

Differences in HIV-1 reservoir size, landscape characteristics and decay dynamics in acute and chronic treated HIV-1 Clade C infection

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Abstract

Background

Persisting HIV reservoir viruses in resting CD4 T cells and other cellular subsets are the main barrier to cure efforts. Antiretroviral therapy (ART) intensification by early initiation has been shown to enable post-treatment viral control in some cases but the underlying mechanisms are not fully understood. We hypothesized that ART initiated during the hyperacute phase of infection before peak will affect the size, decay dynamics and landscape characteristics of HIV-1 subtype C viral reservoirs.

Methods

We studied 35 women at high risk of infection from Durban, South Africa identified with hyperacute HIV infection by twice weekly testing for plasma HIV-1 RNA. Study participants included 11 who started ART at a median of 456 (297-1203) days post onset of viremia (DPOV), and 24 who started ART at a median of 1 (1-3) DPOV. We used peripheral blood mononuclear cells (PBMC) to measure total HIV-1 DNA by ddPCR and to sequence reservoir viral genomes by full length individual proviral sequencing (FLIP-seq) from onset of detection of HIV up to 1 year post treatment initiation.

Results

Whereas ART in hyperacute infection blunted peak viremia compared to untreated individuals ($p < 0.0001$), there was no difference in total HIV-1 DNA measured contemporaneously ($p = 0.104$). There was a steady decline of total HIV DNA in early treated persons over 1 year of ART ($p = 0.0004$), with no significant change observed in the late treated

group. Total HIV-1 DNA after one year of treatment was lower in the early treated compared to the late treated group ($p=0.02$). Generation of 697 single viral genome sequences revealed a difference in the longitudinal proviral genetic landscape over one year between untreated, late treated, and early treated infection: the relative contribution of intact genomes to the total pool of HIV-1 DNA after 1 year was higher in untreated infection (31%) compared to late treated (14%) and early treated infection (0%). Treatment initiated in both late and early infection resulted in a more rapid decay of intact (13% and 51% per month) versus defective (2% and 35% per month) viral genomes. However, intact genomes were still observed one year post chronic treatment initiation in contrast to early treatment where intact genomes were no longer detectable. Moreover, early ART reduced phylogenetic diversity of intact genomes and limited the seeding and persistence of cytotoxic T lymphocyte immune escape variants in the reservoir.

Conclusions

Overall, our results show that whereas ART initiated in hyperacute HIV-1 subtype C infection did not impact reservoir seeding, it was nevertheless associated with more rapid decay of intact viral genomes, decreased genetic complexity and immune escape in reservoirs, which could accelerate reservoir clearance when combined with other interventional strategies.

eLife Assessment

This **important**, clearly written, and timely manuscript links the timing of ART with the kinetics of total and intact proviral HIV DNA. The conclusions are interesting and somewhat novel, and the importance of the work is high because the focus is on African women and clade C virus, both of which are understudied in the HIV reservoir field. The strength of the evidence is **convincing**. Overall, this work will be of very high interest to scientists and clinicians in the HIV cure/persistence fields.

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Introduction

Although antiretroviral therapy (ART) effectively controls HIV, it does not eradicate persisting virus from resting CD4 T cells and other cellular subsets. ART is therefore lifelong, with many side effects and may be financially unsustainable particularly in low-to middle-income countries that are most affected by the HIV epidemic. A cure is therefore necessary but difficult to achieve as the persistent HIV reservoir, which is established soon after infection [1–3] hinders these efforts through its complex nature [4].

The HIV-1 reservoir is heterogenous and dynamic in genotypic, phenotypic and cellular composition and also differs between individuals and populations, making the source of persistent infection difficult to target. The lability of the viral reservoir has been demonstrated by evidence of clonal expansion that occurs by homeostatic proliferation [5, 6], antigenic stimulation [7] or integration into genic regions controlling cell growth [8–10]. Additionally, its size and genetic composition changes over time with intact and defective proviral genomes displaying differences in their rate of decay during suppressive ART [11–13]. The heterogeneity of the HIV-1 reservoir landscape is further obscured by the integration patterns of virus-intact genomes that determine their ability to generate replication competent viral particles when stimulated

[8]. Moreover, recent studies show that the viral reservoir is not restricted to CD4 T cells but persists in other cell types such as monocytes, macrophages [14–19] and follicular dendritic cells [20–23] with each having distinct biological characteristics.

Among the many studies directed towards HIV-1 cure, some have demonstrated that early ART initiation may be a crucial step in limiting the viral reservoir and achieving post-treatment viral control [24, 25]. Even though early treatment does not prevent reservoir establishment, it does result in a rapid decline of viremia and substantially reduces HIV reservoir size [11, 26–28] which delays viral load rebound and results in sustained virological remission following discontinuation of ART [25, 29, 30]. Other described benefits of starting therapy soon after primary infection include preservation of immune function and restriction of viral diversification [26, 28, 31–41]. However, there is a paucity of data that describes the impact of the timing of treatment initiation on the characteristics of the reservoir.

Most studies describing characteristics of the HIV reservoir are from men living with HIV-1 subtype B in the Global North [12, 13, 15, 42] and show that irrespective of treatment timing, intact proviral genomes decay more rapidly compared to defective viral genomes and that defective viral genomes accumulate quickly during acute infection and are predominant within the viral reservoir.

HIV-1 reservoir studies in African populations are limited, particularly in subtype C HIV-1 infection, which is the most prevalent form of HIV-1 globally and predominates in southern Africa. The epidemic in Africa is characterized by extensive viral diversity with multiple subtypes, human genetic heterogeneity which influences immunological and disease outcomes; and unique co-morbidities that modulate HIV reservoirs and immune responses [43–46]. The design of a globally applicable HIV cure strategies and interventions to target the viral reservoir depend on a deeper understanding of the variability in the size, composition and characteristics of the genetic landscapes of persisting reservoir genomes in African populations with non-subtype B HIV infections. Moreover, data are lacking on reservoir characteristics in women, despite known sex differences in immune responses and in viral load during primary infection that could potentially impact the reservoir [47–49].

In this study we performed an extensive longitudinal analysis of HIV-1 subtype C proviral characteristics, that would be informative for understanding mechanisms of reservoir establishment, in a unique hyperacute infection cohort in Durban, South Africa [50, 51]. The cohort was designed to identify acute infection before peak viremia (Fiebig stages I to III) providing HIV-1 testing twice a week to young women at high risk for HIV-1 infection in a region with high population prevalence [50]. Following changes in treatment guidelines in South Africa that allowed for ART initiation regardless of CD4 counts, all study participants were offered ART, including those who were newly detected with acute infection who received ART on average a day after first detection of plasma viremia. Study participants underwent frequent clinical follow-up and sampling following infection and initiation of ART, allowing us to study HIV-reservoir establishment and proviral evolution from the earliest possible stages of infection. We hypothesized that the timing of ART will impact HIV-1 proviral genome characteristics in terms of the size, genetic composition, and decay dynamics. The findings of this study provide insights into HIV-1 proviral characteristics that could inform viral targeting strategies for reservoir control in African populations.

Results

Total proviral DNA load kinetics following early and late treatment

In this analysis we included 35 participants (Supplementary Table 1), of whom 11 first initiated treatment during chronic infection at a median of 456 days (297-1203) post detection of viremia and 24 who were treated during acute infection at a median of 1 day (1-3) post detection of viremia. All participants were female and 31 (89%) were identified with acute infection at Fiebig stage I. Additional participant characteristics are shown in [Table 1](#).

We first quantified total HIV-1 DNA which incorporates all forms of intracellular HIV-1 DNA, both intact and defective, including integrated and unintegrated forms, as well as linear and circularized 2-LTR and 1-LTR forms. Total HIV-1 DNA measurements were performed longitudinally, from baseline (1-3 days following detection of HIV), at the time of peak viral load and was also assessed at 6- and 12-months post-infection for untreated participants and 6- and 12-months post-treatment initiation for late and early treated participants. As expected, treatment during acute infection resulted in a significantly reduced peak plasma viral load (median = 4.18 log copies/ml, IQR, 3.40-4.87) compared to untreated acute infection (median = 7.06 log copies/ml, IQR, 6.83-7.54) ($p < 0.0001$, [Figure 1A](#) top panel). However, at time of peak viremia, the untreated and the early treated groups did not differ in total proviral DNA load ([Figure 1A](#), bottom panel). Longitudinal measurements showed that treatment initiated during chronic infection resulted in a significant decline in plasma viral load to undetectable levels after 1 year ($p < 0.0001$, [Figure 1B](#), top panel) however it did not reduce total proviral load ([Figure 1B](#), bottom panel). In contrast, treatment initiated during acute infection resulted in both a rapid decrease of plasma viremia so that all participants had undetectable viremia at one-year post-ART ($p < 0.0001$, [Figure 1C](#) top panel) and steady decrease of total proviral load over the same time period ($p = 0.0004$) ([Figure 1C](#), bottom panel). Even though treatment initiation during both chronic and acute infection resulted in complete suppression of plasma viral load after 1 year ([Figure 1D](#), top panel), total proviral load was still detectable with the early treated group having 1.3 times lower levels of total proviral HIV DNA compared to the chronic treated group ($p = 0.02$, [Figure 1D](#), bottom panel). These results indicate that early treatment leads to a measurable decline in proviral DNA during the first year of treatment that is not seen when therapy is initiated during chronic infection.

