Characterizing the cellular latent reservoir of HIV-1 and the effect of immune activation on characteristics of the reservoir

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2. **Ismail, SD**, Pankrac, J, *et al.*, "'Addressing an HIV cure in LMIC", *Retrovirology.* 2021;18, 21. https://doi.org/10.1186/s12977-021-00565-1.

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Grateful for promises fulfilled.

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List of abbreviations

°	Degrees
3TC	Lamivudine
ACD	acetate citrate dextrose
AIDS	Acquired immune deficiency syndrome
ANRS	French National Agency for Research on AIDS
APC	Anaphase-promoting complex
APCs	Antigen presenting cells
ART	Antiretroviral therapy
AUC VL	Area under the curve viral load (cumulative viral burden)
bNAb(s)	Broadly neutralizing antibody (-ies)
BV	Brilliant violet
CAPRISA	Centre for the AIDS Programme of Research in South Africa
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) recptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CO2	Carbon dioxide
CRFs	Circulating reconbinant forms
CTL	Cytotoxic T lymphocyte (CD8+ T cell)
CTL	Cytotoxic T lymphocyte (CD8+ T cell)
CXCL-	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
dCA	Didehydro-Cortistatin A
ddPCR	Doroplet digital PCR
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
DTG	Doutegravir
EB	Elution buffer
EFV	Efavirenz
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein isothiocyanate
FTC	Emtricitabine
g	Gram
HDACi	Histone deacetylase inhibitors
HICs	High-income countries
HIV-1	Human immunodeficiency virus type 1
IL-6	Interleukin 6
INSTIs	integrase strand transfer inhibitors
IP-10	Interferon gamma-induced protein 10 (also known as CXCL-10)
IPDA	Intact proviral DNA assay
IQR	Interquartile range
ISG	Interferon-stimulating genes
IUPM	Infectious units per million
L	Litre
LDL	Lower detection limit

LDL	Lower detection limit
LMICs	Low- and middle-income countries
LRAs	Latency-reversing agents
LTR	Long terminal repeat (HIV-1 promoter)
m-	Milli
MHC	Major histocompatability complex
min	Minute
MIP-3β	Macrophage inflammatory protein-3β
MPP	Medicines Patent Pool
n-	Nano
NF-κB	Nuclear factor-κB
NNRTI	Non-nucleoside reverse-transcriptase inhibitor
NRTI	Nucleoside reverse-transcriptase inhibitor
NTC	No-template control
p-	Pico
PBMC	Peripheral blood mononuclear cells
PBS	Phsophate-buffered saline
PCR	Polymerase chaine reaction
PD-1	Programmed death-1
PE	Phycoerythrin
PE-Cy5	Phycoerythrin-Cy5
PE-Cy5.5	Phycoeythrin-Cy5.5
PE-Cy7	Phycoerythrin-Cy7
PEPFAR	U.S. President's Plan for AIDS Relief
PerCP-Cy5.5	Peridinin chlorophyll protein complex-Cy5.5
PI	Protease inhibitor
PKC	Protein kinase C
PLWH	People living with HIV-1
PMA	Phorbol 12-myristate 13-acetate
qPCR	Quantitative PCR
qRT-PCR	Quantitative reverse-trasncriptase PCR
QVOA	Quantitative viral outgrowth assay
RAL	Raltegravir
rCD4+	Resting CD4+ T-cell
RNA	Ribonucleic acid
RNAP II	RNA polymerase II
RTE(s)	Recent thymic emigrant(s)
s	Second
sCD	Soluble cluster of differentiation
STIs	Sexually transmitted infections
sTNFRII	Soluble tumor necrosis factor receptor II
TAR	Trans-activating response
T_{CM}	Central memory T-cells
TCR	T-cell receptor
TDF	Tenofovir disoproxil fumarate
T_{EM}	Effector memory T-cells
Th	T helper
TNF-α	Tumour necrosis factor alpha
T_{SCM}	Stem-cell memory T-cells

T_{TD}	Terminally differentiated T-cells
T_{TM}	Transitional memory T-cells
USD	United States Dollar
ViViD	LIVE/DEAD fixable violet dead cell stain
VL (s)	Viral load (s)
VLP	Virus-like particle
µ-	Micro

List of figures and tables

Abstract

Since the advent of antiretroviral therapy (ART) and the resultant suppression of viraemia in the majority of people living with HIV-1 (PLWH) on ART, HIV-1 infection has become manageable and PLWH have similar life expectancies as uninfected persons. However, ART is not curative, is needed lifelong, and its cessation leads to the recrudescence of viraemia. This is due to the formation of a latent reservoir that is long-lived and stable over time, precluding HIV-1 cure. The factors affecting reservoir formation, establishment, and kinetics are not fully understood. Furthermore, differences exist at the population level in disease progression in PLWH depending on ethnicity, biological sex, and infecting viral subtype. Similarly, differences in the latent reservoir of HIV-1 have been described, although less extensively. Understanding what shapes the latent HIV-1 reservoir is critical for developing strategies for cure. Furthermore, it is imperative that cure research is undertaken in diverse populations to ensure coverage of knowledge across different demographics. The latter will ensure that a cure strategy can be developed that will be globally implementable. In the Introductory chapter of this thesis, I provide a detailed review of the current literature and address the need for cure research in low-and middle- income countries.

If a global cure is to be achieved, the burden of HIV-1 will need to be addressed in many different populations, most notably African women, as women bear the burden of HIV-1 globally. In South Africa, the country in the sub-Saharan African region with the highest prevalence of HIV-1, women are roughly twice as likely to be living with HIV than men (aged 15 to 49), with a prevalence rate 6% higher than the national average of 19%. Since women are underrepresented in HIV-1 research in general and more specifically in cure studies due to the paucity of research in countries outside of the global North, reservoirs and cure strategies

need to be characterized in this context. Furthermore, while early treatment is the WHO standard of care for people diagnosed with HIV, a large majority of PLWH only initiated treatment in chronic infection. Since early ART is known to restrict formation of the latent reservoir of HIV-1, research in both early and late ART initiators is necessary. This research focused on characterising the viral reservoir in South African women in a well-established cohort of women who were recruited during acute HIV infection and followed until treatment initiation (which occurred during chronic infection) and beyond.

Overall, this thesis focuses on characterising immune activation and inflammation during the course of both untreated and treated HIV-1 infection in a cohort of South African women and subsequently determining whether clinical or immune measures influence characteristics of the latent reservoir of HIV-1. T cell activation and the levels of soluble inflammatory cytokines in plasma were determined in forty-six women in the CAPRISA 002 Acute infection cohort. Chapter 2 describes the cellular immune activation and inflammation profiles of these participants throughout the course of infection at the following timepoints: acute infection, one-year post-infection, and within a year preceding ART initiation, and two- and four- years post-ART initiation. T cell activation peaked in chronic infection and reduced dramatically after ART initiation. $CD4^+$ and $CD8^+$ T cell activation reached a post-treatment nadir by two years after ART initiation. Cytokine measures were within the ranges reported in the literature for PLWH. Notably CXCL-10 levels in plasma decreased significantly between two- and four years post-ART, indicating that it may be a sensitive marker of ongoing systemic inflammation in people on ART. In short, the T cell activation and inflammation profiles of the women in this study reflected what has been observed in other cohorts.

The size of the replication-competent HIV-1 reservoir, measured by quantitative viral outgrowth assay after 5 years of suppressive antiretroviral therapy (ART), was quantified in twenty women of the cohort. In Chapter 3, the clinical and immunological correlates of reservoir size were investigated. Predictive modelling showed that the size of the replication-competent reservoir is directly related to viral load and $CD4^+$ T-cell counts over the course of infection, although these measures do not fully predict reservoir size. We found that, in addition to viral load and $CD4^+$ T-cell count, $CD8^+$ T-cell activation within the year preceding ART, nadir $CD4^+$ T-cell count, and baseline as well as on-treatment CD4:CD8 ratio at the time of sizing was associated with replication-competent reservoir size. We provide evidence that the late $CD8^+$ T-cell activation level before treatment, together with viral loads and $CD4^+$ T-cell counts, are directly related to the size of the replication-competent reservoir of HIV-1. Our results are consistent with the hypothesis that the host immune milieu near the time of ART initiation plays an important role in shaping the durable reservoir of HIV infection that persists on ART.

Another characteristic of the HIV-1 reservoir is persistence: the presence of all forms of HIV-1 within cells and tissues that contribute to pathogenesis, including defective, non-induced, and non-integrated forms of HIV-1. In Chapter 4, total HIV-1 DNA levels were measured as a proxy for viral persistence in thirty-one participants, and the correlates thereof investigated. The HIV-1 DNA levels in this cohort were similar to those reported in the literature for other cohorts where participants initiated therapy in late chronic infection. HIV-1 DNA levels did not differ significantly between two- and four years post-ART, but there was a trend to lower HIV-1 DNA when measuring *pol* versus *gag* gene frequencies in peripheral blood mononuclear cells (PBMC). These findings indicate that HIV-1 DNA decay rates may differ depending on the gene being measured, even when using the same assay. A weak significant correlation was

found between $CD4^+$ T cell counts at ART initiation and the change in HIV-DNA levels between two-and four years on ART. There was a significant correlation between residual $CD4^+$ T cell activation at four years post-ART initiation and *gag* copies per million PBMC. A trend towards a correlation was found between $CD4^+$ T cell activation and *pol* copies per million PBMC at the same timepoint. Finally, we found significant correlations between several cytokines at one-year post-infection and within one year pre-ART. These findings further solidify the hypothesis that the immune milieu around the time of ART initiation and after may play a complex role in formation of the viral reservoir of HIV-1.

Our studies show a significant link between chronic immune activation and replication-competent reservoir size, and also ongoing immune activation and viral persistence on ART. Further studies into whether these immune measures affect the timing of establishment and clonality of the reservoir in this cohort are ongoing and will inform the field about whether differences in cure strategies will need to be explored for those PLWH who had high levels of chronic immune activation before treatment initiation and subsequent shaping for the long-lived viral reservoir.

CHAPTER 1

Literature review: Addressing an HIV cure in low- and middle-income countries

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Contribution: Sherazaan D. Ismail provided the outline of the review and wrote the first draft of the manuscript, along with Joshua Pankrac. In particular, aspects directly relevant to this thesis were written by S.D. Ismail including those on pages 13 to 25 and 32-34. S.D. Ismail also extensively edited all other sections. All contributions are stipulated in **Appendix I.**

1.1 Abstract

HIV-1 persists in infected individuals despite years of antiretroviral therapy (ART), due to the formation of a stable and long-lived latent viral reservoir. Early ART can reduce the latent reservoir and is associated with post-treatment control in people living with HIV (PLWH). However, even in post-treatment controllers, ART cessation after a period of time inevitably results in rebound of plasma viraemia, thus lifelong treatment for viral suppression is indicated. Due to the difficulties of sustained life-long treatment in the millions of PLWH worldwide, a cure is undeniably necessary. This requires an in-depth understanding of reservoir formation and dynamics. Differences exist in treatment guidelines and accessibility to treatment as well as social stigma between low- and-middle income countries (LMICs) and high-income countries. In addition, demographic differences exist in PLWH from different geographical regions such as infecting viral subtype and host genetics, which can contribute to differences in the viral reservoir between different populations. Here, we review topics relevant to HIV-1 cure research in LMICs, with a focus on sub-Saharan Africa, the region of the world bearing the greatest burden of HIV-1. We present a summary of ART in LMICs, highlighting challenges that may be experienced in implementing a HIV-1 cure therapeutic. Furthermore, we discuss current research on the HIV-1 latent reservoir in different populations, highlighting research in LMIC and gaps in the research that may facilitate a global cure. Finally, we discuss current experimental cure strategies in the context of their potential application in LMICs.

Keywords: HIV-1, cure, reservoir, low-and-middle income countries, LMICs.

1.2 Introduction

The advent of antiretroviral therapy (ART) has converted the HIV-associated death sentence into a lifelong, manageable illness for those with adequate access. However, for many low-and middle-income countries (LMICs), access to sustained ART for the full population is challenging due to a variety of socio-economic factors. This is especially true in regions with the greatest infection burden, Eastern and Southern Africa, which account for more than half of all the people living with HIV-1 (PLWH). While, globally, more PLWH are aware of their seropositive status (~80%) and are accessing treatment (~67%) [1], this still falls short of the original 90-90-90 goal set forth by the United Nations for awareness of status, accessing treatment, and viral suppression, respectively. In addition, due to the development of drug-resistant strains, the ART failure rate for PLWH on first-line regimens is 5% per year, necessitating constant development of new treatments over time. Lifelong ART also imposes a substantial financial burden on already-constrained public health systems; as PLWH continue to live longer, the overall cost of ART has risen to an estimated 26.2B USD globally in 2020 [1] and could reach 40B USD if goals for 2030 are going to be met. Even a long-term remission of HIV-1 disease [undetectable viral load (VL)] in the absence of ART, as opposed to a sterilizing cure, would save the world from millions of future deaths and trillions of USD in drug and health care costs. Finally, even with full viral suppression there is still a high risk for other long-term morbidities, including an increased risk of heart, bone, and kidney disease [2, 3], and PLWH can be impacted socially, financially, and psychologically due to HIV stigma and discrimination. Thus, there is an urgent need for a cure for HIV-1.

The benefits of an HIV-1 cure to PLWH and the public-health system in LMICs are clear and undeniable (**Figure 1.1**). However, as discussed below, most cure approaches in preclinical

development, or even those tested in clinical trials, may be financially prohibitive and impractical to administer in many LMICs, especially in sub-Saharan Africa. Currently, participation in clinical trials testing cure modalities requires frequent VL monitoring, reservoir quantification (currently developed only for subtype B), and full viral suppression, all readily available in high income countries (HICs)[4], but not in LMICs. Universally, the major barrier for HIV-1 cure is the existence of a long-lived latent reservoir consisting of stably integrated proviruses that persist in the host within various cell types and anatomical locations. Proviruses within the reservoir capable of reactivation and re-establishing plasma viraemia upon ART cessation persist despite years of suppressive ART [5–7]. PLWH exhibit differences in infecting viral subtype, immune responses to HIV-1, and disease progression, notably between individuals in LMICs vs. HICs, as well as between sexes [8–10]. Similarly, differences exist in the size, composition, and turnover in the reservoir between PLWH.

In this review, we introduce HIV-1 cure and the barriers to a cure in LMICs, particularly those in sub-Saharan Africa. This review starts with a description of clinical and immune correlates of the HIV-1 reservoir, which is the focus of this thesis, and provides a detailed summary on the viral and host cellular phenomena contributing to HIV-1 proviral latency establishment and persistence. The most relevant strategies for intervention and cure are discussed, highlighting scientific gaps that may influence generalizability of findings to LMICs. Finally, the rationale, aims and objectives of the studies in this thesis are presented.

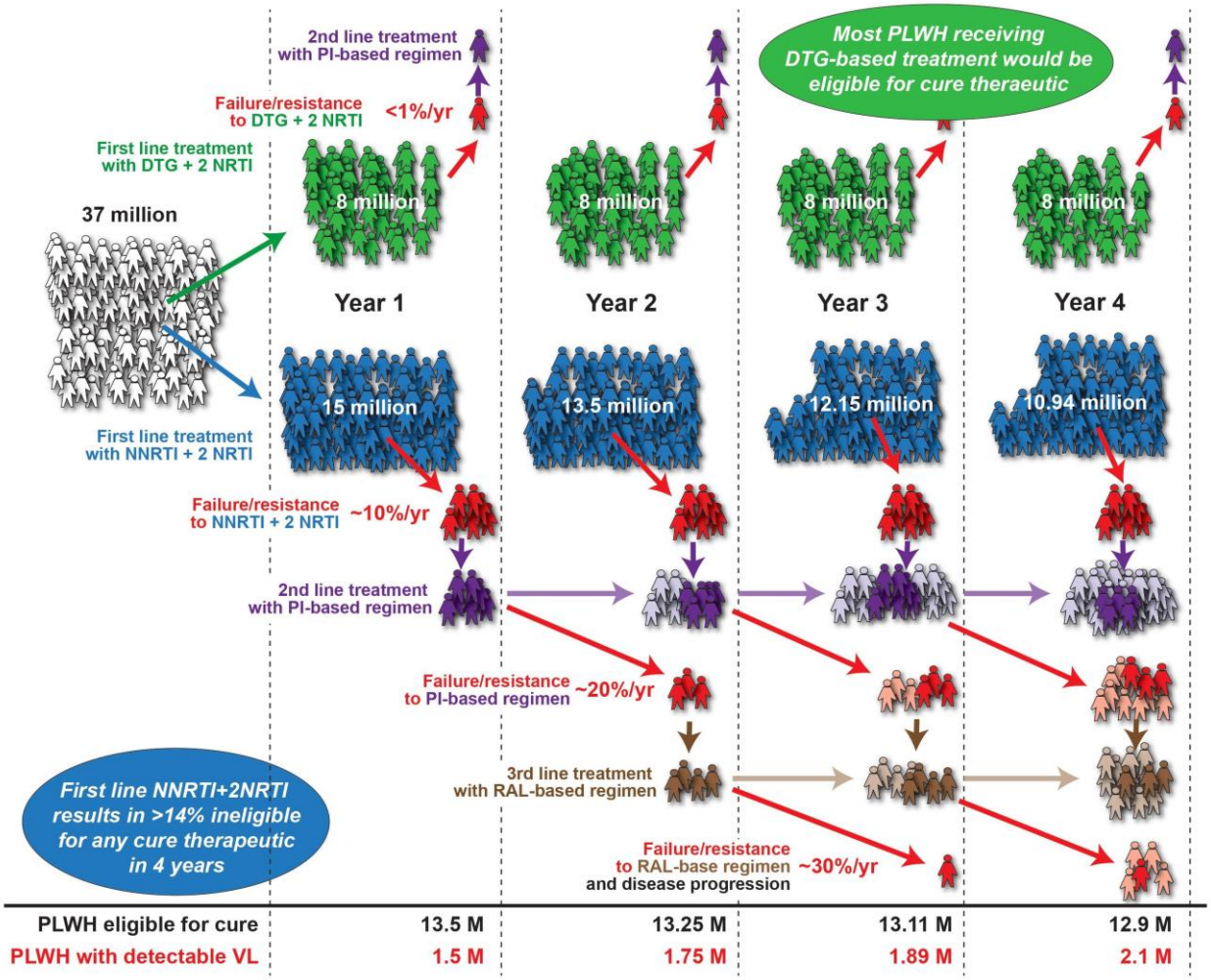

Figure 1.1. ART regimen chosen for first-line therapy can affect eligibility for a therapeutic cure. The incidence of treatment failure and/or drug resistance on an NNRTI+2 NRTI regimen is greatly increased relative to individuals receiving DTG + 2 NRTI treatment. As a result of failing first-line therapy, PLWH may initiate PI- and RAL-based regimens, which have heightened incidence of failure and resistance. Effective ART allows more PLWH to maintain first-line therapy and facilitates initiatives to achieve therapeutic cure. NNRTI=non-nucleoside reverse transcriptase inhibitor; NRTI=nucleoside reverse-transcriptase inhibitor; DT =dolutegravir; PI=protease inhibitor; RAL=raltegravir.

1.3 The current state of ART in LMICs

Early ART has been shown to limit seeding of the HIV-1 reservoir and is associated with post-treatment control. By diminishing the amount of immune activation, $CD4^+$ T-cell depletion and VLs in untreated infection, what results is fewer target cells for HIV-1 infection, preservation of the immune response [11–13], and a lower burden of viraemia over time. Thus, effective HIV-1 treatment in LMICs is necessary to implement a cure therapeutic. This may require use of more potent ART as a functional cure strategy during early stage of HIV infection. However, challenges of limited early HIV diagnosis, and access to potent ART regimens in LMICs need to be addressed. Successful distribution of generic DTG in LMICs through the Medicines Patent Pool (MPP) program [14] coupled with high rate of adherence by patients in LMICs [15] are good indicators that a cure therapeutic in LMIC setting can be achieved. Furthermore, optimization of a cure approach that will be effective in the millions of PLWH who initiated treatment during chronic infection is imperative. Therefore, to establish the potential utility of cure strategies in LMICs, it is essential to understand differences in ART treatment rollout among diverse global populations.

Global access to ART has increased tremendously. By end of 2019, over 26 million people had access to ART of which 17.9 million (69%) were PLWH in sub-Saharan Africa [16]. Despite this staggering number, the proportion of PLWH on ART in sub-Saharan Africa is still well below the UN target of 90%, and this is also true for South America (62%) and Asia and the Pacific (60%). Historically, first-line regimens in sub-Saharan Africa have included two nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI), commonly efavirenz (EFV) or nevirapine [17]. However, due to the combination of frequent drug supply/access issues in LMICs resulting in treatment

interruptions [18] and the low genetic barrier to HIV-1 drug resistance mutations associated with NRTI + NNRTI regimens [19], 10-15% of patients who start ART still fail within one year, and 70-80% of people with virological failure develop acquired drug resistance [20]. In Kampala, Uganda, failure of first-line treatment continues to occur at an annual rate of 9% [21]. This statistic of treatment failure and drug resistance is rarely mentioned in discussion of the 90-90-90 goals by various global enterprises championing these goals.

For patients failing first-line therapy, the guidelines in most sub-Saharan African countries [and supported by the Global Fund and the U.S. President's Emergency Plan for AIDS Relief (PEPFAR)] calls for a second-line ART of an NRTI + protease inhibitor (PI) and, subsequent to the failure of second-line, a third-line four-drug therapy of the NNRTI etravirine, the PI darunavir, and an integrase inhibitor with any suitable NRTI [22–24]. The choice of "salvage" treatment regimens in LMICs is limited due to lack of access to CCR5 antagonists, fusion inhibitors, and second-generation PIs and NNRTIs- agents with a higher barrier to HIV-1 drug resistance employed when patients fail treatment with first-generation PIs and NNRTIs. Limited ART options may exacerbate problems facing ART programs in sub-Saharan Africa, including adherence on ART, which directly translates to increased mortality. Indeed, a recent modelling study shows that 6 months of ART disruption for 50% of people would result in 296,000 more AIDS-related deaths in sub-Saharan Africa over one year [18]. Furthermore, frequent use of suboptimal NRTI + NNRTIs regimens has led to a >10% prevalence of strains resistant to either NNRTIs or NRTIs in treatment-naïve PLWH [25]. Without pre-screening for drug resistance prior to initiation of first-line ART, treatment failure is likely to increase over time. High rates of treatment interruption and/or first-line failure present a huge barrier to cure therapeutic testing in sub-Saharan Africa, as all protocols to date have required participants to be fully virally suppressed. However, this need not be a barrier; provided there is stable drug

supply, effective distribution to clinics/pharmacies, and ease of access to PLWH, adherence and success of first-line treatment is outstanding and often better in LMICs than HICs.

Despite the sombre predictions described above, the roll out of a dolutegravir (DTG)-based regimen (TDF+3TC/FTC+DTG) as a preferred first-line treatment at the end of 2017 has improved the treatment success rates in all LMICs. Current estimates indicate that between 5 and 10 million PLWH in sub-Saharan African countries and other LMICs are receiving a DTG-based regimen [26]. In treatment naïve individuals, DTG-based treatment regimens are extremely well tolerated with minimal adverse events promoting high treatment adherence. Treatment failure and resistance to DTG is extremely rare in clinical studies to date due in part to high genetic barrier for resistance [27] to these second-generation integrase inhibitors (INSTIs). If the combination of DTG, bictegravir and the long-acting cabotegravir were available in dual or triple drug formulations, especially as one-pill-a-day, treatment success would improve dramatically in LMICs and thus provide a population of PLWH in LMICs that could be treated with an affordable and practical cure therapeutic.

Treatment outcome studies in Africa and other LMICs are heavily affected by different sociodemographic factors between countries, regions, and ethnic groups [28–31]. As previously described in several cross sectional and longitudinal analyses on treatment adherence and HIV-1 drug resistance in Uganda and other sub-Saharan African countries, one of the greatest contributors to treatment failure is poor access to care/ARVs and intermittent ARV shortages, even more so than differential sociodemographic factors. Across sub-Saharan Africa and in LMICs during COVID-19 pandemic, ARV shortages, disruption in shipments,

and reduced foreign aid will likely contribute to ART failure by orders of magnitude higher than the impact of HIV-1 types/subtypes, human genetics, and sociodemographic factors.

In a specific area, the co-circulating HIV-1 subtypes and/or CRFs could impact the effectiveness of any proposed cure therapeutic. Like all antiretroviral drugs developed to date, preclinical development and early phase human clinical trials of cure therapeutics have all been based on the ability to inhibit HIV-1 subtype B isolates, i.e., the HIV-1 strains that predominate in HICs. While ARTs have similar inhibitory effectiveness regardless HIV-1 subtype in terms of initial response to treatment [32–35], the effect of subtype on the emergence of drug resistance is not yet fully understood. For instance, despite similar *in vitro* susceptibility of subtype C and B strain-derived HIV-1 integrase enzymes to the currently-approved INSTIs, differential levels of drug resistance were observed between HIV-1 subtype B versus C viruses in cell culture assays, likely resulting from differential mutational pathways being favored between different subtypes [36]. Additionally, *in vivo* development of drug DTG resistance occurs at a slower rate among PLWH infected with subtype B compared to subtypes A/G and C [37]. Drug resistance to NRTIs and PIs has been shown to occur more frequent in subtype B compared to subtype C (26% vs. 8% for NRTIs, and 54% vs. 23% for PI's, respectively)[38]. In Uganda, drug resistance and treatment failure is more prevalent in subtype D compared to A or C-infected patients (**Figure 1.2**) [39]. These differences could be explained by variability of HIV-1 genes at the amino acid level between different subtypes and higher entropy scores observed at sites where drug resistance mutations emerge [40].

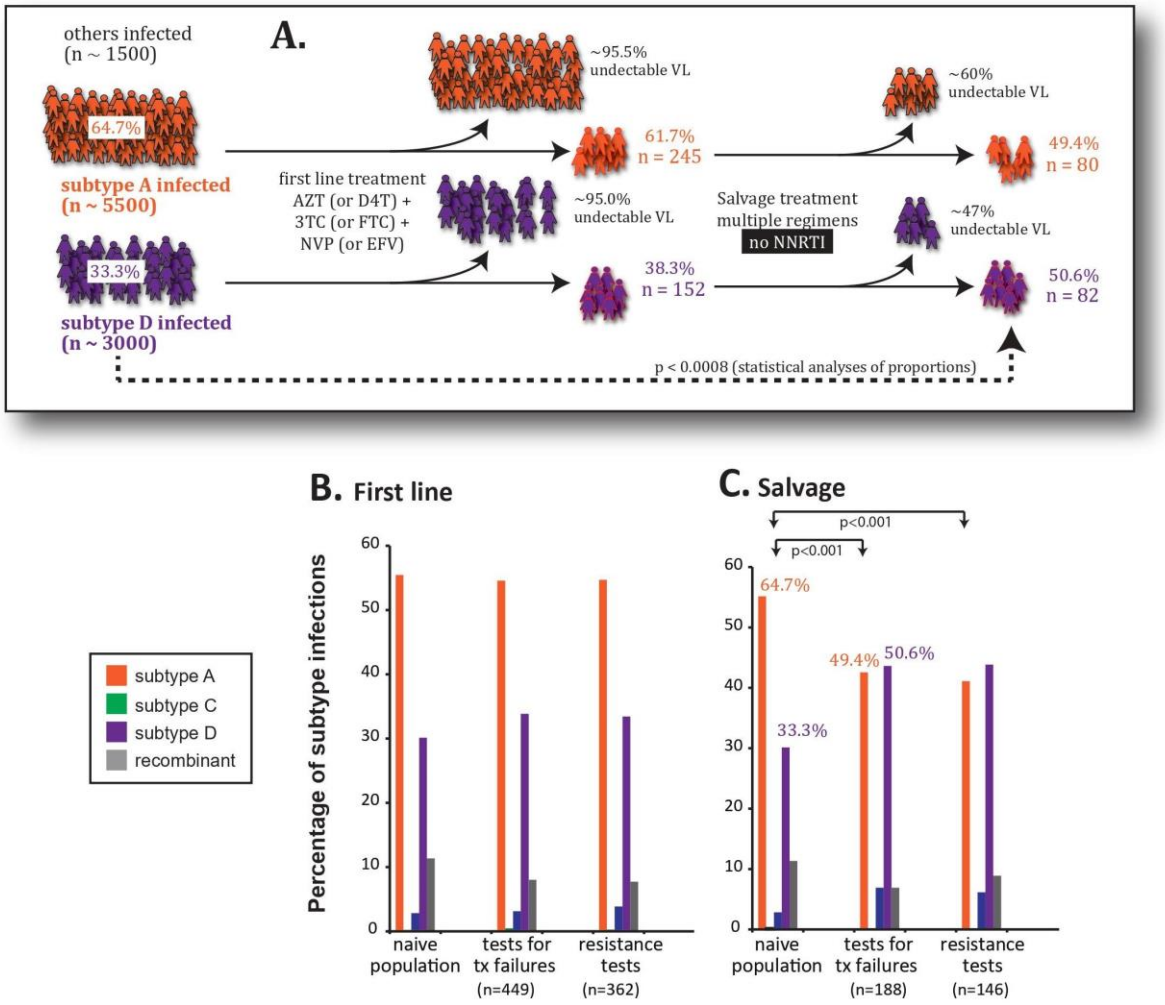

Figure 1.2. Faster subtype D versus subtype A HIV-1 treatment failure observed over first- and second-line treatments. The Joint Clinical Research Centre in Kampala, Uganda follows approximately 12,000 HIV-infected patients treated with antiretroviral drugs. Resistance testing is preformed in cases of treatment failure - VL >1000 copies/ml or two CD4 cell counts <200/ml. Approximately 95% of all patients on first-line treatment maintain undetectable VL in Uganda. <50% reach and maintain undetectable VL on salvage therapy. As part of standard of care, they subtype and analyze drug resistance genotypes of all patients failing treatment on (B) first-line (C) or salvage therapy.

Differences in disease progression exist depending on infecting viral subtype [41, 42]. In Uganda and Zimbabwe, HIV-1 subtype D is associated with faster disease progression [43–46] but lower transmission rates than subtype A [45, 47]. Furthermore, subtype C viruses were linked with rapid disease progression in South African women [48]. The difference in disease progression between HIV-1 subtypes is likely multifactorial [33–35, 39, 46]. However,

functional differences between HIV-1 subtypes may influence reservoir formation, kinetics, and the efficacy of a cure therapeutic. HIV-1 acquisition risk (discussed extensively in [49]) and disease progression is known to differ between the sexes. Women exhibit a faster progression to AIDS-defining illness than men, even at matched viral loads [50] and have higher levels of inflammation and immune activation [51, 52] than men. In addition, women are the most affected by HIV-1 in sub-Saharan Africa and disproportionately contribute to the global burden of disease [49]. Thus, it is imperative that we tailor cure therapeutics to address an HIV-1 cure in this population.

