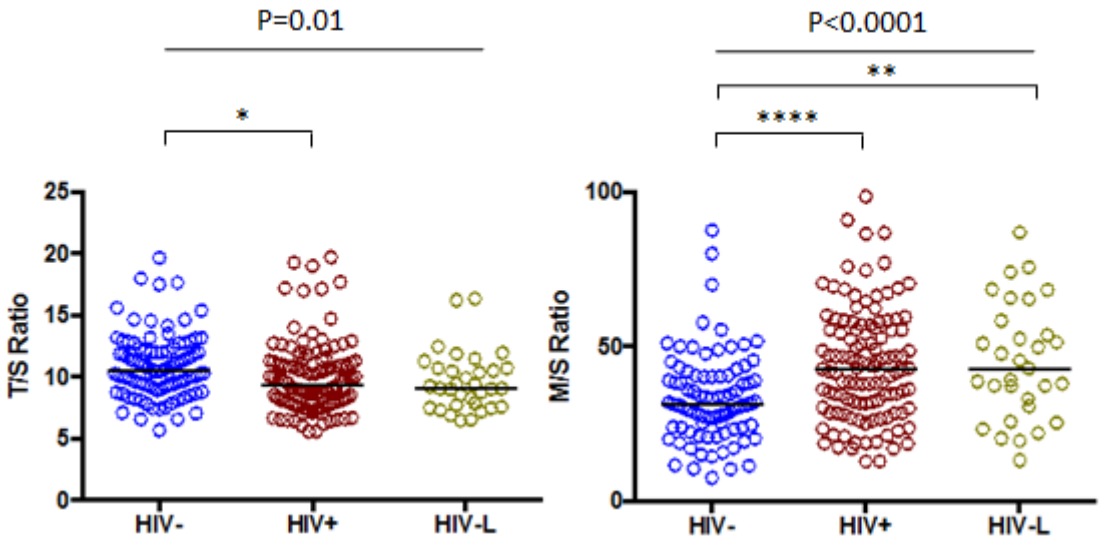

Figure 6.3: mtDNA content from Leucocytes of HIV+ children with and without lung disease. (left) T/S ratio (copies per cell) of children who are HIV- (n=99), HIV+ (n=122) and HIV+ with lung disease (HIV-L (n=33)). (right) M/S ratio of children who are HIV-, HIV+ and HIV-L. The one-way ANOVA with Tukey's multiple comparisons test was used for comparing multiple groups. All statistically significant differences are shown in the figure.

6.3 Discussion

Shorter LTLs are associated with HIV Disease Progression

Leucocyte telomere length (LTL) is closely linked to tissue-specific telomere length, such as in the heart and lung – implicating LTL as a suitable, non-invasive proxy for measuring tissue-related changes in telomere length.[652-653] For example, short LTLs are associated with an increased risk of atherosclerosis and cardiovascular diseases.[647-648] The strongest indication of a causal connection between LTL and systemic diseases is reflected in a large genome wide association study (GWAS) that revealed an increased risk of heart and lung disease with the inheritance of multiple alleles associated with shorter LTLs.[654-655]

In the context of HIV-infection, multiple groups have described shorter LTL in HIV-infected subjects across different age groups – including infants, children and adults.[637-639] Although previous studies have shown a relationship between shorter telomeres and HIV status, CD4 T-cell count and HIV viral loads – only a single study on vertically infected HIV-positive children exists to our knowledge. Since recent clinical evidence have implicated NRTI triphosphate-induced inhibition of telomerase as a mechanism for telomere shortening,[640-641] ART may compound the relationship between telomere shortening and disease progression in the aforementioned Canadian cohort, who were all accessing ART. Hence, this study portrays the impact of longstanding HIV-infection on premature immune senescence in children with greater clarity.

The fact that children who inherit HIV develop ostensible leucocyte telomere attrition despite having an intact thymus underscores the impact of longstanding HIV infection on

immune senescence.[467-468] Since LTL shortening was apparent even in children accessing ART,[638] the prompt initiation of ART is unlikely to reverse telomeric damage. However, the use of telomerase activators or cytokine treatments (such as IL7 administration) that promote haematopoiesis or the early peripheral division of naïve lymphocytes may help to alleviate telomere attrition.[456,656-658] The former strategy could deliver promising results in clinical settings, as the *in vitro* application of telomerase activators on CD8 lymphocytes from HIV-1 infected individuals were sufficient to rescue senescent T-cells from proliferative defects and boost cytokine production and virus suppressive capacity.[456] The implementation of IL7 therapy is more complex – it augments lymphocyte numbers and promotes T-cell reconstitution but has a greater impact on more differentiated cellular phenotypes and therefore, an *ex vivo* approach might be favoured to specifically target naïve T-cells.[656-659]

While the impact of HIV infection on telomere shortening may be indisputable, there is a current lack of data that are directly useful in prescribing clinical solutions to this problem. The accumulation of telomere attrition with age and disease progression indicates that any effort to reverse this process is likely to be time-sensitive and since telomere studies concerning HIV-infection currently offer only temporal snapshots of HIV-induced telomeric damage,[637-639] it remains unclear when the rate of telomere attrition surpasses the threshold of clinical relevance (i.e. progression to AIDS or the development of systemic disease). The design of prospective, longitudinal studies that incorporate LTL or mtDNA measurements; and the precise documentation of clinical indicators related to the loss of systemic function or the onset of HIV progression will be useful for strategising timely interventions that contribute to better clinical outcome.

With regard to lung disease and HIV-infection, independent studies have shown a relationship between LTL and airflow obstruction as well as diffusion capacity impairment in HIV-infected patients – suggesting a link between accelerated immune ageing and obstructive lung disease.[645-648] The lack of a relationship between mtDNA and LTL levels with obstructive lung disease in our study could be due to several reasons. Firstly, baseline spirometric data were lacking for approximately one third of HIV-infected children in ZENITH and this was due either to an inability to perform or grasp the required technique. These children were automatically assigned to the group without lung disease, and the contribution of undiagnosed lung disease in the latter group could mask existing differences in LTL between HIV-infected children with and without lung disease. Although only 11.6% of HIV-infected children were definitively diagnosed with chronic lung disease in our cohort, 66% and 21% of participants reported chronic cough and reduced exercise tolerance respectively. Moreover, more than 40% of HIV-positive ZENITH participants showed symptoms of hypoxia, either at rest or during exercise and 41% had reported multiple respiratory tract infections in the previous year.[116] Overall, the clinical symptoms of ZENITH participants suggest that the percentage of children with lung abnormalities is likely to be underestimated if diagnosis is based solely on the baseline spirometric data in our study. However, we lack other objective baseline measures of lung dysfunction since the abnormalities were discovered during the course of healthcare; together with Ferrand *et al.*, we have started recruitment for a second cohort, INHALE, where changes in the LTL or mtDNA content of the blood from untreated vertically-infected HIV+ children can be more reliably studied in relation to lung disease.