Factors associated with total proviral DNA load after 1 year of suppressive ART

To further understand the impact of host, virological and immunological factors, as well as timing of treatment on the establishment and maintenance of the HIV reservoir, we analysed the associations between virological and immunological markers of clinical disease progression and HIV-1 proviral DNA after 1 year of treatment ([Table 2](#)). Analyses for each treatment group were performed independently using multivariate regression models with HIV-1 DNA levels as the dependent variable and other factors, specifically nadir CD4, pre-infection CD4, baseline CD4 counts and peak viral load, as the independent predictor variables. The analysis showed that when treatment was initiated during acute infection, only peak plasma viral load was significantly associated with levels of HIV-1 proviral DNA after 1 year of treatment ($p = 0.02$). However, when treatment was initiated during chronic infection both baseline CD4 count (measured 1-3 days after detection of HIV) ($p = 0.002$) and peak plasma viral load ($p = 0.03$) positively associated with HIV-1 proviral DNA levels, while there was a significant inverse association with nadir CD4 ($p < 0.0001$). Other factors such as, total viral burden (area under the viral load curves), CD4:CD8 ratio at enrolment, protective HLA alleles and type of treatment regimen were not associated with HIV-1 proviral DNA measured after 1 year of treatment (data not shown). These data indicate that both host and viral characteristics impact the establishment and maintenance of the viral reservoir.

Table 1

Characteristics of study participants

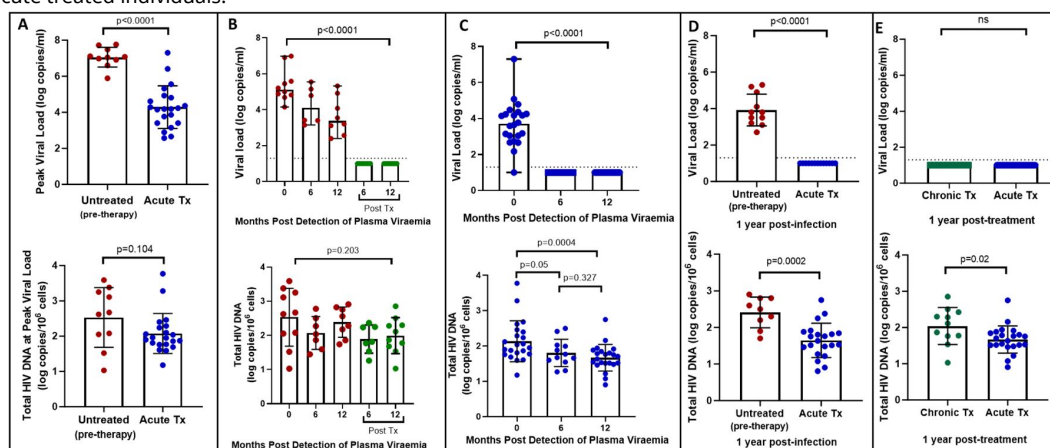
Characteristics	Chronic Treated (n=11)	Acute Treated (n=24)
Age (years)	21 (19-24)	21 (18-24)
Sex		
Female, n (%)	11 (100%)	24 (100%)
Male, n (%)	0 (0%)	0 (0%)
Race/ethnicity, n (%)		
Black	11 (100%)	24 (100%)
Fiebig Stage I at detection, n (%)	10 (91%)	21 (88%)
Treatment Initiation (DPOV)	456 (297-1203)	1 (1-3)
Time to suppression (days)	104 (30-215)	16 (6-116)
CD4 nadir (cells/ μ l)	383 (204-502)	561 (258-859)
CD4 pre-infection (cells/ μ l)	991 (395-1377)	872 (573-1612)
CD4 at study enrollment (baseline) (cells/ μ l)	716 (204-1377)	863 (421-2075)
Peak plasma viral load (log copies/ml)	7.04 (5.89-7.80)	4.21 (2-7.30)
*Protective HLA Allele, n (%)	6 (55%)	11 (46%)
Treatment Regimen Containing, n (%)		
FDC	11 (100%)	24 (100%)
Raltegravir	0 (0%)	16 (67%)

* HLA-B74:01, HLA-B57:02, HLA-B57:03, HLA-B58:01, HLA-B81:01

Figure 1

Plasma viral load and total HIV DNA in acute treated and chronic treated individuals.

A) peak viral load and total HIV DNA measured at peak viral load in untreated (pre-therapy) and acute treated individuals B) longitudinal viral load and total HIV DNA in untreated acute infection and after 6 and 12 months of treatment C) longitudinal viral load and total HIV DNA in acute treated individuals D) viral load and total HIV DNA after 1 year of treatment in chronic and acute treated individuals.



Stage at Treatment Initiation	Variables	Co-efficient	Standard error	t	P value	P value summary	95% confidence interval
Acute Infection	Nadir CD4	-0.0007424	0.00102	0.7277	0.4773	ns	-0.002905 to 0.001420
	Pre-Infection CD4	-0.0001861	0.0004233	0.4395	0.6661	ns	-0.001083 to 0.0007113
	Baseline* CD4	0.0002574	0.0003727	0.6906	0.4997	ns	-0.0005326 to 0.001047
	Peak VL	0.1972	0.07938	2.485	0.0244	*	0.02895 to 0.3655
Chronic Infection	Nadir CD4	-0.006633	0.0007088	9.358	<0.0001	****	-0.008367 to -0.004898
	Pre-Infection CD4	0.0002514	0.0003896	0.6454	0.5425	ns	-0.0007019 to 0.001205
	Baseline* CD4	0.001501	0.0002905	5.166	0.0021	**	0.0007899 to 0.002211
	Peak VL	0.2658	0.09698	2.741	0.0337	*	0.02848 to 0.5031

*at study enrolment

Table 2

Multivariate analysis of factors that predict total HIV-1 proviral DNA load after 1 year of treatment.

Longitudinal genotypic characterisation of HIV-1 DNA

Quantification of total HIV-1 DNA by ddPCR as described above is based on the amplification of a short 127 base pair fragment of the HIV-1 genome, and thus detects defective viruses that are incapable of replication, thereby overestimating the size and functionality of the reservoir. To address this, we next performed single template near full-genome sequencing to determine potential replication competency by establishing the distribution of genome intact and genome defective latent viruses within cells. Viral genome intactness was determined by the HIVSeqinR v2.7.1 computational bioinformatics pipeline [33].

For this analysis we studied 24 participants: The chronic infection (late treatment) group (n=11) consisted of individuals who remained untreated for over one year following infection and before treatment initiation. Longitudinal sampling at untreated time points was available for 9 of these individuals whereas in two individuals, samples were only available post-treatment initiation. The acutely treated group (n=13) received treatment 1-3 days post-detection (Figure 2A). We generated a total of 697 sequences (GenBank accession numbers OR991333-OR991737 and MK643536-MK643827) after sampling a median of 1.4 million PBMC (0.02-4.3 million) per sampling time point. Genome-intact viruses (Figure 2B) accounted for 35% (247/697) of the total pool and were detected in 23 participants (12 from the early treatment group and 11 from the late treatment group), with a median of 8 genome-intact viruses (range=1-60) per study participant. Phylogenetic analysis revealed a significant difference in the mean pairwise distances of intact viral sequences derived from early treated (median=0.12% (IQR, 0.07-0.21) compared to late treated participants (median=0.48% (IQR, 0.16-1.08)) (p=0.04) (Figure 2C). Overall, 56% of the intact genomes collected in this study were obtained from the untreated study arm, while 11% were obtained from late treated chronic infection and 33% from acutely treated infections (Figure 2D).

Longitudinal studies revealed that defective proviruses accumulated rapidly during the course of HIV-1 infection with a relative contribution of 65% (450/697) to the total pool of proviral genomes detected. The majority of defective viral genomes collected in this study were detected during untreated infection (44% (199/450), left-most pie), while 39% (175/450) were detected during late treated chronic infection and 17% (76/450) during acute treated infection. Defective genomes contributed 59% to the proviral population in untreated infection, 87% in late (chronic) treated and 48% in acutely treated infection (Figure 2D). Overall, defective genomes also accumulated quickly after onset of infection and were detectable at a proportion of 47% within the first month irrespective of treatment status (data not shown). Large internal deletions within viral genomes were the most common defect with relative frequencies of 77%, 60% and 79% in untreated infection, chronic treated infection and acute treated infection respectively among the pool of defective genomes. Overall, these gene deletions occurred significantly more frequently between *integrase* and *envelope* in the *integrase* to *envelope* gene segment compared to *gag* (p<0.0001–0.001), with *nef* being similar to *gag* (Supplementary Figure 1). APOBEC induced hypermutations were the second most common defect observed in untreated (16%) and late (chronic) treated (31%) infection. However, in acute treated infection, hypermutations were relatively infrequent, comprising only 7% of the genome-defective pool. Premature stop codons in one of *gag*, *pol* or *env* occurred at a frequency of 5%, 4% and 13% as a percentage of defective genomes in sequences from untreated, chronic, and acute treated infections respectively. Internal inversions (1%, 1%, 1%), and 5' psi defects (2%, 4%, 0%) were other types of genome defects that were detected at minor frequencies in untreated, chronically treated and acutely treated infections respectively.