Predicting the effectiveness of cure strategies against different HIV-1 subtypes will be complicated. Some cure therapeutics aim to activate transcription by targeting specific HIV-1 sequences, which will likely vary between the subtypes. Patterns of recombination sites in the host genome differ by subtype [53]. Integration site may also be relevant in determining HIV-1 persistence and vary by subtype. New data shows that, compared to *in vitro*-derived HIV-1 integration sites, *in vivo*-derived sites are significantly more enriched in transcriptionally silent regions of the genome, which has relevance to reactivation of latent proviruses. With regard to subtype, integration sites from PLWH infected with HIV-1 subtype A, C or D viruses exhibited different preferences for specific genomic features and were more enriched in transcriptionally active regions of the genome compared to subtype B virus [53]. Theoretically, any type of transcriptional activation strategy to induce latency reversal may require greater potency in a subtype B setting compared to subtype A, C and D infected individuals. This will be discussed in further detail in section 1.7.

1.4 Measuring and characterizing the latent viral reservoir

The evaluation of reservoir stability and the efficacy of clinical interventions and/or changes in the reservoir after "drug holidays" or other periods of ART cessation necessitates a reliable method of quantifying the frequency of latently-infected cells that is cost and time effective [54]. This method would ideally not only enable the determination of reservoir size, but also characterize the proviral landscape to monitor qualitative changes in the reservoir. Several methods have been developed to quantify the reservoir to date. Each method measures different aspects of the reservoir and each has caveats on the conclusions that can be drawn. **Table 1.1** provides a summary of current sizing methods and assay examples. However, only the detection of HIV-1 DNA by qRT-PCR could currently be widely implemented in most LMICs and even this methodology would be difficult to apply in some small hospitals and more rural clinics in sub-Saharan Africa. However, this method significantly overestimates the theoretical "true" replication-competent reservoir, which is the frequency of cells harbouring genetically intact proviruses that are capable of recrudescence in the absence of suppressive ART, because resting $CD4^+$ T-cells harbour ~10-fold more defective than intact proviruses [55–58]. This distinction is especially important when assessing efficacy of a potential cure therapeutic, as differences in decay between intact and defective proviruses have been reported, with a slow but significant decline in intact proviruses over time on ART and little to no decline in defective proviruses [59].

In contrast, the quantitative viral outgrowth assay (QVOA) [5, 60, 61] is currently the gold standard for measuring the frequency of cells harbouring replication competent provirus, but underestimates the true reservoir as this assay relies on *ex vivo* reactivation of latently infected

cells, and it has been demonstrated that *in vitro* reactivation methods are not 100% efficient and their efficacy may vary between populations of PLWH [58]. Additionally, this assay is resource-intensive and requires additional biosafety containment for the culture of live HIV-1. As such, the QVOA may only be practical in LMIC sites performing clinical trials of cure therapeutics and this assay would likely not be adopted as standard-of-care assay to monitor the effectiveness of cure therapeutics upon approval.

Using QVOA, the size of the latent reservoir has been estimated to be one infected cell per million resting $CD4^+$ T-cells in PLWH in North American cohorts [62, 63]. Historically in sub-Saharan Africa, treatment has most commonly been initiated in chronic infection, leading to an expectation that the barrier to a cure would to be greater due to lack of restriction of reservoir size through early treatment (as highlighted in the section to follow). However, we have previously reported that PLWH in Uganda (who initiated ART during chronic infection) have reduced replication competent reservoir size (three-fold lower by QVOA) compared to North Americans [8], and that Ugandan females have additionally smaller replication-competent reservoirs than males.

Table 1.1. Methodologies for measuring the HIV-1 latent reservoir.

Biomarker	Feature of the latent reservoir measured	Fraction of the reservoir included in measurement	Under/overestimation of replication competent reservoir size	Assay examples	Caveats	References
HIV-1 DNA	Either total or cell-associated HIV-1 DNA directly ex vivo by polymerase chain reaction (PCR)-based techniques	Both intact and defective HIV-1 DNA (except for IPDA); both inducible and non-induced viruses	Over	Droplet digital PCR (ddPCR); quantitative PCR (qPCR); Intact proviral DNA assay (IPDA); Q4PCR; SGS/NGS/near-full length genome sequencing (with or without integration site analysis)	With total HIV-1 DNA measurement, 2-LTR circles and episomal DNA are quantified along with proviral DNA; only IPDA and near-full length genome sequencing with integration site analysis measure intact provirus	[56, 64–67]
HIV-1 RNA induction	Cell-associated/cell-free RNA produced after *ex vivo* stimulation of infected cells	Both intact and defective (but transcription-competent) provirus; inducible viruses only	Over	Bulk CA-RNA PCR; Single cell RNA PCR; Single copy assay (SCA); SGS/NGS	A single round of maximal stimulation does not induce all transcription-competent proviruses	[68–73]
Induced HIV-1 protein production	Cell-associated HIV-1 proteins induced after a single round of *ex vivo* stimulation	Both intact and defective (but transcription-competent) provirus; inducible viruses only	Over	Flow/mass cytometry; FAST/digital microscopy; ELISA	A single round of maximal stimulation does not induce all transcription-competent proviruses; false positives associated with p24 quantitation	[74–77]
Viral outgrowth	Replication-competent virus that grows out after a single round of *ex vivo* stimulation of resting $CD4^+$ T-cells	Intact, inducible proviruses only	Under	Quantitative VOA (QVOA)	A single round of maximal stimulation does not induce all intact, inducible proviruses as some are in 'deep' latency	[5, 58, 60, 61]

Interestingly, despite lower QVOA outgrowth, HIV-1 DNA levels were similar between males and females [9]. Other studies report lower intracellular HIV-1 DNA in females compared to males [78, 79], highlighting important differences that may need to be considered when assessing latency reversal strategies. These studies in Uganda are two of only a handful of published studies of replication-competent reservoir size in Africans, highlighting the need for

more studies in diverse LMIC settings to examine if other differences in populations exist that may help us to better understand the latent reservoir. Given that close to two thirds of women bear the burden of disease in some LMIC [80], such differences between the sexes need to be established if a global cure is to be achieved. Furthermore, only one study exists showing the association between early ART initiation and HIV-1 DNA levels in children, highlighting a paucity of cure studies in children living with HIV-1 in LMICs.

1.5 Clinical and immunological correlates of the HIV-1 reservoir

The most well-characterized clinical measure that correlates with reservoir size is pre-ART viral load (VL). Several studies show that pre-ART VL setpoint, or even just the VL the time of ART initiation, correlates positively with HIV-1 DNA [13, 81] and QVOA estimates of reservoir size [8, 9], which is consistent with studies showing that early ART restricts reservoir size (both replication-competent and HIV-1 DNA) [82–85]. In ART-treated patients, CD4 counts over time have also been shown to predict replication-competent reservoir size in PLWH who initiated treatment in acute infection [86], and several studies have identified the extent of CD4 depletion as shaping the HIV-1 DNA proviral load [87, 88], with nadir CD4 count and CD4:CD8 ratio shown to be strong negative correlates of reservoir size. Furthermore, time on ART has also been shown to correlate negatively with replication-competent reservoir size [9].

Events soon after the establishment of infection impact disease progression, and there is mounting evidence that this may be the case for characteristics of the latent reservoir. Immune activation and inflammation play an important role in the disease progression of HIV-1 [89,

90]. Immune activation is very strongly correlated with set-point VL, and predicts progression to AIDS-defining illness more robustly than VL [13, 89, 91]. Early initiation of ART not only reduces the cumulative viral burden before ART, preserves $CD4^+$ T-cells, and maintains CD4:CD8 ratios, but also reduces T-cell activation and inflammation [11–13]. T-cell activation may increase the pool of target cells available to sustain HIV-1 replication, but also augments antigen-driven clearance of infected cells and thus increases T-cell turnover. Following ART initiation, many measures of immune activation decline rapidly, but this does not occur equally in all individuals, and residual inflammation is a strong predictor of non-AIDS mortality in the context of successful ART [92]. Understanding upstream contributors to T-cell activation, such as cytokine expression throughout both treated and untreated infection, may shed light on subsequent HIV-1 reservoir dynamics over time. IL-10, and sTNFRII concentrations were positively associated with levels of total HIV-1 DNA in peripheral blood mononuclear cells (PBMC) after 96 weeks of treatment [13], while MIP-3β showed a trend towards a correlation.

Since immune activation and inflammation are known to differ between the sexes, detailed studies into the immune correlates of reservoir size in males and females are needed. A recent study reported a difference in the immune correlates between males and females in PLWH in Uganda [9]. While in males the frequency of PD-1^+ $CD4^+$ T-cells and IL-2^+ $CD8^+$ T-cells were positive and negative correlates of replication-competent reservoir size, respectively, only TNF-α^+ $CD8^+$ T-cells was found to have a positive association with replication-competent reservoir size in females. The lack of a positive association between PD-1 expression and reservoir size in women may be of particular concern, as this association, well-established in men, has led to the development of PD-1 agonists as latency reversal agents. The efficacy of this therapy, and other immune-based cure therapeutics, should be carefully tested in both sexes.

Several studies have suggested that inflammation and immune activation are different in African cohorts [93–97]. High levels of genital inflammation have been observed in a well-characterized cohort of South African females [98] and high levels of inflammation during early infection in this cohort correlated with VL set-point and, to a lesser degree, disease progression (as measured by CD4 depletion). Few studies have addressed ongoing immune activation in the context of Africans receiving ART. Understanding of the impact of immune activation on viral reservoir dynamics at multiple stages of HIV-1 infection, particularly in cohorts where ART was initiated in chronic infection, is imperative to formulating cure strategies in LMIC.

Finally, the paucity of reservoir studies in different geographical settings also means that critical information on reservoir establishment and dynamics in the face of co-infections is overlooked. Moreover, an in-depth discussion of co-infections and the HIV- 1 reservoir is precluded by the absence of studies on the topic. Co-infections have been shown to increase pathogenesis and HIV-1 viral loads, increase immune activation, and inflammation. Thus, our discussion on how increased or decreased viral loads, immune activation, and inflammation impacts reservoir size would extend to the more general effect of co-infections. Effective treatment of co-infections (excluding hepatitis B and C) results in a reduction of plasma viraemia in ART naïve PLWH [1]. As there remains fundamental questions on the timing of viral reservoir seeding prior to ART initiation [10, 99–102], the effects of longer term (e.g. TB) or periodic/sporadic (e.g. malaria or HSV) co-infections on the viral reservoir may depend on the timing of these event(s) during acute and chronic disease and the timing of ART initiation. Furthermore, infections that lower the barrier to HIV-1 acquisition such as bacterial vaginosis

and other sexually transmitted infections, resulting in an increased HIV-1 risk or a higher multiplicity of infection, may influence HIV-1 reservoir size or composition. This topic has been reviewed extensively elsewhere [2], including a detailed summary of knowledge gaps with regards to specific co-infections such as Mycobacterium tuberculosis, Hepatitis B and C, helminth infections, and other STIs. LMICs have a higher burden of co-infections and thus, latent reservoir studies in this context are of great importance for cure strategies in the future.

1.6 HIV-1 reservoir establishment, heterogeneity, and kinetics

Studying the mechanisms that dictate reservoir establishment is challenging. Specifically, cells isolated from an infected individual on ART have already entered into a latent state and therefore can only provide a pseudo-measure of this process of latency establishment. The long-lived reservoir in PLWH displays significant heterogeneity in sequence composition, clonality, genomic integration sites, rates of decay and the proportion of replication-competent to defective proviruses present in cells. Infected resting memory $CD4^+$ T-cells are the most well-characterised of HIV-1 reservoir cells, are highly stable, with a half-life ($t_{1/2}$) of ~44 months, and can theoretically persist for the lifetime of an affected individual [6, 62, 63]. Resting $CD4^+$ T-cells exhibit decreased expression of T-cell activation markers, lower RNA content, and are not cycling [103], resulting in reduced HIV-1 transcription and favouring latency. Activated effector $CD4^+$ T-cells represent a primary target for HIV-1 infection due to their high permissiveness and metabolic state relative to resting cellular subsets. Many of these cells, as well as other non-HIV-specific $CD4^+$ T-cells, are productively infected with HIV-1 and produce viral products, which are then detectable by host immune mechanisms. Such cells are primed for elimination and are unlikely to contribute to persistence *in vivo* [104, 105].

Rather, latency establishment likely occurs as cells that are transitioning towards a long-lived memory phenotype [106]. Alternatively, proviral integration can occur directly in resting CD4 T-cells [107, 108]. However, despite their high abundance in the body, resting cells are relatively resistant to infection, due to low expression of the HIV-1 co-receptor CCR5, limited dNTP availability, and an increase in heterochromatic structures [109, 110].

1.6.1 Differences in integration sites, viral diversity, and decay in the HIV-1 reservoir

Following transmission, HIV-1 variants diversify rapidly [111–115], reaching a plateau in chronic infection [116]. However, not all variants are equally likely to be represented in the long-lived viral reservoir [10]. Rather, variants present at the time of ART initiation are significantly over-represented, measured by both HIV-1 DNA [117, 118] and QVOA [10]. Our South African study (where subtype C predominates) evaluating cells from nine women showed that 17 to 100% (average: 71%) of the replication-competent viruses in the reservoir after five years of suppressive treatment were genetically similar to the viral variants circulating in the patient's plasma the year immediately preceding ART initiation [10]. In comparison, the percentage of viruses seeded into the long-lived reservoir within the first year from the estimated time of infection ranged from 0 to 17% (average: 4%). Similarly, a study in Kenyan women on ART for up to 5 years showed that 59 to 99% (median: 86%; measured by *gag* sequences) of HIV-1 DNA during ART comprised of sequences present in plasma within 2 years of ART initiation [118]. These findings indicate that the long-lived reservoir may not be formed continuously at the same rate. Instead, it is possible that ART initiation establishes an environment favouring latency. Work by Jones *et al*. [119, 120] highlights the persistence of viral variants from earlier time-points in infection, even if in smaller proportion. The

mechanism of persistence of these variants or factors affecting their longevity in the reservoir are still to be elucidated.

Over time the immune response (both CTL and antibody pressure) drives selection of variants with immune escape mutations [111–115, 121, 122]. Given that studies suggests that the reservoir is comprised of HIV-1 variants circulating close to ART initiation, the long-lived reservoir would include variants with escape mutations, particularly in PLWH initiated on treatment in chronic infection [123]. In addition, chronic high viraemia reduces $CD8^+$ T-cell proliferation and promotes exhaustion, leading to CTL dysfunction [124]. Cure strategies looking to boost T-cell responses are reliant on long-lived reservoirs that do not contain a great deal of escape mutations. Recent research has detailed that HIV-specific T-cell responses are maintained in chronical-treated individuals on long-term suppressive ART [125], and that $CD8^+$ T-cells can suppress HIV-1 superinfected $CD4^+$ T-cells both *ex vivo* (isolated cells) [126] and *in vitro* (after latency reversal) [127]. Furthermore, 68% of T-cell epitopes in the replication-competent reservoir were recognized by autologous circulating T-cells [128].

In early treated individuals infected with subtype C viruses, the reservoir was found to have a majority of proviral sequences intact, a low frequency of hypermutated genomes, and a paucity of truncated genomes which are found commonly in chronically-infected, untreated individuals [129]. While this scenario may not represent the majority of PLWH in LMICs due to late initiation of ART, the future of cure in these settings will benefit from more longitudinal studies characterizing reservoir heterogeneity.

1.6.2 Cellular and tissue reservoirs

One area with currently no reported characterization in LMIC studies to our knowledge, is that of cellular and tissue reservoir sites. Specific lineages of memory $CD4^+$ T-cells, the major cellular reservoir of HIV-1, can persist for an individual's lifetime. Their memory differentiation status, as well as their functional polarization dictates longevity, anatomical location, and likelihood of being a stable reservoir. Central memory (T_{CM}) cells are most likely to harbour provirus, followed by transitional memory (T_{TM}) and effector memory (T_{EM}) T-cells [130–132]. A recent study showed that intact proviral DNA copies in each of these subsets varied greatly between individuals [57], but the distribution of relative abundances were similar between subsets and there were no differences in the contribution of each subset to the total pool of intact proviral copies. Less abundant subsets, such as stem cell memory T-cells (T_{SCM}), also contribute to the reservoir, although only substantially in some individuals [133, 134]. Early treatment appears to increase the contribution of T_{SCM} to the total $CD4^+$ reservoir, reducing per-cell HIV-1 DNA levels in T_{EM} and T_{TD} (terminally differentiated) subsets [85]. The contribution of each subset to the reservoir over time on suppressive ART is difficult to study due to the dynamic nature of cell differentiation where a cell can evolve from one subset into others. Thus, the contribution of each subset to rebound virus remains an understudied topic. However, studies have showed that viral rebound likely does not have a singular source of cell type or anatomical location [135–137]. The proportion and number of T-cell subsets during disease and following treatment is poorly understood between viral subtypes and may also differ between early- and late- treated populations. This merits further investigation in the context of the HIV-1 reservoir in LMICs vs. HICs.

1.6.3 Clonality and homeostatic proliferation

The proliferation of $CD4^+$ T-cells brought about by proliferation from homeostatic regulation and in response to antigen stimulation [138] results in large pools of clonal sequences *in vivo* [135, 139–145], comprising both defective and intact proviruses. In our South African study, we reported between 0 and 47% (by sequence identity) of outgrowth viruses form PBMC were clonal in nature [10]. Furthermore, another small study found that clonal sequences were rare over the first year of ART in early treated PLWH, providing insights into the difference in reservoir composition between early- and late-treated individuals infected with subtype C viruses [129]. Replication-competent clonal populations have been found to be distributed across different T-cell memory subsets [57, 135, 146], indicating that infected cells differentiate and proliferate during ART unabated by the immune system [146, 147]. In addition to memory differentiation, differences in functional polarization may influence reservoir size. One study found that functional polarization may lead to preferential clonal expansion of replication-competent HIV-1 in Th_1 cells [148]. Furthermore, these clonal populations are found in different tissues and can contribute to plasma viral rebound when treatment ceases [135, 137]. A caveat with some of these studies is that frequently only a small region of the HIV-1 genome is sequenced, so that it cannot be definitively known if cells with identical sequences in that region are the result of integration by a pool of homogenous viruses, or due to homeostatic proliferation of infected cells, the latter defining a true clonal population. Integration site analysis is required to distinguish between these two possibilities, as the likelihood that integration will occur multiple times in the same location in the human genome is negligible [149]. Nevertheless, it is clear that clonal populations of HIV-1 contribute markedly to reservoir maintenance. Antigenic stimulation and immune modulation are likely different in LMICs vs. HICs, and further studies are needed to assess the contribution of clonal expansion and latency in different cellular subsets in other populations in LMICs.

1.7 Viral factors contributing to HIV-1 persistence

The role of infecting subtype in reservoir formation, size and maintenance is understudied. Particularly in LMIC where the predominant infecting subtypes differ from the most studied cohorts (predominated by subtype B infections), this may result in significant geographical differences in the latent viral reservoir. The viral promoter element of HIV-1, the long terminal repeat (LTR), has been reported to impact latency [150], with 'latency potential' (defined as the ratio of latently infected cells to actively infected) differing between subtype-specific LTR genotypes *in vitro* [151]. In subtype C viruses, increased levels of transcriptional activity resulted in more rapid silencing of the viral promoter due to negative feedback, and was associated with a greater number of NF-κB binding sites within the LTR [152]. Furthermore, the AP-1genotype (a binding motif in the LTR) has been shown to confer greater latency potential (i.e. reduced proportion of cells that lack transcription of HIV-1 genes) to subtype A and C viruses compared to subtype B [153]. Conversely, another study showed no differences in initial latency potential between subtypes with the exception of subtype AE in primary T-cells [154].

The HIV-1 transcriptional switch protein, Tat, is heavily implicated in the establishment and persistence of latent provirus. Tat regulates transcriptional elongation via RNA polymerase II (RNAP II) recruitment in the viral 5' LTR. For viral transcription to occur, Tat must bind the trans-activating response (TAR) hairpin present on the viral transcript. In a study by Razooky *et al.*, Tat-mediated feedback was able to induce proviral reactivity in the absence of cellular stimulation [155]. In fact, in the absence of multiply spliced RNA encoding *tat/rev*, the feedback loop was disrupted and led to non-productive infection [156]. Due to the heavy

implication of HIV-1 Tat on transcriptional status, this protein is critical to many therapeutic strategies being utilized today, highlighting the importance of characterizing *tat* variants in the reservoir. Subtype C Tat (TatC) has been shown to have a higher transcriptional activity in T-cell lines than Tat from subtypes B (TatB) and E [157]. Furthermore, genetic variations in TAR can impact the ability of Tat to facilitate viral transcription [158]. Studies identified intra-subtype C variation in the TAR element as well as Tat that correspond to key functional sites that affect Tat binding and Tat-induced transcriptional activity, respectively [159–161]; including evidence of positive selection in primary infection [160]. In addition to mediating transcription, the viral protein Tat is also responsible for RNA silencing suppressor activity (RSS) in infected cells [162–165]. RSS serves to attenuate translation of HIV-1 transcripts, determining viral load set-point and favouring latency. TatB has been shown to have more potent RSS activity than that of TatC viruses, however, a greater range of RSS activity was observed among TatCs [166].

The HIV-1 accessory protein Nef is an element of interest as a reservoir determinant. Nef facilitates the pathogenesis of HIV-1 by interfering with host protein trafficking [167]. Furthermore, sequestration of major histocompatibility complex-1 (MHC-I) by Nef precludes antigen presentation by infected cells and evasion of the host CTL response as a result [168, 169]. In a recent study, the strain-specific ability of Nef to downregulate MHC-I *in vitro* was associated with *in vivo* reservoir size [81]. Furthermore, in a multivariable analysis adjusting for multiple clinical factors, HIV-1 DNA levels were found to be higher in individuals infected with subtype B compared to those infected with non-B subtypes (CRF_01 AE and G), and this higher abundance of cells harbouring HIV-1 DNA was attributable to the superior Nef function of subtype B viruses. However, this study included only men who initiated treatment in acute/early infection and were on treatment for less than a year [81]. Given that Nef function

has been shown to differ across subtypes, with subtype C exhibiting reduced Nef function compared to other subtypes [170], it will be beneficial to assess Nef function in the context of other HIV-1 subtypes, females, and PLWH who initiated treatment during chronic infection, to determine the generalizability of these findings to LMIC.

1.8 Strategies for HIV-1 Eradication and Cure

In the context of HIV-1, researchers often refer to cure as either 'functional' or 'sterilizing'. In either instance, a cure would allow for PLWH to interrupt ART without experiencing viral rebound. For a functional cure, HIV-1 is durably controlled in the absence of ART, while remaining, in some form, within the body. Alternatively, a sterilizing cure aims to remove all traces of HIV-1, including provirus, from PLWH. The primary strategies being investigated for HIV-1 cure are mentioned below and will highlight the practicality of implementation of these strategies/therapeutics in LMICs based on ease of potential clinical use, cost, and availability.

1.8.1 Hematopoietic Stem Cell Transplant

To date, an apparently sterilizing cure has been achieved in two individuals [171, 172]. These individuals received a homozygous CCR5Δ32 hematopoietic stem cell transplant following immune ablation. The CCR5Δ32 mutation renders cells impervious to strains of HIV-1 that use CCR5 as a co-receptor. These; the ablation eliminates a substantial portion of the cells harbouring replication competent provirus, and the transplanted CCR5Δ32 that reconstitute the immune system are resistant to HIV-1 infection. While these cases of sterilizing cure offer

proof that a cure is possible, this procedure is associated with high mortality and would not be appropriate for an otherwise healthy person living with HIV. For those with treatment access, ART provides a lower risk alternative with relatively fewer co-morbidities, while excluding the possibility of treatment-associated death. Additionally, the high procedure-associated cost (i.e., approaching several hundred thousand dollars per patient for treatment, hospitalization, and follow-up monitoring) limit scalability and global rollout. Aside from the risk to the patients, this approach will never be widely adopted even in HICs based on cost alone.

1.8.2 Shock-and-Kill

The 'shock-and-kill' strategy aims to induce transcriptional reactivation of the replication-competent proviral reservoir, and has been recently reviewed by Kim *et al*. [173]. The intention is that induced cells will produce viral products that are recognizable by host immune mechanisms, prompting their clearance by cytotoxic immune mechanisms. During this process, an individual would maintain daily ART to prevent *de novo* infection of bystander cells by virus produced by reactivated cells. Since post-ART rebound occurs at a frequency of approximately once a week, a substantial proportion of the reservoir must be cleared for this approach to be feasible[174]. For instance, a 1,000-fold reduction in the replication competent reservoir is theoretically required to achieve an average remission of 20 years[175]. Such modeling reveals the innate challenge of developing a functional 'shock-and-kill' therapeutic, while also indicating that it is theoretically possible with the correct approach.

Latency-reversing agents (LRAs) comprise small molecules and biologics designed to induce transcriptional reactivation of virally infected cells. Amongst the most investigated are histone

deacetylase inhibitors (HDACi), which function through direct inhibition of the HDAC enzyme. HDACi can induce highly variable latency reversal in cell lines engineered with latent HIV-1 provirus [176] but fail to induce appreciable HIV-1 from cells isolated from PLWH on stable ART [177–179]. HDACi activate a low level of transcription, but undetectable HIV-1 protein synthesis, which is necessary for immune recognition and elimination of these latently infected cells. They have been utilized with low level, daily dosing in phase I/II clinical trials, but have failed to reduce the latent HIV-1 pool [180]. Increased dosage may provide better latency reversal, but HDACi are not selective for latent, integrated HIV-1 proviral DNA and would result in general transcription upregulation of host genes.

In lieu of epigenetic manipulation, some approaches target cellular activation pathways. Protein kinase C (PKC) agonists, for example, induce global T-cell signalling and transcription factor recruitment [181]. Similarly, toll-like receptors (TLRs) that recognize RNA viruses, such as TLR 3/7/8, can mediate T-cell activation and downstream induction of latent proviral expression, while also enhancing cytolytic activity [182]. One example is the TLR-7 agonist, GS-9620 (Vesatolimod; Gilead), which became a strong candidate after pre-clinical data showed clearance of hepatitis B in several models of infection [183, 184]. In clinical trials, GS-9620 could potently induce expression of interferon-stimulated genes (ISG), leading researchers to investigate the agonist's potential as a latency-reversing agent for HIV-1. A subsequent *ex vivo* study revealed that TLR-7 agonists could promote HIV-associated RNA production by 1.5-2-fold [185]. In a recent Phase Ib study, GS-9620 was well-tolerated at doses sufficient to induce ISG expression (>4mg) [186], providing feasibility for use in future regimens.

Interestingly, several studies have indicated that optimal latency reversal could require stimulation through the T-cell receptor (TCR) [187, 188]. Signalling through the TCR, best demonstrated by the use of PMA/Ionomycin or anti-CD3/anti-CD28 antibodies, results in an intracellular cascade for multi-kinase activation, chromatin remodelling and transcription factor induction necessary for immune activation of memory and naïve T-cell subset [189]. Interestingly, the same activation cascade is required for productive HIV-1 replication in activated T-cells, and reactivation of latent provirus is more potent after stimulation with HIV-1 antigen, as opposed to non-HIV-specific antigen, which may be due to the preferential infection of HIV-specific T-cells [190]. Upon primary HIV-1 infection, antigens consisting of HIV-1 virus particles and proteins are transported to primary and secondary lymphoid tissues/organs by antigen presenting cells. These APCs will ultimately present HIV-1 antigens on MHC class II to $CD4^+$ T-cells with TCR specific to these HIV-1 antigens, leading to activated $CD4^+$ T-cells, which are now susceptible to HIV-1 infection and replication [191]. We hypothesize that this cycle of HIV-1 antigen presentation and activation of HIV-specific T-cells leads to a skewing of the antigen-specificity of latently infected cells towards HIV-1 antigens [192]. This hypothesis is supported by the observation that most variants in the reservoir arose from strains circulating immediately prior to ART initiation [10, 117, 118]: once ART is initiated and HIV-1 antigens are cleared HIV-specific T-cells will revert to a resting state *en masse*. The use of HIV-1 antigens as an immunogen could specifically reactivate and eliminate this substantial portion of the reservoir [192].

A heterogenous virus-like particle (VLP) derived from the quasi-species of five HIV-1 infected patients has recently been developed [192, 193]. This VLP formulation contains all the HIV-1 proteins and is morphologically identical to wild type HIV-1 but lacks genomic RNA and has additional mutations to ensure it is not replication-competent and can be tested as a cure

therapeutic [193]. Heterogenous HIV-1 VLPs have so far out-performed all other LRAs at induction of the latent HIV-1 pool from $CD4^+$ T-cells isolated from patients on stable ART. The level of latent HIV-1 induction by these VLPs is comparable to PMA/ionomycin but the VLPs, unlike PMA/ionomycin, target only a fraction of $CD4^+$ T-cells for activation and therefore is unlikely to create generalize and toxic immune activation. These findings suggest that VLPs may result in targeted activation of a sizeable proportion of the latent pool. However, for this therapeutic to be useful in LMIC, the effect of subtype congruency between VLP and patient must be ascertained. Additionally, while activation of the latent HIV-1 pool in response to VLP has been observed using cells form patients treated during both acute/early [192] and chronic infection, it must be ascertained if the reservoir of individuals treated during chronic infection is as heavily skewed towards HIV-specific T-cells. Individuals who initiated ART soon after infection will have been exposed to fewer non-HIV antigens while viremic, but only a minority of PLWH in LMIC initiated treatment during acute infection. Aside from these remaining uncertainties, compared to the use of small molecules lacking a specific targeting mechanism, the use of VLPs is safe, effective, and is an inexpensive cure therapeutic that could potentially be widely used around the world.

Many of these small molecule or even peptide-based inhibitors/agonists would be a suitable cure therapeutic for testing and potential roll-out in LMICs. Importantly, such strategies would greatly benefit from longitudinal follow-ups to ensure viremia is suppressed in the absence of ART. Currently, their cost remains prohibitive, but with relaxation of the agreement on trade-related aspects of intellectual property rights (TRIPS) agreement/patent regulations for greater drug access for LMIC markets, it is possible that investment by the Global Fund, PEPFAR, and various other foundations, would be offset by the reduction in the burden of ART, the concern of ART drug resistance, and the prevention of new infections.

1.8.3 Block-and-Lock

The ‘block-and-lock’ approach aims to provide a functional cure for HIV-1 through the suppression of transcriptional activity. One way to achieve this is by altering methylation and acetylation status. For a block-and-lock approach, this will typically involve a combination of hyper-methylation and/or hypo-acetylation. Furthermore, many strategies are utilizing small interfering (si) RNA to induce epigenetic changes at sites of transcriptional relevance, such as the HIV-1 NF-kB promoter site [194–196]. An alternative approach is blocking the HIV-1 accessory protein, Tat, which is involved in the recruitment of RNA polymerase II. Blocking its function might greatly limit transcriptional output. Didehydro-Cortistatin A (dCA) is one small molecule capable of engaging with HIV-1 TAR RNA – the binding domain of Tat [197] – and dCA treatment renders cells resistant to the effects of LRAs [198]. Another study proposed the use of “naked” cyclic Tat peptidomimetics that have rapid cellular uptake, that bind HIV-1 TAR RNA with high affinity, and inhibit Tat transactivation/mRNA transcription as well as reverse transcription [199, 200].

For a block-and-lock strategy to be curative, virtually all replication competent provirus would require durable, drug-mediated post-translational modification. This means that, even if a small proportion remains unmodified, the chance of productive re-infection exists. However, studies have shown that reducing the size of the replication competent reservoir can prolong the time to viral recrudescence [175]. This would arguably be true of epigenetic silencing as well. Therefore, the lock-and-block strategy likely reduces risk of re-infection short-term, but will invariably lead to rebound unless complete, sustained reservoir silencing is achieved.