Secondly, although LTL has been shown to be closely related to lung tissue-specific telomere length, the association was demonstrated in adults where the thymus has a

smaller influence on T-cell homeostasis – this relationship might not exist in children where the thymus is more actively contributing to a pool of lymphocytes with long telomeres, as have been observed in older children suffering from vertically-transmitted HIV infection.[467-468] Finally, morphological changes in lung tissue that are specific to OB in HIV-infected children may indeed be independent of leucocyte telomere length or mtDNA content. The lack of association between CD4 T-cell count or duration of ART and pulmonary function in ZENITH suggests that once established, lung disease may be irreversible.[116] Hence, further investigation of the natural history, histopathology, pathogenesis and management of the uniquely high prevalence of HIV-associated CLD in ZENITH is necessary to devise intervention strategies and inform the use of prophylactic antibiotics and corticosteroids.

Increased mtDNA content with HIV Disease Progression

In most HIV-infected cohorts, studies on mtDNA abnormalities corroborate each other and consistently report an observed loss of mtDNA content in the blood from HIV-infected subjects.[643-645] Even in the absence of exposure to NRTIs, which are notorious for damaging mitochondria, HIV-infected adults display marked mtDNA depletion in their blood.[660] Given this context, it was surprising to observe elevated mtDNA content in vertically-infected Zimbabwean children, particularly those with critically low CD4 T-cell counts. However, several mtDNA studies in the HIV-1 literature may provide clues to understanding this phenomenon.

In one longitudinal study which spanned over two years, mtDNA levels were lower in the PBMCs of untreated HIV-1 infected adults than the HIV-1 negative population at baseline, but were elevated together with the respective 4-fold and 0.7-fold increase in

CD4 and CD8 T-cell numbers after treatment – suggesting that mtDNA levels may increase with accompanying changes in T-cell homeostasis or reconstitution.[643] Similarly, Morse *et. al.* demonstrated elevated mtDNA ratios in the adipose tissue of untreated HIV-1 infected adults versus uninfected controls, and the former group also revealed a slight increase in the mtDNA levels of PBMCs – although this difference was not statistically significant. Moreover, they showed that this increase in tissue mtDNA content was positively correlated to the levels of T-cell activation observed in these donors, as determined by the percentage of $CD38^{+}HLADR^{+}$ CD8 T-cells. Finally, Hulgan *et. al.* showed that in treatment-naïve HIV-1 infected adults, specific mtDNA haplotypes were associated with higher CD4 and CD8 T-cell activation,[661] altogether suggesting that mtDNA can influence and be influenced by T-cell signalling and activation in an *in vivo* context, which has been consistently demonstrated in animal models.[414-416] In this perspective, it might be informative to study whether specific mtDNA haplotypes are associated with better survival in paediatric HIV-1 infection. In the setting of ongoing HIV-1 infection, our collaborators, Côté *et al.*, have also observed higher mtDNA in infants born to HIV positive mothers (unpublished), which suggest that HIV infection may in certain environments, promote mitochondrial biogenesis, and that signalling pathways which drive mtDNA replication may be triggered by HIV-related inflammatory signals.

The shortened telomeres of HIV-1 infected children in ZENITH, and data from other HIV-infected children cohorts, provide clear indication that a functioning or hyperactive thymus is insufficient to compensate for the early onset of immunosenescence in vertically-infected children.[467-468,662] Indeed, lower percentages of naïve CD8 T-cells and higher proportions of $CD28^{-}CD57^{+}$ memory CD8 T-cells were observed in treated but viraemic HIV-1 infected children (median age of 14) – who also developed shortened

telomeres – as compared to the uninfected and aviraemic HIV-1 infected children.[639,662] A separate study showed a strong inverse relationship between the percentages of activated ($HLA\text{-}DR^{+}CD38^{+}$) and senescent ($CD28^{-}CD57^{+}$) CD8 T-cells in HIV-infected young children (0-5 years of age) and LTL.[639] Based on the latter observation, and other experimental evidence which demonstrate that telomere shortening occurs as a result of repeated or excessive immune stimulation,[349-350,40] we have strong reason to believe that the increase in leucocyte mtDNA levels is related to the levels of immune activation that accompany chronic paediatric HIV-1 infection. Consistent with this hypothesis, sCD14 levels are significantly elevated in vertically-infected children as opposed to uninfected children, which signals that ongoing microbial translocation continues to be a potent and persistent source of immune activation during paediatric HIV-1 infection.[639]

In children who are rapidly progressing, the exponentially high HIV-1 viral loads contributes directly to excessive immune stimulation,[226-229] which could explain why mtDNA content might be highest in donors with the lowest CD4 T-cell count. Although we currently lack the viral load data to adequately demonstrate this point – once past the viral set point (~5 years for vertically-infected children): HIV-1 viral loads are typically sustained at much higher levels in vertically-infected children than in HIV-1 infected adults, even when slow-progressors are compared.[111] In chronically infected children, mitochondrial biogenesis may be upregulated as a response to bystander activation or to antigen-specific signals in order to support effective viral clearance by both T- and B-cells.[413-416] The measurement of soluble markers of immune activation in the plasma of HIV-1 infected children from the ZENITH cohort will help to sharpen our conclusions, but we expect that leukocyte mtDNA levels of children in ZENITH will correlate closely with plasma levels of sCD14 and LPS.

The increased mtDNA content of circulating leukocytes may have pathologic repercussions. Compromised mitochondria release molecules – including mtDNA – called 'damage-associated molecular patterns' (DAMPs), which contribute to inflammation and oxidative stress – adding to the vicious cycle of chronic immune activation and advanced cellular ageing.[663-664] Several studies have reported increased plasma levels of mtDNA during both acute and chronic phases of untreated HIV infection, and plasma mtDNA concentrations were positively correlated with HIV viral load.[665-666] While these studies did not measure the levels of other DAMPs in the plasma of HIV-infected donors, the high amounts of extracellular mtDNA is by itself an oasis of CpG DNA motifs that can activate immune responses;[664,666-678] while also serving as an indicator of excessive cellular death.

Our preliminary findings warrant a deeper investigation into the relationship between mtDNA and DAMPs and their contribution to disease progression and lung disease. The isolation of specific molecular culprits may not only lead to the identification of biomarkers of disease progression and HIV-associated lung disease, but are essential for designing therapeutic interventions to prevent both outcomes – particularly important since ART is likely to exacerbate mitochondrial damage in HIV-infected children.[640-642] The use of drugs that target specific pathways that regulate mitochondrial biogenesis – including PGC-1α, Tfam, eNOS and SIRT1 – may be useful in this context.[669-671] In addition, drugs that inhibit innate receptors, which activate inflammatory responses upon interactions with DAMPs, may be useful in reducing immune activation and mtDNA dysregulation. For example, molecules that inhibit FPR-1 and TLR-9 – which are activated by mtDNA and other DAMPs – may help to offset HIV-1 induced hyperinflammation in a manner that is relevant to slowing HIV disease progression or the development of lung disease in vertically-infected Zimbabwean children.[672-673] The latter

approach might be more practical to avoid interfering with the high mitochondrial activity that is required to sustain antiviral mechanisms.