To further understand the impact of ART timing on the composition, evolution, and dynamics of the HIV-1 proviral landscape over time, we next performed a stratified analysis of the relative proportions of viral genome sequences in each study arm over 1 year of follow up (Figure 3A-C). Genome-intact viruses were detectable throughout the course of untreated infection while

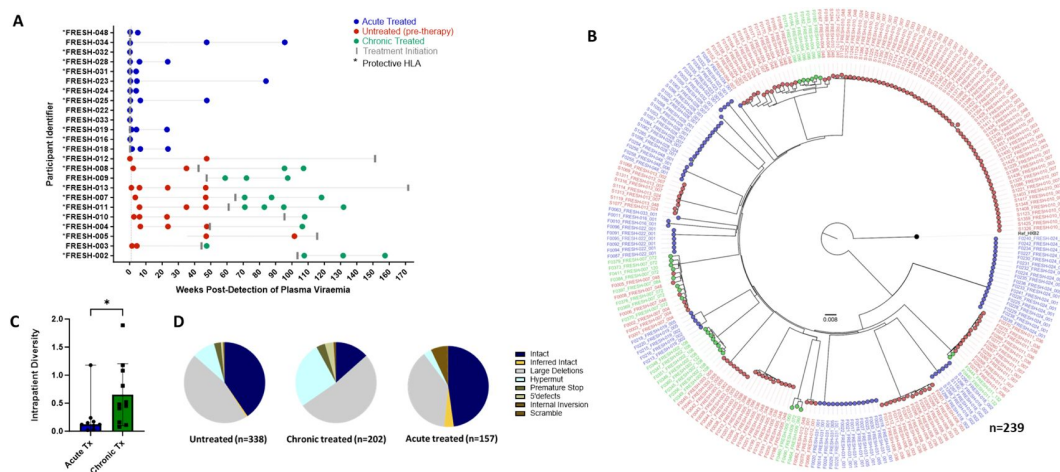


Figure 2

Genotypic characterisation of HIV-DNA sequences.

A) PBMC sequencing timepoints in untreated (red), chronic treated (green) and early treated (blue) study participants where each dot represents a sampling time point. Time of treatment initiation is shown by the vertical grey bar. B) Approximately-maximum-likelihood phylogenetic tree of intact HIV-1 DNA genomes constructed using FastTree2. This method was chosen to resolve full-viral-genome sequences with extreme homology; branch lengths were likely inflated. Viral genomes derived from acute treated participants are marked with (*). C) Comparison of intrapatient mean pairwise distances between early and late treated participants. D) Spectrum of HIV genome sequences detected during untreated acute infection, late treated chronic infection, and acute treated infection.

genome-defective viruses also accumulated over this period (**Figure 3A** [↗](#)). Initiation of ART during chronic infection, at a median of 456 days after detection of plasma viremia, resulted in a decrease in the relative proportion of genome-intact viruses over 1 year of treatment (34% to 14%). However, genome-intact viruses were not completely eradicated and were easily detectable after 12 months of treatment (**Figure 3B** [↗](#)). Additionally, genomes with large deletions and hypermutations became more prominent in the chronic treated group over 1 year of treatment. In contrast, there was a more rapid decrease in the proportion of genome-intact viruses following ART initiation in acute infection such that these viruses were no longer detectable at our sampling depth after 1 year of treatment (57% to 0%) (**Figure 3C** [↗](#)). Hypermutated viruses were also less prominent before 12 months during early treated infection (**Figure 3C** [↗](#)). These data suggest that early treatment initiation facilitates faster clearance of genome-intact viruses in the blood compared to late treatment.

Decay kinetics of intact and defective proviruses

Studies show that the biology and decay dynamics of genome-intact viruses within the viral reservoir likely differ from that of the genome-defective provirus pool [[12](#) [↗](#), [13](#) [↗](#)]. However, the effect of ART timing on the rate of decay of these different pools of viruses is not well known and has not been investigated in African populations where immune responses and viral genetic heterogeneity may well result in population-specific differences. Here we observed that the absolute proportions of both genome-intact and -defective viruses per million PBMCs sampled decreased in both early treated and late treated participants over the 1-year follow-up period of this study (**Figure 4A** [↗](#) and **4B** [↗](#)).

To estimate potential differences in the rate of change between genome-intact and -defective viruses within each treatment group we used a linear mixed effects regression model with random intercepts to account for the correlation between repeated observations from the same individual. We fit a model with log DNA copies as the response variable, and time, treatment group, and a time-group interaction as fixed effects, with participant as a random effect. The analysis was restricted to the first 6 months after starting ART as regular measurements for both groups were available over this period.

Among the acute treated, genome-intact proviruses decreased by 0.308 log copies per month in the first six months after starting ART, corresponding to a decline of 51% per month ($p < 0.001$, **Figure 4C** [↗](#)). In contrast, among the chronic treated, intact proviruses decreased by only 0.059 log copies per month, corresponding to a decline of 13% per month; however, this decrease was not statistically significant ($p = 0.68$) (**Figure 4E** [↗](#)).

Genome-defective proviruses also decreased significantly in the acute treated group by 0.190 log copies per month in the first six months after starting ART, corresponding to a decline of 35% ($p = 0.01$). However, in the chronic treated group the change in the number of log copies of defective provirus in the first 6 months was only 0.015 ($p = 0.88$) corresponding to a decline of just 3.4% per month (**Figure 4D** [↗](#) and **4E** [↗](#)). These results indicate that early treatment is associated with a faster decline of both genome-intact and -defective proviruses compared to late treatment.

Contribution of clonal expansion to maintenance of proviral populations

Studies show that more than 50% of the latent HIV reservoir is maintained by clonal expansion [[52](#) [↗](#)]. We assessed viral genome sequences to determine the extent of persistence of infected cell clones after primary infection. Viral genome sequences sharing 100% identity by FLIP-seq was used as a marker of clonal expansion of infected cells as previous studies have shown that proviral genomes that were 100% identical share the same viral integration site whereas proviruses with different integration sites do not share 100% sequence identity [[53](#) [↗](#)]. At our sampling depth, we

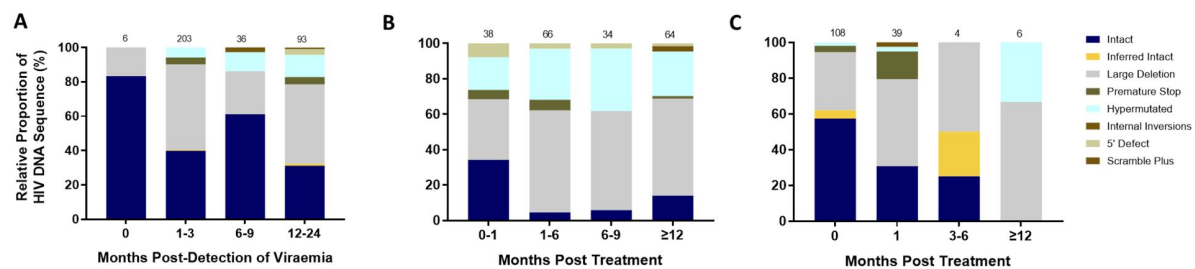


Figure 3

Evolution of the proviral genetic landscape.

Relative proportions of intact and defective viral genomes measured longitudinally in A) untreated acute infection for 2 years B) late (chronic) treated infection for 1 year and C) early (acute) treated infection for 1 year. The number of genomes sampled at each time point is indicated above each vertical bar.

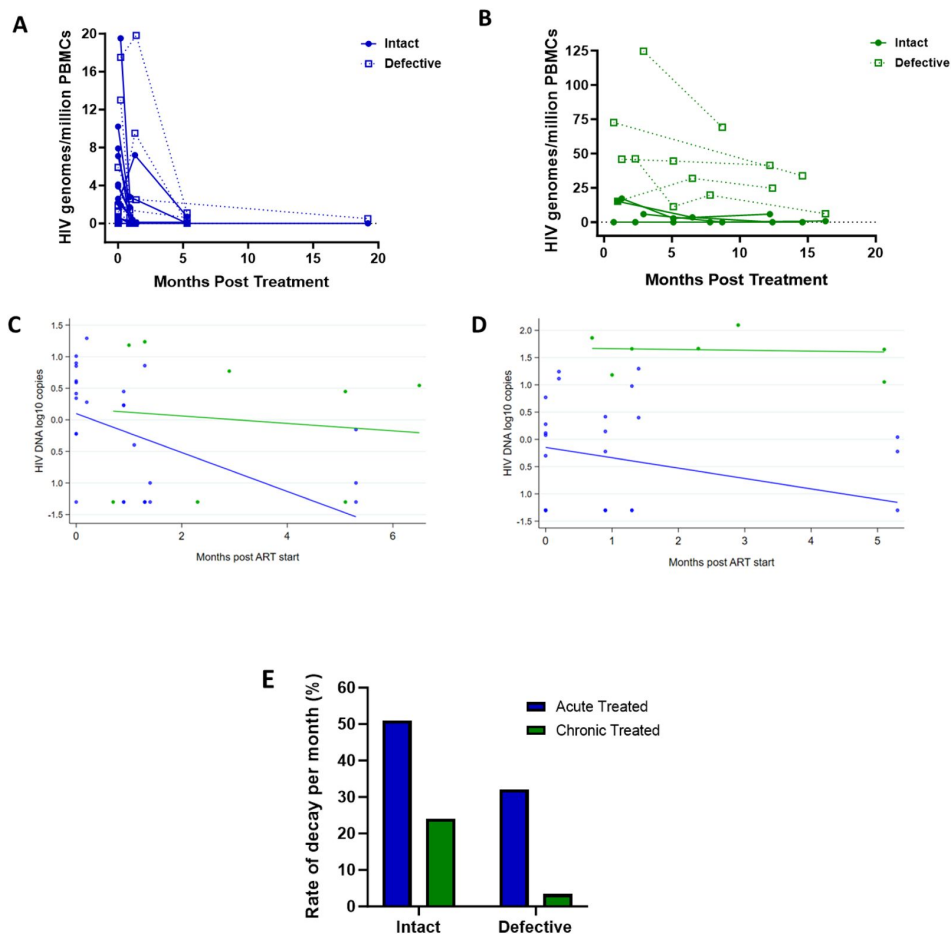


Figure 4

Decay kinetics of intact and defective proviruses.