The block-and-lock approach is uniquely positioned from a therapeutic perspective. Since the goal here is not the removal of the viral genomes but rather their suppression, blocking agents could potentially function on multiple distinct cellular reservoirs. Block-and-lock inhibitors are also easier to design, test, and possibly utilize for treatment. However, long acting formulations are necessary for these inhibitors to be considered as a cure therapeutic, as opposed to a form of ART. Long-acting ART will provide a benefit for those PLWH who have issues with adherence (adolescents, etc.), and was found to be more acceptable than oral ART in the context of pre-exposure prophylaxis [201], but may not replace oral ART completely in the very near future.

One of the drawbacks of long-acting ART administration is having more frequent clinic visits: once a month or every two months as opposed to biannually. In addition, well-resourced settings may be able to handle the operational challenges associated with switching from oral to injectable medications: potentially more staff required on site, more frequent VL testing to ensure viral suppression between visits, increased challenges with regards to ensuring all visits occur timeously, storage and transport of injectables/infusions. In terms of LMICs, this may ease the burden on PLWH who have access to these resources in urban and suburban settings but may not work well in rural settings unless distribution of these interventions can be decentralized (i.e., administered outside of a clinical setting).

More research is needed into whether these treatments can be administered by non-medical personnel or whether self-administration of these drugs is feasible. Despite the challenges, intermittent administration of block-and-lock therapeutics could reduce pill burden and alleviate ART-associated co-morbidities. As with all potential therapeutics, global investment

and robust organizational initiatives are mandatory for proper roll-out and maintenance in LMICs, assuming a clinically successful 'block-and-lock' strategy is realized.

1.8.4 Broadly Neutralizing antibodies as a cure therapeutic

The discovery of monoclonal antibodies capable of potent neutralization of a wide range of HIV-1 isolates, termed broadly neutralizing antibodies (bNAbs), has provided new avenues for functional cure research. In-human trials (passive immunization) have been conducted with several bNAbs and they have been shown to be tolerable and safe, even at high doses and when administered repeatedly in both HIV-1 uninfected [4, 5] and infected adults [6, 7]. Monoclonal bNAb therapy did not result in decrease in residual plasma viraemia [8], HIV-1 DNA/RNA [8, 9] or outgrowth viruses (demonstrated with VRC01) [8] but did result in a delay in viral rebound after analytical treatment interruption (ATI), demonstrated with VRC01 [10, 11], 3BNC117 [12], and UB-241 [13], although results were highly variable across trials (ranging from 4 to 16 weeks median time to rebound). Combination bNAb therapy has also shown promise, with the benefit of a decreased likelihood of developing resistance to multiple antibodies [7]. Trials testing sequential administration of 2G12, 2F5, and 4E10 resulted in 8-10-week delay in viral rebound upon ATI [14, 15]. Administration of 3BNC117 and 10-1074 in combination during ATI resulted in maintained viral suppression for a median of 21 weeks in participants who had antibody sensitive viral reservoirs [6].

A phase I clinical trial is ongoing in South African women (CAPRISA 012), testing CAP256V2LS, a potent bNAb isolated from a subtype C infected individual capable, alone and in combination with VRC07-523LS and PGT121 for use in pre-exposure prophylaxis [16].

However, this trial could pave the way for investigation into CAP256V2LS (likely in combination) as a functional cure in Africans. Finally, the first studies in non-human primates showed efficacy of bNAbs produced in vivo through the administration of adeno-associated viruses encoding bNAbs [17]. Although host-elicited immune responses limited effectiveness of the treatment [18], one macaque was functionally cured. While this approach is still in development and needs refining before in-human trials, it is a proof of concept for the role bNAbs in a functional cure strategy. A disadvantage associated with bNAb therapy in resource-limited settings is the requirement for resistance screening both before administration of therapy to ensure antibody sensitivity of viruses, and during therapy to monitor antibody escape. Similar to long-acting ART, these drawbacks significantly limit the scalability of bNAbs as a cure therapeutic.

1.9 Study rationale, aims, and objectives

The major barrier to an HIV-1 cure is the formation of a stable latent reservoir in people PLWH. The latent reservoir is formed very early during infection, even in people who initiate ART during acute infection prior to seroconversion, and has a long half-life. Little is known about factors influencing latent reservoir formation and maintenance but factors affecting disease progression, such as viral burden during untreated infection, $CD4^+$ T-cell loss, immune activation and inflammation, may impact reservoir formation. Furthermore, if a global cure is to be achieved, the burden of HIV-1 will need to be addressed in many different populations, most notably African women, women who bear the burden of HIV-1 globally. In South Africa, the country in the sub-Saharan African region with the highest prevalence of HIV-1, women are roughly twice as likely to be living with HIV-1 than men (aged 15 to 49) [202], with a prevalence rate 6% higher than the national average of 19%. The studies in this thesis included

the CAPRISA 002 acute infection cohort, a cohort of South African women aged 19 to 49 [48, 203]. The cohort was established for the long-term follow-up of at-risk women who became infected with HIV-1 and were then monitored throughout infection until ART initiation according to prevailing South African guidelines.

The overall aim of this study was to investigate the influence of immune activation and inflammation on the size and persistence of the latent reservoir of HIV-1, in African women on long-term suppressive ART initiated during chronic infection. This study contributes to a growing body of knowledge on HIV infection in African women, with the aim of informing HIV cure strategies in an African context.

Hypothesis: High levels of immune activation and inflammation in women living with HIV results in seeding of a larger latent reservoir, due to an expansion in the number of HIV target cells and/or increased viral replication when the reservoir is laid down. Furthermore, sustained inflammation throughout the course of infection, in the presence or absence of treatment, will expand the reservoir.

Aim 1

To characterize immune activation, measured by the frequency of activated memory $CD4^+$ and $CD8^+$ T-cells, and levels of plasma markers of inflammation longitudinally during HIV infection in PLWH.

Rationale: The role of immune activation over the course of untreated and treated infection in influencing the latent reservoir over time is still being defined. Immune activation plays an important role in viral control during acute infection but can also lead to a greater number of

activated target cells for HIV. Sustained immune activation results in exhaustion of the T-cell response to HIV, leading to a dysfunctional phenotype which could contribute to reservoir size. This aim of our study serves to describe longitudinal immune activation and inflammation in a clinically well-characterized cohort of South African women, the CAPRISA 002 cohort.

Aim 2

To determine the influence of immune activation and inflammation on the size of the replication-competent latent reservoir in PLWH

Rationale: The replication-competent reservoir is the proportion of cells infected with viruses that remain quiescent in the presence of ART but are capable of recrudescence when ART is ceased. This proportion of cells represents the primary target for HIV cure strategies as it can reinitiate viral replication in PLWH regardless of the duration on ART. While early ART initiation and viral burden prior to ART are described determinants of reservoir size, the role of immunological factors such as immune activation and inflammation remains largely unknown. This aim serves to fill the gap in cure research by defining the effects of higher levels of immune activation and inflammation on the replication-competent latent reservoir.

Aim 3

To determine the impact of immune activation and inflammation on the frequency of total HIV DNA, a marker of HIV persistence, in PLWH

Rationale: Total HIV DNA is another measure of the latent reservoir and encompasses intact HIV-1 genomes that are replication-competent, intact HIV-1 genomes that are not able to be reactivated, and defective HIV-1 genomes that may impact the host immune response and viral

clearance during treatment. Thus, total HIV DNA represents a marker of HIV persistence and decay kinetics of intact versus defective genomes have been shown to differ. In this aim, the effect of immune activation and inflammation on total HIV DNA is elucidated. Total HIV DNA will be measured at 2 time-points post-ART initiation to determine whether this portion of the reservoir decays significantly during this time.

CHAPTER 2

T-cell activation and inflammation in HIV-1 chronically infected South African women

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2.1 Introduction

Sub-Saharan Africa bears the greatest burden of HIV-1 infection worldwide, with South Africa being one of the world's top affected countries. While new infections in South Africa have more than halved over the last 10 years to ~200 000 new infections in 2019, there are 7.5 million adults and children living with HIV-1 [202], requiring substantial resources to provide lifelong ART. Furthermore, females aged 15 to 49 years are three times more likely to be living with HIV-1 than their age-matched male counterparts (prevalence of 10.2% and 3.4%, respectively) [202]. Thus, despite the stabilization of HIV-1 incidence in South Africa over the past 10 years, women continue to bear a disproportionate burden of HIV-1 infection [204].

Recent data from South Africa found that 32% of PLWH are diagnosed late in infection, at a $CD4^+$ T-cell count <200 cells/μL [202], indicating that a substantial number of people are still initiating ART during at a late stage of chronic infection. The timing of ART is an important determinant of the HIV-1 reservoir as firstly, studies have shown a restriction in reservoir seeding in individuals treated early [82–85] and, secondly, viral variants in the long-lived HIV-1 reservoir are dominated by viruses in plasma just before the initiation of ART [10, 117, 118]. This was shown recently by Abrahams *et al*. in study participants from the same cohort presented in this thesis [10]. These findings that the long-lived latent reservoir is established proximal to ART initiation has been observed in different populations, infecting subtypes, and treatment initiation times.

Several host factors that impact disease progression also impact reservoir establishment and can differ significantly between early and chronic infection including the temporal expression

of immune mediators of inflammation [89], T-cell activation and exhaustion [205]. Viral factors influencing disease progression also differ over time as well as interactions between virus and host. Examples of these are infecting viral subtype [41, 42, 48], co-receptor tropism [206] and the propensity of early versus chronic Envelope proteins to elicit pro-inflammatory cytokine production [207]. Thus, it is imperative that studies characterizing the HIV-1 latent reservoir occur in diverse settings and include time points at this pivotal period of treatment initiation during chronic infection.

The CAPRISA 002 Acute infection cohort presents an ideal opportunity to gain novel insights into reservoir formation and maintenance in the context of subtype C infection and treatment initiation during chronic infection [203]. This longitudinal cohort followed up women at a high risk of HIV-1 acquisition, from pre-infection and up to 5 years after ART initiation. In these participants, ART was initiated during the chronic stage of HIV infection, according to South African National Treatment Guidelines at the time [203]. A previous study showed that rapid disease progression occurred in approximately 50% of the participants in the original cohort infected with subtype C viruses [48]. Since factors associated with both infecting subtype and disease progression, such as $CD4^+$ T cell counts, $CD4^+$ T-cell nadir and VLs, have been shown to impact the HIV-1 latent reservoir, it is conceivable that HIV-associated immune activation and inflammation (either directly or through their relationships with these clinical markers of disease progression) may also influence the formation of the latent reservoir and the frequency of circulating latently infected cells.

The overall aim of this study was to: 1) characterize immune activation and inflammation in untreated and treated HIV-1 infection, and 2) determine which immunological characteristics

over the course of infection impact the formation of the HIV-1 latent reservoir. This chapter comprehensively characterized immune activation and inflammation before and after ART initiation in participants from the CAPRISA 002 cohort.

2.2 Materials and methods

2.2.1 Study participants and study ethics

Forty-six women from the CAPRISA 002 acute infection cohort [48, 114, 203] from Kwa-Zulu Natal, South Africa were recruited into this study. Briefly, participants were enrolled and, upon seroconversion, were followed up weekly for the first three months, monthly thereafter until 12 months post-infection, and then every three months until ART initiation. The date of infection was estimated as the midpoint between the last antibody negative test and the first antibody positive test, or as 14 days prior to the first HIV RNA positive test where the enzyme-linked immunosorbent assay (ELISA) test was negative. ART was initiated according to the prevailing South African National HIV Treatment Guidelines: a $CD4^+$ T-cell count of <200 cells/mm^3 prior to October 2012, <350 cells/mm^3 until March 2015, then <500 cells/mm^3 until universal test and treat was adopted in September 2016. After ART initiation, follow-up visits occurred at week one, months one, three and six, and then every six months thereafter. Peripheral blood mononuclear cells (PBMC), plasma samples and sera were collected at each follow up visit and $CD4^+$ and $CD8^+$ T-cell absolute counts and plasma viral load (VL) measured.

VLs were determined using a PCR-based diagnostic assays. The specific assay used for VL determination changed throughout the duration of the study. The initial assay was Roche

AMPLICOR HIV-1 Monitor test version 1.5 (lower detection limit (LDL) of 400 RNA copies/mL) which was switched to Roche 42 Taqman version 1.0 in June 2010 (LDL of 40 RNA copies/mL), and then Roche Taqman version 2.0 in January 2012 (LDL of 20 RNA copies/mL). The FACSCalibur TruCOUNT method (BD Biosciences) was used to determine absolute blood $CD4^+$ and $CD8^+$ T-cell counts.

For this study, PBMC were obtained at the following five time points for each participant, when a sample was available: acute infection (median: 2.2 months post-estimated time of infection [IQR: 1.9-3.3]), one-year post-infection (median: 11.9 months post-infection [IQR: 10.6-13.9] and 3.3 years post-ART initiation [IQR: 2.1-4.6]), and late chronic infection (within one year of ART initiation; median: 7.9 months pre-ART [IQR: 5.0-10.7]), as well as two- (median: 2.4 years post-ART initiation [IQR: 2.0-2.5]) and four years post-ART initiation (median: 4.5 years post-ART initiation [IQR: 4.0-4.5]). $CD4^+$ and $CD8^+$ T-cell counts and VL were available for all time points. All participants provided written informed consent prior to enrolment into the CAPRISA 002 cohort (University of KwaZulu-Natal HREC BE178/150). The parent protocol as well as this sub-study, including laboratory protocols and the use of participant samples and information, was reviewed, and approved by the University of Cape Town's Faculty of Health Sciences Human Research Ethics Committee (HREC 025/2004 and HREC 719/2017).

2.2.2 Thawing and resting cryopreserved PBMC

PBMC vials (of approximately 10 million cells/mL) were thawed rapidly in a 37˚C water bath and added to a 50mL tube. RPMI 1640 medium (Sigma) supplemented with 1% heat

inactivated FBS (subsequently referred to as R1) was added dropwise to the thawed cells up to a volume of 10mL. R1 medium was topped up to 30mL and cells were centrifuged (1200 rpm) for 10 min at room temperature. The supernatant was discarded, and cells were resuspended in 500µL of R1 containing 0.002% of DNAse and incubated for 3 min at room temperature to prevent the clumping of cryopreserved cell suspensions following thawing. R1 was added up to 25mL and then samples were centrifuged (1200rpm) for 10 min at room temperature. After discarding the supernatant, cells were resuspended in 3mL RPMI 1640 supplemented with 10% heat inactivated FBS and penicillin-streptomycin (50 U/mL) (subsequently referred to as R10). Cell counts were determined using Trypan Blue exclusion staining. Briefly, 20µl of cell suspension was diluted 1:2 in Trypan Blue (Invitrogen) and counted using a Countess cell counter and slides (Invitrogen). Cells were then diluted in R10 to a final concentration of 2 to 4 million cells/mL and rested by incubating them overnight (maximum of 16h) in a 5% CO_2 incubator set to 37˚C.

2.2.3 Ex vivo staining and flow cytometry

Fluorescently labelled monoclonal antibodies (**Table 2.1**) were used for surface staining of rested, *ex vivo* PBMC. All antibodies were titrated to determine the optimal staining concentration to achieve maximal separation between the negative and positive population in a 50µL final staining volume. The titer was selected based on saturation, signal-to-noise ratio and staining index. Delineating the positive and negative populations was informed by the inclusion of a fluorescence minus one (FMO) control (i.e., a sample stained with all antibodies excluding the one of interest). Each new lot of antibody was titrated alongside an FMO sample before use in the panel.

Table 2.1. Antibody cell surface staining panel for flow cytometry.

	Marker	Fluorochrome	Clone	Manufacturer
Exclusion/Viability	ViViD	Pacific Blue*		Invitrogen
Exclusion (monocytes)	CD14	Pacific Blue	Tuk4	Invitrogen
Exclusion (B cells)	CD19	Pacific Blue	SJ25-C1	Invitrogen
Lineage	CD3	PE-Cy7	SK7	BD
Lineage	CD4	PE-Cy5.5	S3.5	Invitrogen
Lineage	CD8	Qdot 705	3B5	Invitrogen
Memory differentiation	CD45RA	BV570	HI100	Biolegend
Memory differentiation	CCR7	PE-CF594	150503	BD
Activation	CD38	FITC	HIT2	eBioscience
Activation	HLA-DR	APC-Cy7	L243	BD

*ViVid is a fixable dead cell stain detected in the Pacific Blue channel

For surface staining, rested, *ex vivo* PBMC were transferred into a 96-well v-bottom plate and centrifuged (1800 rpm) for 3 min (as for all subsequent centrifugation steps in this section). Cells were then washed twice using 200µL PBS followed by centrifugation. Thereafter, cells were stained using a LIVE/DEAD Fixable Violet Dead Cell marker (ViViD, Invitrogen) for 20 min at room temp in the dark, washed thrice with 200µL FACS wash (1% FCS in PBS), stained with a pre-mixed cocktail containing antibodies targeting surface molecules (**Table 2.1**) for 30 min in the dark at room temperature, followed by three washes with FACS wash solution. Stained cells were resuspended in 150µL of 1x CellFIX solution (BD) and kept at 4˚C until acquisition. Samples were acquired on the same day on a BD LSRFortessa using FACSDiva software (version 8.01) and analysed using FlowJo (version 10.5.3; TreeStar)

2.2.4 Gating strategy

T-cells were identified and categorised into memory subsets using the gating strategy presented in **Figure 2.1**. A "time gate" was applied to exclude any potential fluorescent intensity shifts, followed by sequential gating for singlets, lymphocytes, live $CD3^+$ cells. While gating for the latter, CD14 and CD19, detected in the same channel as the dead cell stain (ViViD), were used to exclude monocytes and B cells, respectively. $CD4^+$ and $CD8^+$ T-cells were gated from the $CD3^+$ subset. Memory phenotypes were delineated using the markers CD45RA, CD27, CCR7, CD95 and CD127, while HLA-DR and CD38 were used for identifying activated cells. **Figure 2.1** describes the cell surface expression profile of each of the memory markers for each T-cell memory subset. Since naïve T-cells constitutively express CD38, T-cell activation was assessed on memory cells only ($CCR7^+$ $CD45RA^+$ cells excluded).

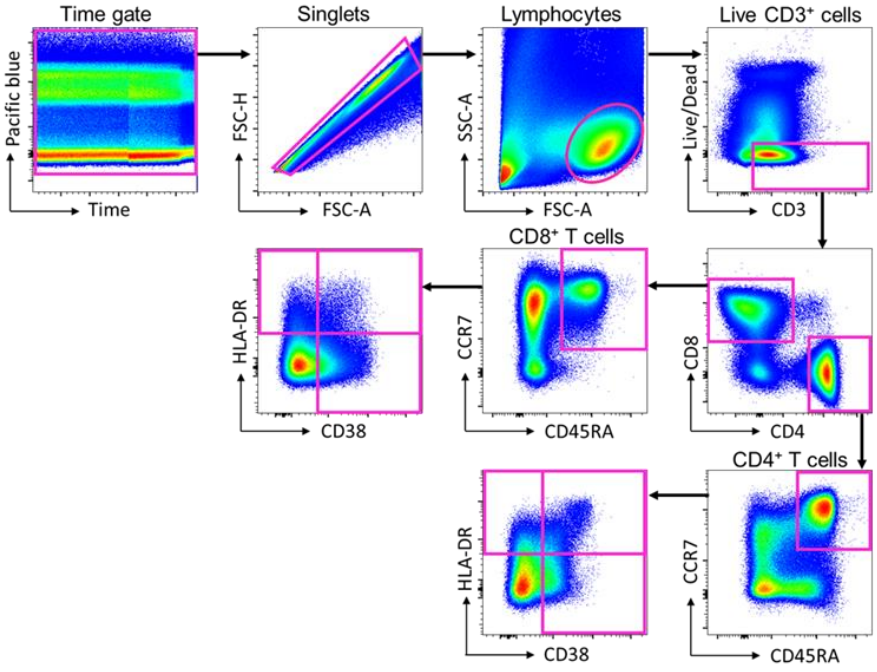

Figure 2.1. Gating Strategy for the analysis of T-cell activation and memory differentiation. Cells were gated against time and then singlets, lymphocytes, live $CD3^+$ T-cells, and the frequency of $CD38^+$ and HLA-DR^+ $CD4^+$ and $CD8^+$ memory T-cells was determined by excluding naïve cells ($CCR7^+CD45RA^+$ cells).

2.2.5 Measuring markers of inflammation

The concentrations of five mediators of inflammation: Interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-α), chemokine ligand 10 (CXCL-10) also known as interferon gamma-induced protein 10 (IP-10), soluble cluster of differentiation 14 (sCD14), and soluble cluster of differentiation 163 (sCD163) were measured in plasma samples by ELISA (DuoSet ELISA kits and DuoSet ancillary reagent set 2, R&D), according to the manufacturer's instructions. Samples were run in duplicate, and the mean absorbance (minus background absorbance) was used for calculating each analyte concentration. Background absorbance was measured in wells that had been coated and washed but did not contain any sample (blank). Concentrations of each inflammatory mediator were measured using a VersaMax microplate reader (Molecular Devices) and SoftMax Pro software (version 5.3). The kit sensitivities for all measured inflammatory mediators ranged from 0.6 pg/mL to 10 ng/mL. The data were analysed using a five-parameter logistic regression applied to calculate sample concentrations from the standard curve on each plate. Soluble marker concentrations below the lower detection limit (LDL) of each assay were reported as half the value of the LDL.

2.2.6 Statistical analysis

Flow cytometry plots and resulting cell population frequencies, including Boolean frequencies, were generated using FlowJo (version 10.5.3, BD Biosciences). GraphPad Prism version 8.0.2 (GraphPad Software, Inc.) was used to generate figures, calculate area under the curve VLs (AUC VL), perform t tests and correlations. For AUC VL calculations, peak viraemia (<3 months post-infection) were not included since these data were not available for all

participants. The Mann Whitney U Test and Wilcoxon Signed Rank Test were used to compare groups of unmatched and matched samples, respectively. All correlations were tested using the non-parametric Spearman's Rank test. A *P*-value of <0.05 was considered significant for individual comparisons. Where multiple comparisons were made, a Bonferroni *P*-value cut-off correction was employed and is stipulated in the figure legend.

2.3 Results

2.3.1 Participant characteristics

Stored PBMC and plasma were obtained for 46 participants in the CAPRISA 002 Acute Infection cohort. The cohort consists of women at a high risk of HIV-1 acquisition enrolled during acute infection and followed-up up to 5 years after ART initiation, which occurred based on South African National Treatment Guidelines at the time. **Table 2.2** shows the demographic and clinical characteristics of participants included in this study. The median age of participants included in this study was 28.8 years (Interquartile range (IQR): 25.6-32.8). Participants were ART-naïve for a median of 3.8 years after their HIV positive diagnosis (IQR: 2.6-5.0) and initiated ART at a median $CD4^+$ T-cell count of 292 cells/mL (IQR: 203-339). Of the 46 participants, 16/46 (35%), 20/46 (43%), and 10/46 (22%), initiated ART at $CD4^+$ T-cell counts <250, <350, and <500 cells/mL, respectively. All participants included in this study were on ART for at least 2 years.

Table 2.2. Participant characteristics.

PID	Age	Estimated time of untreated infection		$CD4^+$ T-cell count		CD4:CD8	Log_{10}(VL)	Log_{10}(AUC VL)		
	At ART initiation	Acute infection to ART initiation		Nadir	At ART initiation	At ART initiation	At ART initiation	Acute infection to ART initiation	Within 1y p.i.	Within 1y pre-ART
	(months)	(months)	(years)	(cells/mL)		-	(copies/mL)	(months.copies/mL)		
100303	28.8	26	2.2	60	60	0.0	4.8	6.7	6.3	6.4
100336	21.8	32	2.7	74	74	0.1	5.4	6.4	4.9	6.1
100276	26.3	16	1.3	94	94	0.3	3.9	5.5	5.4	5.5
100340	27.2	52	4.3	97	97	0.1	4.7	5.9	5.4	-
100065	45.8	80	6.7	121	121	0.3	5.0	6.5	5.9	5.7
100286	23.1	34	2.8	142	142	0.1	4.7	6.5	6.0	6.0
100268	25.3	50	4.2	163	163	0.2	3.6	5.7	5.1	4.8
100257	32.8	57	4.8	170	170	0.2	4.7	6.2	5.3	5.8
100008	41.7	40	3.3	175	185	0.3	5.0	6.3	5.7	5.8
100279	32.1	32	2.7	170	193	0.4	4.3	6.0	5.5	5.4
100358	26.3	13	1.1	165	194	0.2	3.9	5.3	5.3	5.3
100177	42.8	68	5.7	201	206	0.2	4.8	6.7	5.8	5.9
100333	26.5	44	3.7	218	218	0.2	4.8	5.6	3.7	5.5
100037	25.9	66	5.5	199	222	0.4	4.4	6.8	5.7	5.5
100329	22.5	18	1.5	243	243	0.2	4.7	5.8	5.5	5.8
100313	24.4	40	3.3	207	247	0.4	3.2	6.7	6.2	4.3
100321	19.0	2	0.2	241	267	0.3	5.9	-	-	-
100267	30.3	65	5.4	269	269	0.3	4.6	5.9	5.2	4.9
100328	40.9	32	2.7	238	280	0.5	3.0	4.6	3.6	4.1
100282	32.8	58	4.8	170	285	0.2	4.1	5.5	5.3	4.6
100217	27.3	82	6.8	256	287	0.3	4.9	6.7	5.9	5.8
100206	41.5	62	5.2	240	289	0.2	5.3	7.6	6.0	6.9

100337	26.1	38	3.2	267	290	0.5	4.8	6.2	5.8	5.5
100244	29.9	87	7.3	241	293	0.3	4.4	6.3	5.4	5.5
100386	32.7	19	1.6	304	304	0.3	4.8	5.7	-	5.7
100222	25.6	73	6.1	305	305	0.6	3.7	5.5	4.1	4.3
100302	29.2	37	3.1	204	307	0.4	4.7	6.7	6.4	5.7
100372	25.6	41	3.4	309	309	0.5	5.2	6.2	5.4	6.0
100088	29.1	60	5.0	248	311	0.1	5.8	7.3	5.7	7.2
100341	29.5	54	4.5	250	312	0.3	4.1	5.4	4.2	5.0
100345	27.6	11	0.9	156	314	0.5	3.1	7.2	7.2	7.2
100288	29.1	48	4.0	288	318	0.4	4.3	6.1	5.6	4.9
100277	40.8	59	4.9	281	322	0.2	3.9	5.3	4.0	4.7
100237	25.6	63	5.3	183	325	0.6	3.2	5.7	5.2	4.4
100326	24.3	35	2.9	332	336	0.3	3.7	5.3	4.8	4.6
100289	37.7	42	3.5	235	347	0.4	5.5	7.1	6.3	6.7
100266	28.5	81	6.8	363	363	0.3	4.7	6.2	5.1	5.9
100188	37.8	56	4.7	267	364	0.1	5.4	7.0	5.8	6.0
100353	22.4	11	0.9	271	400	0.5	4.3	5.3	5.3	5.3
100280	32.0	68	5.7	174	406	0.3	4.8	6.5	5.6	6.0
100380	36.3	30	2.5	377	407	0.6	4.7	6.1	5.8	5.5
100287	26.2	59	4.9	216	411	0.3	4.2	6.1	5.4	5.4
100256	23.4	54	4.5	245	435	0.3	4.7	6.9	6.7	5.4
100316	28.7	49	4.1	278	436	0.3	3.7	5.6	5.4	4.4
100395	30.8	24	2.0	436	436	0.3	4.1	4.8	-	4.8
100352	39.8	24	2.0	333	451	0.4	4.1	5.9	5.8	5.2
Median	**28.8**	**46**	**3.9**	**239**	**292**	**0.3**	**4.7**	**6.1**	**5.5**	**5.5**
IQR	**25.6-32.8**	**32-61**	**2.7-5.1**	**170-273**	**203-339**	**0.2-0.4**	**4.0-4.8**	**5.6-6.7**	**5.2-5.8**	**4.9-5.9**

2.3.2 Clinical parameter trajectories before and after ART initiation

The clinical parameters of the 46 participants were studied before and after ART initiation. Representative VL and $CD4^+$ T cell count trajectories for two participants are shown in **Figure 2.2,** illustrating the frequency of sampling, and the dynamics of viral suppression and absolute $CD4^+$ T-cell reconstitution after ART initiation. Median pre-ART VL over time (AUC VL) was 6.1 time.copies/mL (IQR: 5.6-6.7) (**Table 2.2**) and 16/46 participants initiated ART in the first 3 years post-estimated time of infection, indicating rapid progression on 35% of the cohort. Nadir CD4+ T-cell count was 239 cells/μL (IQR: 170-273), indicating that most of the cohort initiated ART at low $CD4^+$ T-cell counts.

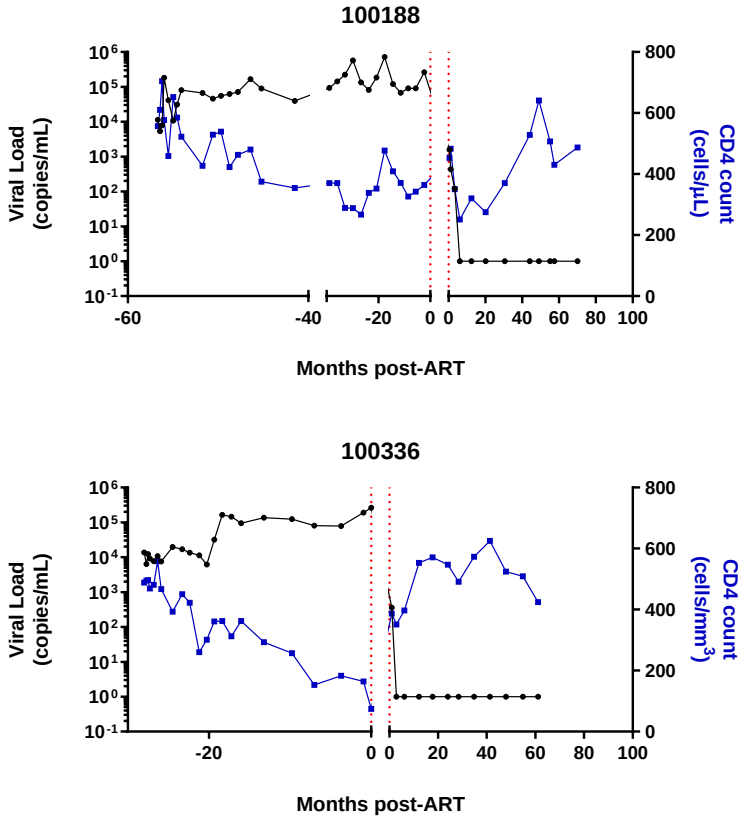

Figure 2.2. Representative viral load and $CD4^+$ T-cell trajectories. Viral load (left y-axis; black dots with connecting line) and $CD4^+$ T-cell counts (right y-axis; blue dots and connecting line) for two participants in this study. The vertical dotted red lines indicate ART initiation.