Chapter 7: General Discussion and Concluding Perspectives

7.1 Preservation of T-cell Homeostasis and Function in HIC2

In summarising our HIV-2 data, we have conclusively shown that the development trajectory from haematopoiesis and thymopoiesis to the preservation of naïve T-cells is intact in asymptomatic HIV-2 infected donors; we also demonstrated that the representation of HPCs, naïve T-cells, memory T-cells and senescent T-cells in HIC2 are very similar to the numbers observed in HIV uninfected donors. As expected of HIV negative populations,[530] the maintenance of the naïve T-cell pool remains closely linked to thymic output in our cohort of French HIC2.[530] Moreover, we show that HIV-2 specific CD4 and CD8 T-cells are highly polyfunctional, and retain potent Gag-specific cytokine responses as well as HIV-2 suppressive activities that strongly relate to the CD4 T-cell numbers of our HIV-2 controllers.

On the question of whether T-cell homeostasis remains unperturbed by HIV-2 infection, our data are generally compatible with descriptions from previous HIV-2 studies, even though we did not directly compare markers of T-cell activation – or plasma markers of immune activation – between HIV-2 infected donors and uninfected or HIV-1 infected donors. We agree that the levels of systemic immune activation levels are likely to be higher in HIV-2 infected donors than uninfected donors, albeit to lower levels than observed in HIV-1 infection.[285,315-316] Generally, HIC2 presented with more memory CD8 and CD4 T-cells, and more CD57+ CD4 T-cells than uninfected donors, although the difference in the numbers of memory CD4 T-cells were not statistically significant. Moreover, for both CD4 and CD8 T-cells, we observed higher naïve and smaller memory T-cell populations – CD57+ or otherwise – in HIC2 than viraemic HIV-1 donors or HIC1.

These conclusions support the findings of Hegedus et. al., whose team reported incremental changes in the expression of immune activation (HLA-DR/CD38) on T-cells and an increased turnover rate of naïve T-cells into memory T-cells based on HIV-infection status, where HIV negative controls < HIV-2 infected donors with low viral loads < HIV-1 infected donors < HIV-2 infected donors with high viral loads in their manifestation of heightened T-cell activation status and signs of memory T-cell inflation..[529]

An important question remains unanswered in our study, and in the current HIV-2 literature, which pertains to whether HIV-2 continues to modulate T-cell dynamics directly in both progressive and asymptomatic chronic HIV-2 infection. A major limitation of our study is the lack of plasma and tissue samples from HIC2, which prevents us from addressing the question of whether HIV-2 is actively replicating in the tissues of HIV-2 controllers. HIV-1 is known to replicate actively in the GALT throughout all stages of infection, even in the absence of plasma viraemia.[161,232] The persistent high levels of sCD14 and LPS during chronic HIV-2 infection suggests continuous GALT compromise,[316,325] which may be mediated by active HIV-2 replication during chronic infection. Alternatively, HIV-2 antigens may persist in the form of exosomes and circulate in the periphery, continuously activating HIV-2 specific T-cells that contribute to systemic immune activation by responding with the secretion of pro-inflammatory cytokines.[267] HIV-1 proteins, such as Nef and Gag, have also been shown to traffic in exosomes during HIV-1 infection, and lead to the activation of virus-specific T-cells as well as the bystander activation and apoptosis of T-cells with other specificities.[160,180,266,674-675]

The observation of high frequencies of HIV-2 specific CD4 and CD8 T-Cells with preserved anti-HIV-2 suppressive capacities despite decades of asymptomatic infection supports our hypothesis of a persistent source of HIV-2 antigens, as repeated encounters with antigens are usually required for the preservation of antigen-specific T-cells and their functionality.[551-555] The preservation of high frequencies of HIV-2 specific CD4 and CD8 T-cells has also been reported in previous HIV-2 studies.[281,290,296-297,305] More significantly, the close coupling between the percentages of HIV-2 Gag-specific CD4 and CD8 T-cells; the maintenance of close correlations between the ability of CD8 T-cells to mediate HIV-2 suppression and the magnitude of CD4-specific cytokine responses; the relationships between p27-specific cytokine responses and the CD4 T-cell counts of HIC2; and finally, the clear associations between the magnitude of HIV-2 specific T-cell IFNγ responses with CD4 naïve T-cell decline and memory inflation further substantiates our view – that HIV-2 continues to modulate T-cell homeostasis directly despite asymptomatic infection. The exact source of HIV-2 antigens could be determined in future experiments by purifying exosomes from the plasma of HIC2 from other cohorts, and probing for the presence of HIV-2 proteins; or by examining the lymph nodes or tonsils of HIC2 for signs of active HIV-2 replication.

Next, high proviral loads have been reported in HIV-2 infected individuals across multiple HIV-2 cohorts, and this poses the question of whether the integrated provirus can serve as a reservoir for allowing the recrudescence of HIV-2.[306,308,515] Recent publications indicate that the majority (>90%) of HIV-1 proviral DNA in HIV-1 infected individuals is defective and cannot contribute to productive HIV-1 replication.[676-677] This raises a possibility that the longer asymptomatic phase or high proportions of HIV-2 controllers associated with HIV-2 infection could be due to an even higher incidence of defective provirus in HIV-2, which warrants further investigation. The detection of high levels of

APOBEC3F/G editing activity within the HIV-2 proviral genome suggests a strong possibility of the latter scenario.[312]

With our data, we were able to make educated speculations on how the majority of HIV-2 infected individuals achieve long-term control over HIV-2 viraemia. We believe that a major component of HIV-2 control hinges on the ability of HIC2 continuously to generate robust HIV-2 specific T-cell responses that suppress viral replication, which are much rarer in HIV-1 infection (Figure 7.1).[290,297,305] The preservation of high numbers of HPCs and naïve T-cells in HIC2 supports the continuous renewal of exhausted or depleted HIV-2 specific CD4 and CD8 T-cells – altogether supporting the mounting of *de novo* or recall immune responses against HIV-2 when required. Nevertheless, a major limitation of our study is the lack of HIV-2 progressors, which would otherwise provide a more accurate and valuable counterpoint than HIC1 to verify our conclusions against.

