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AIDS Rev. 2021;23:65-73

How Does HIV Persist Under Antiretroviral Therapy: A Review of the Evidence

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Abstract

HIV-1 is a retrovirus capable of establishing viral reservoirs that remain stable for extended periods under suppressive antiretroviral therapy (ART). Immune dysfunction and latency are well known to contribute to this longevity, but the respective roles of viral replication and latently infected (LI) cell proliferation under suppressive antiretroviral therapy (ART) have long been controversial. This historical review critically appraises the body of evidence regarding possible viral replication and proliferation of infected cells under ART. An ever-growing body of genetic and phylogenetic studies has demonstrated that HIV-infected cells are able to proliferate and contribute to the longevity of the reservoir in ART-treated patients. The role of ongoing replication remains controversial: it has been well established that HIV does not undergo evolution during ART or develop drug resistance, but some genetic, phylogenetic, and in vivo imaging studies have suggested that there may be ongoing replication despite this. The respective roles of viral replication and cellular proliferation in maintaining the LI reservoir remains an area of controversy. Elucidating these processes may allow us design interventions to reduce the size of the LI reservoir, increasing the length of treatment interruptions during which the virus will remain adequately suppressed, bringing us closer to a functional cure. Novel experimental techniques such as immuno-PET and digital droplet PCR (ddPCR) are increasingly being employed, and these, along with rapid particle sorting techniques currently in development, will be necessary to fully answer this question. (AIDS Rev. 2021;23:65-73)

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Key words

HIV reservoir. Antiretroviral therapy. Replication. Proliferation. Latent infection.

Introduction

Antiretroviral therapy (ART) is effective in rapidly suppressing viremia in patients with HIV. It was predicted that 3.1 years of therapy would result be curative[1], however, even after extended therapy, a reservoir of replication competent (RC) HIV remains meaning that viremia rebounds ~2 weeks after cessation of treatment[2]. Viral reservoirs remain stable over extended periods of treatment through induction of immune dysfunction and latency[3]. The degree to which ongoing viral replication and proliferating latently infected (LI) cells also contribute to this is controversial topic. There is now compelling evidence that proliferation of LI cells plays a role in the maintenance of the reservoir, although how this is controlled, and whether any role is played by viral replication remains controversial.

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Received in original form: 26-01-2021
Received in final form: 21-02-2021
DOI: 10.24875/AIDSRev.21000004

What is the evidence that LI cells can proliferate?

Originally, the idea that LI cells could proliferate without being rapidly eliminated was considered unlikely as the half-life of productively infected cells is very short[4,5] due to cytopathy and immune elimination. A series of papers using partial genome sequencing reported a lack of viral evolution overtime[6-9] and the presence of identical viral sequences within patient samples overtime[8,9] The implication of a lack of viral evolution and multiple identical sequences is the provirus which has been copied by host polymerases, rather than the error-prone viral replication machinery which would introduce mutations into the HIV genome[10], and therefore, the conclusion drawn was that the reservoir must be maintained by cellular proliferation, not replication. There were, however, several limitations to this body of literature:

1. The papers relied on a limited range of techniques, sequencing either integrated HIV DNA, plasma RNA, or both. Only selected segments of the genetic material were sequenced; failure to identify mutations in the rest of the genome may lead to underestimation of genetic diversity. One study noted a prolonged delay in restoration of viral diversity after treatment interruption[11], which they argued represented an evolutionary bottleneck, however, it could be interpreted as a failure of the partial genome sequencing to detect the increase in genetic diversity that would be expected with ART interruption.
2. There is no assessment of replicative capacity; replication incompetent viruses, which comprise most of the reservoir, are less likely to cause cytopathy, making them more likely to enrich overtime through host cell proliferation, meaning that their inclusion will overestimate proliferation.
3. This technique cannot rule out the possibility that multiple cells are infected by a dominant viral variant[12].
4. The papers mostly only consider one or two compartments and, therefore, can only draw conclusions concerning an absence of evolution in those compartments. Replication may occur in an isolated compartment.

With the recognition that integration into the same chromosomal locus during different infection events is unlikely[13], integration site analysis was incorporated into later studies to more accurately evaluate clonality. Wagner et al. found multiple shared integration points within each patient throughout follow-up, with none shared between patients, but found that, in all patients > 50% of integration sites were unique, providing a more conservative estimate of the importance of proliferation than the partial sequencing body of literature would suggest[14]. Two similar papers reported 57%[15] and 60%[16] unique integration sites. It remains an issue, however, that the proportion of viruses with repeated or unique integration sites that are RC is not determined, which means that one cannot conclude which, if either, of these populations are a reservoir of virus with proliferative capacity.