Plasma VLs, $CD4^+$ T-cell counts, and CD4:CD8 ratios were compared longitudinally at three time points: at ART initiation and at two- and four-year post-ART initiation (**Figure 2.3**). At two years post-ART initiation, VLs had decreased to undetectable levels from a median of 4.6 (IQR: 4.0-4.8) $\log_{10}$ copies/mL at ART initiation (P<0.0001). Absolute $CD4^+$ T-cell counts increased from a median of 292 cells/µL (IQR: 203-339) at ART initiation to 609 cells/µL (IQR: 476-753) at two years post-ART (P<0.0001), and then further increased to a median of 638 cells/µL (IQR: 522-758) at four years post-ART initiation (P=0.033 between two and four years on ART). Absolute CD8+ T-cell counts also decreased significantly between ART initiation (median: 990 cells/µL (IQR: 631-1292) and after two years on ART (median: 686 cells/µL (IQR: 505-893), P=0.0014), and then remained stable four years post-ART (median: 653 cells/µL, IQR: 486-847). CD4:CD8 ratios, a measure of immune reconstitution, increased from a median of 0.3 (IQR: 0.2-0.4) at ART initiation to 0.8 (IQR: 0.6-1.0) at two years post-ART (P<0.0001) and then to a median of 1.0 (IQR: 0.7-1.3) at four years post-ART (P<0.0001 between two and four years on ART).

VL at ART initiation correlated positively with estimated time to viral suppression (P=0.004 r=0.42) and AUC VL both over the entire course of infection (P<0.0001, r=0.62) and within one year before ART initiation (P<0.0001, r=0.77; data not shown). VL at ART initiation also correlated negatively with CD4:CD8 ratio at ART initiation (P=0.013, r=-0.36). $CD4^+$ T-cell count at ART initiation correlated positively with CD4:CD8 ratio at ART initiation (P=0.001, r=-0.49) but not with contemporaneous VL (P=0.263, r=-0.17).

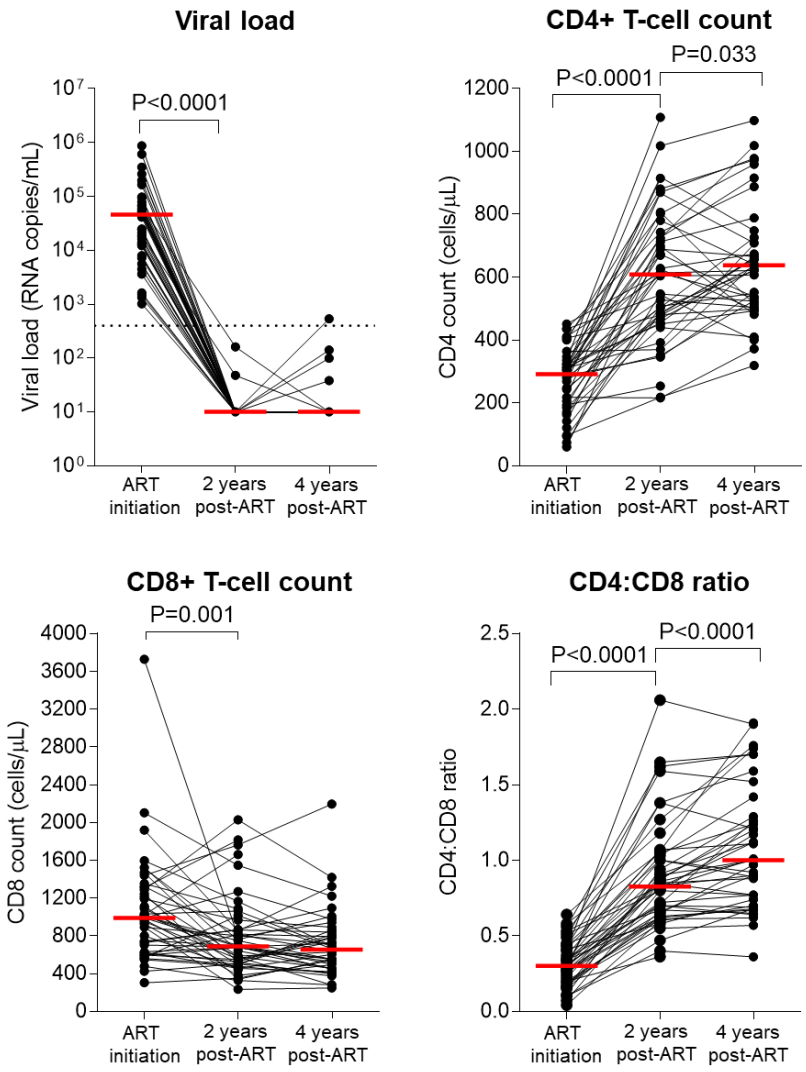

Figure 2.3 Clinical parameters before and after ART initiation. Plasma viral load, absolute $CD4^+$ T-cell count, absolute $CD8^+$ T-cell count and CD4:CD8 ratio in participants before ART initiation and at two- and four-years after treatment (n=46). The horizontal dashed line indicates the detection limit of the assay. Red horizontal bars indicate the median values. Statistical significance was calculated using a Wilcoxon matched pairs signed rank test. Only statistically significant *P*-values ($P<0.05$) are annotated, and *P*-values were not corrected for multiple comparisons. All but the comparison of $CD4^+$ T-cell count between two- and four years post-ART initiation remained significant after Bonferroni correction (Adjusted *P*-value cut-off of 0.025).

2.3.3 Immune activation trajectories throughout untreated HIV-1 infection and after ART initiation

HIV-associated immune activation impacts disease progression by driving $CD4^+$ T-cell loss and exacerbating pathogenesis. Excessive T-cell activation results in increased cell turnover, cell depletion [11–13] and cellular exhaustion and dysfunction [124]. Furthermore, sustained activation of memory cells may impact the formation and dynamics of the HIV-1 latent reservoir. For example, the presence of more activated memory $CD4^+$ T-cells may facilitates HIV-1 replication, as these are the primary targets of HIV-1 [208], and could lead to an increase in reservoir seeding, or increased immune activation may lead to a higher rate of turnover of infected cells in the latent reservoir. In addition, chronic $CD8^+$ T-cell activation leads to immune dysfunction that may also impact reservoir formation.

To evaluate memory T-cell activation over time in this cohort, we measured CD38 and/or HLA-DR expression on memory T-cells (**Table 2.3**) during acute infection, one-year post-infection, within one year pre-ART, and at two- and four years post-ART. As expected, the frequencies of memory $CD4^+$ T-cells expressing either $CD38^+$, HLA-DR^+, or co-expressing both markers- increased significantly from acute infection (median 2.2 months post-estimated time of infection) to chronic infection (i.e., within a year before ART initiation, median 7.9 months pre-ART) (**Figure 2.4**; P=0.003, 0.008, and 0.022, respectively). After ART initiation, the frequency of activated memory $CD4^+$ T-cells decreased significantly (P<0.0001 for all subsets). The frequencies of $CD4^+CD38^+$, $CD4^+$HLA-DR^+ and $CD4^+CD38^+$HLA-DR^+ T-cells did not differ significantly between two- and four years on ART.

Table 2.3. T-cell activation before and after ART initiation.

			% of memory $CD4^+$ T-cells		% of memory $CD8^+$ T-cells	
		n	**Median**	**IQR**	**Median**	**IQR**
$CD38^+$	**Acute infection**	29	21.5	13.6-30.7	52.3	45.3-72.4
	1y p.i.*	32	31.4	20.0-40.2	71.0	54.6-78.2
	1 year pre-ART	37	33.4	21.7-46.1	69.2	53.4-78.9
	2 years post-ART	42	20.0	15.0-27.2	27.8	22.2-39.7
	4 years post-ART	33	21.9	15.6-24.7	23.3	15.8-37.9
$HLA\text{-}DR^+$	**Acute infection**	15	5.4	3.4-11.5	27.8	19.6-39.8
	1y p.i.*	14	6.1	3.2-11.6	26.9	19.1-33.7
	1 year pre-ART	21	9.4	7.0-15.75	29.2	22.7-39.8
	2 years post-ART	24	4.5	2.2-5.815	14.2	11.0-19.6
	4 years post-ART	24	2.7	1.3-4.0	10.8	6.6-18.2
$CD38^+$ $HLA\text{-}DR^+$	**Acute infection**	15	2.1	0.9-6.4	23.9	13.6-32.1
	1y p.i.*	14	3.5	1.2-5.0	21.6	15.9-26.7
	1 year pre-ART	21	4.3	2.6-8.2	22.8	14.1-32.7
	2 years post-ART	24	1.3	0.5-1.6	7.2	4.0-9.7
	4 years post-ART	24	0.60	0.3-1.0	4.6	2.5-7.8

*p.i.: post-infection

The memory $CD8^+$ T-cell activation profile remained similar from acute infection to the year before ART initiation (**Figure 2.4**), but the memory $CD4^+$ T-cell activation, $CD8^+CD38^+$, $CD8^+HLA\text{-}DR^+$, and $CD8^+CD8^+HLA\text{-}DR^+$ cell frequencies significantly decreased at two years after ART initiation ($P<0.0001$). Although it did not reach statistical significance, memory $CD8^+$ T-cell activation further decreased between two and four years after ART initiation (between 1.2- and 1.6-fold lower).

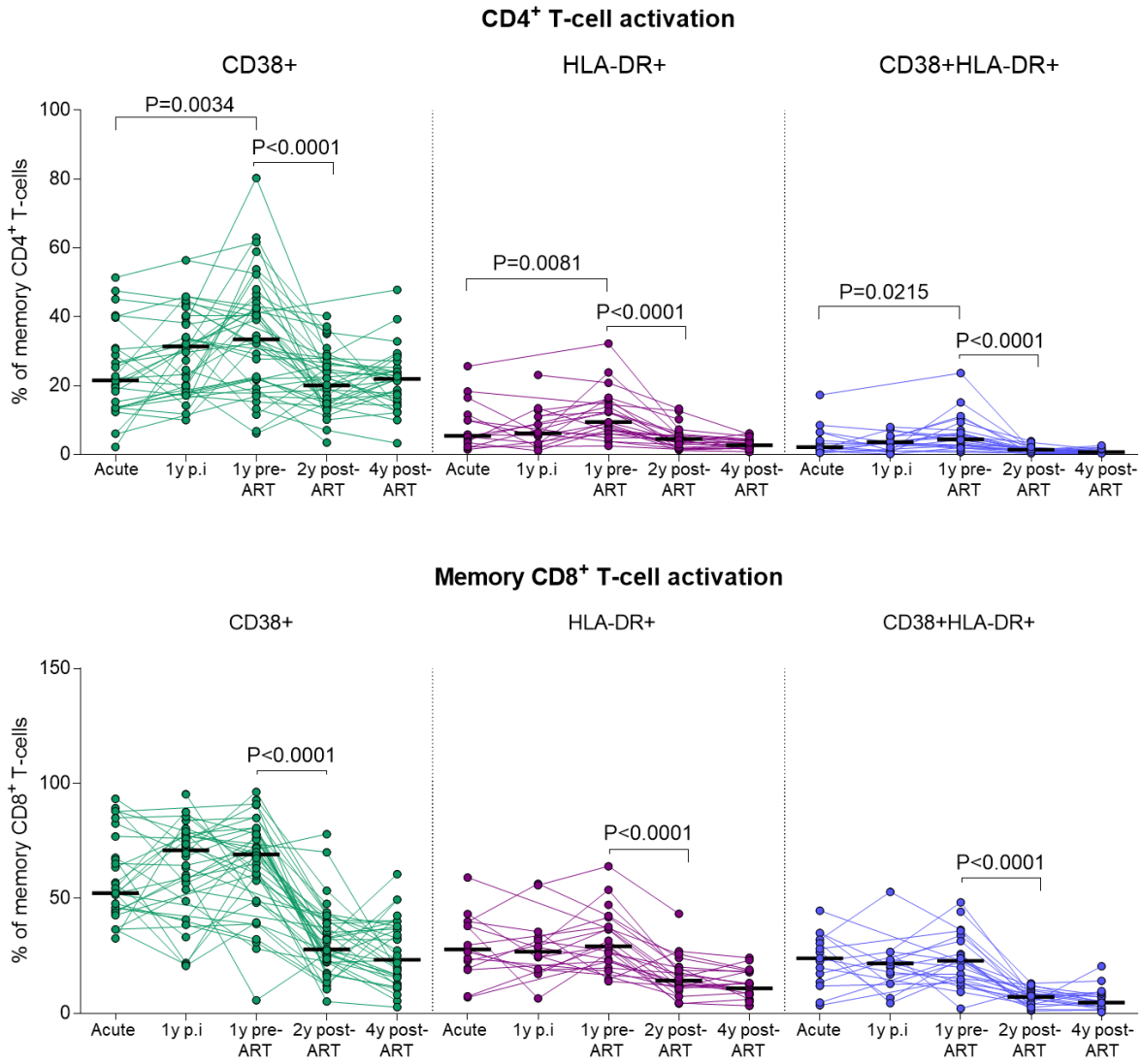

Figure 2.4. Memory T-cell activation before and after ART initiation. Frequencies of $CD38^+$ (green), $HLA\text{-}DR^+$ (purple), and $CD38^+HL\text{-}DR^+$ (blue) $CD4^+$ and $CD8^+$ T-cells during untreated and treated infection. Untreated infection time points were at acute infection, 1 year post-infection (p.i.), and within 1 year pre-ART initiation, while on-treatment time points were at two- and four years post-ART initiation. Wilcoxon Signed Rank tests were used to compare median T-cell activation between acute infection and within 1 year pre-ART, between 1 year pre-ART and two years post-ART initiation, and between two- and four years post-ART initiation. Only significant *P*-values were indicated on the figure. All but the difference in median $CD4^+$ $HLA\text{-}DR^+$ and $CD4^+CD38^+HLA\text{-}DR^+$ frequency between acute infection and 1 year pre-ART remained statistically significant after Bonferroni P-value cut-off correction ($P<0.005$).

These results show that memory $CD4^+$ T-cells become more activated over time during untreated HIV-1 infection and that there is a high degree of memory $CD8^+$ T-cell activation starting early in acute infection and persisting until treatment initiation. T-cell activation in both the memory $CD4^+$ and $CD8^+$ compartments decrease significantly after ART is initiated and reaches a plateau by two years post-ART initiation that persists up until at least four years after the start of treatment. However, even after years of ART, T-cell activation does not normalize to those levels seen in HIV-uninfected counterparts, possibly due to the fact that treatment was initiated in chronic infection [92].

2.3.4 Relationship between $CD4^+$ and $CD8^+$ T-cell activation before and after ART initiation

We next investigated the relationships between $CD4^+$ and $CD8^+$ memory T-cell activation. Positive associations were observed between the frequency of $CD4^+$ and $CD8^+$ memory T-cells expressing CD38, HLA-DR and co-expressing CD38 and HLA-DR at all time-points before and after ART except for the timepoint within one year pre-ART (**Figure 2.5**). After Bonferroni *P*-value cut-off correction, the associations that remained statistically significant ($P<0.003$) were those between memory $CD4^+$ and memory $CD8^+$ T-cells expressing CD38 at acute infection ($P<0.0001$; r=0.73), one-year post-infection ($P<0.0001$; r=0.65), within one year pre-ART ($P<0.0001$; r=0.75), and at two- ($P=0.0002$; r=0.54) years post-ART initiation. Only a trend towards a positive correlation was observed for cells expressing CD38 at four years post-ART initiation ($P=0.0084$; r=0.45) after employing the corrected *P*-value cut-off. Furthermore, at four years post-ART initiation the frequencies of memory $CD4^+$ and memory $CD8^+$ T-cells expressing HLA-DR ($P<0.0001$; r=0.77) and co-expressing CD38 and HLA-DR ($P<0.0001$; r=0.72) also correlated significantly.

Overall, these findings show that memory $CD4^+$ T-cell activation mirrors the activation profile of memory $CD8^+$ T-cells. This suggests that common systemic factors drive peripheral T-cell activation.

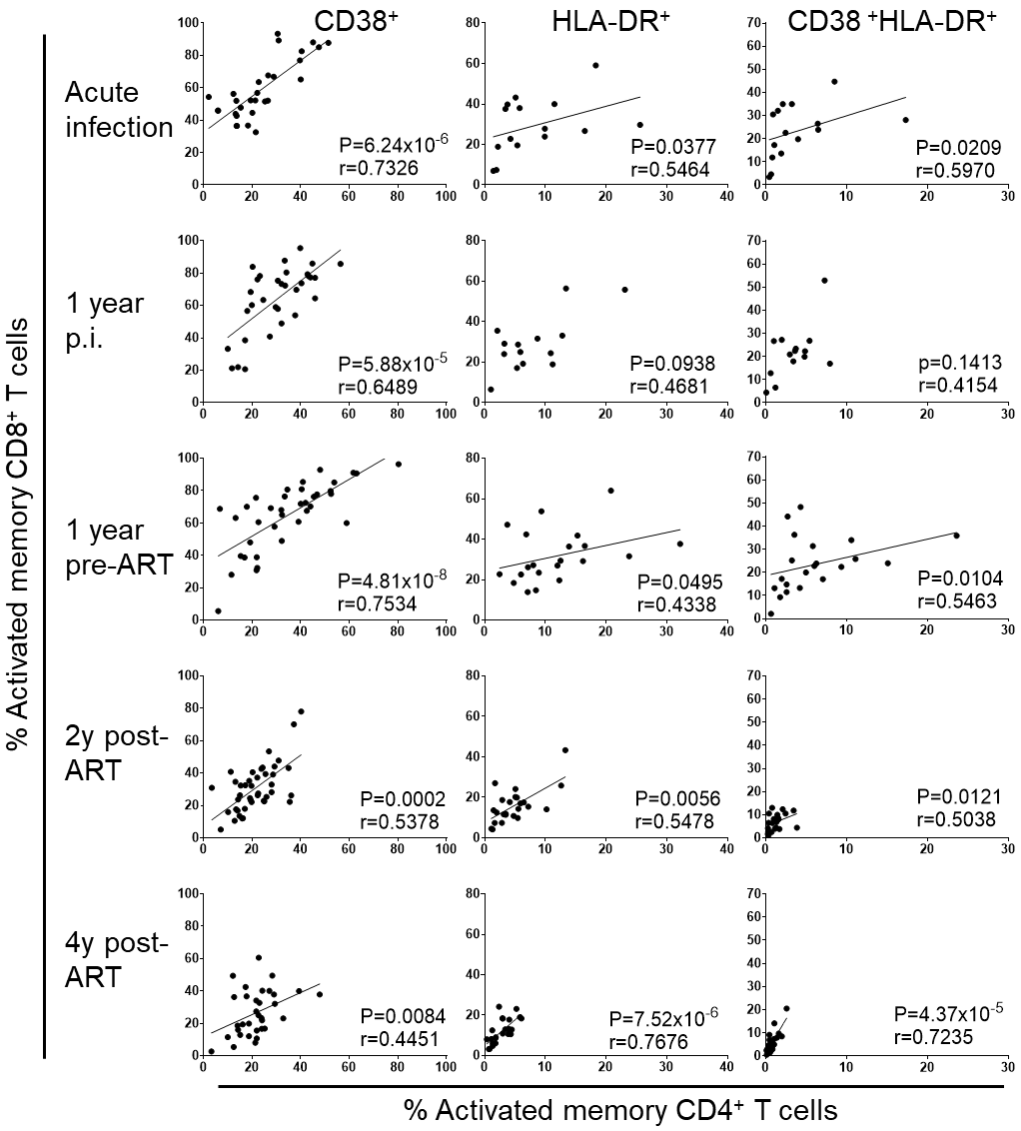

Figure 2.5. Association between $CD4^+$ and $CD8^+$ memory T-cell activation before and after ART initiation. Spearman correlations assessing the relationship between the frequency of $CD38^+$, HLA-DR^+ and $CD38^+$HLA-DR^+ cells in the memory $CD4^+$ T-cell compartment versus the memory $CD8^+$ T-cell compartment. P-values and Spearman r coefficients are displayed in the bottom right corner of each graph. Associations of significance (P<0.05) were also fitted with a best-fit line. The Bonferroni-corrected P-value cut-off was 0.003. After P-value cut-off correction only the $CD38^+$ associations, and all associations at four years post-ART remained statistically significant.

2.3.5 Profiles of soluble markers of inflammation in plasma throughout natural infection and during ART

While ART reduces inflammation [11–13], the cytokine milieu is not restored to pre-infection levels [209]. Persistently elevated cytokines in the face of ART may impact reservoir dynamics. Furthermore, there is a need to characterize the expression of inflammatory mediators over the course of infection in Africans as there is a paucity of descriptive studies in these populations. Here, we measured a range of soluble inflammatory markers (including IL-6, TNF-a, CXCL-10, sCD163 and sCD14), and assessed their changes over time. Concentrations of each marker at each of the sample time points are reported in **Table 2.4.**

Over the course of untreated infection, all five markers remained at similar levels over the three time points measured (**Figure 2.6**). Plasma TNF-α, CXCL-10, and sCD163 concentrations decreased significantly after two years of ART by 4.3-fold (P=0.0003), 2.9-fold (P<0.0001), and 1.9-fold (P<0.0001), respectively. Concentrations of TNF-α and sCD163 were not statistically different after four years of ART compared to two years on ART, while CXCL-10 concentrations decreased a further 1.4-fold (P=0.0075). In contrast, plasma concentrations of sCD14 and IL-6 did not differ significantly pre- and post-ART and did not decrease even after four years of treatment. Overall, these results show only a partial dampening of the systemic inflammatory milieu after long-term ART.

Table 2.4. Concentration of inflammatory mediators before and after ART initiation.

Analyte (pg/mL)	Pre-ART initiation			Post-ART initiation		HIV-uninfected*
	Acute infection (n=42)	1 year p.i. (n=40)	1 year pre-ART (n=39)	2 years (n=46)	4 years (n=39)	
IL-6						
Median	3.6	2.0	2.6	1.3	1.7	0.62
IQR	0.3-10.8	0.3-10.0	0.9-6.6	0.3-8.2	0.3-7.7	0.04-6.5
Detectable %	66.7	67.5	79.5	71.7	69.2	N/A
TNF-α						
Median	3.1	3.5	4.3	1.0	1.0	unavailable
IQR	1.0-8.1	1.0-6.9	2.5-6.7	1.0-2.4	1.0-3.3	-
Detectable %	66.7	70.0	79.5	26.1	28.2	-
CXCL-10						
Median	118.9	95.7	135.5	46.4	34.1	45.2
IQR	66.9-179.9	61.0-140.8	72.8-180.2	27.4-84.0	18.1-62.2	39.0-65.9
Detectable %	100.0	100.0	100.0	100.0	97.4	N/A
sCD163						
Median	$5.1x10^5$	$5.4x10^5$	$5.9x10^5$	$3.1x10^5$	$3.1x10^5$	$4.4\ x10^5$
IQR	$3.3x10^5$-$9.2x10^5$	$3.8x10^5$-$8.1x10^5$	$4.1x10^5$-$7.4x10^5$	$2.2x10^5$-$3.9x10^5$	$2.3x10^5$-$4.0x10^5$	$2.9x10^5$-$7.9x10^5$
Detectable %	100.0	100.0	100.0	100.0	100.0	
sCD14						
Median	$1.9x10^6$	$1.9x10^6$	$1.8x10^6$	$1.9x10^6$	$1.8x10^6$	$1.2x10^6$
IQR	$1.5x10^6$-$2.5x10^6$	$1.6x10^6$-$2.4x10^6$	$1.4x10^6$-$2.3x10^6$	$1.4x10^6$-$2.4x10^6$	$1.5x10^6$-$2.6x10^6$	$1.0x10^6$-$1.4x10^6$
Detectable %	100.0	100.0	100.0	100.0	100.0	

Detectable %: the percentage of samples above the lower limit of detection for the assay.
p.i.: post-infection.
*Plasma concentrations of analytes in HIV-uninfected individuals determined in a large African cohort described by Kroeze *et al.* [210] included as a reference.

The plasma concentrations of all analytes (except for TNF-α for which data was unavailable) during untreated infection were higher than in HIV-uninfected controls (**Table 2.4**). While concentrations of CXCL-10 and sCD163 effectively normalized to the levels reported for HIV-uninfected controls, sCD14 concentrations in treated individuals remained higher than controls. Notably, median plasma IL-6 concentration in HIV-infected individuals during untreated infection fell within the IQR of IL-6 concentrations in HIV-uninfected individuals.

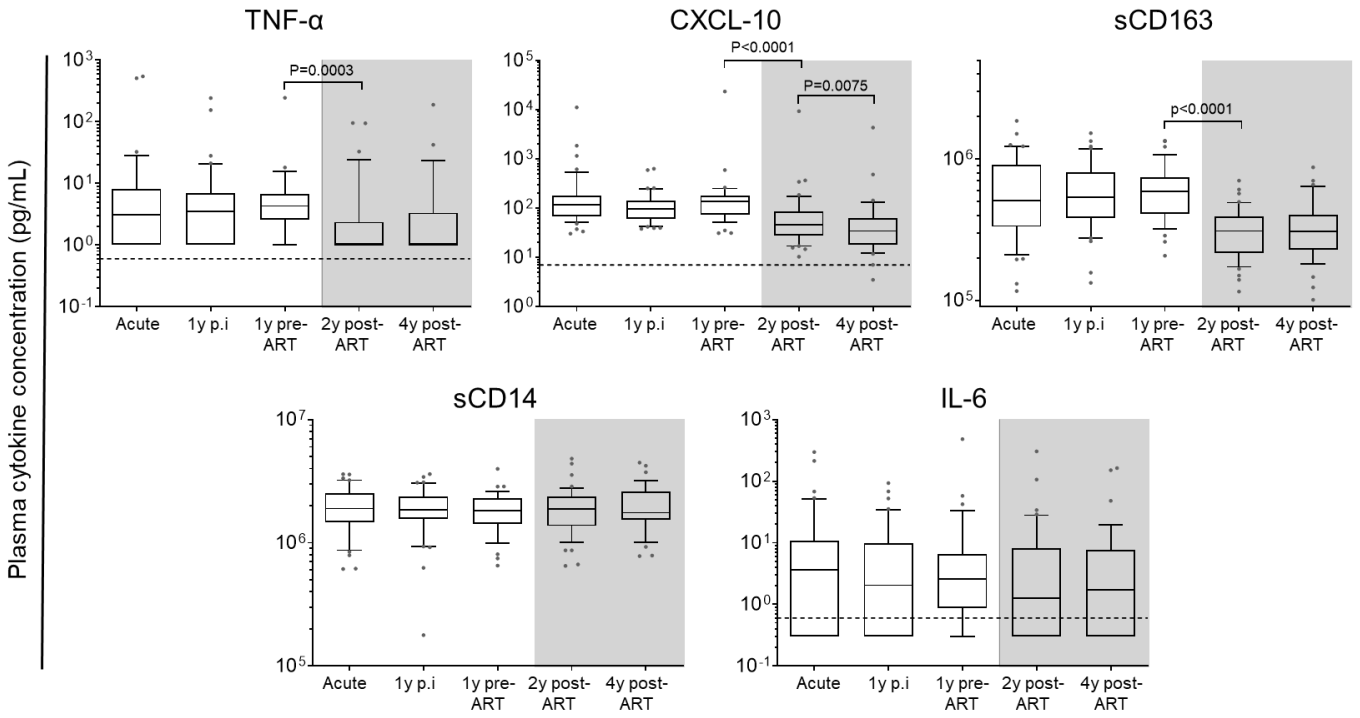

Figure 2.6. Plasma marker concentrations over time in women living with HIV-1 treated during chronic infection. Concentrations of plasma markers of inflammation during untreated and treated infection are shown. Untreated infection time points were at acute infection, 1 year post-infection (p.i.), and within 1 year pre-ART initiation, while on-treatment time points were at two- and four years post-ART initiation (shaded area). Wilcoxon Signed Rank tests were used to compare median marker concentrations between acute infection and within 1 year pre-ART, between 1 year pre-ART and two years post-ART initiation, and between two- and four years post-ART initiation. Only significant *P*-values were indicated on the figure and were considered significant at $P<0.016$ (after Bonferroni cut-off correction).

2.3.6 Relationships between plasma mediators of inflammation

Next, we compared inflammatory marker expression at each timepoint to evaluate the relationships between markers, as their expression may be co-regulated. Statistically significant associations were considered at a $P<0.005$ after Bonferroni cut-off correction. Several markers correlated significantly with one another consistently across most time points, including IL-6 and TNF-α ($P< 0.002$ with only a trend for the one-year pre-ART time point), IL-6 and CXCL-10 ($P<0.0001$ except for the one-year pre-ART time point), TNF-α and CXCL-10 ($P< 0.003$), and sCD14 and sCD163 ($P< 0.0004$ with only a trend at four years post-ART) (**Table 2.5**). Only trends towards correlations were observed between CXCL-10 and sCD163 ($P=0.0478$; r=0.31) or sCD14 ($P=0.0358$; r=0.32) during acute infection, and between IL-6 and sCD14 at two years post-ART initiation ($P=0.0257$; r=0.33). These findings indicate that several inflammatory markers may be influenced by the same stimuli during HIV-1 infection, or that the expression of some markers influences the expression of others. There were no statistically significant correlations between contemporaneous VL or AUC VL over the course of infection and plasma concentrations of any of the analytes measured ($P>0.005$; Bonferroni-corrected *P*-value cut-off).

Table 2.5. Spearman correlation coefficients and *P*-values of plasma markers of inflammation with each other at each timepoint.

			IL-6	TNF-a	CXCL-10	sCD163
Acute infection	**TNF-a**	**P**	0.0019			
		r	0.4651			
	CXCL-10	**P**	0.0001	<0.0001		
		r	0.5571	0.6818		
	sCD163	**P**	0.7142	0.1388	0.0478	
		r	-0.0582	0.2323	0.3072	
	sCD14	**P**	0.5383	0.0539	0.0358	<0.0001
		r	0.0977	0.2996	0.3249	0.6255
1y p.i.	**TNF-a**	**P**	0.0001			
		r	0.5645			
	CXCL-10	**P**	0.0000	<0.0001		
		r	0.6502	0.7027		
	sCD163	**P**	0.3347	0.7495	0.7215	
		r	-0.1565	0.0521	0.0582	
	sCD14	**P**	0.7309	0.5975	0.8776	0.0001
		r	-0.0561	0.0861	0.0251	0.5719
1 year Pre-ART	**TNF-a**	**P**	0.0315			
		r	0.3449			
	CXCL-10	**P**	0.3241	0.0029		
		r	0.1621	0.4637		
	sCD163	**P**	0.6646	0.2012	0.0555	
		r	-0.0717	0.2092	0.3091	
	sCD14	**P**	0.8322	0.0711	0.0869	0.0004
		r	-0.0351	0.2921	0.2777	0.5379
2 years Post-ART	**TNF-a**	**P**	<0.0001			
		r	0.5763			
	CXCL-10	**P**	<0.0001	<0.0001		
		r	0.5699	0.6020		
	sCD163	**P**	0.7143	0.4492	0.9180	
		r	0.0555	-0.1144	0.0156	
	sCD14	**P**	0.0257	0.5637	0.0582	0.0002
		r	0.3288	0.0874	0.2814	0.5223
4 years Post-ART	**TNF-a**	**P**	0.0001			
		r	0.5728			
	CXCL-10	**P**	<0.0001	<0.0001		
		r	0.6830	0.6214		
	sCD163	**P**	0.3295	0.1958	0.7522	
		r	-0.1603	-0.2117	0.0522	
	sCD14	**P**	0.6456	0.5372	0.4246	0.0122
		r	-0.0760	-0.1019	0.1316	0.3978

Yellow shading indicates significance at $P<0.05$ (uncorrected P-value cut-off) and red indicates significance at $P<0.005$ (Bonferroni-corrected P-value cut-off)

2.3.7 Relationships between T-cell activation and inflammation before and after ART initiation

Finally, we explored the relationships between soluble markers of inflammation in plasma and T-cell activation measured previously (**Table 2.6**). We identified weak positive relationships between the frequency of activated memory $CD4^+$ T-cells expressing CD38 and CXCL-10 (*P*=0.0288; r=0.37) at one year pre-ART, and both IL-6 (*P*=0.0388; r=0.32) and TNF-α (*P*=0.0458; r=0.31) at two years post-ART initiation. In addition, weak positive relationships between the frequency of activated memory $CD8^+$ T-cells expressing CD38 and sCD163 at both acute infection (*P*=0.0321; r=0.41) and one-year post-infection (*P*=0.0189; r=0.43), and also TNF-α within one year pre-ART initiation (*P*=0.0380; r=0.33). These relationships may be stochastic or spurious as relationships between the same cytokine-T-cell activation pairs were not maintained across time points, relationships between cytokines and different activated subsets were not consistent, and none of these correlations remained statistically significant after Bonferroni *P*-value cut-off correction.