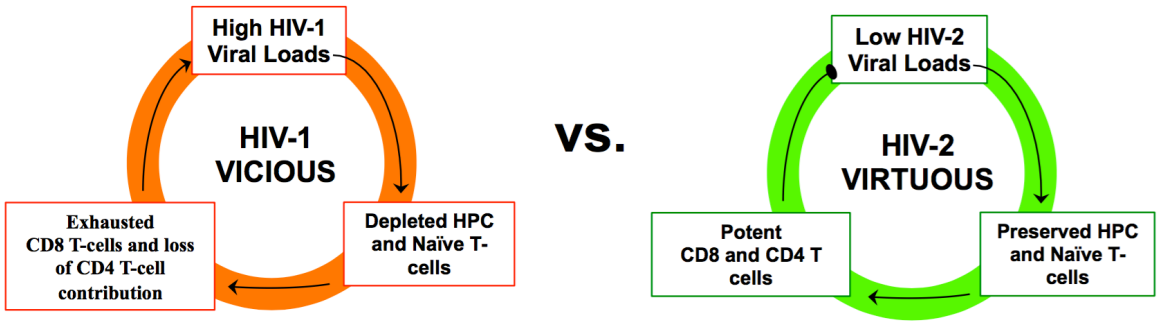

Figure 7.1: Virtuous cycle of HIV-2 infection versus vicious cycle of HIV-1 infection. (left) During the chronic phase of HIV-1 infection, the depletion of HPCs and naïve T-cells from cellular infection by HIV-1 and HIV-1 associated immune activation fails to replenish the growing numbers of exhausted and senescent CD8 T-cells. The latter results in high HIV-1 viral loads, which perpetuates the vicious cycle of cellular death and destruction through excessive immune activation, ultimately leading to AIDS. (right) During chronic HIV-2 infection, the low HIV-2 viral loads – perhaps due to better innate control – and accompanying low levels of systemic immune activation lead to the preservation of high HPC and naïve T-cell populations, ultimately sustaining potent T-cell responses that result in durable viraemic control.

7.2 HIV-1 vertically-infected children in Zimbabwe – a case of Emergency?

Although both HIC2 and perinatally infected HIV-1 positive children have been shown to possess an intact or even hyperactive thymus;[461-462,467-468] the prognosis is more optimistic for untreated HIV-2 infection than vertically-infected children.[31,99,100-104,280] Comparing the two cohorts of slow progressing HIC2 (median time since diagnosis: 13.6 years) and paediatric HIV-1 (median age: 10.5 years) cases, it is clear, even in terms of CD4 T-cell counts, that the former group fare better, even though both groups have been infected with a human retrovirus.

55% of ZENITH participants had CD4 T-cell counts < 400 cells/μl, and 24% had CD4 T-cell counts < 200 cells/μl. The lung-associated morbidities associated with HIV-1 children in the ZENITH cohort are debilitating; 41% reported multiple respiratory tract infections, 40% had hypoxaemia at rest or on exercise, and 47% revealed chest radiography abnormalities. A significant proportion of ZENITH children also present with pubertal delay (21%) and stunting (34%).[113-114,116] Altogether, the data indicate that it may be beneficial to initiate treatment in these children regardless of CD4 T-cell counts. Current WHO guidelines for ART initiation in children assume that treatment can be safely deferred until CD4 T-cell counts fall below 350 cells/μl or meet the criteria for WHO Stage 3-4.[678] However, the deferral of treatment in slow progressing children has revealed an unacceptably high risk of chronic complications and suboptimal immune function.

Our data indicate that for ZENITH children, immune dysfunction can be traced to the molecular level. Leucocytes from vertically-infected children in the ZENITH cohort present with shortened telomeres and abnormally high mtDNA levels. The lack of cellular samples have prevented us from demonstrating how the molecular data translate into cellular or tissue dysfunction, but the range of morbidities that accompany the infection suggests that these molecular biomarkers, which are relatively simple to sample, may have significant prognostic value or clinical relevance. At this point, it is important to obtain a clearer clinical picture, including information on viral loads, the extent of systemic or tissue inflammation, immune cell competence and function, and prospective data that document the impact of treatment in reversing these adverse clinical symptoms.

We reveal a trend where HIV-1+ children with lung disease present with even shorter telomeres than HIV-1+ children without lung abnormalities, but this difference was not statistically significant. The observation that CXCR4-tropic HIV is able to directly infect airway epithelial cells (but not replicate in them) and impair cell-cell adhesion and stimulate the production of inflammatory cytokines probably accounts for the latter,[679] as direct cellular damage caused by HIV is likely to have a greater impact in accelerating the presentation of pathological lung phenotypes than tissue damage caused by cellular senescence; although the latter may severely impair immune function and the mounting of anti-viral capabilities.[335] A more rigorous clinical and scientific documentation of the characteristics of ZENITH children is critical to the implementation of appropriate treatment regimens to improve the health of these children.

7.3 Constrants and Limitations of Study

A core limitation of our HIV-2 study lies in the lack of HIV-2 progressors, which would have allowed us to ascertain whether the loss of primary immune resources would be accelerated by active HIV-2 replication, as is the case in HIV-1 infection.[191] The data from HIV-2 progressors would be useful in determining whether the modest influence of HIV-2 infection on accelerated immunosenescence was due to the nature of the virus itself or the lack of active viral replication. Next, we crudely classified memory cells crudely as CD45RA- T-cells, which simplified the analysis but precluded the contribution of TEMRA cells, which are terminally differentiated T-cells that reexpress CD45RA and exhibit high CD57 expression. The latter was not consistently observed to be present in substantial numbers in the majority of our small cohort of HIC2 and was therefore, excluded from our analysis but should be considered in larger studies. The small size of our HIV-2 cohort limits the statistical power of our analyses, however, we have endeavoured to eliminate incidences of Type 1 error through the use of multiple post-hoc tests, such as the Dunn's and Tukey's test.

In our HIV-1 vertically-infected adolescent study, our participants were recruited from major suburban clinics, which may potentially contribute to selection bias towards more severe cases of infection. Moreover, we currently lack the resources to perform viral load measurements on the collected blood samples, which in addition to CD4 T-cell counts, would have been useful to determine the clinical relevance of telomere attrition. As a result of difficulties in shipping, we only had access to DNA and plasma samples from our HIV-1 infected adolescent donors, which limited the scope of our telomere assays to qPCR techniques.Ideally, we would have liked to substantiate our conclusions with other

forms of telomere length measurements, such as with the Flow-FISH technique, which would be discussed later.

7.4 Can Immunosenescence be treated?

We have attempted a very comprehensive study of immunosenescence in this work. Together with the data that was reviewed, we have demonstrated how HIV (HIV-1 more than HIV-2) is able to impose immunosenescence prematurely at the molecular, cellular and systemic level. Altogether, there is a substantial amount of evidence that relate to how immunosenescence can be accelerated during chronic disease conditions that impose a prolonged systemic state of high inflammatory stress, which leads to a further question of whether immunosenescence can be treated or reversed. Since immunosenescence operates at the functional, clonal, and systemic level, therapeutic interventions may target any of these stages.