Absolute frequencies of intact and defective HIV-1 DNA sequences per million PBMCs during the 1st year of infection following treatment during A) acute infection and B) chronic infection. Longitudinal analysis of the change in (C) intact and (D) defective provirus copies in the 6 months after ART initiation, comparing the acute treated (blue) and chronic treated (green) groups. Dots represent a measurement from a given participant; solid lines are slopes estimated from linear mixed effect model. (E) Comparison of the monthly rate of decay of intact and defective proviruses in acute and chronic treated infection.

detected clonal expansion in 3/11 (27%) participants who were treated during chronic infection and 4/13 (30%) participants treated during acute infection, showing that in subtype C infection clonal expansion of infected cells occurred as early as one day post detectable viremia (Supplementary Figure 2). Defective clones were detected in two late treated participants at proportions of 6% and 13% of total proviral population, while intact clones were identified in one late treated participant at a proportion of 6% of the total proviral pool. In contrast, a higher proportion of intact clones were detected in early treated participants at 33%, 30%, 37% and 24% of the total proviral pool. Although the data is limited and needs to be interpreted with caution, this suggests that clonal expansion of intact proviral genomes is more likely to occur when treatment is initiated early, likely due to the early inhibition of viral replication that prevents the accumulation and seeding of defective viral genomes into the viral reservoir.

CTL epitope diversity in the latent reservoir

The emergence of escape mutations in viral epitopes as a mechanism to evade human leukocyte antigen (HLA) class I-restricted immune responses, specifically of CD8⁺ cytotoxic T lymphocytes (CTL), drives viral diversification and is a significant challenge in developing effective therapies against HIV [54–57]. We investigated the impact of late compared to early ART initiation on CTL epitope diversity and escape in the HIV proviral genomes by longitudinally analysing Gag, Nef and Pol CTL epitopes, [58] from single genome viral sequences (excluding only hypermutated sequences), that are restricted by HLA genotypes B*57:02, B*57:03, B*58:01, B*81:01 and A*74:01. These HLA genotypes have been associated with protection against disease progression in HIV-1 subtype C infection [59]. CTL epitope mutations were classified according to the Los Alamos HIV Molecular Immunology Database [58]. Protective HLA genotypes were present in 7/11 (64%) late treated participants and in 7/13 (54%) early treated participants. In the presence of relevant restricting HLA genotypes, mutations compared to the Clade C consensus were detected in 12% of participants with Gag, 23% with Pol and 27% with Nef targeted epitopes after 1 year of follow-up when treatment was initiated late (Figure 5A–C) in contrast to 0%, 0% and 8% respectively when treatment was initiated early (Figure 5G–I) suggesting that chronic treatment is associated with the retention of a wide spectrum of CTL escape mutations within proviral genomes compared to early treatment. Escape mutations detected at baseline (up to 1 month after infection) in the presence of restricting HLA genotypes were present in 3% of participants within Gag, 19% within Pol and 23% (Figure 5A–C) within Nef targeted epitopes when treatment was initiated later compared to 0%, 13% and 11% respectively (Figure 5G–I) with early treatment. Escape mutations observed in early treated participants were present in the earliest sequences that were derived close to the time of infection and therefore likely represent transmitted escape variants. Similar proportions of transmitted escape mutations were present in participants who did not have a protective HLA genotype and remained unchanged after 1 year. (Figure 5D–F and 5J–L).

Discussion

In this study we used a well characterised acute HIV infection longitudinal cohort of untreated, early treated and late treated study participants, to perform an extensive quantitative and qualitative analysis of the HIV-1 subtype C proviral landscape. Our aim was to determine whether the timing of treatment has an impact on the viral reservoir size, genetic landscape, and decay kinetics. This was a longitudinal study where we measured total proviral load levels by ddPCR and characterised the genetic landscape of the proviral genomes by next generation sequencing using FLIP-Seq. Total HIV-1 DNA is an important biomarker of clinical outcomes [30, 60–62]. We found that HIV DNA was detectable at high levels during primary infection and even in participants who were treated during acute infection, total HIV DNA levels measured at peak viremia was very similar to untreated participants. In contrast, we found that early but not late treatment was associated with steady decline of total proviral load over the first year of ART. These observations confirm previous data that the viral reservoir is seeded at the earliest stages of

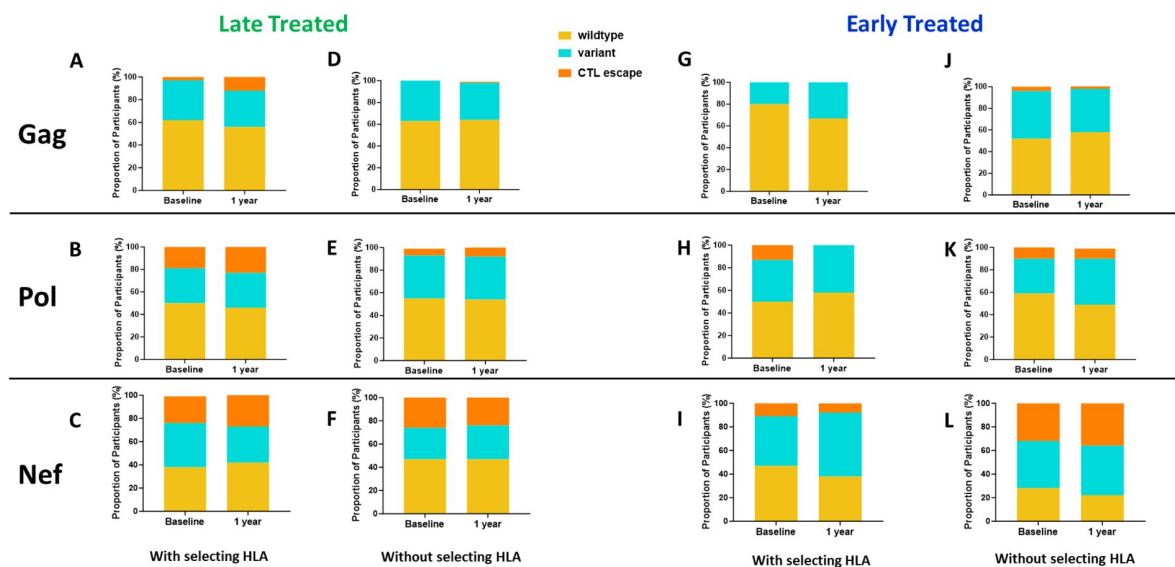


Figure 5

Comparison of CTL epitope diversity in late compared to early treated participants.

Proportion of participants with wildtype, variant and CTL escape at baseline (within 1 month of infection) and up to 1 year of infection in Gag (A, D, G, J), Pol (B, E, H, K) and Nef (C, F, I, L) epitopes in participants with protective HLA genotypes (A, B, C, G, H, I) and without protective HLA genotypes (D, E, F, J, K, L).

infection, possibly before peak viremia [2, 42, 63–65]. However, in contrast, studies have suggested that early ART intervention restricts the seeding of the HIV reservoir in long-lived central memory CD4 T cells [27]. Moreover, it has recently been demonstrated that a small fraction of deeply latent genetically intact proviruses are archived in CD4 T cells during the very first weeks of infection [66]. However, even though early ART initiation has been associated with continued HIV DNA reduction during long-term ART after 10 years of follow up [28], it remains detectable in most individuals indicating that early ART alone is insufficient to achieve viral eradication.

We also examined the association of clinical and virological factors with the levels of proviral DNA after 1 year of treatment. In both early and late treatment groups, peak plasma viral load was associated with HIV DNA levels. Moreover, in the late treatment group there was a positive association with CD4 at enrolment (baseline) and an inverse correlation with nadir CD4 counts. Considering, that the majority of CD4 T cells remain uninfected it is likely that this does not represent a higher number of target cells, and this warrants further investigation. Similar associations with nadir CD4 have been reported previously [67–69] suggesting that during untreated progressive HIV infection, ongoing viral replication may drive the accumulation of long-lived latently infected cells that repopulate the immune system by expansion during successful ART.