Table 2.6. Association between inflammation and T-cell activation before and after ART initiation.

			%CD4+CD38+	%CD4+HLA-DR+	%CD4+CD38+HLA-DR+	%CD8+CD38+	%CD8+HLA-DR+	%CD8+CD38+HLA-DR+
Acute infection		**n**	**27**	**14**	**14**	**27**	**14**	**14**
	IL-6	**P**	0.8572	0.5818	0.5299	0.7348	0.7603	0.9787
		r	0.0363	0.1611	0.1832	-0.0684	-0.0905	-0.0088
	TNF-a	**P**	0.6476	0.7556	0.7092	0.7141	0.7092	0.7075
		r	0.0922	0.0911	0.1089	0.0739	0.1089	0.1090
	CXCL-10	**P**	0.2468	0.7156	0.5733	0.5239	0.9035	0.6510
		r	0.2308	0.1077	0.1648	0.1282	0.0374	0.1320
	sCD163	**P**	0.6939	0.9155	0.7616	0.0321	0.2149	0.3039
		r	0.0794	-0.0330	-0.0901	0.4133	0.3538	0.2948
	sCD14	**P**	0.5542	0.7966	0.8676	0.0611	0.0779	0.1223
		r	0.1190	0.0769	0.0505	0.3651	0.4901	0.4334
1y p.i.		**n**	**30**	**13**	**13**	**30**	**13**	**13**
	IL-6	**P**	0.8527	0.7573	0.5691	0.8657	0.2536	0.0775
		r	0.0354	-0.0947	-0.1727	0.0322	-0.3399	-0.5098
	TNF-a	**P**	0.7067	0.5691	0.9743	0.8170	0.8069	0.3055
		r	-0.0717	0.1727	0.0111	-0.0441	-0.0752	-0.3064
	CXCL-10	**P**	0.3936	0.3734	0.5786	0.8877	0.6960	0.4043
		r	0.1616	0.2692	0.1703	0.0269	-0.1209	-0.2527
	sCD163	**P**	0.7918	0.1695	0.0673	0.0189	0.2886	0.1695
		r	0.0503	0.4066	0.5275	0.4261	0.3187	0.4066
	sCD14	**P**	0.1601	0.7647	0.7370	0.7552	0.3939	0.6167
		r	-0.2631	0.0934	0.1044	-0.0594	0.2582	0.1538
1y Pre-ART		**n**	**35**	**20**	**20**	**35**	**20**	**20**
	IL-6	**P**	0.4088	0.4022	0.3199	0.1889	0.4682	0.3250
		r	-0.1441	-0.1982	-0.2344	-0.2274	-0.1721	-0.2320
	TNF-a	**P**	0.6746	0.8369	0.8469	0.9981	0.7475	0.8019
		r	0.0735	0.0492	0.0461	0.0004	-0.0768	-0.0599
	CXCL-10	**P**	0.0288	0.1952	0.1271	0.1227	0.9749	0.4125

		r	0.3697	0.3023	0.3528	0.2658	0.0075	0.1940
	sCD163	**P**	0.8517	0.8011	0.4877	0.4927	0.9699	0.7865
		r	-0.0328	-0.0602	-0.1647	0.1199	0.0090	-0.0647
	sCD14	**P**	0.6485	0.1180	0.2131	0.2869	0.1053	0.5522
		r	0.0798	-0.3609	-0.2911	0.1852	-0.3729	-0.1414
2y Post-ART		**n**	**41**	**24**	**24**	**41**	**24**	**24**
	IL-6	**P**	0.0388	0.2916	0.1197	0.0689	0.7748	0.0923
		r	0.3239	0.2245	0.3263	0.2870	0.0616	0.3513
	TNF-a	**P**	0.0458	0.5106	0.2169	0.0380	0.1813	0.9303
		r	0.3137	0.1412	0.2616	0.3253	-0.2823	0.0189
	CXCL-10	**P**	0.0674	0.8591	0.3725	0.0246	0.8370	0.1147
		r	0.2884	0.0383	0.1905	0.3505	0.0443	0.3305
	sCD163	**P**	0.3899	0.3552	0.2488	0.0953	0.9069	0.3671
		r	-0.1379	-0.1974	-0.2449	-0.2640	-0.0252	-0.1927
	sCD14	**P**	0.8797	0.1398	0.0839	0.8558	0.4117	0.4970
		r	0.0244	-0.3104	-0.3602	-0.0293	-0.1757	-0.1457
4y Post-ART		**n**	**32**	**23**	**23**	**32**	**23**	**23**
	IL-6	**P**	0.1548	0.0174	0.1504	0.1708	0.3054	0.9909
		r	-0.2575	0.4908	0.3097	-0.2482	0.2235	-0.0025
	TNF-a	**P**	0.8953	0.0341	0.0878	0.3331	0.0808	0.2514
		r	0.0242	0.4434	0.3639	-0.1768	0.3717	0.2493
	CXCL-10	**P**	0.7741	0.0304	0.2301	0.3012	0.1996	0.8914
		r	-0.0528	0.4518	0.2604	-0.1886	0.2777	-0.0301
	sCD163	**P**	0.9104	0.6390	0.2841	0.7347	0.8755	0.3659
		r	-0.0207	-0.1033	-0.2333	-0.0623	0.0346	-0.1977
	sCD14	**P**	0.8844	0.4923	0.4260	0.3197	0.2759	0.4180
		r	-0.0268	0.1508	0.1745	0.1817	0.2372	0.1774

Yellow shading indicates significance at $P<0.05$ (uncorrected P-value cut-off) and red indicates significance at $P<0.00028$ (Bonferroni-corrected P-value cut-off).

2.4 Discussion

Chronic HIV-1 infection results in hyperactivation of the immune system, resulting in $CD4^+$ T-cell depletion, T-cell dysregulation, and disease progression. Persistent immune dysregulation is likely a major contributor to increased mortality in treated individuals compared to HIV-uninfected counterparts [211]. ART reduces T-cell activation and restores some T-cell functions [212–214] but not all immune functions are restored to a 'pre-infection' state. Although studies indicate that the majority of reconstitution occurs within the first year of treatment [210, 213, 215], there is evidence that some measures of immune activation and inflammation continue to decrease after the first year on ART [216]. PLWH who initiate ART earlier in infection are more likely to have more favourable treatment outcomes such as higher $CD4^+$ T-cell counts, a CD4:CD8 ratio closer to two, a reduced occurrence of transient detectable viraemia (or VL "blips") and a lower risk of treatment failure. Thus, the timing of ART plays a key role in preserving immunity and delaying disease progression, and these factors may, in turn, affect the latent reservoir of HIV-1. This study characterized T-cell activation and inflammation in PLWH to gain an understanding of how these measures change over the course of both treated and untreated HIV-1 infection in chronically treated individuals and the impact on the HIV reservoir, described in the next Chapters.

We explored the changes in T-cell activation over time and observed a significant increase in memory $CD4^+$ T-cell activation over the course of untreated infection. Activation of memory $CD4^+$ and $CD8^+$ T-cells decreased significantly after two years on ART but did not differ significantly between two- and four years on ART. Other studies have shown previously that HIV-induced immune activation is not fully normalized to pre-infection levels, including studies of treatment during chronic infection [213, 217]. The degree of normalization is related

to the timing of ART, an important determinant in reservoir formation. Thus, we hypothesize that higher levels of $CD4^+$ T-cell activation are associated with a larger HIV-1 reservoir (i.e., a greater frequency of latently infected cells during ART). This hypothesis will be explored further in Chapters 3 and 4.

HIV-1 also induces sustained systemic inflammation characterized by elevated levels of circulating pro-inflammatory cytokines, chemokines, and other mediators of inflammation such as soluble receptors. These alterations in the inflammatory milieu may in turn activate cells that become available as targets for infection. The concentration of certain plasma cytokines during early infection were predictive of set-point VL, $CD4^+$ T-cell loss, and disease progression [98]. Numerous studies have reported that the levels of pro-inflammatory cytokines observed in HIV-infected, ART-naïve individuals are significantly higher compared to HIV-uninfected controls [210, 214, 215]. We found that plasma concentrations of several markers of inflammation during untreated HIV-1 infection were comparable to those reported in other studies [210, 215]. While ART decreases the levels of inflammatory markers in plasma, these are not reconstituted to pre-ART levels despite suppression of VLs, particularly if ART is initiated at low $CD4^+$ T-cell counts (<200 cells/μL) [210, 215]. In this study, we found that while TNF-α, CXCL-10 and sCD163 concentrations decreased significantly after ART initiation, those of sCD14, and IL-6 remained statistically similar to those before ART initiation, even after four years on ART. While some studies have reported a reduction in the levels of IL-6 after ART initiation [210, 218], a statistically significant decrease was not observed in this cohort, similar to one other study [215]. Notwithstanding the challenge of comparing cytokine concentrations between studies, it appears that these apparent discrepancies could be explained by lower overall IL-6 levels in our cohort, i.e., pre-ART plasma IL-6 concentrations to those in HIV-uninfected individuals which we have shown.

Indeed, in the same study that reported similar levels of IL-6 in HIV-infected individuals on ART compared to HIV-uninfected controls, plasma IL-6 concentrations were lower in in HIV-1 infected South Africans compared to their Kenyan, Ugandan, and Nigerian counterparts [210]. Residual CXCL-10 expression within the first year of ART has also been described [210, 216, 218]. Here, we report that CXCL-10 levels continue to decrease during ART, even up to four years on treatment, consistent with a large study performed in European participants [216]. In addition, we have sustained high concentrations of sCD14 after ART. Consistent with this, numerous studies have reported persistently elevated sCD14 despite suppressive ART [210, 219, 220]. sCD14 is shed from activated monocytes [221], suggesting that sustained epithelial damage to the gut and incomplete deactivation of monocytes persists after ART. Persistent inflammation has been linked to an increased risk of cardiovascular disease in treated PLWH, and strategies to reduce inflammation are being pursued [222].

This study had several limitations. We did not assess the impact of co-infections on these measures of immune activation and inflammation. *Mycobacterium tuberculosis* co-infection has been associated with persistently elevated sCD14 levels [210, 219]. Thus, in countries with high prevalence of co-infections (such as tuberculosis, helminths, etc.), further studies are warranted to decipher the potential synergistic impact of other co-infections on HIV-associated chronic immune activation. Another factor our study did not account for is the effect of sex differences on clinical markers of disease [223], and persistent immune activation and inflammation [51, 52, 220]. Earlier studies have found discordant results, some showing that immune activation [52, 224] and inflammation [220] are higher in women compared to men, and others finding no difference [210]. There are also a number of factors that would result in immune differences between our cohort and others, including population genetics, environmental stimuli, smoking status and lifestyle differences, to name a few. Nevertheless,

this study provides an important longitudinal perspective of immune activation and inflammation in South African women before and after prolonged ART. Finally, our analysis did not include HIV-1 specific cells but rather the total memory $CD4^+$ or $CD8^+$ T-cell subset, an important consideration for future studies as this may alter the findings.

In conclusion, this study describes the immune environment in women who initiated ART at different times during the course of HIV-1 infection, predominantly chronic infection. Although viremia was successfully suppressed on ART, there was evidence of persistent innate cell activation. This Chapter provides the context on which the remaining thesis is based, namely determining the effect of T-cell activation and inflammation on shaping the long-lived HIV-1 reservoir.

CHAPTER 3

Immunological correlates of the HIV-1 replication-competent reservoir size

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3.1 Abstract

The main challenge for curing HIV is the formation of a stable latent viral reservoir in infected individuals. Determinants that shape this reservoir are understudied in women in general and African populations in particular. It is well established that the majority of this reservoir is composed of defective viral genomes, with only a small proportion capable of replication and rebound following treatment interruption. Understanding correlates of the replication-competent reservoir is critical for developing strategies for curing HIV-1. The size of the replication-competent HIV-1 reservoir, measured by a quantitative viral outgrowth assay (QVOA), was previously determined for twenty South African women; all of whom initiated antiretroviral therapy (ART) during chronic infection. We investigated clinical and immunological correlates of reservoir size in this cohort. Predictive modelling showed that the size of the replication-competent reservoir was directly related to viral load and $CD4^+$ T-cell counts over the course of infection, although these measures did not fully predict reservoir size. As shown previously, viral load and absolute $CD4^+$ count associated with reservoir size. In addition, we found that $CD8^+$ T-cell activation within the year preceding ART, nadir $CD4^+$ T-cell count, and baseline as well as on-treatment CD4:CD8 ratio at the time of QVOA also associated negatively with reservoir size. The frequency of activated $CD8^+$ T-cells in the year before treatment, together with viral loads and absolute $CD4^+$ counts, were positively associated with the size of the replication-competent reservoir of HIV-1. Our results are indicative that the host immune milieu near the time of ART initiation plays an important role in shaping the HIV-1 reservoir that persists on ART.

3.2. Introduction

The greatest challenge to eradicating HIV-1 is the long-lived latent viral reservoir that is seeded early in infection, decays slowly after treatment initiation (half-life of 44 months [62, 63, 86]) and results in viremia if ART is interrupted. There is an urgent need to understand factors contributing to the formation and maintenance of the reservoir to inform strategies for an HIV-1 cure. Furthermore, if a global cure is to be achieved, we need to expand the number of studies on the durable HIV reservoir are needed in different populations, particularly in low- and middle-income countries which bear the greatest burden of HIV infection. One purpose of this study was to diversify the body of knowledge on the HIV-1 reservoir in different demographics. Most North American and European HIV-1 cure studies are majority male cohorts. There is a paucity of HIV-1 reservoir studies in African cohorts, particularly in African women who bear the greatest burden of disease globally.

A number of clinical and immunological factors influencing the HIV-1 latent reservoir have been described previously. These include early initiation of ART, which results in a smaller HIV-1 reservoir [86], reducing the cumulative viral burden, preserving $CD4^+$ T-cells, and maintaining CD4:CD8 ratios, while reducing T-cell activation [11, 12]. Nadir absolute $CD4^+$ count and CD4:CD8 ratio have been found to correlate inversely with the size of the HIV-1 latent reservoir, as measured by proviral DNA copy number [87][88]. Furthermore, pre-ART plasma viral loads (VL) correlate positively with HIV-1 DNA levels in $CD4^+$ T-cells on ART [88]. A range of assays have been developed to quantify the latent HIV-1 reservoir, the reference standard for measuring reservoir size being the quantitative viral outgrowth assay (QVOA) [61]. HIV-1 DNA and cell-associated RNA measures are more easily quantifiable, requiring lower blood volumes, but overestimate the true replication-competent reservoir size

since most infected cells harbour defective proviruses [55]. In contrast, QVOA underestimates the true size of the reservoir because not all intact proviruses grow out after a single round of cell stimulation [58]. Some measures of HIV-1 DNA, such as intact proviral DNA, correlate with the number of infectious units per million $CD4^+$ T-cells (IUPM) and are therefore an excellent proxy for replication-competent reservoir size [225].

As part of a collaborative NIH-funded study to understand the dynamics of the establishment of the HIV-1 replication-competent reservoir in African women, investigators at the University of North Carolina at Chapel Hill performed a QVOA on PBMC from 20 women from the CAPRISA 002 acute infection cohort, and these data were made available for this study. Taking advantage of clinical data and samples spanning approximately 5 years before and after ART initiation, this study investigated the association between clinical and immunological factors and replication-competent HIV-1 reservoir size in these virally suppressed South African women on long-term ART.

3.3 Materials and methods

3.2.1 Study participants and study ethics

Twenty women from the CAPRISA 002 acute infection cohort [48, 114, 203] in Kwa-Zulu Natal, South Africa were recruited into this study. The study cohort and ethics are described in **Chapter 2, section 2.2.1**.

3.2.2 Thawing PBMC, staining and flow cytometry

Thawing, resting, staining of PBMC, and flow cytometry were performed as described in **Chapter 2, sections 2.2.2** and **2.2.3**. The gating strategy employed has been described in **Chapter 2, section 2.2.4**.

3.2.3 Measuring markers of inflammation

The concentrations of five mediators of inflammation, IL-6, TNF-α, CXCL-10 (IP-10), sCD14 and sCD163, were measured in plasma samples, as described in **Chapter 2, section 2.2.5**.

3.2.4 Quantitative viral outgrowth assays

QVOAs and bias-corrected maximum likelihood estimation were carried out by the Swanstrom and Archin laboratories, respectively (Department of Medicine, University of North Carolina at Chapel Hill). Resting CD4+ T cells (HLA-DR$^-$CD69$^-$CD25^{lo}) were isolated from PBMC obtained ~5 years after ART initiation by negative selection with a CD25 depletion kit according to the manufacturer's instructions (custom kit, STEMCELL Technologies, BC, Canada). The purity of the resulting cell fraction was assessed by flow cytometry (BD LSRII Fortessa) with the following markers: CD69-PE, CD25-APC, CD8-FITC, CD4-PerCP-Cy5.5 (All from BD Biosciences), and Aqua LIVE/DEAD viability stain (Thermo Fisher Scientific, MA, USA). QVOAs were performed as described [10]. Briefly, purified resting cells were seeded at 100,000 cells per well and incubated with highly purified phytohemagglutinin (PHA, 1.5 μg/mL) (Remel, Thermo Fisher Scientific, MA, USA), IL-2 (60 U/mL), and irradiated

PBMC from a seronegative donor. Irradiated PBMC were used as feeder cells; that is, they produce necessary cytokines to support T cell expansion but do not proliferate hence will not contaminate the culture after a few days. HIV-1 p24 capsid protein ELISAs were used to test for production of HIV on days 15 and 19. Culture supernatants positive for p24 were stored at −80°C. Bias-corrected maximum likelihood estimates for infectious units per million resting $CD4^+$ T-cells (IUPM) were calculated in R using the SLDAssay package [226] based on the frequency of HIV-1 p24 capsid-positive wells on day 15 of the assay.

3.2.7 Modelling the replication-competent reservoir size

Modelling was performed by the Perelson group (Theoretical Biology and Biophysics Group) at the Los Alamos National Laboratory, Los Alamos, New Mexico, USA. The model given by the equation three from Archin *et al.* [86] was employed:

$$L(t_F) = e^{-\mu t_F} L_0 + e^{-\mu t_F} f\beta \int_0^{t_H} V(\tau)T(\tau)e^{\mu\tau} d\tau + e^{-\mu t_F} f(1-\varepsilon)\beta \int_{t_H}^{t_F} V(\tau)T(\tau)e^{\mu\tau} d\tau,$$

It was assumed that the latent reservoir half-life is 25 months before ART and 44 months during ART, to predict reservoir size in this cohort. To calculate the area under the VL times $CD4^+$ T cell count curve, a piecewise cubic Hermite interpolant was fit to both the VL and the $CD4^+$ T-cell count data using the MATLAB function "pchip". The trapezoidal rule and the product of these two cubic interpolants were used to calculate the IUPM. The predicted IUPM is given in the arbitrary units 10^{13} f β. The initial VL was assumed to be 1×10^{-3} copies/mL and used 20 copies/mL for VL measurements below the limit of quantification. These values do not impact our results as most of the AUC occurs between acute infection and ART.

3.2.5 Statistical analysis

Spearman rank correlation tests and area under the curve calculations for the VL (AUC VL) used in correlations and linear regression models were performed in Prism version 8 (GraphPad). Uni- and multivariable linear regression models were used to determine the clinical and immunological factors associated with replication-competent reservoir size (performed by Farzana Osman at CAPRISA). All multivariable models adjusted for AUC VL and/or nadir $CD4^+$ T-cell count and/or CD4:CD8 ratio at ART initiation. A *P*-value < 0.05 was considered statistically significant for reporting and a Benjamini-Hochberg *P*-value cut-off correction with a false discovery rate of 25% was used to adjust for multiple comparisons. Regression analyses were performed in SAS version 9.4.

3.4 Results

3.4.1 Cohort characteristics

This study utilized samples from twenty participants living with HIV-1 from the CAPRISA 002 Acute Infection cohort (median age: 33 years [IQR, 30-39] who had been infected for a median of 4.4 years (IQR, 3.3-5.2) prior to treatment initiation (**Table 3.1**). Of the 20 women, 5/20 (25%), 9/20 (45%), and 6/20 (30%) participants initiated ART at $CD4^+$ T-cell count <250, <350, and <500 cells/uL, respectively. Median absolute $CD4^+$ T-cell counts at nadir and ART initiation were 243 cells/μL (IQR: 182-280) and 306 cells/μL (IQR: 235-396), respectively. The median plasma VL at ART initiation was 4.7 $\log_{10}$ copies/mL (IQR: 4.2-4.9). The median cumulative (AUC) VL was 6.2 $\log_{10}$ (months x copies/mL) (IQR:5.8-6.6).

ART was initiated at according to the prevailing South African National Guidelines and thus first- and second-line therapies in the cohort reflected changes in Treatment Guidelines over time. Seven participants switched ART regimens during the study period.

QVOA was performed a median of 5.1 years post-ART initiation (IQR: 4.7-5.5) at which time all patient VLs were undetectable. At the time of QVOA, the median $CD4^+$ T-cell counts were 655 cells/μL (IQR: 545-881). Eighteen (90%) women achieved $CD4^+$ T-cell count >500 cells/μL after five years on ART, while the remaining two (10%) had $CD4^+$ T-cell count >400 cells/μL. CD4:CD8 ratios had also normalized to a median of 1.0 (IQR: 0.8-1.3). Thus, all participants demonstrated immune reconstitution during the five years of ART, with no virological failures.

Table 3.1. Cohort characteristics

		Time (years)			$CD4^+$ T-cell count (cells/μL)		CD4:CD8		Log_{10} VL (copies/mL)	Log_{10} AUC VL (months x copies/mL)
PID	**Age at QVOA**	**ART naïve**	**On ART[a]**	**Virally suppressed**	**Nadir**	**QVOA**	**ART initiation**	**QVOA[b]**	**ART initiation**	**Infection to ART initiation**
100277	46	4.9	5.1	4.8	281	926	0.2	1.2	3.9	5.3
100222	31	6.1	4.6	5.5	305	717	0.6	1.7	3.7	5.5
100316	33	4.1	4.1	3.7	278	888	0.3	1.1	3.7	5.6
100333	32	3.7	5	4.8	218	524	0.2	0.8	4.8	5.6
100268	33	4.2	7.3	7.2	163	502	0.2	0.8	3.6	5.7
100287	30	5	4.3	4	216	599	0.3	0.8	4.2	6.1
100288	34	4	5.3	5	288	860	0.4	1.2	4.3	6.1
100380	41	2.5	5.1	4.6	377	774	0.6	1.3	4.7	6.1
100257	39	4.8	6.1	5.6	170	596	0.2		4.7	6.2
100337	31	3.2	5.3	4.8	267	955	0.5	0.8	4.8	6.2
100372	30	3.5	4.7	4.3	309	897	0.5	1.4	5.2	6.2
100244	35	7.3	5.1	4.6	241	652	0.3	1	4.4	6.3
100336	27	2.7	5	4.7	74	558	0.1	1.2	5.4	6.4
100280	37	5.7	4.8	3.8	174	736	0.3	0.9	4.8	6.5
100286	29	2.8	6	3.5	142	474	0.1	0.4	4.7	6.5
100217	32	6.9	4.6	4.3	256	657	0.3	0.7	4.9	6.7
100302	34	3.1	5.2	4.7	204	541	0.4		4.7	6.7
100256	30	4.5	6.1	5.9	245	567	0.3	1.4	4.7	6.9
100188	43	4.7	4.7	4.2	267	430	0.1	0.6	5.4	7
100206	47	5.3	5.5	5.2	240	978	0.2		5.3	7.6
median	**33**	**4.4**	**5.1**	**4.7**	**243**	**655**	**0.3**	**1.0**	**4.7**	**6.2**
IQR	**30.3-38.5**	**3.3; 5.2**	**4.7; 5.5**	**4.2; 5.2**	**182; 280**	**545; 881**	**0.2; 0.4**	**0.8; 1.3**	**4.2; 4.9**	**5.8; 6.6**

[a]ART was offered according to the South African National HIV treatment guidelines (at a CD4 count of <200 cells/mm^3 prior to October 2012; <350 cells/mm^3 from October 2012 to March 2015). The majority of participants (17/20) were taking standard first-line therapy, namely TDF/3TC/EFV (n=10); TDF/FTC/EFV (n=4); TDF/3TC/NVP (n=2) or DTG/3TC/TFV (n=1). Three individuals were on a second-line regimen, consisting of AZT/3TC/Lpvr/r; AZT/EFV/3TC or EFV/3TC/Lpvr/r. Seven participants switched drug regimens during the study period, namely 100188, 100222, and 100333 (TDF/3TC/EFV to TDF/FTC/EFV at months 24, 22, and unknown, respectively), 100206 (TDF/3TC/EFV to TDF/FTC/EFV at month 36 with drug holidays between months 26 to 29, and 30 to 36, respectively, and no recorded VL rebound at ART re-initiation visits), 100257 (TDF/3TC/NVP to TDF/3TC/EFV at month 12 to TDF/FTC/EFV at ~month 48), 100302 (AZT/3TC/EFV to TDF/FTC/EFV at month 24), 100302 (AZT/EFV/3TC to TDF/FTC/EFV at month 34), and 100316 (AZT/3TC/ Lpvr/r to TDF/FTC/EFV at month 13).

[b]CD8$^+$ T-cell counts not available at the QVOA timepoint for 100206, 100257, and 100302.

3.4.2 The frequency of latently infected cells in South African women on ART

The frequency of resting $CD4^+$ T-cells harbouring replication-competent HIV-1 was determined by QVOA after a median of 5.1 years (IQR, 4.7-5.5) of ART, and 4.7 years (IQR, 4.2-5.2) of viral suppression (**Table 3.1**). The cohort had a median of 0.75 IUPM resting $CD4^+$ T-cells (IQR: 0.43-1.85) while the minimum and maximum were 0.07 (too low to be reliably determined) and 4.40 IUPM resting $CD4^+$ T-cells, respectively (**Figure 3.1**).

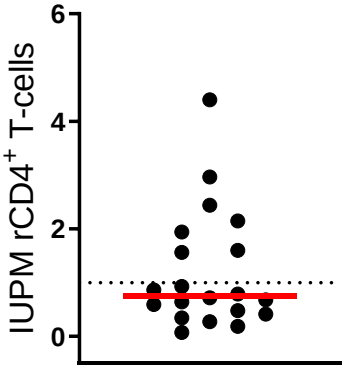

Figure 3.1. The frequency of latently infected cells in South African women on ART. Infectious units per million (IUPM) resting $CD4^+$ ($rCD4^+$) T-cells as determine by quantitative viral outgrowth assay in n=20 women of the CAPRISA 002 cohort after 5 years of ART. The median is indicated by a red bar and the estimated frequency according to the literature is indicated with a dotted line at 1 IUPM $rCD4^+$ T-cells. QVOA data and bias-corrected maximum likelihood estimation were provided by the Swanstrom and Archin laboratories, Department of Medicine, University of North Carolina at Chapel Hill.

3.4.3 Clinical correlates of the replication-competent reservoir size

Several studies have associated VL [8, 9] and absolute $CD4^+$ count dynamics with the frequency of latently infected cells as measured by QVOA [86]. We investigated the

association of latent cell infection with demographic and clinical characteristics in our cohort (**Table 3.2**). Reservoir size, represented as IUPM, correlated positively with overall viral burden (AUC VL) from infection to ART initiation, and also with viral burden within the 12 months prior to ART initiation (*P*=0.012, r=0.549; and *P*=0.026, r=0.498; respectively; **Figure 3.2 A-C**). Furthermore, reservoir size correlated inversely with nadir $CD4^+$ T-cell count (*P*=0.005, r=-0.597; **Figure 3.2 D**), CD4:CD8 ratio at the time of ART initiation (*P*=0.004, r=-0.610; **Figure 3.2 E**) and CD4:CD8 ratio at the time post-ART when the blood sample was collected for QVOA (*P*=0.007, r=-0.643; **Figure 3.2 D-F**). The significant inverse relationship between nadir $CD4^+$ T-cell count and IUPM remained significant in a multivariate linear regression model adjusting for AUC VL (**Table 3.3,** discussed further in **section 3.4.4**).

Table 3.2. Demographic and clinical factors associated with replication-competent HIV reservoir size [as Log_{10}(IUPM resting $CD4^+$ T-cells)].

	Spearman correlation		**Univariate linear regression**	
n=20	**Spearman's r**	***P*-value**	**Coefficient**	***P*-value**
Age at QVOA (years)[a]	-0.1916	0.4185	-	-
Duration (months)[b]				
• Infection to ART initiation	-0.0361	0.8799	-	-
• from ART initiation to QVOA	0.0286	0.9046	-	-
• virally suppressed on ART before QVOA	-0.1734	0.4647	-	-
Plasma VL (log_{10} copies/mL)				
• at ART initiation[c]	0.3098	0.1838	-	-
• Cumulative (AUC VL)[d]				
Infection to ART initiation	0.5489	***0.0122***	0.3931	***0.0220***
1 year pre-ART	0.4977	***0.0255***	0.3047	0.0528
$CD4^+$ T-cell count (cells/μL)[e]				
• Nadir	-0.5972	***0.0054***	-0.0040	***0.0037***
• ART initiation	-0.4271	0.0604	-	-
• at time of QVOA	-0.2947	0.2071	-	-
CD4:CD8 ratio[f]				
• ART initiation	-0.6102	***0.0043***	-1.7576	***0.0017***
• at time of QVOA	-0.6426	***0.0065***	-0.7612	***0.0142***

[a]Age of cohort: median 33 years, interquartile range (IQR) 30.3-38.5.