As the age-associated defects caused by HIV infection can be traced all the way to the progenitor level,[191,380] therapeutics that improve the quality and production of progenitors or naïve lymphocytes can be considered. In human and animal studies, the administration of growth factors and cytokines such as IL-7, IL-15, and IGF-I vastly improves immune reconstitution;[680-682] it is worth exploring whether these treatments can be combined with cART to bridge the gap in mortality between treated HIV-1 subjects and the uninfected population. Another approach may be to enhance thymic output through the use of gonadal steroids, which have a substantial influence on thymus size and function, and have been shown to promote the proliferation and survival of lymphocytes.[683-685]

Since immune activation is a key driver of immunosenescence,[223,255] the neutralisation of innate immune receptors, inflammatory cytokines and inhibitory molecules that interfere with immune function could also be an effective strategy.[563,672-673] However, the latter approaches must be finely balanced and calibrated, such that the benefits outweigh the cons in immunocompromised patients.[686] An emerging yet promising area of research proposes that senescent cells, which are key players in driving age-related cellular or tissue damage, may be specifically targeted for deletion – a strategy referred to as senotherapy. For example, the use of small-molecule inhibitors to block cell death-resistance pathways that relieve apoptotic resistance, or immune-based clearance that rely on antibodies or cytotoxic T-cells to remove senescent cells could be beneficial in treating immunosenescence;[339,687] the latter could be achieved by raising antibodies against senescence-specific surface antigens, such as CD44 or CD57,[454,688] which then direct cytotoxic killing or the delivery of cytotoxic nanoparticles. Again, it is important to reduce the risk of off-target effects, as senescent cells may be beneficial in certain settings, such as in directing wound repair.[689]

7.5 Novel approaches to studying immunosenescence

Monitoring of CD4 T-cell and B-cell subsets

Due to the sustained high interest in the study of immunosenescence as a mechanism that is involved in driving disease, we now have sufficient information and accompanying advancement in technology to broaden our approach of study, but the latter resources have remained relatively untapped in the current literature. In the context of T-cell senescence, our understanding is growing in specificity, such that we are able to identify specific CD4 or CD8 T-cell subsets in which homeostasis is dysregulated during disease. For example, recent studies have begun to show a decrease in Th17 cells and an increase in Treg cells with age.[356-360,690] In addition, the Th1/Th2 balance is altered in aged individuals,[691] and older people further present with altered T follicular helper (Tfh) / regulatory (Tfr) cells frequencies that impair antibody production and vaccination responses.[691-694] These compositional changes are also directed by HIV-1 infection, in a manner that influence, or is influenced by HIV-1 viraemia.[695-697] During chronic HIV-1 infection, an increased frequency of Tfh cells was highly correlated with HIV-1 viral load and also associated with an increase in the number of germinal centre B-cells and plasma cells, ultimately contributing to dysregulated antibody production.[698] A further understanding of how these pathological changes relate to immune activation will be important for improving the health of HIV-infected populations.

Unlike T-cell senescence, the subject of B-cell senescence has remained relatively unexplored in humans, especially in the context of disease. Apart from declining lymphopoiesis and alterations to the composition of the peripheral B-cell compartment, which becomes increasingly skewed towards populations of marginal zone and $CD5^+$ type

B1 cells with age, little is known about B-cell senescence.[698-699] For example, there are no reliable markers that can identify senescent B-cells with the consistency of CD57 in T-cells. Furthermore, the influence of class switching, B-cell maturation, ageing, and immune activation on telomere length is not known. It is appropriate and important to define the impact of these different factors on B-cell senescence, as this could help explain the variation in vaccine responses that is observed between individuals. The study of B-cell immunosenescence becomes particularly important when we consider that successful vaccination becomes increasingly difficult with age, and that the development of strong adjuvants is necessary to support vaccination in people beyond 65 years of age.[700] Considering the advanced immunosenescence that also accompanies HIV-infection,[335] a concrete understanding of changes in B-cell biology will also be useful in the advancement of HIV vaccines.

In order to avoid a parochialism that limits our understanding of immunosenescence and how it pertains to HIV pathology, we need to develop strategies that widen our approach of study. For this purpose, we have optimized antibody panels that can be used to identify specific CD4 T-cell and B-cell subsets; with these panels, we are able to monitor and determine how compositional changes in their representation relate to immune activation or pathology. Moreover, we may use these panels to isolate specific cellular populations by fluorescence-activated cell sorting for the quantification of mtDNA content or telomere length by qPCR (Figure S1 and S2). With our B-cell panel, we have compared HIV-2 donors at different stages of disease progression. At a preliminary level, our data suggest that HIV-2 progressors may have a higher representation of more differentiated B-cell subtypes. For example, the percentages of marginal zone B-cells, transitional B-cells and plasma cells - appear to be elevated within a HIV-2 progressor (Figure S3).

Measuring telomere length of specific cellular subsets by imaging flow cytometry

The advent of imaging flow cytometry has allowed the high throughput visualisation of cellular behaviour; this can be employed to the detailed study of cell cycle and proliferation events, phagocytosis, and also quantify chromosomal damage.[701-705] In the ten years since its commercialisation, the platform has grown and so has its scope for potential applications, and the technique is now featured in approximately 500 peer-reviewed scientific articles.[685] Despite its steady growth, the range of applications is still limited and has rarely been employed in immunological or immune senescence related studies. This could be due to previous technical limitations that prevent the study of a broad range of immune markers, as is necessary for the identification of specific subsets of interest, but the advent of the ImageStream X® (Merck Millipore) now allows for the study of up to ten fluorescent markers, which exponentially broadens our ability to exploit the technology in the study of immunology.

We have adapted the use of imaging flow cytometry and combined it with a Flow-FISH technique to measure telomere length in specific T-cell subsets – an application that has hitherto been unexplored in the current scientific literature. With this technique, we show that the difference in telomere length between CD57+ and CD57- T-cells can be examined at a higher resolution than conventional flow cytometry approaches (Figure S4 cf. Figure 7.2). As the PNA probe is specific for telomeric repeats, the PNA-FITC intensity provides an indicator of telomere length, with higher intensities signalling longer telomeres. Figure 7.2 shows representative data from one donor; here, we show that regardless of CD27 expression, CD57+ T-cells have shorter telomeres than CD57- T-cell populations. For example, CD27-CD57- CD8 T-cells have approximately 34% longer

telomeres than CD27-CD57+ cells; while CD27+CD57- cells had approximately 29% longer telomeres than CD27+CD57+ cells.