Even though total HIV DNA has been shown to be a clinically significant marker of the HIV reservoir it does not distinguish between replication-competent and -defective viruses that contribute to the viral reservoir. The unique design of the FRESH cohort based on frequent HIV screening and sampling intervals of high-risk uninfected participants allowed us to examine the dynamics of the proviral landscape from the earliest stages of infection (Fiebig I) up to 1 year of ART by near-full-length viral genome sequencing. We performed a comparison of the proviral populations between study participants who were treated during the acute phase of infection and those initiating ART during chronic infection. Defective genomes accumulate rapidly after the onset of infection and contributed to almost half of the proviral population within the first four weeks of infection irrespective of treatment status. Consistent with other studies [33, 42, 70] the overall proviral landscape in the untreated and late treated participants was dominated by defective viruses suggesting that prolonged ongoing viral replication before treatment initiation leads to the accumulation of defective viral genomes.

A further analysis of the composition of defective viral genomes revealed that genome deletions were most frequently observed between *integrase* and *envelope*. A previous study showed that large deletions are non-random and occur at hotspots in the HIV-1 genome with *envelope* being a hotspot for large deletions [42, 71]. Additionally, we noted differences in the frequencies of hypermutations compared to other studies in subtype B cohorts suggesting that the timing of ART initiation and sex-or race-based differences in immunological factors that impact the reservoir may play a role [42].

Genome-intact viruses were easily detectable throughout untreated infection but decreased after treatment. In participants who were treated during chronic infection, genome-intact viruses were still detectable after one year of ART compared to early treated individuals where they were no longer detectable. With our limited sampling size and depth, of a median of 1.4 million PBMC (0.02–4.3 million) per sampling time point, we cannot rule out that intact genomes may be retrieved with further sampling and investigation into tissue reservoirs of the participants who initiate ART during acute infection [72]. However, these findings do provide further evidence that introducing ART during acute HIV infection limits the size of the HIV reservoir considerably compared to treatment during chronic infection [11, 24–28].

The intact proviral DNA assay (IPDA) which is a more scalable method that uses multiplexed ddPCR to measure individual proviruses and differentiates intact from defective proviruses without the need for long-distance PCR, has been suggested to provide more accurate quantitative information about the size and composition of the latent reservoir compared to near full genome sequence methods [73]. However, this assay has not yet been developed and optimised for quantification of subtype C HIV.

Studies have shown that during suppressive ART, intact and defective proviruses have different rates of decay that occurs in a biphasic manner [11–13]. Our analysis of decay kinetics was limited to a linear mixed effects regression model as we were unable to fit a model for biphasic decay which requires frequent proviral DNA measurements. Our analysis was further restricted to the first six months of ART as viral genomes were difficult to detect by FLIP-seq in early treated participants after this time. We found that indeed intact genomes decay faster than defective genomes in both early and late treatment groups. Cells containing intact viral genomes likely represent productively infected cells that may be preferentially targeted for clearance by the host immune response or eliminated by viral cytopathic effects [12, 13]. Moreover, early treatment results in a faster decline of both intact and defective genomes compared to treatment initiated during chronic infection and is suggestive of a more effective immune clearance mechanism from preserved immune function. Despite this, it is estimated that 226 years of effective ART is necessary to decrease intact proviral DNA levels by $4 \log_{10}$ [12]. This further indicates that early ART in combination with novel interventional strategies will be needed to achieve a faster viral eradication.

Several studies show that clonal expansion of HIV-infected cells plays an important role in maintaining the HIV reservoir further contributing to the challenge of eradicating HIV [53, 74–76]. Our findings suggest that clonal expansion of intact viral genomes detected predominantly in early treated infection may contribute to the maintenance of the HIV reservoir in these study participants. Further studies that extend beyond 1 year of treatment will help elucidate whether these clones expand further after several years of treatment. Our analysis of CTL epitope escape mutations, known to drive viral diversification, revealed that early treatment minimises the emergence of CTL escape in Gag, Pol and Nef epitopes despite these participants having protective HLA alleles. In contrast CTL escape was detected when treatment was initiated during chronic infection specifically in the well characterised TW10 gag epitope restricted by HLA B57/58. Transmitted CTL escape mutations detected within the first few weeks of infection were common in both treatment groups confirming previous data from this study population [77]. Moreover, the rapid rate of clearance of viral genomes observed with early treatment could be attributed to the lower proportion of cytotoxic T lymphocyte escape mutations in the proviral genomes of these participants compared with those treated later.

To our knowledge this is the first study in an African population, dominated by subtype C HIV infection, that examined the impact of the timing of ART initiation on HIV reservoir establishment in a longitudinal setting. Moreover, our data focused on women who are underrepresented in reservoir and cure studies globally. Our data showed that early ART initiation does not blunt proviral DNA seeding in immune reservoirs, but it nevertheless results in a more rapid decay of intact viral genomes, decreases genetic complexity and immune escape. Although early ART alone may not be sufficient to eradicate the persisting viral reservoir, our results suggest that when combined with interventional strategies, it is more likely to achieve an effective HIV cure.

Materials and methods

Ethics Statement

The Biomedical Research Ethics Committee of the University of KwaZulu-Natal and the Institutional Review Board of Massachusetts General Hospital approved the study. All participants provided written informed consent.

Study Design and Participants

This was a longitudinal study of the Females Rising through Education, Support, and Health (FRESH) cohort, a prospective, observational study of 18–23-year-old HIV uninfected women at high risk for HIV acquisition, established in Umlazi, Durban, South Africa [50, 51]. Finger prick blood draws were collected from FRESH study participants twice a week and subjected to HIV-1 RNA testing, with the aim of detecting acute HIV infection during Fiebig stage I. The study included a socioeconomic intervention program and HIV prevention interventions including PrEP that coincided with study visits to address challenges faced by the young women that likely contribute to the increased risk of HIV acquisition in this setting. If a participant acquired HIV-1 infection while on the study, blood samples were collected weekly for a month, then monthly until 3 months post infection, then monthly for one year and every 3 months thereafter. Days post onset of viremia (DPOV) was calculated as the interval between the first positive HIV test and the date of sample collection. Unique participant identifier numbers were assigned to the participants and are only known to the research group.

Study participants recruited during the first 19 months of the study did not receive antiretroviral (ARV) treatment immediately after detection of acute HIV infection but were monitored and referred for treatment when they became eligible according to national treatment guidelines at the time. The South African national treatment eligibility criteria subsequently changed allowing the immediate initiation of ART for all people living with HIV (PLWH), including those with acute HIV infection as recommended under the World Health Organisation's universal test and treat policy [78]. The treatment schedule was a three-drug daily oral regimen of 300 mg tenofovir disoproxil fumarate, 200 mg emtricitabine, and 600 mg efavirenz. Additionally, following the change in South African first line treatment guidelines to include an integrase inhibitor, raltegravir (400 mg twice a day) was introduced as a fourth drug and was continued for 90 days after viral suppression (<20 copies per mL).

For this study participants were categorized into 3 groups (untreated, late (chronic) treated, and early (acute) treated) where, 11 remained untreated during acute infection and later started ART during chronic infection at a median of 456 (297–1203) days post onset of viremia (DPOV), while 24 started ART at a median of 1 (1–3) DPOV. Participants were studied at 0-, 1-, 3-, 6-, 9-, 12- and 24-months post onset of viremia and up to 12 months post treatment. Peak viraemia refers to the highest recorded viral load in all participants.

Quantification of total HIV-1 DNA

Measurement of total HIV-1 DNA was performed as previously described [79]. Total DNA was extracted from total PBMC samples using DNeasy Blood & Tissue Kits (QIAGEN). Droplet digital PCR (ddPCR) (Bio-Rad) was used to measure total HIV-1 DNA and host cell concentration with primers and probes covering HIV-1 5' LTR-*gag* HXB2 coordinates 684–810 (forward primer 5'-TCTCGACGCAGGACTCG-3', reverse primer 5'-TACTGACGCTCTCGACC-3' probe/56-FAM/CTCTCTCCT/ZEN/TCTAGCCTC/31AbkFQ/, and human RPP30 gene38 forward primer 5'-GATTGACCTGCGAGCG-3', reverse primer 5'-GCGGCTGTCTCCACAAGT-3', probe/56-FAM/CTGACCTGA/ZEN/AGGCTCT/31AbkFQ/). Thermocycling conditions for ddPCR were: 95°C for 10 min, 45 cycles of 94 °C for 30 s and 60 °C for 1 min, 72 °C for 1 min. Thereafter droplets from each sample were analyzed on the Bio-Rad QX200 Droplet Reader and data were analysed using QuantaSoft software (Bio-Rad).