One group noted a replication incompetent clone present in many T cells[9] which expanded overtime as evidence of clonal proliferation of LI cells. While this is compelling evidence that integrated HIV DNA can be copied during normal cellular proliferation processes[13], it does not indicate whether or not cells infected with RC virus are capable of proliferating. One patient has been reported as carrying an HIV clone was both heavily expanded and RC[17]. This provides good evidence that it is possible for RC HIV to undergo clonal expansion *in vivo*. However, if, as the authors theorized, tumor antigens were stimulating the proliferation of this clone, it is likely that this immune stimulation was stronger and more sustained than would be the case for most infected cells, thereby allowing the clones to proliferate despite cytopathy and immune destruction. A survey of 75 heavily expanded clones found that none of them was an intact, integrated provirus, suggesting that extensive proliferation of cells infected with RC virus is rare[16].

Near full-length genome sequencing has been employed more recently; it does not underestimate mutation rates and allows at least qualitative assessment of replicative capacity. Hiener et al., 2017, reported that all six of their participants' samples contained identical proviral sequences, however, 92% of the identical sequence expansions contained non-replicative provirus[18]. In three participants, they identified genetically intact, identical proviruses, providing further evidence that proliferation of RC HIV-infected cells is possible. Lee et al., 2017, also identified multiple identical HIV sequences in ART-treated individuals, of which 62% were believed to be replication and infection competent[19]. This evidence, while compelling, is not definitive as the authors did not perform integration site analyses, which, if it had shown the sequences to be at the same locus on the human genome, would prove definitively that they are the result of proliferation rather

than the product of multiple infections by a dominant variant. Although they assessed replicative capacity qualitatively, it would be more rigorous to confirm these assessments with viral outgrowth assays (VOAs).

Some groups sequenced the products of VOAs, thereby ensuring that the genomes sequenced were from viruses capable of replicating. Hosmane et al. reported that 57% of *env* sequences from RC virus derived from different infected cells were identical within each sample[20]. They demonstrated that this was unlikely to be accounted for by infection with a dominant variant, as this would be dependent on replication by the error-prone HIV reverse transcriptase, would be expected to result in multiple closely related sequences to the dominant variant, which were not found and Bui et al. confirmed multiple identical viral sequences from different wells in the VOAs using near full-length sequencing, with a median of 57% of the RC viruses having identical sequence matches in other wells, with an identical near full-length sequence being very unlikely to result from replication[21]. While these probabilistic calculations likely do exclude widespread infection by a dominant variant, this could be further confirmed with integration site analysis. Overall, these papers provide compelling evidence that it is possible for LI cells with RC HIV to proliferate.

How can proliferation contribute to maintaining the reservoir?

It was thought that the proliferation of latently infected cells would result in the cells becoming productive, rendering them subject to cytopathy or immune elimination. A recent study found that multiple rounds of maximal T-cell stimulation were needed to trigger viral replication, despite > 99% dividing with each stimulation; only a mean of 60% was activated after the first round[20] This suggests that LI cells can divide without becoming productive and although the extended culture conditions may alter the results from the later rounds, the results from the early rounds of stimulation are sufficient to make this argument alone. If LI cells can proliferate without becoming active, they would not be subject to the short half-lives of productively infected cells, thereby making proliferation a viable explanation for the maintenance of the reservoir. Multiple mechanisms have been suggested for why proliferation does not always lead to viral replication including integration into transcriptionally inactive areas, and expression of genes inhibiting viral transcription or suppressing apoptosis[22]. This also has implications for the "kick and kill" approach to cure; as multiple rounds of maximal activation would be required to activate all infected cells, although any activation leading to a reduction in reservoir size would still increase the time needed for viremia to rebound, which is a more realistic goal[23].

Why do LI cells proliferate?

There are three main possibilities for the mechanisms driving proliferation: integration site-driven proliferation, antigen-driven proliferation, and homeostatic proliferation.