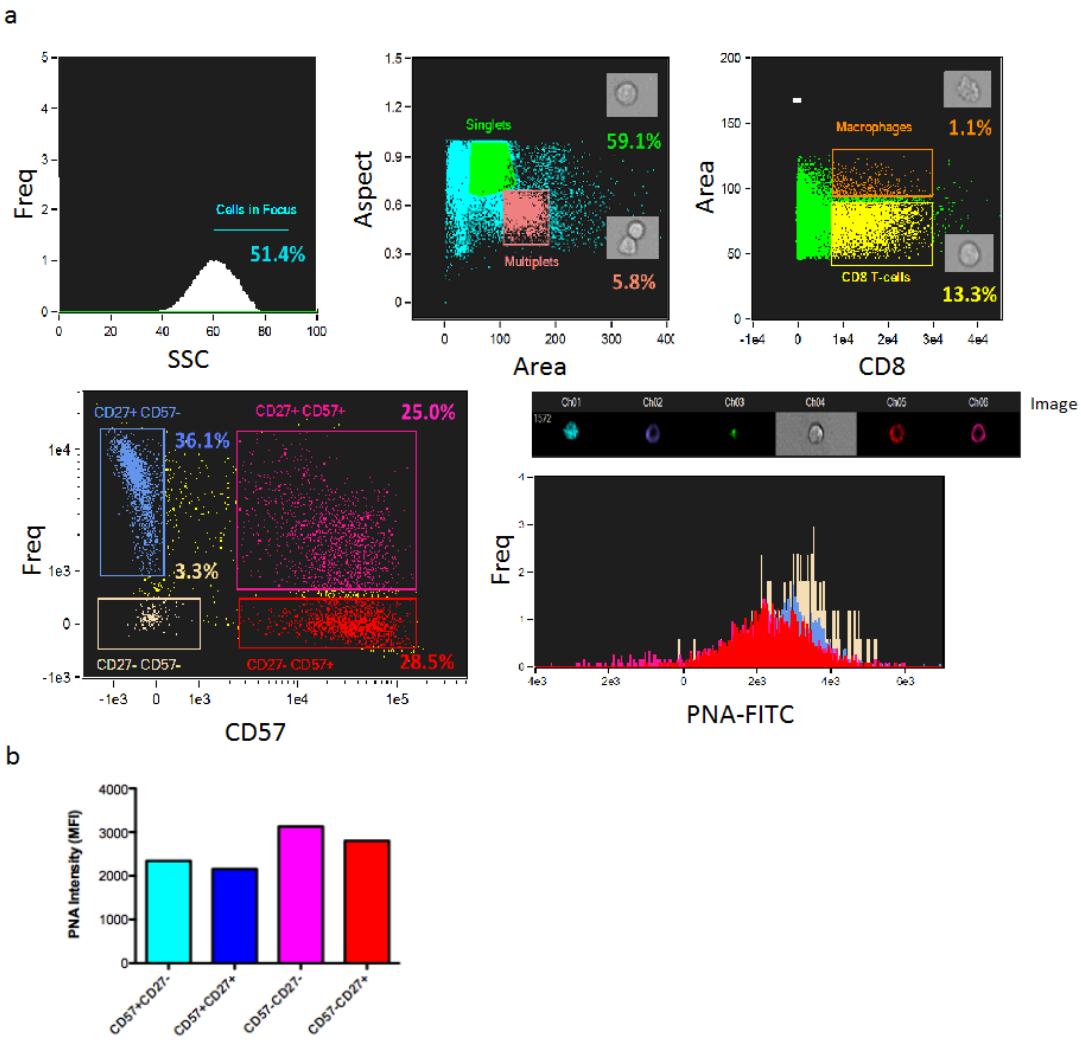

Figure 7.2: FLOW-FISH Telomere Length Assay by Imaging Flow Cytometry. (**a**) (top) After gating for cells in focus and singlets, CD8 T-cells were separately gated from macrophages based on size and area (cellular images shown in insets). (bottom) Gating of CD57+ and CD27+ T-cells. An image of a CD8+CD27+CD57+ cell is shown on the right, together with a histogram with an overlay of all four populations and their expression of PNA-FITC. (**B**) CD57+ cells have shorter telomeres, regardless of CD27 expression.

In addition to PNA fluorescence, the ImageStream allows for the analysis and quantification of bright PNA-FITC spots, or foci, which presumably forms when chromosomes have telomeres that extend beyond a threshold length. Therefore, we expect PNA foci count to provide an indication of the number of chromosomes with long telomeres. In our analysis of PNA foci formation (Figure 7.3), we observed an identical trend to the data obtained with PNA fluorescence, where CD57 expression is associated with cells that form fewer PNA foci. CD27+CD57+ CD8 T-cells had the lowest foci count (2.172), while CD27-CD57- CD8 T-cells had the highest foci count (2.728).

We further employed the ImageStream-based Flow-FISH assay to study the telomere length of various B-cell subsets. We focused on the identification of IgG1 B-cells, as the latter are the most abundant isotype in blood and also the main focus of HIV vaccine research.[706] For example, the efficacy of the relatively successful RV144 vaccine has been largely attributed to IgG recognition of gp120.[707] We observed that IgG1 B-cells had longer telomeres (approximately 20%) than non-IgG B-cell isotypes (Figure 7.4). This was unexpected because naïve IgM and IgD B-cells represents the majority of non IgG1 B-cells, and we expected the more antigen-experienced IgG B-cells to have a more substantial proliferative history and therefore, reveal signs of telomere shortening. However, we rationalised that the proliferative requirements of somatic hypermutation and class switching in germinal centres are likely to be compensated by the upregulation of telomerase activity. Indeed, it was observed that germinal centre B-cells expressed 128-fold higher telomerase than other B-cell subsets, including naïve and plasma B-cells.[708]

From our data, we also observed that circulating IgG1 B-cells could be grouped into two populations based on PNA intensity. This observation is probably due to differences in the biology of IgG1 plasma cells as compared to other non-terminally differentiated B-cells; the former is expected to exhibit shorter telomeres as plasma cells tend to be relatively long-lived and prolific.[709] This hypothesis can be further validated by using CD138 as a marker to separate IgG1 plasma cells from other IgG1 B-cells.

Overall, the Imagestream has provided a sensitive and useful system for measuring the telomere length of specific T- and B-cell subsets by Flow-FISH. This technique can be employed to perform the high throughput imaging analysis of donor PBMC samples, while simultaneously collecting typical flow cytometry data. Moreover, we can expand the study to incorporate the detailed imaging of cell cycle and proliferation events, enumerate cytotoxic granules, and also quantify chromosomal damage;[701-705] the latter are all relevant in the study of immunosenescence, and the incorporation of all these analyses into one assay maximises the amount of information that can be obtained from a single donor sample.

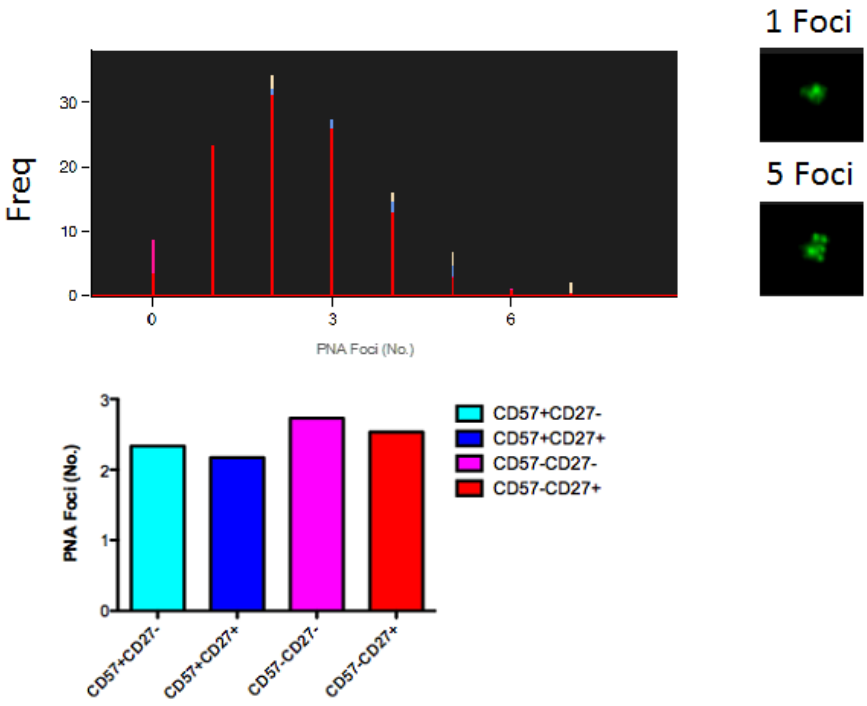

Figure 7.3: CD57+ Cells Display a Lower PNA Foci Count. (top) The distribution of PNA Foci (No.) across four populations of cells that have been gated on CD27 and CD57 expression is shown. Representative images displaying cells with different foci count are shown on the right. (bottom) The graph shows the mean PNA Foci for these four populations of CD8 T-cells; and indicates that CD8 T-cells expressing CD57 have fewer PNA foci, with the CD27-CD57- population exhibiting the highest foci count.