Single genome amplification and deep sequencing of near-full length HIV-1 DNA

Near full-length proviral sequences were generated as previously described [79]. Total HIV-DNA copy number as determined by ddPCR was used to calculate the DNA sample dilution to achieve one PCR-positive reaction in every three reactions. This method of limiting dilution gives a Poisson probability of 85.7% that each PCR amplicon originated from a single HIV-DNA template [80]. A nested PCR approach was used to amplify the near-full genome using one unit of Platinum™ Taq DNA Polymerase High Fidelity (Invitrogen) with the following primers: first round PCR: forward primer 5'-AAATCTCTAGCAGTGGCGCCCGAACAG-3', reverse primer 5'-TGAGGGATCTCTAGTTACCAGAGTC-3'; second round PCR: forward primer 5'-GCGCCCGAACAGGGACYTGAAARCGAAAG-3', reverse primer 5'-GCACTCAAGGCAAGCTT TATTGAGGCTTA-3' (HXB2 coordinates 638-9632, 8994 bp). The 20 µL reaction mix contained 1× reaction buffer, 2 mM MgSO₄, 0.2mM dNTP, 0.4 µM each of forward and reverse primers. The thermal cycling conditions were 2 min at 92 °C, 10 cycles [10 s at 92 °C, 30 s at 60 °C, 10 min at 68 °C], 20 cycles [10 s at 92 °C, 30 s at 55 °C, 10 min at 68 °C], 10 min at 68 °C, 4 °C infinite hold.

Illumina Mi-Seq and Bioinformatics Analysis

All PCR amplicons detectable by gel electrophoresis were subjected to Illumina MiSeq sequencing and thereafter the resulting small reads were *de novo* assembled using in-house UltraCycler v1.0. (Brian Seed and Huajun Wang, unpublished) [79]. Viral genome-intactness was inferred by the computational bioinformatics pipeline HIVSeqinR v2.7.1 [33].

HLA Typing

HLA typing was performed using a targeted next-generation sequencing method as previously described [81].

Statistical methods

GraphPad Prism 10 was used to perform summary statistical analyses and comparisons among study groups using Fishers' Exact, Mann-Whitney and Kruskal-Wallis and multiple linear regression analysis.

Data availability

The data that support the findings of this study are available from the corresponding author (T.N.) upon reasonable request.

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Author Contributions

The study was conceptualized and designed by K.R., G.Q.L., M.L., X.G.Y and T.N. and PBMC samples and clinical/demographical data were collected by K.L.D., B.D.W., K.R., and T.N. HIV-1 genotyping laboratory work was done by K.R., G.Q.L., N.R., and T.J.B.C. Results were analyzed by K.R., G.Q.L., and T.N. K.R., G.Q.L. and T.N. wrote the manuscript; all authors contributed to and approved the manuscript. T.N. supervised the study.

Supplementary Table 1

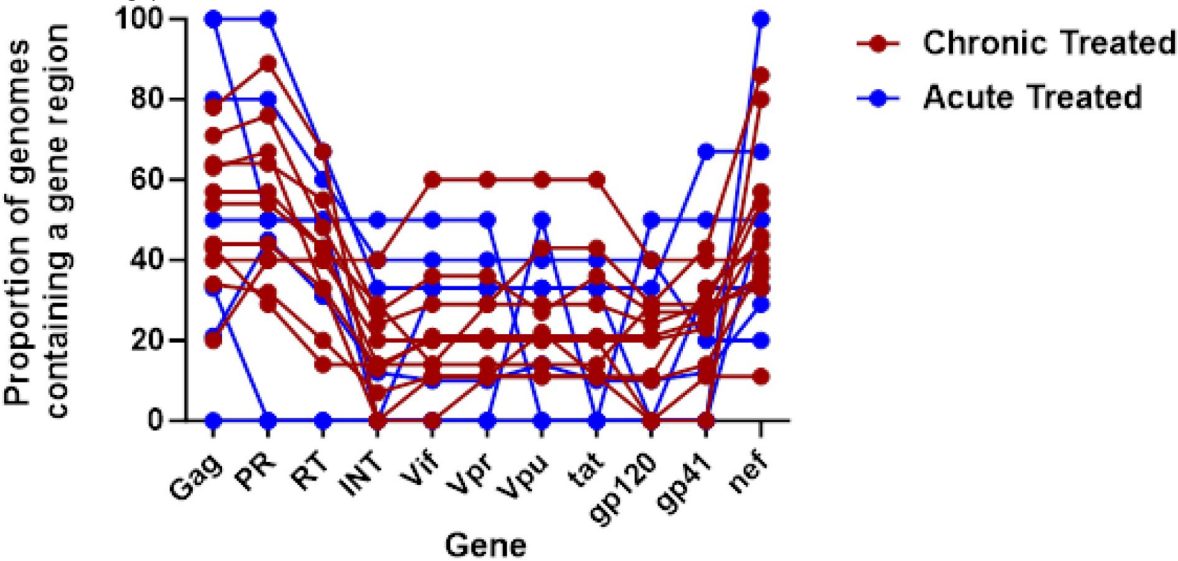
Clinical and biological characteristics of 35 study participants

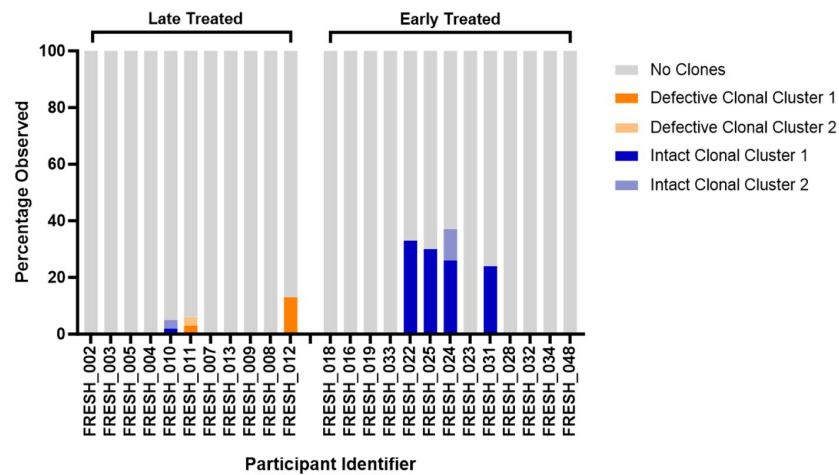
Identifier	HLA-A1	HLA-A2	HLA-B1	HLA-B2	HLA-C1	HLA-C2	Fiebig Stage at Detection	Timing of Treatment Initiation (DPOV)	Peak Viral Load	ddPCR	FLIP Seq
FRESH_002	02:01	30:01	44:03	58:02*	04:01	06:02	I	724	5,70E+07	✓	✓
FRESH_003	02:01	30:01	15:10	42:02	08:04	17	I	310	8,20E+06	✓	✓
FRESH_005	24:02	29:02	07:02	44:03	07:01	07:02	I	809	5,20E+07	✓	✓
FRESH_004	03:01	74:01**	15:03	58:02	02:10	06:02	I	346	3,00E+07	✓	✓
FRESH_010	23:01	30:01	15:10	58:01	03:02	16:01	III	668	5,70E+07	✓	✓
FRESH_011	23:01	68:02	08:01	58:01	03:04	07:01	I	427	1,10E+07	✓	✓
FRESH_007	23:01	74:01	35:01	58:01	04:01	06:02	I	456	4,00E+06	✓	✓
FRESH_013	01:01	66:01	39:10	81:01	12:03	18	I	1202	7,70E+05	✓	✓
FRESH_009	43:01	43:01	07:02	15:03	02:10	18	I	416	9,90E+06	✓	✓
FRESH_008	02:05	02:05	58:01	58:01	07:01	07:01	I	297	9,50E+06	✓	✓
FRESH_012	23:01	29:02	42:01	53:01	03:04	17	I	1059	1,20E+07	✓	✓
FRESH_015	30:02	34:02	08:01	44:03	04:01	07:01	I	1	2,30E+04	✓	✗
FRESH_018	68:02	74:01	15:03	57:02	02:10	18	V	2	1,50E+02	✓	✓
FRESH_016	01:23	30:02	42:01	58:01	06:02	17	I	1	3,40E+04	✓	✓
FRESH_029	30:01	30:01	39:10	42:02	12:03	17:01	I	1	1,40E+04	✓	✗
FRESH_019	02:01	03:01	58:01	58:02	03:02	06:02	I	1	3,60E+05	✓	✓
FRESH_033	68:02	68:02	07:02	15:10	03:04	07:02	I	1	4,40E+02	✓	✓
FRESH_022	30:01	30:02	18:01	53:01	04:01	07:04	I	1	2,10E+05	✓	✓
FRESH_025	01:01	30:01	42:01	47:01	06:02	17	I	3	8,90E+04	✓	✓
FRESH_024	01:01	29:01	44:03	81:01	08:04	18	I	1	5,90E+03	✓	✓
FRESH_030	02:05	29:02	42:01	58:01	07:01	17:01	III	1	2,00E+07	✓	✗
FRESH_023	02:05	23:01	08:01	14:01	07:01	08:04	I	3	4,10E+04	✓	✓
FRESH_036	02:05	68:01	35:01	58:01	04:01	07:01	I	1	1,90E+04	✓	✗
FRESH_031	01:01	68:02	14:01	81:01	08:02	18	I	1	1,70E+04	✓	✓
FRESH_038	30:01	30:02	08:01	15:03	02:10	07:01	I	1	4,60E+02	✓	✗
FRESH_028	03:01	29:02	44:03	58:02	06:02	07:01	I	2	2,60E+06	✓	✓
FRESH_035	29:02	33:03	07:02	44:03	07:01	07:02	I	1	7,70E+02	✓	✗
FRESH_032	30:01	74:01	15:03	18:01	02:10	07:04	I	1	7,30E+03	✓	✓
FRESH_034	30:02	33:03	53:01	58:02	04:01	06:02	I	2	8,60E+04	✓	✓
FRESH_039	02:01	02:05	08:01	58:02	06:02	07:01	I	1	2,50E+03	✓	✗
FRESH_048	30:01	30:02	15:03	57:03	02:10	07:01	I	1	1,50E+04	✓	✓
FRESH_041	29:02	74:01	44:03	57:03	07:01	07:01	I	1	1,40E+03	✓	✗
FRESH_046	23:01	30:01	15:10	18:01	07:04	16:01	I	1	2,50E+03	✓	✗
FRESH_042	29:02	30:02	07:02	44:03	07:01	07:02	III	1	1,50E+04	✓	✗
FRESH_044	01:01	02:05	42:02	81:01	17:01	18	I	1	6,50E+04	✓	✗

*Deleterious HLA class I alleles (red), **protective HLA class I alleles (green).