Two of the early papers that analyzed integration sites sought to assess the hypothesis that integration into genes associated with cancer would make infected cells more likely to proliferate. Maldarelli et al. reported that in one patient, a much greater proportion of the integrations were into two cancer associated genes as compared to integration libraries of acutely infected HeLa cells and CD34+ hematopoietic stem cells[15]. The integrations were reported to be consistent with altering the expression or structure of these proteins, putatively influencing cell survival and proliferation. Similarly, Wagner et al. reported a greater frequency of integrations into cancer-associated genes between proliferating patient cells and the controls; acutely infected CD4+ cells[14]. Since proliferating cells under ART are latently, not acutely, infected, the acutely infected HeLa cells and CD4+ T cells are not good controls as acute infection substantially alters gene expression[24]. Despite this, integration into cancer genes increasing the likelihood of cell survival and proliferation is a common feature of retroviruses[25], and may help contribute to the development of lymphomas associated with HIV. Conversely, Cohn et al. reported that the preference for integration into cancer genes was not significant, being merely similar to the preference for highly expressed genes. Furthermore, they reported a lack of overrepresentation of clonally expanded cells with integration sites in cancer genes, suggesting that integration into cancer genes is unlikely to contribute to proliferation[16]. Although this area remains controversial, if future studies reveal that disruption of cancer genes is an important cause of HIV-infected cell proliferation we should investigate repurposing drugs developed to reverse these changes in cancer cells. RITA, a small molecule which reactivates p53 causing apoptosis in cells, such as cancer cells, with proapoptotic signalling[26] may be one potential avenue – p53 has previously been reported to suppress HIV infection by multiple mechanisms[27].

CD4+ T cells latently infected with RC provirus can proliferate *in vitro* in response to cytokines and T-cell receptor agonists[28]. This proliferation was shown to occur without becoming productive, while retaining the capacity for viral replication, which could be induced in daughter cells. It will likely be very difficult to target this mode of proliferation, if it occurs *in vivo*, without also disrupting normal immune function unless biomarkers specific to latently infected cells can be identified and utilized to target treatments[29]. As this study was performed *in vitro*, under extended culture conditions, cellular stress may have influenced the results, as may the absence of other cell types. The *in vivo* picture is harder to establish. A recent high profile ddPCR study suggested that cells infected with intact proviruses are less likely to proliferate *in vivo* in response to T-cell receptor stimulation, however, even if antigen proliferation is less likely *in vivo*, it may contribute significantly in at least some patients[30]. A recent study by Mendoza et al.[22] demonstrated intact HIV proviruses found in CD4+ T cells that respond to antigens from common chronic or recurrent viruses in 3/8 patient samples, some of which were demonstrated to be part of clonal populations and could be matched to replication competent proviruses identified in the same patient by VOA.

The proliferation of LI stem cells has recently been suggested to be another mechanism driving proliferation. Hematopoietic stem and progenitor cells can serve as long-term reservoirs of HIV[31], can produce infected daughter cells[32], and are a source of clonally amplified residual plasma virus in treated patients[33].

One recently developed model suggests that extracellular vesicles carrying viral RNA and pro-inflammatory factors released by HIV-infected cells interacting with uninfected cells may create a feedback loop of pro-inflammatory factors, leading both to increased proliferation and reactivation of viral transcription[34].

Does replication occur *in vivo* under suppressive ART?

The lack of evidence of viral evolution in the early partial sequencing literature[6-9] led some groups to argue that replication does not occur in any significant capacity but later integration site analyses[14-16] and sequencing of VOAs reported more conservative estimates of infected cell proliferation (all < 60%)[18-21] potentially implying a greater role for replication in maintenance of the viral reservoir. Crucially, a recent paper employing multiple displacement amplification to more fully map viral ancestry suggests that identical proviruses can result not only from cellular proliferation but also from genetic bottlenecks occurring either before or under ART[35].

Two recent genetic modeling studies have suggested that there is negligible contribution to the reservoir by replication. One group reanalyzed genetic sequencing literature, generating a model predicting that the larger the samples taken, the greater the findings of clonality were likely to be, and that after 1 year of ART, > 99% of infected cells would be shown to be members of clonal populations if a sufficiently large sample was taken[13]. Being extrapolated from genetic sequencing literature, the model is subject to the same caveats, perhaps most crucially the underestimation of diversity resulting from sequencing only partially. Another group performed phylogenetic modeling on blood samples from two patients to estimate integration dates[36]. In participant 1, they did not identify single HIV sequence integrating into the genome during the period of suppression, suggesting that there may be some patients in which no significant replication occurs during ART. Some new sequence integration was detected in the second participant; this difference may be due to sampling methods (e.g., having only used partial genome sequencing). While the authors account for variable evolutionary rates in participant 2, they acknowledge various shortcomings in their chosen model (e.g., not accounting for potential multiple latent periods). The use of only blood samples means that, even if no new virus integrated the blood in participant 1, the findings do not necessarily apply to other compartments which are a significant issue as the latent reservoir is known to reside in peripheral tissues. Finally, sampling from only two participants limits the generalizability of the results.