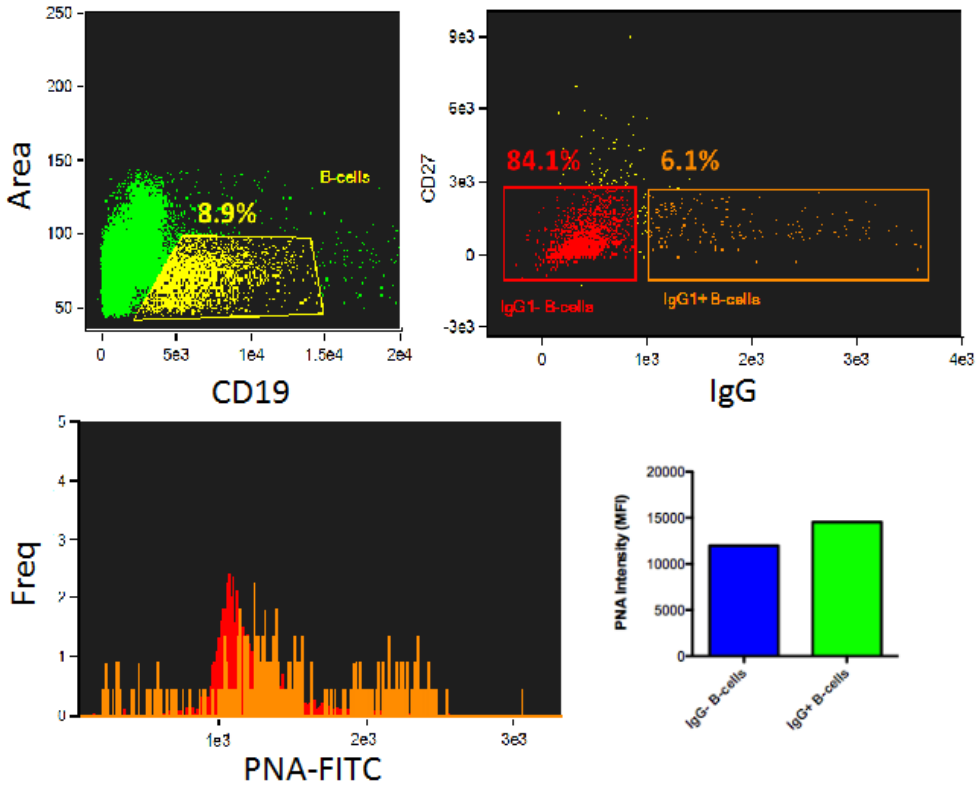

Figure 7.4: IgG1 B-cells Display Longer Telomeres. (top) Gating of IgG1+ B-cells. After gating for cells in focus and singlets (not shown), B-cells were gated based on size and CD19 expression and subsequently on IgG1 expression. (bottom) IgG1 B-cells display longer telomeres. The PNA fluorescence of IgG1+ and IgG1- B-cells are shown in the histogram and accompanying graph on the right.

Appendix

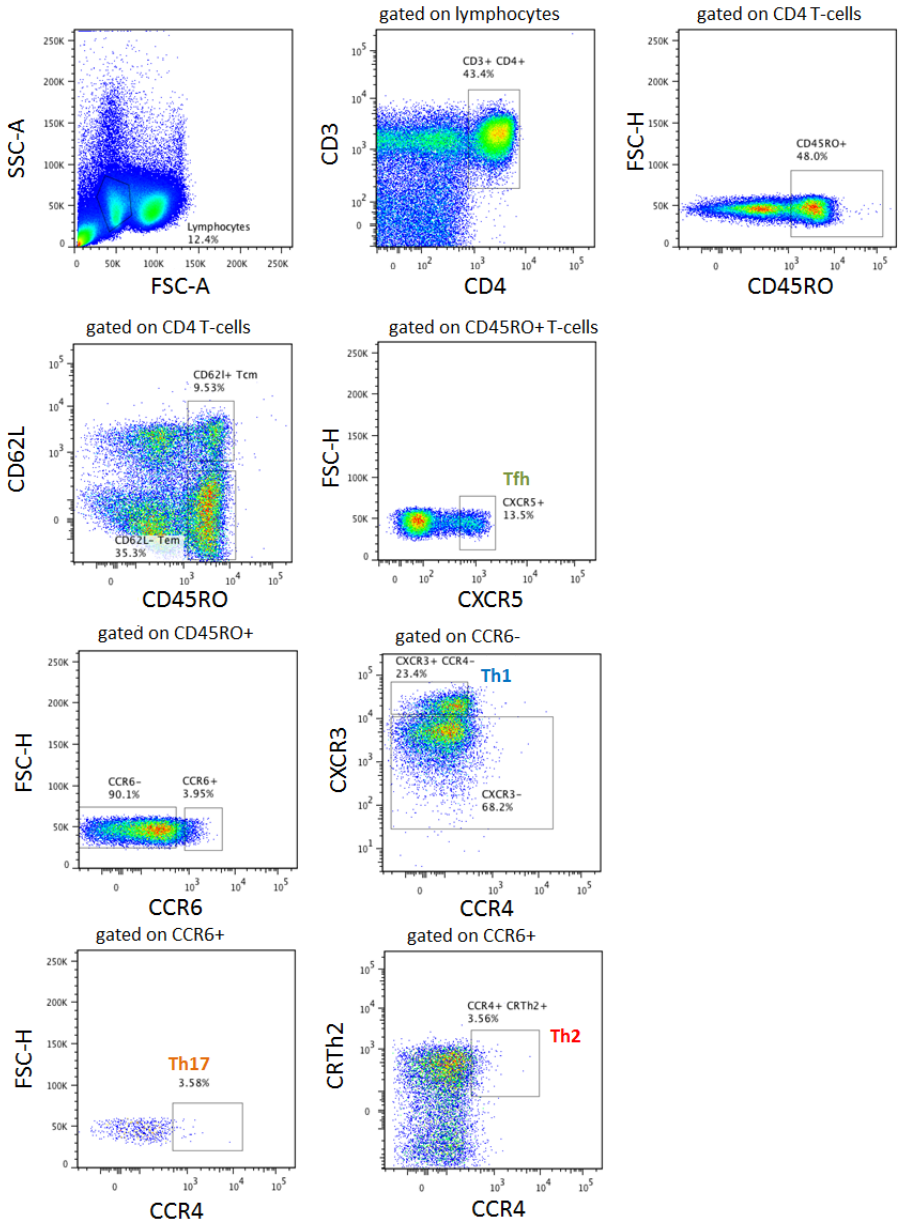

Figure S1: Identification of CD4 T-cell subsets. Flow cytometry events are first gated to identify lymphocytes, then CD3+CD4+ cells, then CD45RO+ CD4 T-cells. From the latter, the gating strategy for Th1, Th2, Th17 and Tfh subsets in peripheral blood are shown.