Supplementary Figure 1

In this cohort of HIV-1 subtype C, genome deletions were most frequently observed between between *integrase* and *envelope* relative to *Gag* ($p<0.0001-0.001$).





Supplementary Figure 2

Clonal expansion of infected cells was detected in both defective (orange) and intact (blue) genomes in late and early treated study participants. This analysis was performed with all sequences available for each participant at all time points.

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Reviewer #1 (Public review):

The authors sought to determine the impact of early antiretroviral treatment on the size, composition, and decay of the HIV latent reservoir. This reservoir represents the source of viral rebound upon treatment interruption and therefore constitutes the greatest challenge to achieving an HIV cure. A particular strength of this study is that it reports on reservoir characteristics in African women, a significantly understudied population, of whom some have initiated treatment within days of acute HIV diagnosis. With the use of highly sensitive and current technologies, including digital droplet PCR and near full-length genome next-generation sequencing, the authors generated a valuable dataset for investigation of proviral dynamics in women initiating early treatment compared to those initiating treatment in chronic infection. The authors confirm previous reports that early antiretroviral treatment restricts reservoir size, but further show that this restriction extends to defective viral genomes, where late treatment initiation was associated with a greater frequency of defective genomes. Furthermore, an additional strength of this study is the longitudinal comparison of viral dynamics post-treatment, wherein early treatment was shown to be associated with a more rapid rate of decay in proviral genomes, regardless of intactness, over a period of one year post-treatment. While it is indicated that intact genomes were not

detected after one year following early treatment initiation, sampling depth is noted as a limitation of the study by the authors, and caution should thus be taken with interpretation where sequence numbers are low. Defective genomes are more abundant than intact genomes and are therefore more likely to be sampled. Early treatment was also associated with reduced proviral diversity and fewer instances of polymorphisms associated with cytotoxic T-lymphocyte immune selection. This is expected given that rapid evolution and extensive immune selection are synonymous with HIV infection in the absence of treatment, yet points to an additional benefit of early treatment in the context of immune therapies to restrict the reservoir.

This is one of the first studies to report the mapping of longitudinal intactness of proviral genomes in the globally dominant subtype C. The data and findings from this study therefore represent a much-needed resource in furthering our understanding of HIV persistence and informing broadly impactful cure strategies. The analysis on clonal expansion of proviral genomes may be limited by higher sequence homogeneity in hyperacute infection i.e., cells with different proviral integration sites may have a higher likelihood of containing identical genomes compared to chronic infection.

Overall, these data demonstrate the distinct benefits of early treatment initiation at reducing the barrier to a functional cure for HIV, not only by restricting viral abundance and diversity but also potentially through the preservation of immune function and limiting immune escape. It therefore provides clues to curative strategies even in settings where early diagnosis and treatment may be unlikely.

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Reviewer #2 (Public review):

HIV infection is characterized by viral integration into permissive host cells - an event that occurs very early in viral-host encounter. This constitutes the HIV proviral reservoir and is a feature of HIV infection that provides the greatest challenge for eradicating HIV-1 infection once an individual is infected.

This study looks at how starting HIV treatment very early after infection, which substantially reduces the peak viral load detectable (compared to untreated infection), affects the amount and characteristics of the viral reservoir. The authors studied 35 women in South Africa who were at high risk of getting HIV. Some of these women started HIV treatment very soon after getting infected, while others started later. This study is well designed and has as its focus a very well characterized cohort. Comparison groups are appropriately selected to address proviral DNA characterization and dynamics in the context of acute and chronic treated HIV-1. The amount of HIV and various characteristics of the genetic makeup of the virus (intact/defective proviral genome) was evaluated over one year of treatment. Methods employed for proviral DNA characterization are state of the art and provide in-depth insights into the reservoir in peripheral blood.

While starting treatment early didn't reduce the amount of HIV DNA at the outset, it did lead to a gradual decrease in total HIV DNA quantity over time. In contrast, those who started treatment later didn't see much change in this parameter. Starting treatment early led to a faster decrease in intact provirus (a measure of replication-competence), compared to starting treatment later. Additionally, early treatment reduced genetic diversity of the viral DNA and resulted in fewer immune escape variants within intact genomes. This suggests that collectively having a smaller intact replication-competent reservoir, less viral variability, and less opportunity for virus to evade the immune system - are all features that are likely to facilitate more effective clearance of viral reservoir, especially when combined with other intervention strategies.

Major strengths of the study include the cohort of very early treated persons with HIV and the depth of study. These are important findings, particularly as the study was conducted in HIV-1 subtype C infected women (more cure studies have focussed on men and with subtype B infection)- and in populations most affected by HIV and in need of HIV cure interventions. This is highly relevant because it cannot be assumed that any interventions employed for reducing/clearing the HIV reservoir would perform similarly in men and women or across different populations. Other factors also deserve consideration and include age, and environment (e.g. other comorbidities and coinfections).

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Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public reviews):

(1) Given that this is one of the first studies to report the mapping of longitudinal intactness of proviral genomes in the globally dominant subtype C, the manuscript would benefit from placing these findings in the context of what has been reported in other populations, for example, how decay rates of intact and defective genomes compare with that of other subtypes where known.

Most published studies are from men living with HIV-1 subtype B and the studies are not from the hyperacute infection phase and therefore a direct head-to-head comparison with the FRESH study is difficult. However, we can cite/highlight and contrast our study with a few a few examples from acute infection studies as follows.

- a. Peluso et. al., JCI, 2020, showed that in Caucasian men (SCOPE study), with subtype B infection, initiating ART during chronic infection virus intact genomes decayed at a rate of 15.7% per year, while defective genomes decayed at a rate of 4% per year. In our study we showed that in chronic treated participants genomes decreased at a rate of 25% (intact) and 3% (defective) per month for the first 6 months of treatment.
- b. White et. al., PNAS, 2021, demonstrated that in a cohort of African, white and mixed-race American men treated during acute infection, the rate of decay of intact viral genomes in the first phase of decay was <0.3 logs copies in the first 2-3 weeks following ART initiation. In the FRESH cohort our data from acute treated participants shows a comparable decay rate of 0.31 log copies per month for virus intact genomes.
- c. A study in Thailand (Leyre et. al., 2020, Science Translational Medicine), of predominantly HIV-1 CRF01-AE subtype compared HIV-reservoir levels in participants starting ART at the earliest stages of acute HIV infection (in the RV254/SEARCH 010 cohort) and participants initiating ART during chronic infection (in SEARCH 011 and RV304/SEARCH 013 cohorts). In keeping with our study, they showed that the frequency of infected cells with integrated HIV DNA remained stable in participants who initiated ART during chronic infection, while there was a sharp decay in these infected cells in all acutely treated individuals during the first 12 weeks of therapy. Rates of decay were not provided and therefore a direct comparison with our data from the FRESH cohort is not possible.
- d. A study by Bruner et. al., Nat. Med. 2016, described the composition of proviral populations in acute treated (within 100 days) and chronic treated (>180 days), predominantly male subtype B cohort. In comparison to the FRESH chronic treated group, they showed that in

chronic treated infection 98% (87% in FRESH) of viral genomes were defective, 80% (60% in FRESH) had large internal deletions and 14% (31% in FRESH) were hypermutated. In acute treated 93% (48% in FRESH) were defective and 35% (7% in FRESH) were hypermutated. The differences frequency of hypermutations could be explained by the differences in timing of infection specifically in the acute treated groups where FRESH participants initiate ART at a median of 1 day after infection. It is also possible that sex- or race-based differences in immunological factors that impact the reservoir may play a role.

This study also showed that large deletions are non-random and occur at hotspots in the HIV-1 genome. The design of the subtype B IPDA assay (Bruner et. al., Nature, 2019) is based on optimal discrimination between intact and deleted sequences - obtained with a 5' amplicon in the Ψ region and a 3' amplicon in Envelope. This suggest that Envelope is a hotspot for large while deletions in Ψ is the site of frequent small deletions and is included in larger 5' deletions. In the FRESH cohort of HIV-1

subtype C, genome deletions were most frequently observed between Integrase and Envelope relative to Gag ($p < 0.0001-0.001$).

e. In 2017, Heiner et. al., in Cell Rep, also described genetic characteristics of the latent HIV-1 reservoir in 3 acute treated and 3 chronic treated male study participants with subtype B HIV. Their data was similar to Bruner et. al. above showing proportions of intact proviruses in participants who initiated therapy during acute/early infection at 6% (94% defective) and chronic infection at 3% (97% defective). In contrast the frequencies in FRESH in acute treated were 52% intact and 48% defective and in chronic infection were 13% intact and 87% defective. These differences could be attributed to the timing of treatment initiation where in the aforementioned study early treatment ranged from 0.6-3.4 months after infection.