[b]Duration of HIV infection to ART initiation: median 52 months, IQR 38.8-61.3; from ART initiation to the time of sizing by QVOA: median 61 months, IQR 56.3-65.8; from viral suppression to QVOA: median 56, IQR 50.3-61.5.
[c]Median VL at ART initiation: 4.7 log_{10} copies/ml, IQR 4.2-4.9.
[d]Area under the curve VL expressed as log_{10} time-copies/mL; from infection to ART initiation (excluding peak VL during acute infection): 6.2, IQR 5.8-6.6; over the year prior to ART initiation: median 5.5, IQR 5.0-6.0.
[e]Absolute CD4+ T-cell count at nadir: median 243 cells/μL, IQR 182-280; at ART initiation: median 306, IQR 235-396; at time of sizing by QVOA: median 655, IQR 545-881.
[f]CD4:CD8 ratio at ART initiation: median 0.3, IQR 0.2-0.4; at QVOA: median 1.0, IQR 0.8-1.3

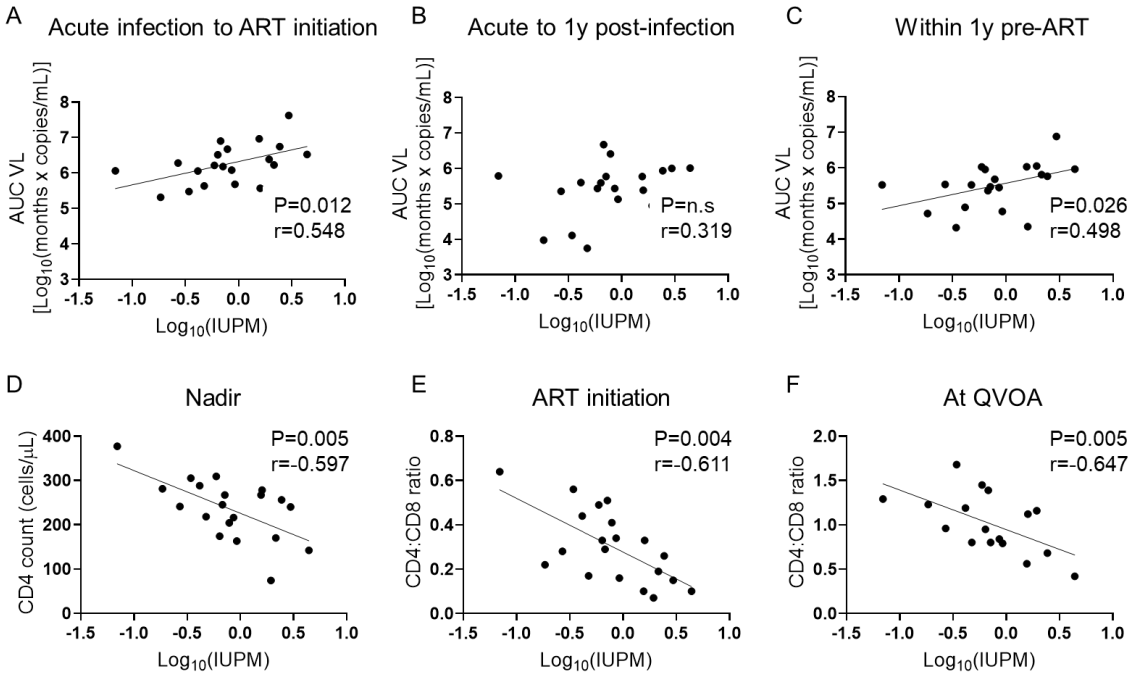

Figure 3.2. Correlates of the HIV replication-competent latent reservoir. (A-C) Association between replication-competent HIV in resting memory $CD4^+$ T-cells [log_{10} (infectious units per million, IUPM)] and cumulative viral burden [area under the curve viral load (AUC VL), in log_{10} (months x copies/mL)], over the time period from acute infection to antiretroviral therapy (ART) initiation, acute infection to 1 year post HIV infection, and over the final year prior to ART initiation (n=20). **(D)** Association between replication-competent HIV [log_{10} (IUPM)] and absolute nadir $CD4^+$ T-cell count (cells/μL; n=20), and **(E)** CD4:CD8 ratio at the time of ART initiation (n=20) or **(F)** at the time point where the quantitative viral outgrowth assay (QVOA) was performed (n=17). Non-parametric Spearman rank correlations were performed, and statistically significant correlations were fitted with a regression line.

3.4.3 Modelling the frequency of latently infected cells from viral load and CD4$^+$ T-cell counts over time

Next, we modelled the frequency of latently infected cells containing replication-competent HIV-1, similar to a previous study in early treated individuals reporting on determinants of the frequency of latently infected cells [86]. Here, we sought to determine whether longitudinal patient data could be used as a reliable predictor of the frequency of latently infected cells even after 5 years on ART. This model makes use of CD4$^+$ T-cell count as the estimate of target cells, while VL is a measure of viral replication, each of which contribute to seeding of the reservoir. We also investigated whether these measures during the year prior to ART initiation contributed disproportionately to replication competent reservoir size, compared to these measures over the entire course of untreated infection. Since the model produced results in arbitrary units such that reservoir size in each participant could be evaluated relative to others in the cohort, the predicted IUPM was compared to measured IUPM to determine the association between the two and evaluate the sensitivity of the model. We observed a correlation between predicted and measured IUPM (**Figure 3.3**) for VL and CD4$^+$ T-cell counts modelled from acute infection to ART initiation (P=0.012, r=0.555; **Figure 3.3 A**) and from the year before ART initiation (P=.013, r=.552; **Figure 3.3 B**). These correlations do not differ when extending the analyses to include on-ART VL and CD4$^+$ T-cell count (**Figures 3.3 C** and **D**).

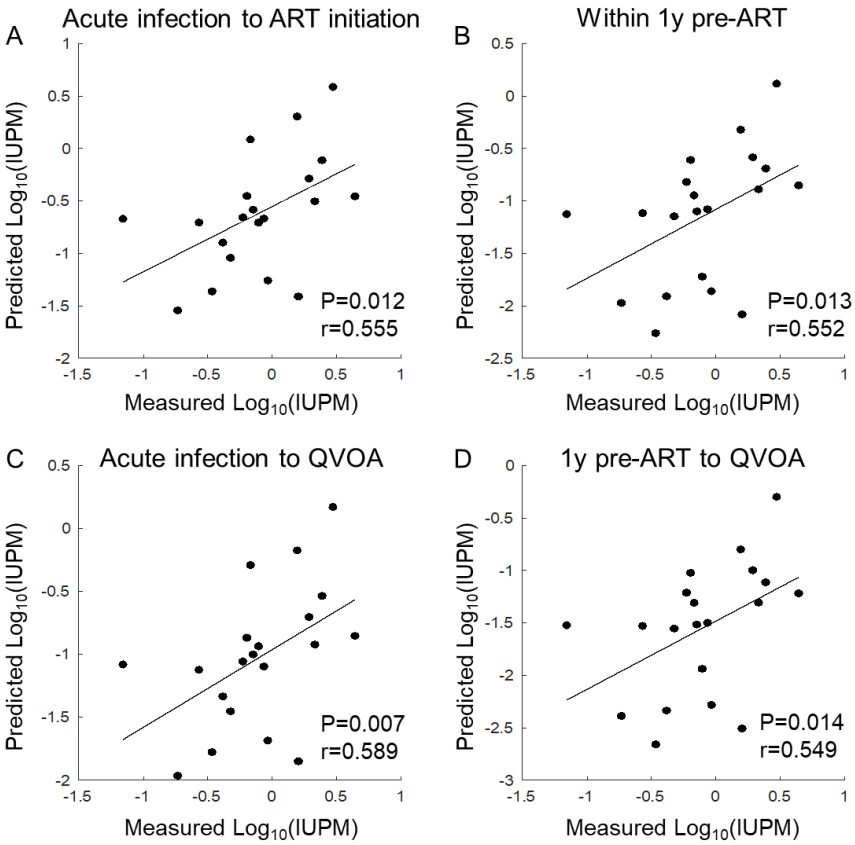

Figure 3.3. Correlations between model predictions and measured frequency of latently infected cells. Correlation between replication-competent HIV-1 in resting memory $CD4^+$ T-cells [log_{10} (infectious units per million, IUPM)] and the predicted IUPM resting $CD4^+$ T-cells over the time period from **(A)** acute infection to ART initiation (n=20), **(B)** over the final year prior to ART initiation (n=20), **(C)** acute infection to QVOA (n=20), and **(D)** from a year prior to ART initiation to QVOA (n=20). Non-parametric Spearman rank correlation coefficients and the *P*-value of the correlation are given at the bottom of each panel. Modelling was performed by the Perelson group at the Los Alamos National Laboratory, Los Alamos, New Mexico, USA

3.4.4 Relationships between T-cell activation and replication-competent reservoir size

We next wanted to investigate whether T-cell activation during the course of untreated infection, or residual T-cell activation after ART initiation associated with replication-competent reservoir size. Extensive longitudinal sampling of PBMC in the CAPRISA 002 cohort allowed us to investigate whether memory $CD4^+$ or $CD8^+$ T-cell activation at multiple

time points before and after ART initiation correlated with reservoir size. We measured the surface expression of CD38 and HLA-DR on memory T-cells during acute infection, one year post infection, one year pre-ART initiation, and at two- and four years post-ART initiation. Reservoir size correlated positively with the frequency of memory $CD8^+$ T-cells co-expressing HLA-DR and CD38 as well as those expressing HLA-DR alone, only in the year preceding treatment initiation (*P*=0.018, r=0.709; and *P*=0.040, r=0.636, respectively; **Figure 3.4**). These findings remained statistically significant after adjusting for multiple comparisons. We found no correlations between the frequency of latently infected cells and the frequency of memory $CD4^+$ T-cells expressing CD38, HLA-DR or co-expressing both activation markers at any time point (**Table 3.3**).

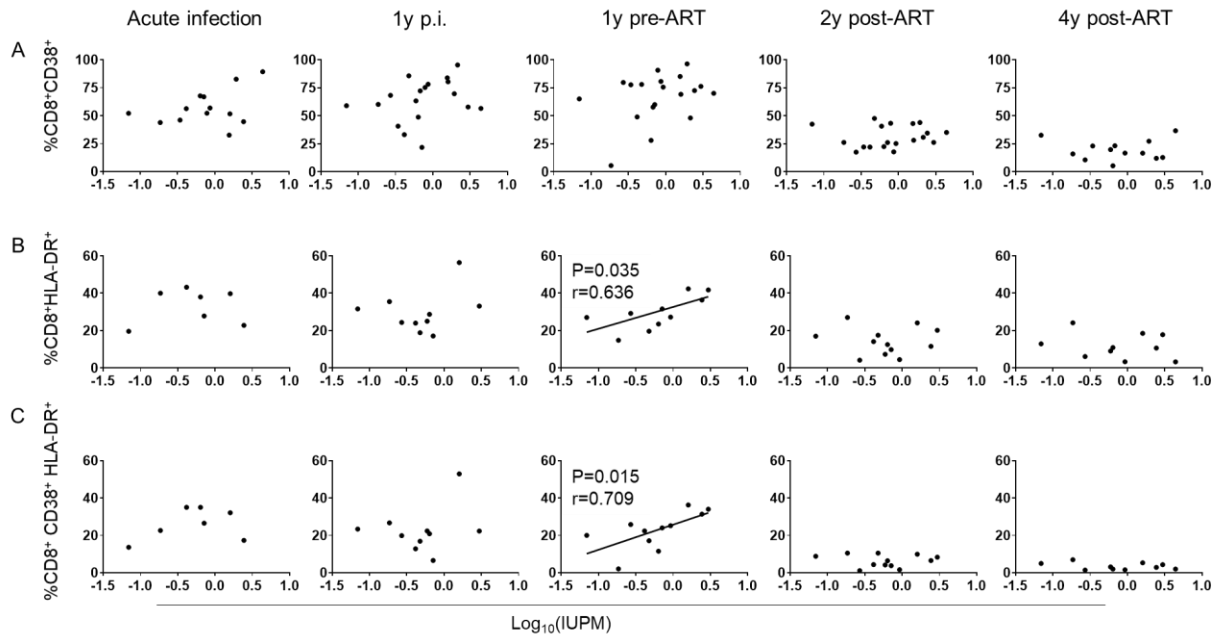

Figure 3.4. Associations between memory $CD8^+$ T-cell activation and reservoir size. Relationship between infectious units per million (IUPM) estimates of replication-competent reservoir size in resting memory $CD4^+$ T-cells and the frequency of **(A)** $CD38^+$ (n=13-19), **(B)** HLA-DR^+ (n=7-12), and **(C)** $CD38^+$HLA-DR^+CD8^+ T-cells (n=7-12), at each of the five time-points assessed in this study. Non-parametric Spearman rank correlations were performed, and statistically significant correlations were fitted with a regression line.

Table 3.3. Associations between $CD4^+$ T-cell activation and reservoir size.

Activation marker	Time point	% of memory $CD4^+$ T-cells	
		Spearman's r	***P*-value**
$CD38^+$	Acute infection	0.4231	0.1517
	1y post-infection	0.2530	0.3112
	1y pre-ART	0.0158	0.9488
	2y post-ART	-0.0754	0.7589
	4y post-ART	-0.1706	0.5747
$HLA\text{-}DR^+$	Acute infection	-0.5357	0.2357
	1y post-infection	0.4424	0.2044
	1y pre-ART	-0.0364	0.9241
	2y post-ART	-0.1119	0.7329
	4y post-ART	-0.1636	0.6567
$CD38^+HLA\text{-}DR^+$	Acute infection	-0.3214	0.4976
	1y post-infection	0.3091	0.3869
	1y pre-ART	0.1455	0.6731
	2y post-ART	-0.0839	0.8004
	4y post-ART	-0.1515	0.6821

Given that nadir $CD4^+$ T-cell count and $CD8^+$ T-cell activation may be influenced by viral burden over the course of untreated infection, we performed multivariable linear regression analyses (**Table 3.3**) and found that nadir $CD4^+$ T-cell count and memory $CD8^+$ T-cell activation within a year prior to treatment initiation were significantly associated with reservoir size, even after adjusting for AUC VL (**Table 3.4**). These models predict that a 1% increase in the frequency of $CD38^+HLA\text{-}DR^+$ memory $CD8^+$ T-cells would result in a 0.033 $\log_{10}$ increase in IUPM (*P*=0.0422) when adjusting for CD4:CD8 ratio at ART initiation and AUC VL, while a unit increase in nadir $CD4^+$ T-cell count resulted in a 0.004 $\log_{10}$ decrease in IUPM (*P*=0.0420) after adjusting for the frequency of $CD38^+HLA\text{-}DR^+CD8^+$ memory T-cells and AUC VL (**Table 3.4**).

Table 3.4. Multivariable linear regression of predictors of reservoir size [Log_{10}(IUPM resting $CD4^+$ T-cells)].

Effect			**95% CI**		
Model 1:	**Estimate**	**Standard error**	**Lower**	**Upper**	***P*-value**
% $CD38^+HLA\text{-}DR^+$ memory $CD8^+$	0.02831	0.01089	0.002550	0.05407	***0.0355***
Log_{10}(AUC VL)	0.1415	0.1684	-0.2568	0.5398	0.4286
Nadir CD4+ T-cell count	-0.00414	0.001667	-0.00808	-0.00020	***0.0420***
Model 2:					
%$CD38^+HLA\text{-}DR^+$ memory $CD8^+$	0.03251	0.01311	0.001521	0.06350	***0.0422***
Log_{10}(AUC VL)	-0.00262	0.2259	-0.5368	0.5315	0.9911
CD4:CD8 at ART initiation	34.5742	20.4826	-13.8594	83.0078	0.1353
Model 3:					
% $HLA\text{-}DR^+$ memory $CD8^+$	0.03826	0.01181	0.01033	0.06619	***0.0143***
Log_{10}(AUC VL)	0.05463	0.1597	-0.3230	0.4323	0.7423
Nadir CD4+ T-cell count	-0.00499	0.001519	-0.00858	-0.00140	***0.0134***
Model 4:					
% $HLA\text{-}DR^+$ memory $CD8^+$	0.04658	0.01522	0.01059	0.08257	***0.0183***
Log_{10}(AUC VL)	-0.1769	0.2304	-0.7217	0.3680	0.4679
CD4:CD8 at ART initiation	49.0998	19.9969	1.8146	96.3850	***0.0438***

3.4.5 3.4.5 Association of soluble markers of inflammation and replication-competent reservoir size

Finally, in order to determine whether soluble mediators of inflammation associate with replication-competent reservoir size, the relationship between inflammatory marker concentrations in plasma and viral persistence was evaluated (**Figure 3.5**). We identified a positive correlation between replication-competent reservoir size and CXCL-10 during acute infection (*P*=0.0071, r=0.582) In addition, we identified an inverse correlation between reservoir size and the concentration of sCD14 after four years on ART (*P*=0.017, r=-0.56). These associations remained statistically significant after adjustment for multiple comparisons.

There were also correlations between reservoir size and TNF-α during acute infection (*P*=0.031, r=0.48) and sCD14 at one-year post-infection (*P*=0.042, r=-0.48), however these did not remain significant after P-value cut-off correction.

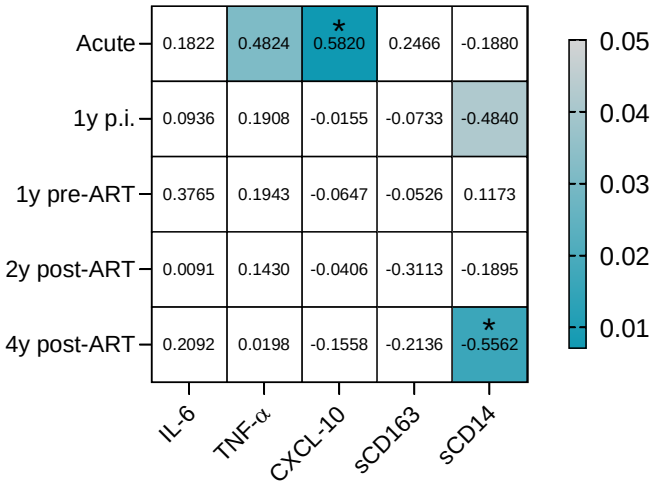

Figure 3.5. Heatmap of the associations between the frequency of latently infected cells and plasma levels of inflammatory mediators measured at different time points over the course of infection. Spearman correlation coefficients for each correlation between the analyte concentration (pg/mL in plasma) and the frequency of latently infected cells (IUPM). The bar on the right of the figure shows the colour scale for uncorrected *P*-values of significance between 0 and 0.05. *P*<0.05 before correction are coloured according to the scale, with non-significant correlations in white. An asterisk (*) above the correlation co-efficient indicates significance after Benjamini-Hochberg *P*-value cut-off correction. The number of participants in each correlation were as follows: acute infection n=20; one-year post-infection (p.i.) n=18; within one year pre-ART n=20; two years post-ART initiation n=20; and four years post-ART initiation n=18.

3.4 Discussion

Factors influencing HIV-1 reservoir formation and dynamics are understudied, particularly in African populations. In this study, we provide insights into the immunological characteristics correlating with the size of the replication-competent reservoir in a cohort of South African women who initiated treatment in late chronic infection.

We show that the cumulative viral burden measured over the entire course of HIV-1 infection or in the year prior to treatment directly predicted reservoir size. In addition, nadir $CD4^+$ T-cell count and CD4:CD8 ratio before treatment initiation correlated inversely with replication-competent HIV-1 after prolonged viral suppression. Indeed, the extent of CD4 depletion has also been shown to shape the HIV-1 DNA proviral load [87, 88]. Here, we show that this is true for replication-competent reservoir size as well. Furthermore, our findings build on previous work that modelled VL and pre-ART $CD4^+$ T-cell dynamics as key predictors of replication-competent reservoir size in the context of early ART [86]. We found that VL and $CD4^+$ T-cell counts over time could be used to predict the frequency of latently infected cells in the context of ART initiated in chronic infection, and that cumulative VL within the year before ART initiation predicted the size of the replication-competent reservoir similar to the cumulative VL over the entire course of infection. Finally, we observed that predicted IUPM values, when including VL and $CD4^+$ T-cell counts after ART in the model, correlated similarly with measured IUPM values when only pre-ART measures were used in the model. These findings indicate that VL and $CD4^+$ T-cell count before ART initiation are robust predictors of replication-competent reservoir size and that events preceding the year of treatment initiation are critical to the establishment of the long-lived reservoir.

We hypothesized that greater T-cell activation pre-ART may enhance establishment of the HIV-1 reservoir by providing a larger pool of target $CD4^+$ T-cells for infection. However, the frequency of activated $CD4^+$ T-cells was not significantly associated with IUPM at any time-point before or after treatment initiation. A limitation of this analysis is that $CD4^+$ T-cell activation is highly dynamic due to continual depletion and migration of these cells, possibly limiting our ability to detect a significant relationship between $CD4^+$ T-cell activation and reservoir size. However, we unexpectedly observed that the frequency of activated memory

$CD8^+$ T-cells in late chronic infection was positively associated with reservoir size. Immune hyperactivation, a strong predictor of HIV-1 disease progression, drives $CD8^+$ T-cell dysfunction, and may explain the relationship between $CD8^+$ T-cell activation and reservoir size [227]. CD38 and HLA-DR co-expression on $CD8^+$ T-cells has been linked to an exhausted cell state and reduced cytotoxic ability [228], resulting in diminished clearance of infected $CD4^+$ T-cells during the transition to latency upon ART initiation in chronic infection, and potentially a larger latent reservoir.

A limitation of our study was that T-cell activation was measured at too few time points to use these measures in our model to predict IUPM. Nevertheless, our multivariate linear regression analysis found that the link between $CD8^+$ T-cell activation and reservoir size remained significant after controlling for viral burden, providing additional support for a direct link between $CD8^+$ T-cell activation within the year prior to ART and reservoir size. Of note, a recent study in a subset of the same women in this cohort showed that 71% of unique viral outgrowth variants from the QVOA were genetically similar to viruses in circulation during the year before ART initiation [10]. Together, the results suggest that immunological events proximal to the time of ART initiation may disproportionately shape the size and composition of the HIV-1 reservoir.

The impact of inflammation on the HIV-1 latent reservoir has been investigated by measuring the association with HIV-1 DNA and cell-associated RNA [13, 229, 230], with most studies showing no associations between the markers of interest included in our study and these measurements of the reservoir. To date, there are a limited number of studies describing the relationship between measures of inflammation and the replication competent reservoir [9, 231]. Thus, we explored the associations between inflammatory markers in plasma and the

replication-competent reservoir size in our cohort. We found that the concentration of CXCL-10 during acute infection correlated positively with the frequency of latently infected cells. CXCL-10, also known as IP-10, is involved in immune cell trafficking to sites of inflammation, having a role in clonal proliferation [232]. This chemokine is induced by IFN-γ and produced by several cell types including monocytes (the main producer of CXCL-10), NK cells, and T-cells. The receptor for CXCL-10, CXCR3, is expressed on Th_1-polarized T-cells and NK cells [233]. CXCL-10 levels in chronic infection have been associated with a higher VL, lower $CD4^+$ T-cell counts, [234] and faster disease progression [232, 235]. Given that higher VLs and lower $CD4^+$ T-cell counts are associated with a larger replication-competent reservoir, and that a large proportion of the reservoir is present in Th_1-polarized cells [148, 236], CXCL-10 concentrations in early infection may result in recruitment of target cells and/or the expansion of infected cells, increasing the frequency of latently infected cells. Additionally, higher CXCL-10 in plasma has been associated with increased infection of resting $CD4^+$ T-cells *in vitro* [237]. As infection of resting $CD4^+$ T-cells has been shown to occur early during the course of infection [238], elevated CXCL-10 concentrations may be facilitating reservoir seeding in this manner.

We also found a negative association between sCD14 concentrations at four years post-ART and the frequency of latently infected cells. sCD14, a soluble receptor, is a marker of systemic inflammation and has been shown to be positively associated with disease progression [239], co-morbidities [240], and mortality [241]. One possible hypothesis for this negative relationship is increased cell turnover or a sequestration of HIV-1 specific cells (shown to be preferentially infected by HIV-1 [190]) in the gut in individuals with high levels of plasma sCD14. A recent study has shown that plasma sCD14 concentrations and the frequency of Gag-specific $CD4^+$ T-cells in rectal tissue are both positively associated with HIV-1 Gag p24 protein

expression in rectal and lymphoid tissues in individuals virally suppressed on ART [242]. Furthermore, HIV-1 Gag p24 protein expression was distinct between ART-suppressed immunological responders ($CD4^+$ T-cell count >500 cells/μL) and non-responders ($CD4^+$ T-cell count ≤350 cells/μL), potentially allowing for use of sCD14 concentrations on ART as a surrogate marker of ongoing Gag expression. In this subset of participants, however, $CD4^+$ T-cell counts were all >350 cells/μL. We could not explore this indirect link between sCD14 and HIV-1 protein expression in lymphoid tissues. This study brings up questions relating to temporal expression of inflammatory markers and their impact on formation of the replication-competent reservoir that are pertinent to investigate in future studies.

While we could not explore the differences in the HIV-1 latent reservoir between males and females due to our access to samples only from women in our retrospective study, the question of whether differences exist is an interesting one. Two recent publications have performed a comparison of replication-competent reservoir size (by QVOA) in males and females with discordant findings [9, 243]. Prodger *et al*. [9] found a significantly smaller HIV-1 replication competent reservoir size by QVOA in Ugandan females compared to males. Falcinelli *et al*. [243] found no significant differences in reservoir size measured by QVOA or intact proviral DNA (IPDA) between females and males in the U.S.A, having matched participants in each group. These findings highlight the fact that further studies are needed. However, similar to our findings, neither of the aforementioned studies found a correlation between $CD4^+$ T-cell activation and IUPM. Furthermore, Prodger *et al*. [9] found no associations between cytokines (IL-6 and CXCL-10) and HIV-1 replication competent reservoir size by QVOA in Ugandan participants, with no significant differences in plasma concentrations of either cytokine in females compared to males.

In conclusion, we demonstrate that replication-competent HIV-1 reservoir size during long-term suppressive ART associates with multiple immunological measures prior to ART, underscoring the need for early diagnosis and treatment. This new knowledge may assist with the design of novel cure and/or therapeutic vaccine approaches.

CHAPTER 4

Clinical and immunological correlates of HIV-1 DNA levels in South African women on antiretroviral therapy initiated during chronic infection

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Table of Contents

4.1 Introduction

HIV-1 DNA has been shown to be a reliable measure of viral persistence [244, 245]. Measuring HIV-1 DNA copy number typically involves the use of economical and rapid PCR-based techniques to estimate the frequency of latently infected cells. As a large portion of the DNA reservoir is defective, measuring HIV-1 DNA proviral copy number is an overestimate of the size of the replication-competent latent reservoir [58], however it has relevance as it is a practical and broadly applicable assay [54]. As such, measuring HIV-1 DNA has been widely used to describe HIV-1 persistence across different cell types and tissues [130, 246]. The most common methods for quantifying HIV-1 DNA are quantitative PCR (qPCR) and droplet digital PCR (ddPCR) that target conserved regions of the genome such as *pol*, *gag*, or the long terminal repeat (LTR). In this chapter, we have estimated the size of the DNA reservoir using a ddPCR assay whereby a droplet oil emulsion is generated and the amplification signal of HIV *pol* and *gag* targets for each droplet is reported separately.

Total intracellular HIV-1 DNA includes all forms of HIV-1 contributing to persistence including intact and defective copies of provirus, as well as unintegrated linear DNA episomal forms (1- and 2- LTR circles). Episomal forms cannot be transcribed or translated, and cannot integrate [245], and the stability of unintegrated HIV-1 DNA is still being debated [54]. The episomal DNA contribution to the total HIV-1 DNA reservoir is thus considered negligible [54, 65, 245]. Total HIV-1 DNA is a relevant clinical marker of HIV-1 disease and in individuals on treatment is positively correlated with integrated HIV DNA [247, 248] and with unspliced RNA, a marker of ongoing transcription that persists despite long-term ART [248]. HIV-1 DNA load provides complementary information regarding treatment success and viral rebound [244].

Pre-ART HIV-1 DNA levels can be used as a predictor of both disease progression [249, 250] and time to viral rebound after ART interruption [78, 79, 87, 251–254], and correlates positively with HIV-1 DNA levels during ART [229]. Lower peak plasma VL and higher $CD4^+$ T-cell count nadir have been found to be associated with lower HIV-1 DNA levels during ART as well as the absence of virological failure [79]. In addition, the frequency of HIV-1 DNA in peripheral blood mononuclear cells (PBMC) is lowest in long term non-progressors [248], and is associated with virological control in post-treatment controllers [255]. Thus, frequency of HIV-1 DNA in people living with HIV (PLWH) is an important marker for a functional cure.

To date, there are only two studies that have characterized the HIV-1 DNA reservoir in Africans [9, 118]. The first assessed HIV-1 DNA decay in a cohort of superinfected Kenyan participants. Pankau *et al*. [118] found that the DNA reservoir of HIV-1 decays slower than the replication-competent reservoir, with a half-life of 11.7 years (140 months) [256] compared to 3.7 years (44 months) [62, 63], respectively. The second study compared HIV-1 DNA levels between men and women in Uganda [9]. Two early studies reported lower total HIV-1 DNA levels in PBMC from women compared to men [78, 79], while two recent publications, one being the study in Ugandan women and men, found no significant difference in HIV-1 DNA between the sexes in resting $CD4^+$ T-cells [9, 224].

The most extensive characterization of HIV-1 DNA in different cohorts has been performed in European studies, most notably by the French National Agency for Research on AIDS and Viral Hepatitis (ANRS) [244]. These studies provide a good basis for comparison of HIV-1 DNA levels in PBMC between cohorts with different ART statuses, treatment initiation times, and disease progression due to the use of the same methodology to measure HIV-1 DNA across studies. In individuals treated during chronic infection, median HIV-1 DNA levels ranged from

2.14 to 2.84 $\log_{10}$ copies per million PBMC [78, 79, 248, 255, 257], with 3.44 $\log_{10}$ copies per million PBMC reported in patients who had progressed to AIDS prior to beginning treatment [258].

Given the paucity of studies in Africans, in particular African women, we aimed to quantify the persistence of intracellular HIV-1 DNA in PBMC from women in the CAPRISA 002 cohort. Subsequently, we explored associations of immune activation, inflammation, and clinical markers of disease progression with total HIV-1 DNA levels.

4.2. Materials and methods

4.2.1 Study participants and study ethics

Thirty-one women from the CAPRISA 002 acute infection cohort [48, 114, 203] in Kwa-Zulu Natal, South Africa were recruited into this study. The study cohort and ethics are described in **Chapter 2, section 2.2.1**.

4.2.2 Thawing PBMC, staining and flow cytometry

Thawing, resting, staining of PBMC, and flow cytometry were performed as described in **Chapter 2, sections 2.2.2** and **2.2.3**. The gating strategy employed has been described in **Chapter 2, section 2.2.4**.

4.2.3 Measuring markers of inflammation

The concentrations of five soluble markers of inflammation, IL-6, TNF-α, CXCL-10 (IP-10), sCD14 and sCD163, were measured in plasma samples, as described in **Chapter 2, section 2.2.5**.

4.2.6 DNA isolation

Cellular DNA and RNA were extracted simultaneously using the Allprep DNA/RNA Mini Kit (Qiagen, Venlo, Netherlands), according to the manufacturer's instructions. Briefly, ~5 million PBMC from thawed cell pellets were lysed in the kit lysis buffer supplemented with β-mercaptoethanol to stabilize nucleic acids. Lysate was passed through QIAShredder columns (Qiagen) to remove cell membrane material and proteins. The flow-through was passed through an AllPrep DNA spin column by centrifuging at 14 000 rpm for 2 min. The flow-through was then added to an RNeasy spin column and the protocol followed to extract RNA while the DNA spin column was placed at 4˚C.