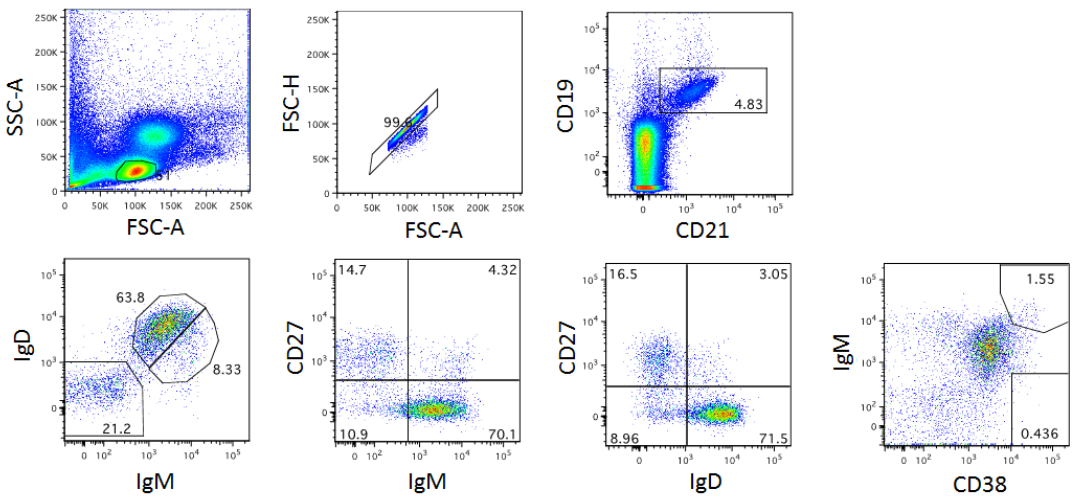

Figure S2: Identification of B-cell subsets for the study of immunosenescence. Gating of CD19+CD21+ B-cells and naïve, class-switched and marginal zone B-cells. The average of the two percentages of IgM+CD27- and IgD+CD27- B-cells is taken to represent the naïve B-cell population; the percentage of memory, class-switched B-cells (Bsw) is averaged from the percentages of IgM-CD27+ and IgD-CD27+ populations; and the average percentage between the two IgM+CD27+ and IgD+CD27+ populations represents the marginal zone B-cell (Bmz) population. Transitional B-cels (Btr) are $IgM^{hi}CD38^{hi}$ and plasma cells (Bpl) are $IgM\text{-}CD38^{hi}$.

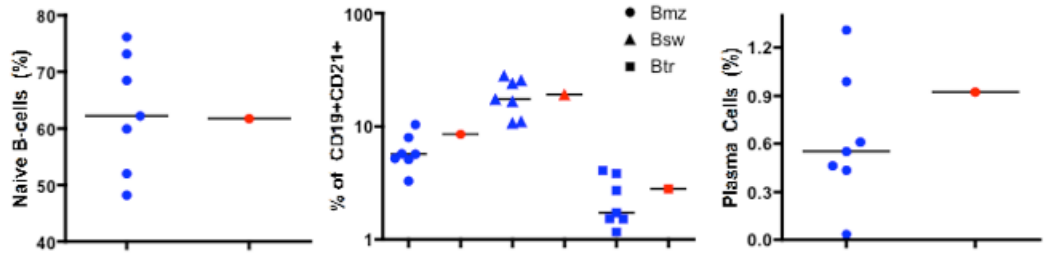

Figure S3: B-cell compartments in HIC2 (blue) and HIV-2 progressors (red). (left and middle) HIV-2 progressors (n=1) and non-progressors (n=7) appear to possess comparable percentages of Naïve B-cells and Bsw; but the remaining data are suggestive of differences in the percentages of marginal zone B-cells (Bmz), transitional B-cells (Btr) and plasma B-cells (Bpl) in HIC2 and HIV-2 progressors. Bars indicate the median

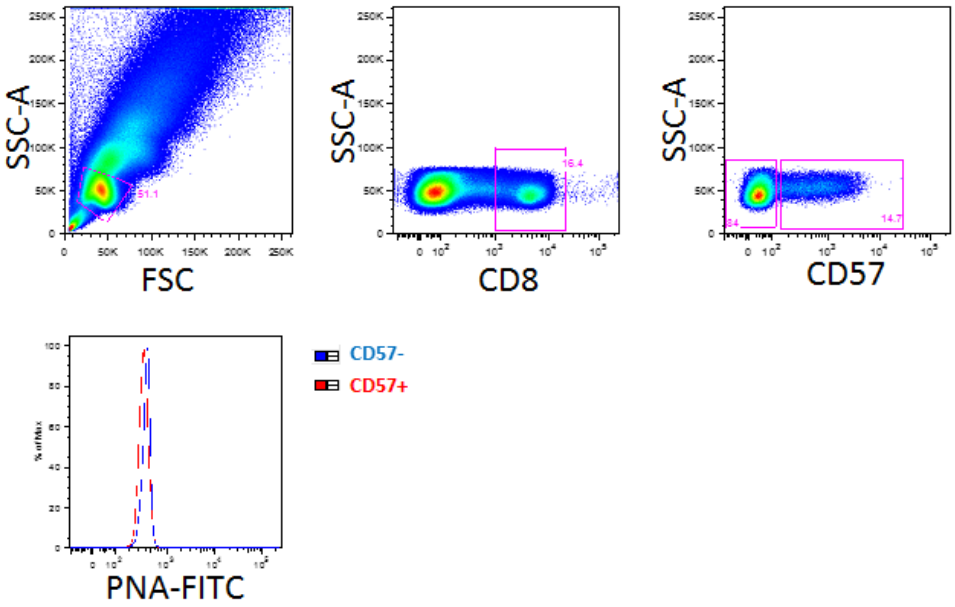

Figure S4: FLOW-FISH analysis of Telomere Length by Flow Cytometry. (top) Gating of CD8 T-cells followed by CD57+ CD8 T-cells. (bottom) PNA intensity of CD57+ vs CD57- CD8 T-cells from two different donors. CD57- CD8 T-cells exhibit marginally higher PNA MFI.

The Journal of Immunology

Preservation of Lymphopoietic Potential and Virus Suppressive Capacity by CD8 $^{+}$ T Cells in HIV-2–Infected Controllers

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