(2) Indeed, in the abstract, the authors indicate that treatment was initiated before the peak. The use of the term 'peak' viremia in the hyperacute-treated group could perhaps be replaced with 'highest recorded viral load'. The statistical comparison of this measure in the two groups is perhaps more relevant with regards to viral burden over time or area under the curve viral load as these are previously reported as correlates of reservoir size.

We have edited the manuscript text to describe the term peak viraemia in hyperacute treated participants more clearly (lines 443-444). We have now performed an analysis of area under the curve to compare viral burden in the two study groups and found associations with proviral DNA levels after one year. This has been added to the results section (lines 162-163).

Reviewer #2 (Public reviews):

(1) Other factors also deserve consideration and include age, and environment (e.g. other comorbidities and coinfections.)

We agree that these factors could play a role however participants in this study were of similar age (18-23), and information on co-morbidities and coinfections are not known.

Reviewer #3 (Public reviews):

(1) The word reservoir should not be used to describe proviral DNA soon after ART initiation. It is generally agreed upon that there is still HIV DNA from actively infected cells (phase 1 & 2 decay of RNA) during the first 6-12 months of ART. Only after a full year of uninterrupted ART is it really safe to label intact proviral HIV DNA as an approximation of the reservoir. This should be amended throughout.

We agree and where appropriate have amended the use of the word reservoir to only refer to the proviral load after full viral suppression, i.e., undetectable viral load.

(2) All raw, individualized data should be made available for modelers and statisticians. It would be very nice to see the RNA and DNA data presented in a supplementary figure by an individual to get a better grasp of intra-host kinetics.

We will make all relevant data available and accessible to interested parties on request. We have now added a section on data availability (lines 489-491).

(3) The legend of Supplementary Figure 2 should list when samples were taken.

The data in this figure represents an overall analysis of all sequences available for each participant at all time points. This has now been explained more clearly in the figure legend.

Recommendations for the authors:

Reviewer #1 (Recommendations for The Authors):

(1) It is recommended that the introduction includes information to set the scene regarding what is currently reported on the composition of the reservoir for those not in the immediate field of study i.e., the reported percentage of defective genomes and in which settings/populations genome intactness has been mapped, as this remains an area of limited information.

We have now included summary of other reported findings in the field in the introduction (lines 89-92, 9498) and discussion (lines 345-350). A more detailed overview has been provided in the response to public reviews.

(2) It may be beneficial to state in the main text of the paper what the purpose of the Raltegravir was and that it was only administered post-suppression. Looking at Table 1, only the hyperacute treatment group received Raltegravir and this could be seen as a confounder as it is an integrase inhibitor. Therefore, this should be explained.

Once Raltegravir became available in South Africa, all new acute infections in the study cohort had an intensified 4-drug regimen that included Raltegravir. A more detailed explanation has now been included in the methods section (lines 435-437).

(3) Can the authors explain why the viral measures at 6 months post-ART are not shown for chronic-treated individuals in Figure 1 or reported on in the text?

The 6 months post-ART time point has been added to Figure 1.

(4) Can the authors indicate in the discussion, how the breakdown of proviral composition compares to subtype B as reported in the literature, for example, are the common sites of deletion similar, or is the frequency of hypermutation similar?

Added to discussion (lines 345-350).

(5) Do the numbers above the bars in Figure 3 represent the number of sampled genomes? If so, this should be stated.

Yes, the numbers above the bars represent the number of sampled genomes. This has been added to the Figure 3 legend.

(6) In the section starting on line 141, the introduction implies a comparison with immunological features, yet what is being compared are markers of clinical disease progression rather than immune responses. This should be clarified/corrected.

This has been corrected (line 153).

(7) Line 170 uses the term 'immediately' following infection, however, was this not 1-3 days after?

We have changed the word “immediately” to “1-3 days post-detection” (line 181).

(8) Can the sampling time-points for the two groups be given for the longitudinal sequencing analysis?

The sequencing time points for each group is depicted in Figure 2.

(9) Line 183 indicates that intact genomes contributed 65% of the total sequence pool, yet it's given as 35% in the paragraph above. Should this be defective genomes?

Yes, this was a typographical error. Now corrected to read “defective genomes” (line 193).

(10) The section on decay kinetics of intact and defective genomes seems to overlap with the section above and would flow better if merged.

Well noted, however we choose to keep these sections separate.

(11) Some references in the text are given in writing instead of numbering.

This has been corrected.

(12) In the clonal expansion results section, can it be indicated between which two time-points expansion was measured?

This analysis was performed with all sequences available for each participant at all time points. We have added this explanation to the respective Figure legend.

Reviewer #2 (Recommendations for The Authors):

(1) The statement on line 384 "Our data showed that early ART...preserves innate immune factors" - what innate immune factors are being referred to?

We have removed this statement.

(2) HLA genotyping methods are not included in the Methods section

Now included and referenced (lines 481-483).

(3) Are CD4:CD8 ratios available for the cohorts? This could be another informative clinical parameter to analyse in relation to HIV-1 proviral load after 1 year of ART – as done for the other variables (peak VL, and the CD4 measures).

Yes, CD4:CD8 ratios are available. We performed the recommended analysis but found no associations with HIV-1 proviral load after 1 year of ART. We have added this to the results

section (lines 163-164).

(4) Reference formatting: Paragraph starting at line 247 (Contribution of clonal expansion...) - the two references in this paragraph are not cited according to the numbering system as for the rest of the manuscript. The Lui et al, 2020 reference is missing from the reference list - so will change all the numbering throughout.

This has been corrected.

Reviewer #3 (Recommendations for The Authors):

(1) To allow comparison to past work. I suggest changing decay using % to half-life. I would also mention the multiple studies looking at total and intact HIV DNA decay rates in the intro.

We do not have enough data points to get a good estimate of the half-life and therefor report decay as percentage per month for the first 6 months.

(2) Line 73: variability is the wrong word as inter-individual variability is remarkably low. I think the authors mean "difference" between intact and total.

We have changed the word variability to difference as suggested.

(3) Line 297: I am personally not convinced that there is data that definitively shows total HIV DNA impacting the pathophysiology of infection. All of this work is deeply confounded by the impact of past viremia. The authors should talk about this in more detail or eliminate this sentence.

We have reworded the statement to read “Total HIV-1 DNA is an important biomarker of clinical outcomes.” (Lines 308-309).

(4) Line 317; There is no target cell limitation for reservoir cells. The vast majority of CD4+ T cells during suppressive ART are uninfected. The mechanism listing the number of reservoir cells is necessarily not target cell limitation.

We agree. The statement this refers to has been reworded as follows: “Considering, that the majority of CD4 T cells remain uninfected it is likely that this does not represent a higher number of target cells, and this warrants further investigation.” (lines 325-326).

(5) Line 322: Some people in the field bristle at the concept of total HIV DNA being part of the reservoir as defective viruses do not contribute to viremia. Please consider rephrasing.

We acknowledge that there are deferring opinions regarding total HIV DNA being part of the reservoir as defective viruses do not contribute to viremia, however defective HIV proviruses may contribute to persistent immune dysfunction and T cell exhaustion that are associated comorbidities and adverse clinical outcomes in people living with HIV. We have explained in the text that total HIV-DNA does not distinguish between replication-competent and -defective viruses that contribute to the viral reservoir.

(6) Line 339: The under-sampling statement is an understatement. The degree of under-sampling is massive and biases estimates of clonality and sensitivity for intact HIV. Please see and consider citing work by Dan Reeves on this subject.

We agree and have cited work by Dan Reeves (line 358).

(7) Line 351: This is not a head-to-head comparison of biphasic decay as the Siliciano group's work (and others) does not start to consider HIV decay until one year after ART. I think it is important to not consider what happens during the first year of ART to be reservoir decay necessarily.

Well noted.

(8) Line 366-371: This section is underwritten. In nearly all PWH studies to date, observed reservoirs are highly clonal.

We agree that observed reservoirs are highly clonal but have not added anything further to this section.

(9) It would be nice to have some background in the intro & discussion about whether there is any a priori reason that clade C reservoirs, or reservoirs in South African women, might differ (or not) from clade B reservoirs observed in different study participants.

We have now added this to the introduction (lines 94-103).

(10) Line 248: This sentence is likely not accurate. It is probable that most of the reservoir is sustained by the proliferation of infected CD4+ T cells. 50% is a low estimate due to under-sampling leading to false singleton samples. Moreover, singletons can also be part of former clones that have contracted, which is a natural outcome for CD4+ T cells responding to antigens &/or exhibiting homeostasis. The data as reported is fine but more complex ecologic methods are needed to truly probe the clonal structure of the reservoir given severe under sampling.

Well noted.

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