Once the RNA was extracted, the DNA column was brought to room temperature and the DNA extraction protocol completed. DNA was eluted by adding 200µL buffer EB (Qiagen) to the DNA spin column, incubating for 1 min and centrifuging for 1 min at 14 000 rpm. The elution step was performed twice for a total volume of 400µL eluent. Two parts of 100% ethanol and 0.1 parts 3M sodium acetate (NaOAc) was added to the extracted DNA volume in order to precipitate the DNA. The mixture was incubated at -20˚C overnight and then centrifuged at 14 000 rpm for 30 min at 4˚C. The supernatant was discarded carefully, using a fine 10µL pipette tip to remove the residual supernatant. The pellet was resuspended in 1mL 70% ethanol

(freshly prepared) and centrifuged at 14 000rpm for 30 min at 4˚C. The supernatant was removed as previously described, and the pellet left to air dry for at least 2 min. The precipitated DNA pellet was resuspended in 30µL buffer EB. DNA concentration and quality were evaluated using a Nanodrop 2000 (ThermoScientific, MA, USA).

4.2.7 Quantifying total HIV-1 DNA in PBMC

Similar to a previously described method [65], droplet digital PCR was used to determine the frequency of HIV-1 copies in PBMC, through the detection and quantification of HIV-1 *pol* and *gag* gene copy numbers in 1 µg of DNA extracted from PBMC. The detection of a cellular housekeeping gene, *rpp30,* in 100ng of genomic DNA was used to quantify the number of genomes in each reaction volume. HIV-1 *pol* and *gag* ddPCRs were multiplexed and performed in triplicate in each experiment, while *rpp30* reactions were performed in duplicate. A master mix was made containing 1X Bio-Rad ddPCR Supermix for Probes (no dUTP), 0.25 U/µL BanII restriction enzyme (New England Biolabs, NEB), and gene-specific primers and probes. The primer/probe sets used to detect HIV-1 *gag* and *pol* genes are listed in **Table 4.1**.

A volume of 5 µL of template DNA was added to wells of a 96-well plate containing 15 µL of the master mix. Template DNA was allowed to digest at 37˚C for 30 min in an AB 2720 Thermal Cycler (Applied Biosystems). Thereafter, the PCR reaction mix containing digested DNA, along with ddPCR droplet generator oil (Bio-Rad), was loaded into the Bio-Rad QX200 droplet generator and droplets generated according to the manufacturer's instructions. Once droplet formation was complete, the samples were transferred to a ddPCR 96-well plate and the plate was sealed with pierceable foil heat seals (both Bio-Rad) using a pre-heated Bio-Rad PX1 PCR plate sealer (3s at 180˚C). Cycling conditions, performed in a Bio-Rad C1000 Touch

thermal cycler, were as follows: 95˚C for 10 min; then 94˚C for 30s and 60˚C for 1 min, repeating for 50 cycles; then 98˚C for 10 min. Samples were held at 4˚C until they were analysed on the same day using a Bio-Rad QX200 Droplet Reader.

Table 4.1. ddPCR primers and probes

Primer/Probe name	Sequence	HxB2 numbering
***pol* Fwd 6**	TCG GGT TTA TTA CAG AGA CAG CAG AGA	4898-4924
***pol* Rev 4**	AGC ICC TGC CAT CTG TTT TCC AT	5041-5062
***pol* probe 3 FAM ZEN**	/56-FAM/AAG GAC CAG/ZEN/CCA ARC TAC TCT GGA AAG GTG/3IABkFQ*/	4936-4965
***gag* Fwd**	GTT GGA GGA CAT CAA GCA GCC ATG CA	1360-1385
***gag* Rev**	TTC CTG CTA TGT CAC TTC CCC T	1483-1504
***gag* probe HEX ZEN**	/5HEX/ACC ATC AAT/ZEN/GAR GAG GCT GCA GAA TGG GA/3IABkFQ*/	1399-1427
***rpp30* Fwd**	GAT TTG GAC CTG CGA GCG	-
***rpp30* Rev**	GCG GCT GTC TCC ACA AGT	-
***rpp30* probe HEX ZEN**	/5HEX/CTG ACC TGA/ZEN/AGG CTC T/3IABkFQ*/	-

Each reaction used 1 µg of genomic DNA and HIV-1 DNA quantification was performed in triplicate on samples, unless <1 µg gDNA was recovered in which case duplicate reaction were performed. A no-template control (NTC) was included on each plate. As a negative control for HIV-1 DNA and positive control for *rpp30*, genomic DNA from PBMC from HIV-uninfected controls was included in each run. Individual wells were considered 'negative' for the detection of HIV-1 DNA when the reading was below the Poisson confidence interval maximum for the negative and NTC controls and excluded if the well contained fewer than 8000 droplets. Samples were excluded from the analysis if *rpp30* quantification failed. Thresholds were set conservatively using results from negative controls across all experiments. In addition, a positive control (0.25 pg/well subtype C HIV-1 infectious molecular clone) was included to ensure that primers and reagents were functional and effective in detection of HIV-1 DNA. The limit of detection for this assay was 10 copies per million PBMC for both *pol* and *gag*.

4.2.8 Statistical analysis

Flow plots and resulting cell population frequencies, including Boolean frequencies, were generated using the FlowJo software (version 10.5.3, BD Biosciences). GraphPad Prism version 8.0.2 (GraphPad Software, Inc.) was used to generate figures, perform t tests and correlations, and calculate area under the curve VLs (AUC VL). For AUC VL calculations the viral, peak viraemia (<3 months post-infection) were not included since these data were not available for all participants. The Mann Whitney U Test and Wilcoxon Signed Rank Test were used to compare groups of unmatched and matched samples, respectively. All correlations were tested using the non-parametric Spearman's Rank test. A P-value of <0.05 (two-sided) was considered significant for individual comparisons. Adjustments for multiple comparisons were performed using a Benjamini-Hochberg *P*-value cut-off correction with a false discovery rate of 25%.

4.3 Results

4.3.1 Cohort characteristics

Thirty-one women from the CAPRISA 002 Acute Infection cohort were included in this study (**Table 4.2**). Participants initiated therapy according to the prevailing South African treatment guidelines at a median of 3.4 years post-estimated date of infection (IQR: 2.0-5.0) and at a median age of 29 years (IQR: 25-32). Of the 31 women, 9/31 (29%), 13/31 (42%), and 9/31 (29%) initiated ART at $CD4^+$ T-cell count <250, <350, and <500 cells/uL, respectively. Median $CD4^+$ T-cell counts at nadir and ART initiation were 241 cells/μL (IQR: 170-304) and 304 cells/μL (IQR: 194-364), respectively.

Following treatment initiation, the median $CD4^+$ T-cell counts were 622 cells/μL (IQR: 503-786) after two years of ART and 641 cells/μL (IQR: 623-904) after four years of ART. Twenty-three (74%) women achieved $CD4^+$ T-cell count >500 cells/μL after two years on ART, while the remaining 8/31 (26%) had $CD4^+$ T-cell count >350 cells/μL. Twenty-two of the 31 (71%) women had a PBMC sample from the two year post-ART time point only, 1/31 (3%) had a PBMC sample from the four year post-ART time point only, and 8/31 had samples available at both two- and four-years post-ART time points.

Table 4.2. Cohort characteristics

PID	Log_{10} AUC VL (months x copies/mL)	Log_{10} VL (copies/mL)	$CD4^+$ T-cell count (cells/μL)				CD4:CD8			Time (months)	
	Infection to ART initiation	ART initiation	Nadir	ART initiation	2 years post-ART	4 years post-ART[a]	ART initiation	2 years post-ART	4 years post-ART[a]	2 years post-ART	4 years post-ART[a]
100328	4.6	3.0	238	280	526		0.5	0.6		32	
100395	4.8	4.1	436	436	1737		0.3	0.9		30	
100326	5.3	3.7	332	336	1109		0.3	0.6		27	
100353	5.3	4.3	271	400	881		0.5	1.3		25	
100381	5.3	3.9	165	194	754		0.2	0.5		33	
100277	5.3	3.9	281	322	545	493	0.2	1.1	1.2	35	60
100282	5.5	4.1	170	285	468		0.2	0.6		37	
100222	5.5	3.7	305	305	781	641	0.6	1.6	1.7	30	54
100276	5.5	3.9	94	94	486		0.3	1.4		35	
100316	5.6	3.7	278	436	743		0.3	0.9		25	
100268	5.7	3.6	163	163	626		0.2	0.9		29	
100386	5.7	4.8	304	304	919		0.3	0.7		21	
100329	5.8	4.7	243	243	508		0.2	0.9		35	
100352	5.9	4.1	333	451	613		0.4	1.1		36	
100267	5.9	4.6	269	269	614	622	0.3	0.8	1.1	28	54
100279	6.0	4.3	170	193	486		0.4	1.4		16	
100380	6.1	4.7	377	407	802		0.6	1.2		22	
100287	6.1	4.2	216	411	604		0.3	0.6		22	
100266	6.2	4.7	363	363	670		0.3	0.6		18	
100372	6.2	5.2	309	309	618		0.5	0.8		11	
100257	6.2	4.7	170	170	440		0.2	0.5		29	
100244	6.3	4.4	241	293	689	666	0.3	0.6	1.0	24	60
100008	6.3	5.0	175	185	483		0.3	0.7		36	

100336	6.4	5.4	74	74	547		0.1	0.8		23	
100280	6.5	4.8	174	406	479	635	0.3	0.8	1.0	24	48
100286	6.5	4.7	142	142	713	789	0.1	0.4	0.4	42	54
100188	7.0	5.4	267	364	370		0.1	0.5		30	
100345	7.2	3.1	156	314		624	0.5		0.8		54
100088	7.3	5.8	248	311	1018	1099	0.1	0.7	0.9	29	47
100206	7.6	5.3	240	289	724	1019	0.2	0.8	0.8	36	48
100321		5.9	241	267	915		0.3	1.6		30	
Median	**6.0**	**4.4**	**241**	**304**	**622**	**641**	**0.3**	**0.8**	**1.0**	**29**	**54.0**
IQR	**5.5-6.3**	**3.9-4.8**	**170-304**	**194-364**	**503-787**	**623-904**	**0.2-0.4**	**0.62-1.07**	**0.81-1.18**	**23.8-35.0**	**48.0-57.0**

[a]22/31 (71%) participants only had a single sample from two year post-ART, 1/31 (3%) participants only had a single sample from four years post-ART, and 8/31 (26%) participants had samples at both two- and four-years post-ART time points.

4.3.2 Quantification of HIV-1 subtype C pol and gag DNA in the CAPRISA 002 cohort

Quantification of intracellular HIV-1 DNA is a broadly applied measure of latent reservoir size in PLWH on ART, that correlates with clinical outcomes of disease including time to viral rebound after treatment interruption [244]. We initially compared total HIV-1 *pol* and/or *gag* DNA levels (copies per million PBMC) at two years (median: 2.4 years, IQR: 2.0-2.9) and four years post-ART initiation (median: 4.5 years, IQR: 4.0-4.7) in the 31 CAPRISA 002 women using the highly sensitive and reproducible ddPCR platform. Not all participants had samples available for both the two- and four-year time points (**Table 4.2**). *Pol* DNA was measured in 28/31 (90%) and 9/31 (26%) women at two- and four-years post-ART, respectively, whereas *gag* DNA was measured in 27/31 (87%) and 8/31 (23%) women at two- and four-years post-ART, respectively. In addition, three samples had detectable *pol* DNA but not *gag* DNA (all three at two years post-ART) and four samples had detectable *gag* DNA but not *pol* DNA (three at 2 years post-ART and one at four years post-ART). The *pol* and *gag* DNA copy number per million PBMC correlated significantly with one another at both two- ($P<0.0001$; r=0.71) and four-years ($P=0.0004$; r=0.98) post-ART (**Figure 4.1**).

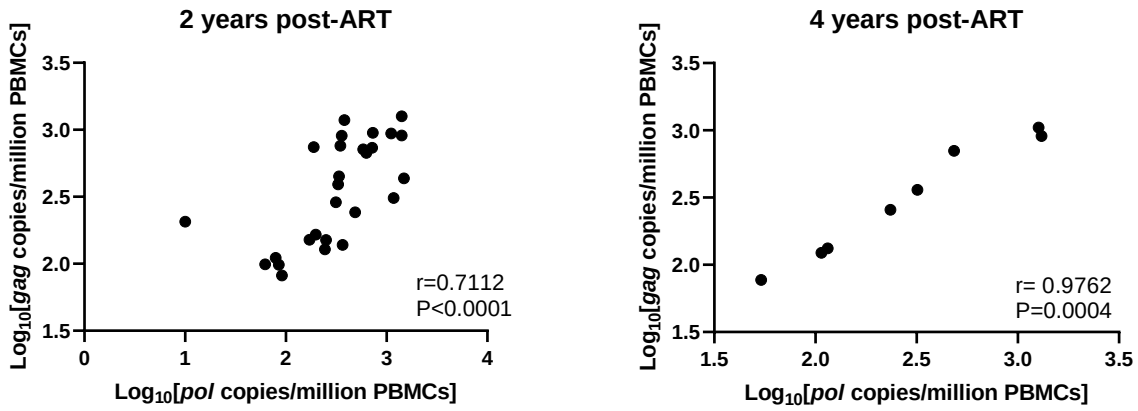


Figure 4.1. HIV-1 DNA frequencies measured by *pol* and *gag* correlate. The frequency of *pol* and *gag* copies per million PBMC correlate with one another at both two- and four-years post-ART initiation. Each black dot represents one participant. Spearman rank correlations were performed.

To determine if there was a change in the size of the DNA reservoir over time, we compared DNA copy number between two- and four years post ART initiation in our cohort. There was no statistically significant change in DNA copies per million PBMC for either gene region over this period. The median *pol* DNA frequency was 2.52 $\log_{10}$copies per million PBMC (IQR: 2.25-2.84) and 2.37 $\log_{10}$copies per million PBMC (IQR: 1.91-2.89) at two- and four- years post-ART initiation, respectively (**Figure 4.2**). Similarly, the median *gag* DNA frequency was 2.49 $\log_{10}$copies per million PBMC (IQR: 2.14-2.88) and 2.48 $\log_{10}$copies per million PBMC (IQR: 2.10-2.93) at two- and four- years post-ART initiation, respectively.

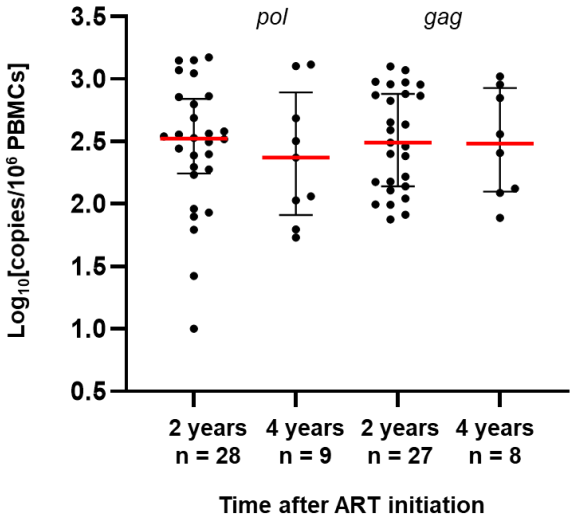


Figure 4.2. Total HIV-1 DNA levels in the CAPRISA 002 participants. Droplet digital PCR was performed to detect *pol* and *gag* copy numbers in peripheral blood mononuclear cells obtained at two- and four-years post-ART initiation. Medians (red line) and interquartile ranges (black vertical bars) of HIV-1 DNA levels in the study participants are shown.

To further assess the degree of decay of total HIV-1 DNA, we assessed the change in the frequency of cells harbouring HIV-1 DNA in matched samples from two- and four years after ART initiation in a subset of women. We found no significant difference between *pol* (n=8) or *gag* (n=7) DNA levels in this subset of participants (**Figure 4.3**). However, there was a trend towards lower total HIV-1 DNA at four years post-ART when measuring *pol* gene copies, indicating that different genes may undergo deletion at varying rates, or that deletions in the primer binding sites hampered detection.

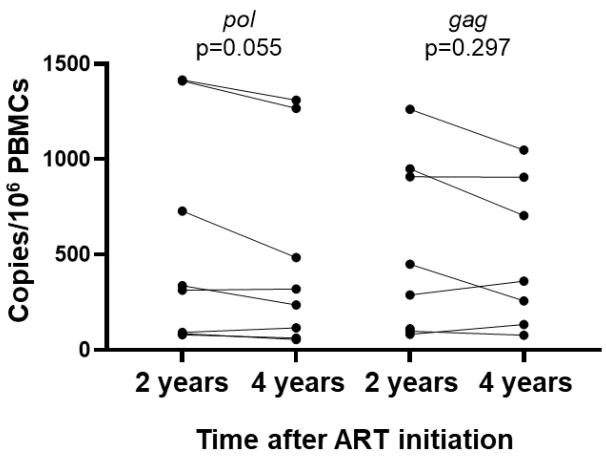


Figure 4.3. HIV DNA levels do not differ significantly betweeen two- and four-years after ART initiation. The frequency of *pol* (n=8) and *gag* (n=7) gene copies per million PBMC measured by digital droplet PCR. A Wilcoxon matched pairs signed rank test was used to compare HIV DNA levles between time points.

Finally, we investigated the relationship between HIV-1 DNA levels and the size of the replication competent reservoir (IUPM estimates) in a subset of 13 participants for whom QVOA had previously been performed at ~5 years post-ART initiation (as described in Chapter 3). Similar to other studies [244, 245], we found no correlation between IUPM estimates and HIV-1 DNA copies per million PBMC for either *pol* DNA (*P*=0.0812; r=0.51) or *gag* DNA (*P*=0.1822; r=0.40) levels (**Figure 4.4**). However, the limited overlap between study participants in the two studies may be too small to accurately identify a correlation between these two measurements.

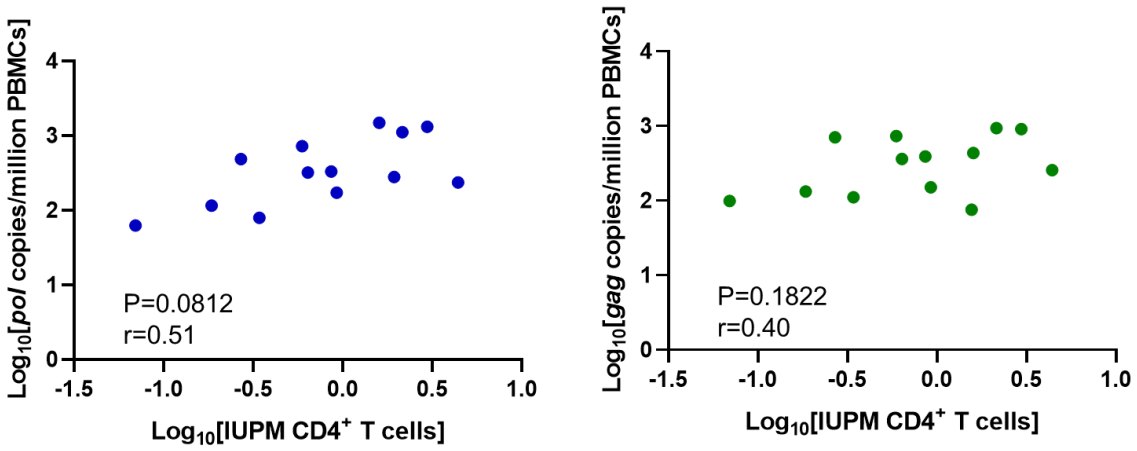
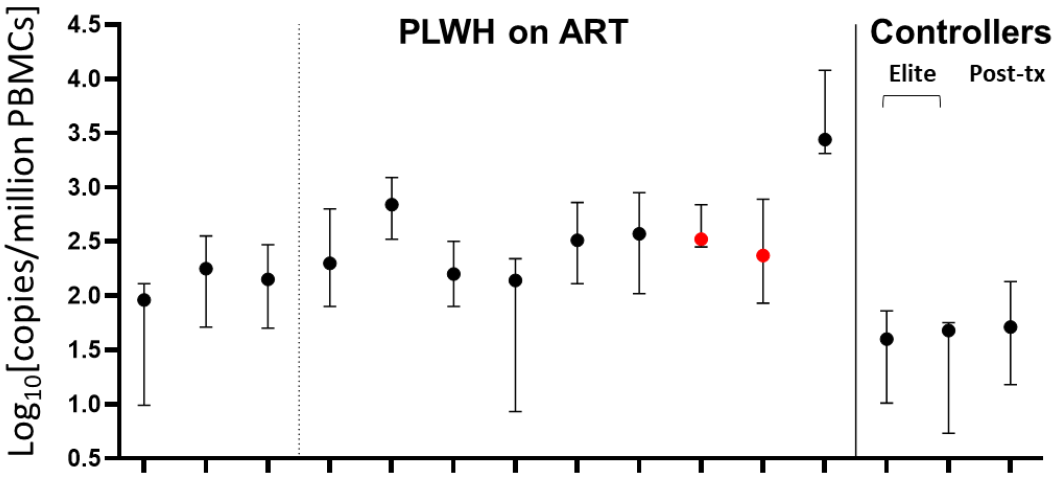


Figure 4.4. Association between HIV-1 DNA levels and infectious units per million (IUPM) CD4$^+$ T-cells. The frequency of *pol* (n=13) and *gag* (n=13) copies per million PBMC do not correlate with IUPM estimates measured by quantitative viral outgrowth assay (QVOA) at both two- and four-years post-ART initiation. Spearman rank correlations were performed.

4.3.3 Comparison of HIV-1 DNA levels of the CAPRISA 002 cohort with other cohorts

To determine whether viral persistence in this cohort was similar to what has been described in other cohorts, total HIV-1 DNA levels were compared to those measured across different ANRS studies using similar methodology (**Figure 4.5**). Total HIV-1 DNA levels fell within the range of those in the ANRS cohorts where treatment was initiated during early- (SALTO cohort; **Figure 4.5 D**) [257] and late chronic infection [83] (**Figure 4.5 E-I**), with lower medians identified compared to those in the late chronic treatment cohort. As expected, HIV-1 DNA levels in the CAPRISA 002 cohort were higher than in those who initiated therapy in primary HIV-1 infection (**Figure 4.5 A- C**) [83, 259] and in controllers (elite controller and VISCONTI cohorts; **Figure**

4.5 M-O) [255, 260], but lower than those who had progressed to AIDS-defining illness (ETOILE cohort; **Figure 4.5 L**) [258].



Tx initiation (stage of HIV-1 infection)	PHI			CHI								AIDS	Un-tx		PHI
Median ART duration (y)	10.8	2	3.6	5.3	7.3	5.6	9.8	13	9	2	4.5	-	-	-	3
n	25	45	35	116	272	451	32	522	243	28	9	21	15	17	14
Legend	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O

Figure 4.5. A comparison of HIV-1 DNA levels in different ANRS cohorts with the measurements in the CAPRISA cohort. Medians and interquartile ranges of $\log_{10}$ HIV-1 DNA copies per million PBMC measured in different European studies (black circles) compared to the HIV-1 DNA levels in the women of the CAPRISA 002 acute infection cohort enrolled into this study (red circles depicting *pol* DNA copies per million PBMC). This figure summarizes data from individuals treated in primary HIV infection (PHI): **A)** AmfAR study [248], **B-C)** OPTIPRIM cohort, ANRS [83, 259]; individuals treated from chronic HIV infection (CHI): **D)** SALTO cohort, ANRS (early chronic treatment) [257], **E)** ANRS study (later chronic treatment) [83], **F)** LoViReT study [261], **G)** AmfAR study [248], **H)** ANRS study [79], **I)** ANRS/CHAIN study [78], **J-K)** CAPRISA 002 cohort; those who had reached AIDS-defining illness: **L)** ETOILE cohort (ANRS) [258]; elite controllers: **M)** ANRS study [260], **N)** AmfAR study [248]; and post-treatment (tx) controllers: **O)** VISCONTI cohort (ANRS) [255]. Figure adapted from Avettand-Fenoel *et al*. 2016 [244].

4.2.4 Clinical correlates of HIV-1 persistence

To investigate predictors of HIV-1 DNA reservoir size, we tested the associations between total intracellular HIV-1 DNA levels (copies per million PBMC), demographic, and clinical measures (**Table 4.3**). There was a correlation between both *pol* (*P*=0.0369; r=0.72) and *gag* (*P*=0.0458; r=0.74) DNA levels at four years post-ART and VL at ART initiation. There was also a trend towards a correlation between *pol* DNA levels at two years post-ART (*P*=0.0832, r=0.34) and four years post-ART (*P*=0.0589, r=0.67) with cumulative AUC VL from acute infection to ART initiation, and between *pol* DNA levels at two years post-ART with AUC VL within one-year pre-ART (*P*=0.0827, r=0.34). There were no correlations between *gag* DNA levels and AUC VL at any time point. Furthermore, when examining $CD4^+$ T-cell counts, *pol* (*P*=0.0255, r=0.75) and *gag* (*P*=0.0107, r=0.86) levels at four years post-ART correlated with contemporaneous $CD4^+$ T-cell count but not with nadir $CD4^+$T-cell count nor the $CD4^+$T-cell count at ART initiation **(Table 4.3)**. Unexpectedly, *pol* (*P*=0.0120, r=0.47) and *gag* (*P*=0.0112, r=0.48) DNA levels at two years post-ART initiation correlated with contemporaneous $CD8^+$ T-cell count, while *pol* (*P*=0.0214, r=0.77) and *gag* (*P*=0.0458, r=0.074) levels at four years post-ART initiation correlated with $CD8^+$ T-cell count at ART initiation.

Next, the association between clinical measures and the fold-change in HIV-1 DNA frequencies between two- and four years post-ART was evaluated. It was found that there was a weakly significant positive correlation of both *pol* (*P*=0.0458; r=0.74) and *gag* (*P*=0.0480; r=0.79) fold

change in frequencies in PBMC with $CD4^+$ T-cell count at ART initiation (**Figure 4.6**). These analyses did not include correction for multiple associations.

Table 4.3. Demographic, clinical, and immunological factors associated with Log_{10}(HIV-1 DNA copies per million PBMC), a measure of viral persistence.

	Log_{10}(*pol* gene copies/10^6 PBMC)				**Log_{10}(*gag* gene copies/10^6 PBMC)**			
Time after ART initiation	**2 years**		**4 years**		**2 years**		**4 years**	
	Spearman r	***P***	**Spearman r**	***P***	**Spearman r**	***P***	**Spearman r**	***P***
Duration (months)[a]								
• Infection to ART initiation[b]	0.0249	0.8998	0.0667	0.8801	-0.1475	0.4628	0.3095	0.4618
• from ART initiation to ddPCR[b]	-0.1928	0.3256	-0.4480	0.2392	0.0459	0.8200	-0.5312	0.1881
Plasma VL[c] (log_{10} copies/mL)								
• at ART initiation	*0.3716*	*0.0515*	***0.7167***	***0.0369***	0.0464	0.8182	***0.7381***	***0.0458***
• Cumulative[d] (AUC VL)								
Infection to ART initiation	*0.3394*	*0.0832*	*0.6667*	*0.0589*	0.0961	0.6406	0.5952	0.1323
One year pre-ART	*0.3400*	*0.0827*	0.5833	0.1080	0.2328	0.2524	0.5000	0.2162
$CD4^+$ T-cell count[e] (cells/µL)								
• Nadir	-0.0019	0.9923	-0.3333	0.3853	-0.1094	0.5871	-0.0476	0.9349
• ART initiation	0.1508	0.4436	0.0333	0.9484	0.0409	0.8394	0.04762	0.9349
• at time of ddPCR	0.2929	0.1304	***0.7500***	***0.0255***	0.2393	0.2293	***0.8571***	***0.0107***
$CD8^+$ T-cell count (cells/µL)								
• ART initiation	0.2266	0.2462	***0.7667***	***0.0214***	0.0037	0.9855	***0.7381***	***0.0458***
• at time of ddPCR	***0.4680***	***0.0120***	*0.6333*	*0.0760*	***0.4805***	***0.0112***	0.5000	0.2162
CD4:CD8 ratio[f]								
• ART initiation	-0.1253	0.5251	-0.5000	0.1777	0.0513	0.7995	-0.4048	0.3268
• at time of ddPCR	-0.2504	0.1992	-0.5500	0.1328	-0.1569	0.4345	-0.3571	0.3894

[a]Age of cohort at ART initiation: median 29 years, interquartile range (IQR) 25-32.
[b]Duration of HIV infection to ART initiation: median 40 months, IQR 24-59; from ART initiation to the time of ddPCR at two years post-ART: median 29 months, IQR 23.75-35; from ART initiation to the time of ddPCR at four years post-ART: median 54 months, IQR 48-57.

[c]Median VL at ART initiation: 4.4 log_{10} copies/ml, IQR 3.9-4.8.
[d]Area under the curve VL expressed as log_{10} time-copies/ml; from infection to ART initiation (excluding peak VL during acute infection): 6.0, IQR 5.2-5.7; over the year prior to ART initiation: median 5.5, IQR 4.8-6.0.
[e]Absolute $CD4^+$ T-cell count at nadir: median 241 cells/μL, IQR 170-304; at ART initiation: median 304, IQR 194-364; at time of ddPCR at two years post-ART: median 622, IQR 503-786; at time of ddPCR at four years post-ART: median 641, IQR 623-904.
[f]CD4:CD8 ratio at ART initiation: median 0.3, IQR 0.2-0.4; at time of ddPCR at two years post-ART: median 0.8, IQR 0.6-1.1; at time of ddPCR at four years post-ART: median 1.0, IQR 0.8-1.2.

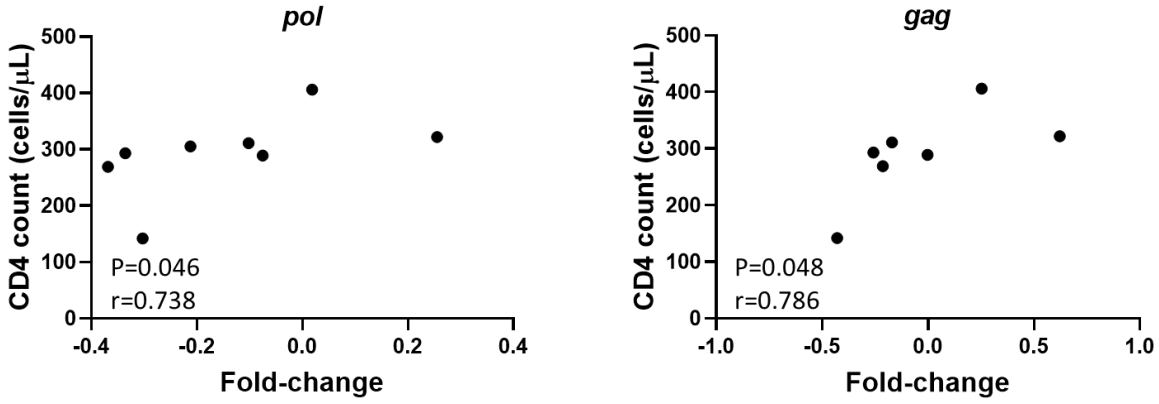


Figure 4.6. HIV-1 DNA fold change between two- and four-years after ART initiation correlated positively with $CD4^+$count at ART initiation. A Spearman Rank correlation was used to investigate the relationship between HIV-1 DNA fold-change in *pol* (n=8) and *gag* (n=7) copies per million PBMC. Negative x-values indicate a decrease in the frequency of HIV-1 DNA copies.

4.3.5 Immunological correlates of total HIV-1 DNA load

Next, the relationship between measures of immune activation at various time points and viral persistence on ART was evaluated. T-cell activation was measured by the cell surface expression of CD38 and/or HLA-DR at the following time points: acute infection, one-year post-infection, within one-year pre-ART, and then at two- and four years post-ART initiation (**Table 4.4**).

Table 4.4. Median time post-infection and relative to ART for participants where both T-cell activation and HIV-1 DNA levels were available.

Time point name	n	Median time post-infection (months)	IQR (months)	Median time post-infection (years)	Median time pre/post-ART initiation (months)	IQR (months)	Median time pre/post-ART initiation (years)
Acute infection	20	**2.0**	**2.0 - 3.0**	**0.2**	30.0	16.8 - 54.0	2.5
One year post-infection	20	**11.0**	**10.3 - 12.8**	**0.9**	32.0	20.3 - 47.0	2.7
One year pre-ART	24	41.0	21.0 - 54.3	3.4	**8.0**	**5.0 - 10.8**	**0.7**
Two years post-ART initiation	29	76.0	54.0 - 91.0	6.3	**29.0**	**24.5 - 35.0**	**2.4**
Four years post-ART initiation	23	94.0	77.0 - 112.0	7.8	**53.0**	**48.0 - 54.0**	**4.4**

*Values in bold represent timing taken into account in inclusion criteria for samples.

HIV-1 DNA levels at four years post-ART initiation was associated positively with the contemporaneous frequency of $CD4^+$ T-cells co-expressing CD38 and HLA-DR (**Figure 4.7**). This correlation did not reach statistical significance for *pol* DNA levels (P=0.0583, r=0.83) but was significant for *gag* DNA levels (P=0.0333, 0.089). There were no significant correlations with activated $CD4^+$ T-cells and HIV-1 DNA levels at two-years post-ART initiation. Furthermore, there was no relationship between $CD8^+$ T-cell activation and HIV-1 DNA levels.

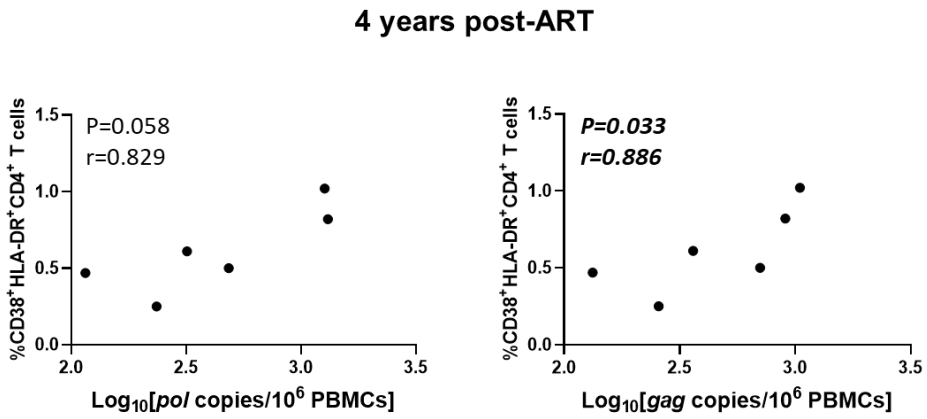


Figure 4.7. The relationship between CD4$^+$ T-cell activation and HIV DNA levels at 4 years post-ART. There was a significant correlation between contemporaneous CD4$^+$ T-cell activation (measured as the frequency of CD38$^+$HLA-DR$^+$CD4$^+$ T-cells) and *gag* copies per million PBMC (n=6). A trend towards a correlation was found between CD4$^+$ T-cell activation and *pol* copies per million PBMC (n=6). A Spearman rank correlation was used to evaluate these relationships.

The impact of T-cell activation on the change in HIV-1 DNA levels between two- and four years post-ART initiation was assessed. The frequency of HLA-DR-expressing CD8$^+$ T-cells at both two- and four years post-ART initiation did not correlate with the fold-change in HIV-1 DNA (**Figure 4.8**), although there was a trend towards a correlation. No other activation markers showed any significant associations with HIV-1 DNA levels.

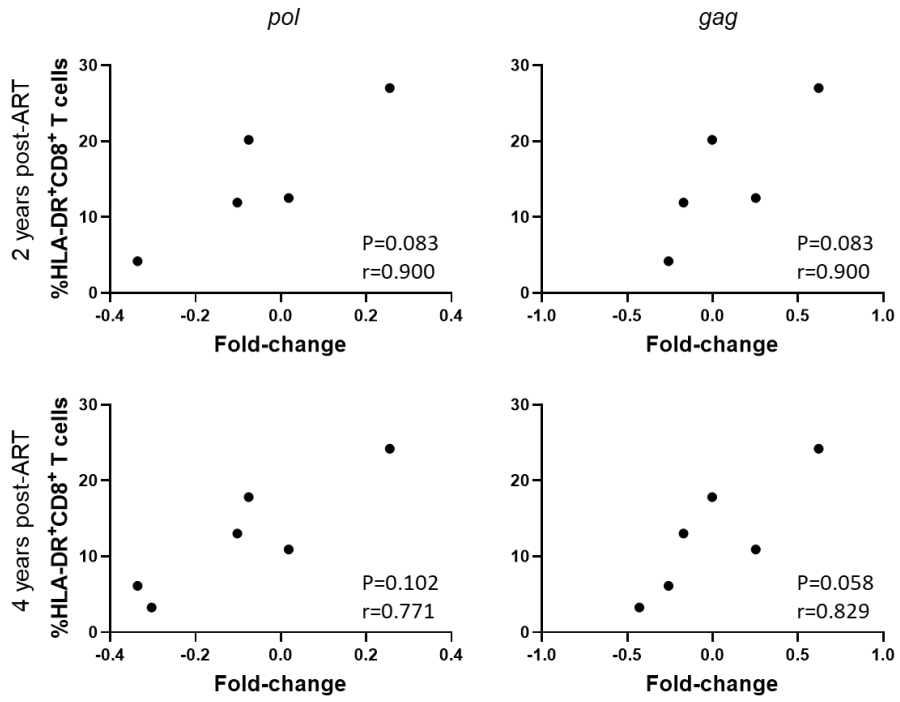


Figure 4.8. The relationship between CD8$^+$ T-cell activation and fold-change in HIV-1 DNA levels. There were trends towards a correlation between the frequency of activated CD8$^+$ T-cells at both two- and four years post-ART initiation and HIV-1 DNA levels (both *pol* and *gag*). Spearman correlations were performed to test the significance of these relationships.

4.3.6 Plasma concentrations of inflammatory mediators correlate with HIV-1 DNA load

Finally, the relationship between plasma concentrations of inflammatory markers and viral persistence as measured by DNA reservoir size was evaluated. Samples were selected for each of the five time points in this study (**Table 4.5**). Median and interquartile ranges differ slightly from those in **Table 4.4** (immune activation time points) due to samples being available for a greater number of participants.

Table 4.5. Median time post-infection and relative to ART for participants where both inflammation and HIV-1 DNA levels were available.

Time point name	n	Median time post-infection (months)	IQR (months)	Median time post-infection (years)	Median time pre/post-ART initiation (months)	IQR (months)	Median time post-infection (years)
Acute infection	27	**2.0***	**1.0 - 3.0**	**0.2**	46.0	27.0 - 57.0	3.8
One year post-infection	27	**11.0**	**10.0 - 13.0**	**0.9**	30.0	20.0 - 47.0	2.5
One year pre-ART	27	38.0	20.0 - 55.0	3.2	**8.0**	**4.0 -11.0**	**0.7**
Two years post-ART initiation	30	75.0	52.8 - 90.5	6.3	**29.5**	**24.8 - 35.0**	**2.5**
Four years post-ART initiation	28	95.0	73.3 - 111.0	7.9	**53.5**	**48.0 - 54.0**	**4.5**

*Values in bold represent timing taken into account in inclusion criteria for samples.

Spearman correlations were performed to test the relationship between the concentrations of IL-6, TNF-α, CXCL-10 (also known as IP10), soluble CD163 (sCD163) or soluble CD14 (sCD14) and HIV-1 DNA frequencies measured by *pol* and *gag* copies per million PBMC at two- and four years post-ART initiation (**Figure 4.9**). HIV-1 *pol* copy number at four years post-ART initiation correlated strongly with plasma concentrations of TNF-α (P= 0.007; r= 0.929; n= 7) and CXCL-10 (P= 0.003; r= 0.964; n= 7) at one-year post infection, and with TNF-α (P= 0.022; r= 0.810; n= 8) within one-year pre-ART.

Similarly, HIV-1 *gag* copy number at four years post-ART initiation correlated with plasma concentrations of CXCL-10 at one-year post infection (P= 0.017; r= 0.943; n= 6), and within one-year pre-ART (P= 0.024; r= 0.857; n= 7), respectively. HIV-1 *gag* copy number at 2 years post-ART also correlated with contemporaneous sCD163 (P= 0.05; r= 0.383; n= 27). None of the relationships remained significant after P-value cut-off correction.

Lastly, there were no significant relationships (after *P*-value cut-off correction) between plasma analyte levels at any time point and HIV-1 DNA levels measured at two years post-ART. There were also no significant correlations between contemporaneous plasma analyte concentrations and HIV-1 DNA levels at both two- and four years post-ART initiation.

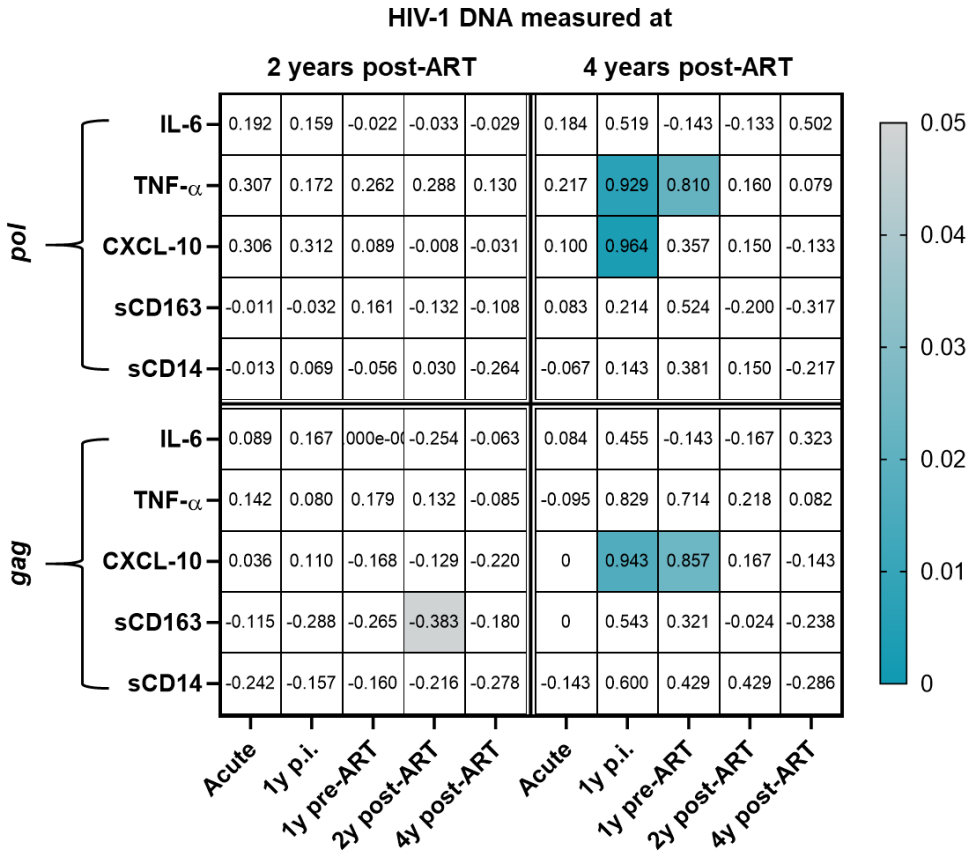


Figure 4.9. Heatmap of the associations between on-ART HIV-1 DNA levels and plasma inflammatory marker levels measured at different time points over the course of infection. The heatmap shows Spearman correlation coefficients for each correlation between the cytokine concentration (pg/mL in plasma) and either *pol* or *gag* frequency (log_{10}copies per million PBMC). The bar on the right of the figure shows the colour scale for uncorrected P-values of significance between 0 and 0.05. Significant correlations are coloured according to the scale, with non-significant correlations in white. The number of participants in each correlation were as follows (n=*pol 2 year, pol 4 year, gag 2 year, gag 4 year,* respectively): acute infection n=26, 9, 25, 8; one-year post-infection (p.i.) n=25, 8, 24, 7; within one year pre-ART n=23, 8, 24, 7; two years post-ART initiation n=28, 9, 27, 8; and four years post-ART initiation n=23, 9, 22, 8.

4.4 Discussion

Given the burden of HIV-1 in sub-Saharan Africa, this is an important setting for cure studies if a global cure is to be achieved. We aimed to address the gap in knowledge of correlates of viral persistence, of which HIV-1 DNA is a marker, in the context of HIV-infected African women. While there have been studies reporting differences in HIV-1 DNA levels between different populations in the global North, data from African cohorts is limited. To our knowledge this is only the second study to characterize total HIV-1 DNA in an African cohort, and the first in South African women infected predominantly with subtype C. An advantage of characterizing this specific cohort was that ART was initiated at different $CD4^+$ T-cell counts, reflective of the change in treatment guidelines in South Africa over time, although all participants initiated ART during chronic infection. This scenario is applicable to many sub-Saharan African countries where early treatment has only recently been adopted, if at all, and reflect a great majority of PLWH.

In this study, viral persistence in a cohort of South African women was characterized by measuring HIV-1 DNA levels (*pol* and *gag* copy number) in PBMC after two- and four years of ART. The assay used was adapted from a subtype B based ddPCR protocol described by Strain *et al*. [65]. ddPCR is more precise than qPCR and is less sensitive to primer/probe mismatches. However, a drawback of ddPCR is the occurrence of false-positives, which are less frequent in qPCR [65, 262]. In our study, this was controlled for by setting conservative thresholds based on uninfected control samples. Infected cell frequencies did not differ significantly between two- and four years post-ART initiation for both *gag* and *pol* DNA levels. However, there was a trend towards lower *pol* copies per million PBMC at four-years post-ART initiation compared to two years. The lack

of a difference in HIV-1 DNA levels between time points is not unexpected. The total HIV-1 DNA reservoir has been shown to have a half-life of 140 months [256], longer than that reported for the replication-competent reservoir [62, 63], thus the degree of decay over two years would be minimal. Furthermore, a study in Kenyan women evaluating decay of *gag* and *env* sequences found that decay rates differed between the two genes [118], although not significantly.

In some participants, *pol* and *gag* DNA levels were discordant, either with a large discrepancy in copy number or where one gene was detectable but the other undetectable. Discordant *pol* and *gag* DNA levels were reported in one other study reporting on HIV-1 DNA dynamics in a large cohort [256]. Intact genomes have been found to decay more rapidly than defective genomes in the first seven years of treatment [263], which may be contributing to the observed discordance if genome deletions are more common on some regions compared to others. Supporting this, deletion mapping in PBMC shows an accumulation of large deletions over time in acutely (subtype C) infected individuals [129]; and subtype B-infected [148] individuals treated during chronic infection more frequently in *pol* than in *gag*. Although we cannot be sure that deletions in chronically infected individuals map similarly in a subtype C context compared to subtype B, the differences in deletion patterns are likely to play a role in susceptibility of total HIV-1 DNA measures to variation depending on the gene region targeted.

Due to the similarity in methodology used, results from this study could be compared to published studies quantifying HIV-1 DNA levels post-ART [78, 79, 83, 248–250, 255, 257–261]. Furthermore, these studies have been conducted in cohorts where participants initiated treatment

at different times post-infection, in elite controllers, and also post-treatment controllers, providing a spectrum for comparison. HIV-1 DNA levels in our study cohort were similar to those reported in the European studies where the participants initiated ART during chronic infection. While the AmfAR [248] and LoViReT [261] studies used ddPCR to quantify *gag* copies per million PBMC (and the LTR in LoViReT), allowing for a more acceptable comparison with our results, a caveat of the comparison with most of the ANRS studies is that qPCR was used to quantify LTR copies per million PBMC [264]. All studies, however, used unfractionated PBMC as a sample type. One other study characterized HIV-1 DNA in men and women from a Ugandan cohort [9] but our findings cannot be compared directly due to the fact that HIV-1 DNA levels were measured in isolated resting $CD4^+$ T-cells. We acknowledge that the list of comparator studies is not exhaustive. Nevertheless, the similarity in HIV-1 DNA levels between European and African cohorts treated from chronic infection may indicate that the time of ART initiation is a more significant determinant of HIV-1 DNA persistence than population.

This study found a positive correlation between contemporaneous memory $CD4^+$ T-cell activation and HIV-1 DNA levels at four years post-ART initiation. We hypothesize that residual memory $CD4^+$ T-cell activation that persists for longer than two years on ART results in a greater HIV-1 DNA load in chronically treated individuals. This could be related to the degree of homeostatic proliferation or clonal expansion occurring in $CD4^+$ T-cells, expanding the HIV-1 DNA copy number.

In addition, memory $CD8^+$ T-cell activation at both two- and four years post-ART trended towards a positive correlation with the fold-change in HIV-1 DNA levels between two- and four years post-ART initiation. This finding is unexpected as $CD8^+$ T-cell activation would more likely be associated with clearance of cells with low-level HIV expression, although a positive relationship was observed between memory $CD8^+$ T-cell activation and replication-competent reservoir size in Chapter 3. It is important to note that these measures were not in HIV-specific $CD8^+$ T-cells, and the association requires further exploration with larger sample numbers. Moreover, we did not consider the co-infection burden in these participants and whether this may have an effect on memory T-cell count and activation, which should be addressed in future studies.

In a mixed-sex cohort, one study reported no association between HIV-1 DNA at one-year post-ART initiation and $CD4^+$ T-cell activation (measured as the frequency of $CD4^+CD38^+$ T-cells) [88]. However, it is important to contextualize the findings in our cohort as studies comparing the reservoir between biological sexes note differences in immune correlates of the reservoir, even when no difference in HIV-1 DNA levels were observed. The study in a Ugandan cohort by Prodger *et al*. [9] found a difference in replication-competent reservoir size but not HIV-1 DNA levels in resting $CD4^+$ T-cells between men and women. Similar to Prodger *et al.* [9], both Falcinelli *et al*. [243] and Scully *et al.* [224] report no difference in HIV-1 DNA per million resting $CD4^+$ T-cells and total $CD4^+$ T-cells, respectively. Although, the latter study reported a trend towards lower inducible HIV-1 RNA in women, both studies attributed these differences to unique immune correlates between the sexes. Scully *et al*. showed a difference between males and females in correlates of both total and integrated HIV-1 DNA (measures that correlate with one another),

with $CD4^+$ T-cell count nadir being a strong negative correlate in males but not statistically relevant in females.

Our study found no association between HIV-1 DNA levels in PBMC and $CD4^+$ T-cell nadir, but we did identify a correlation with contemporaneous $CD4^+$ T-cell count at four years post-ART. This is in keeping with our hypothesis of increased $CD4^+$ T-cell activation, also positively correlated with HIV-1 DNA levels at this time point, leading to increased clonal expansion. As expected, we found a trend towards a correlation between HIV-1 DNA levels and plasma VL at ART initiation (with only trends towards positive associations observed between AUC VL and HIV-1 *pol* DNA levels). Relationships between HIV-1 DNA measures and T-cell activation have also been described and found to differ between the sexes. Scully *et al*. found no correlations between $CD38^+HLA\text{-}DR^+$ $CD4^+$ subsets and integrated HIV-1 DNA in $CD4^+$ T-cells in females, but strong correlations of these same subsets in males [224]. Furthermore, Falcinelli *et al*. found no difference in $CD38^+HLA\text{-}DR^+$ $CD4^+$ frequency between matched males and females [243] and no relationship between the frequency of $CD38^+HLA\text{-}DR^+$ $CD4^+$ T-cells and proviral HIV-1 DNA (*gag* DNA copies per million resting $CD4^+$ T-cells) in men or women. There seems to be differences in correlates of HIV-1 DNA levels between cohorts when comparing males and females, however this is confounded by differences in methodologies across studies, an issue that needs to be addressed by exploring assays that can be used in clinical trial settings so that results are directly comparable.

Interestingly, there was a weak positive association between $CD4^+$ T-cell count at ART initiation and fold-change in HIV-1 DNA levels. That is, a higher fold-reduction in the detection of HIV-1 DNA was associated with a lower CD4 count. This could likely be attributed to thymic output after ART initiation and $CD4^+$ T-cell recovery resulting in a reduction in infected cell frequency. We were not able to assess the levels of recent thymic emigrants (RTEs) in this study. However, in adults and in children, an increased frequency of RTEs, indicating a higher thymic output, is observed in individuals on ART compared to baseline (ART initiation) even in chronic infection [265, 266]. Moreover, in children, this increase in RTE frequency was associated with a decline of cell-associated HIV-1 DNA independent of age or $CD4^+$ T cell count at ART initiation [267]. RTE frequency in both adults and children at baseline correlates with RTE frequency on ART [265, 266]. Thus, it is likely that the same relationship between RTE frequency and HIV-1 DNA exists in adults. A limitation of our findings was that multiple comparisons were not corrected for due to the small sample size where HIV-1 DNA and immune activation measures overlapped.

Finally, we tested associations between soluble markers of inflammation in plasma and HIV-1 DNA levels. HIV-1 DNA levels at four years post-ART initiation were associated with the concentrations of TNF-α and CXCL-10 in plasma during early and late chronic infection and also sCD163 during late chronic infection. In a study in Thai individuals (predominantly males) who received early treatment (RV254 cohort), pre-treatment levels of soluble biomarkers, including TNF-α and CXCL-10, were determined to correlate with contemporaneous VL and also HIV-1 DNA levels at treatment initiation. However, neither TNF-α nor CXCL-10 associated with HIV-1 DNA 96 weeks after treatment, while other markers such as MCP-1, sTNRF-II, MIP-3β, and MIG

did [13]. Firstly, this may point to differences in biomarkers associated with reservoir formation between early and chronically treated individuals. A recent study from Spain showed that a range of cytokines, including CXCL-10, were not significantly different in PLWH with low versus higher (>50 copies per million PBMC) levels of HIV-1 DNA, although no correlation analysis was performed. Secondly, these differences between inflammatory correlates of HIV-1 DNA levels in PBMC may be confounded by differences in cytokine profiles between males and females [52]. Even so, both studies point to the cytokine milieu close to ART initiation being associated with HIV-1 DNA levels in PLWH on treatment. This finding is in accordance with our observations in the previous chapter. Lastly, in one study, authors found no correlations between HIV-1 DNA levels and levels of IL-6, sCD14 or sCD136 at four years on ART [229], consistent with our findings that contemporaneous inflammation is not associated with HIV-1 DNA levels on ART.

In Chapter 3 of this thesis, the clinical and immunological correlates of the replication-competent HIV-1 reservoir were described for twenty of the women in the CAPRISA 002 cohort. We found a difference in the correlates of HIV-1 DNA in PBMC and replication-competent reservoir size. Furthermore, total HIV-1 DNA levels in PBMC did not correlate with the frequency of infectious units per $CD4^+$ T-cells, similar to other studies [245]. Thus, while findings of this study provide insight into reservoir formation and dynamics in African cohorts, they cannot be applied to the replication-competent fraction of the reservoir. In addition, a limitation of this study was the small sample size in some of the comparisons, which means that our results should be taken as hypothesis-forming in nature and that further studies are needed to confirm these findings. The latter is important particularly with regards to the differences in correlates of HIV-1 DNA at two

versus four years post-ART, where the sample sizes differ significantly (n= 28 and 9, respectively) and may influence these statistics. Finally, the intact proviral DNA assay (IPDA) has recently been developed to differentiate intact and defective copies of HIV-1 DNA using ddPCR [56]. This method differentiates between intact proviruses and defective proviruses with 3' and 5' deletions. IPDA estimates correlate with QVOA estimates for the frequency of replication-competent HIV-1 in resting $CD4^+$ T-cells [268, 269]. However, this assay is yet to be optimized for non-B subtypes of HIV-1. Future work will evaluate both intact and defective portions of the HIV-1 DNA reservoir using IPDA, and will provide further resolution on correlates of importance in cell-associated DNA dynamics on ART.

CHAPTER 5

Discussion

Forty years after the discovery that HIV-1 causes acquired immunodeficiency syndrome (AIDS), we still have not found an effective preventative vaccine or cure, resulting in an accumulating number of people on treatment. ART has resulted in HIV-1 infection becoming a manageable chronic disease however HIV-1 persists in a latent viral reservoir requiring lifelong ART and thus a growing number of people needing treatment. This is placing an increased burden on society, necessitating the need for an HIV cure.

The latent reservoir is influenced by the time of ART initiation, infecting viral subtype, immune responses to HIV-1, and disease progression, and sexes [8–10]. Millions of individuals globally only initiate ART during chronic infection, many of whom may also have difficulties in continuity of accessing treatment. In South Africa, approximately a third of individuals are diagnosed during chronic infection at a low $CD4^+$ T-cell count [202]. Thus, it is imperative that cure research takes place in diverse settings to ensure that cure interventions are globally applicable. The overall focus of this thesis was the characterization of immune activation and inflammation both before and after ART in PLWH treated during chronic infection, and to assess their impact on the frequency of HIV-infected cells after long-term ART. We undertook this research in the CAPRISA 002 acute infection cohort, consisting of women from a South Africa where subtype C infection predominates [203]. This is a well-characterized cohort having disease progression [48], adaptive and innate immunity [89, 212, 213, 217, 270, 271], host genetics and viral factors described in

previous studies [115, 272]. We have now added a study on correlates of the HIV-1 latent reservoir. We measured HIV-1 DNA levels at two- and four years post-ART initiation. Despite minor differences in methodology, HIV-1 DNA levels in our study cohort were similar to those reported in the European studies where ART was initiated during chronic infection [78, 79, 83, 248, 257, 261]. Notably, to our knowledge, this is only the second study reporting these frequencies in an African cohort [8, 9]. This suggests that despite differences in viral subtype, demographic characteristics, and methodologies, the size of the HIV-1 reservoir likely has conserved features across different populations.

We characterized memory T-cell activation over time in both untreated and treated HIV-1 infection. As expected, memory $CD4^+$ and $CD8^+$ T-cell activation increased significantly over the course of untreated infection. Upon ART initiation, the frequency of activated memory $CD4^+$ and $CD8^+$ T-cells decreased significantly and reached a plateau between two and four years on ART. While we did not measure T-cell activation in these subjects before infection, studies suggest that T-cell activation does not normalize to levels observed in HIV-1 uninfected individuals [213, 217]. There were no correlations between memory $CD4^+$ T cell activation at any time point and replication-competent reservoir size. However, this study found a positive correlation between contemporaneous memory $CD4^+$ T-cell activation and HIV-1 DNA levels at four years post-ART initiation. We hypothesize that residual $CD4^+$ T-cell activation is related to proliferation of cells containing HIV-1 DNA, expanding the DNA reservoir. We found that the frequency of activated memory $CD8^+$ T-cells was a positive predictor of replication-competent reservoir size. In addition, memory $CD8^+$ T-cell activation at both two- and four years post-ART correlated positively with

the fold-change in HIV-1 DNA levels between two- and four years post-ART initiation. Together, these findings indicate an inadequate clearance of infected cells, potentially due to T-cell dysfunction [227] and a reduced cytotoxic ability [228].

We went on to characterize systemic inflammation by measuring plasma concentrations of five analytes: IL-6, TNF-α, CXCL-10, sCD163 and sCD14. Similar to other studies, we showed a decrease in TNF-α, CXCL-10, and sCD163 upon ART initiation, and a persistently elevated level of sCD14 after ART. Interestingly, plasma IL-6 concentrations during untreated infection in this cohort were similar to levels reported for HIV-1 uninfected individuals. Also of note was the significant decrease in CXCL-10 between two and four years on ART, providing evidence for ongoing immune cell activation well after treatment initiation. Plasma CXCL-10 levels during acute infection were a positive correlate of the replication-competent reservoir. We hypothesize that this finding is attributable to infection of resting $CD4^+$ T-cells [237, 238] or an expansion of already-infected Th_1-polarized $CD4^+$ T-cells. We also found that plasma sCD14 concentration at four years on ART was a negative correlate of replication-competent reservoir size. We hypothesize that the latter finding is due to ongoing systemic inflammation, possibly driven by production of Gag p24 in the gut and lymph nodes of infected individuals [242], resulting in increased cell turnover and a reduced reservoir size. Finally, there was only a trend towards a correlation between chronic inflammation (from one-year post-infection through to one year pre-ART) and HIV-1 DNA levels at four years post-ART initiation, namely CXCL-10 and TNF-α concentrations. Our findings were differed from other studies identifying correlates of HIV-1 DNA [13, 229], that either found a different set of markers of inflammation to be correlates of the

reservoir or found no correlations between the markers measured in our study and the reservoir. Despite this, these findings may highlight potential differences between early and late treated individuals or differences in inflammatory mediator expression between females and males [52]. Further studies into the abovementioned hypotheses are necessary, including potential studies into the mechanisms underlying the link between expression of inflammatory mediators and the latent reservoir of HIV-1.

A limitation of this study was the small sample size, making this research exploratory. Hence, this research should be taken as hypothesis-generating rather than elucidating mechanisms of reservoir formation and maintenance. This study has identified several avenues for future work. Firstly, the correlates of the replication-competent reservoir and the total HIV-1 DNA reservoir differ. Our study was not able to measure these in the same samples. Employing an assay such as the IPDA [56] will allow simultaneous measurement of both intact and defective genome frequencies in the latent reservoir, while differentiating between defective copies with 5' and 3' defects. This will provide further resolution on correlates of importance in cell-associated DNA dynamics on ART. Secondly, elucidating the mechanisms behind the association of T-cell activation and replication-competent reservoir is paramount. Early ART is not accessible to everyone in LMICs. Interventions to mitigate T-cell dysfunction until ART can be initiated may result in a smaller reservoir being established. Ongoing exploratory research also includes evaluating the associations between T-cell activation, inflammation and timing of establishment of the latent reservoir, and other characteristics such as the drivers of clonality. Finally, these findings need to be confirmed

in other African cohorts, and in cohorts including male participants, to determine whether these findings are generalizable to other populations.

Lastly, our sample size precludes the evaluation of the contribution of other chronic co-infections to T-cell activation in PLWH on long-term ART. Nevertheless, we can speculate that co-infections that result in an increased VL set-point or increase the VL significantly within the year preceding ART initiation would result in the establishment of a larger replication-competent reservoir. Further, large-scale studies would be needed to evaluate the roles of different co-infections on reservoir formation.

In conclusion, we find that in South African women who initiate therapy during chronic infection, VL and $CD4^+$ T-cell dynamics before treatment initiation, as well as immune reconstitution, are important determinants of replication-competent reservoir size. Our research also supports previous findings that the year preceding ART is an important period in reservoir formation [10, 117, 118], with our findings that both cumulative VL and memory $CD8^+$ T-cell activation within the year preceding ART predict replication-competent reservoir size. Furthermore, the correlates of replication-competent reservoir size and HIV-1 DNA levels differ, with ongoing T-cell activation on ART (both memory $CD4^+$ and memory $CD8^+$ cells) being predictors. Our study is the first to undertake a detailed study on immune and clinical correlates influencing reservoir size in a cohort of African women, paving the way for future cure studies in this cohort. Our findings provide insights for possible cure interventions in future.

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