Conversely, another recent high-profile genetic analysis study concluded that significant viral replication does continue during ART. The group used deep sequencing of HIV DNA from blood and lymph nodes, which they argued would more reliably detect low-frequency HIV variants than the partial sequencing techniques used previously[37,38]. They reported new mutations in multiple compartments and phylogenies consistent with random mutation occurring at a constant rate matching the estimated rate of viral mutation. They also set out a model explaining the lack of ART resistance[39] if the fitness cost of drug resistance mutations means that non-drug-resistant virus is selected for in drug sanctuaries, and the drug concentration

outside a sanctuary is too high even for resistant strains to replicate. The authors do, however, note difficulties in differentiating between low-level replication in lymph nodes and reactivation of LI cells. Alternative explanations for the appearance of evolution in this study include failure to account for PCR resampling and hypermutation[40] or differential decay of viral populations which are replenished when untreated but have different half-lives under ART; unintegrated provirus (days), infected resting cells (weeks), and integrated proviruses in blood and lymph (4 years)[41].

Animal models can an alternative line of evidence for viral replication *in vivo*, which may help to clarify many controversies of the viral genetics literature. Tissue reservoirs have long been known to exist in animal models of HIV and are being increasingly well characterized, with modern techniques such as next-generation *in situ* hybridization allowing us to identify HIV DNA and RNA levels in postmortem samples from multiple potential reservoirs[42]. In an SIV macaque model, immuno-PET using[64]. Cu-labeled antibodies against SIV gp120 revealed active viral replication in lymphoid tissue, gut, nasal turbines, lungs, and genital tract, even in ART treated, aviremic monkeys[43]. The macaques had only been on ART for 40 days so it is questionable whether this replication continues to any relevant level after years of therapy, or even if the level of replication detected is of any pathological significance, as well as whether this holds true for HIV given that there are differences between how the viruses respond to treatment[44]. Longer term follow-up of these experimental animals would be of significant benefit to determine if the rate of replication appears to change overtime, but the ultimate aim of this technology would be to develop *in vivo* nuclear imaging of HIV in humans[45].

If replication does continue to occur, how does it do so?

A popular theory is that viral replication occurs in a sanctuary into which ART is unable to penetrate at fully effective concentrations. One particularly influential early study reported that, even after 6 months of therapy, ART concentrations are lower in the lymph nodes than in the blood[46]. This was found to be significantly correlated with a slowing of the rate of decay of the follicular dendritic cell virion pool and detection of viral RNA in productively infected cells in 4/9 patients, providing a mechanism that may explain continual replication in the lymph nodes of some patients. This finding appears consistent with phylogenetic mapping studies which suggested that replication in lymph nodes gives rise to viral lineages which then migrate into the blood[37]. A subset of lymph node T follicular helper cells displaying viral transcription even in viremic patients after 12 years of ART treatment has been identified[47], however, this does not necessarily mean that they are producing virions or that any virions produced are capable of infection; this could be confirmed by VOA. A large body of papers has shown a lack of genetic compartmentalization between blood and putative sanctuary sites, which fails to address the argument that unique sequences may be generated by replication within sanctuaries and then escape. A recent paper showing a lack of evidence of evolution within lymph nodes[48] is more compelling, but this may be a result of a genetic bottleneck rather than a lack of replication[35], meaning that these findings represent a lack of evidence for replication, rather than being evidence against it.

There is good evidence to support compartmentalization within the CNS, and some evidence of compartmentalization in vaginal, testicular, gut, and lymphoid tissues[49], but there is currently no phylogenetic evidence[50] and an insufficiency of other evidence to conclude whether or not there is ongoing replication under ART (Table 1)[51-58].

Multiple simultaneous infections, by cell-cell spread, have been suggested to allow for ongoing intermittent replication *in vitro* despite clinical ART concentrations[59] They predict that replication would be insufficient to sustain itself, in part due to increased cytopathy with multiple infections, but that with input from other reservoirs such as reactivating LI cells, this would create a steady state in which many new cells are infected, but without substantial accumulation of mutations per cell and therefore little viral evolution. A more recent study modeling the possibility concluded that multiple infection may be able to attain a low steady state, even with effective ART, if cells are at reduced risk of death due to being at low density[60], however, given that cell-to-cell transmission will result in a higher density of infected cells, it seems unlikely that this condition would be fulfilled. These models do not go on to consider the roles of macrophages which have a unique role in cell-cell transmission of HIV. Macrophages use chemotaxis to seek out and phagocytose infected T cells, making themselves susceptible to high multiplicity infection, and then interact with their target $CD4^+$ T cells, forming virological synapses for ongoing multiple infection[61]. Macrophages should, therefore, be considered a potentially key contributor to cell-cell transmission

Table 1. A brief summary of literature regarding ongoing HIV replication in potential ART sanctuaries

Tissues	Evidence	Caveats
Testicles	Jenabian et al., 2016[51], identifies viral DNA in testicular samples from treated, aviremic individuals, and hypothesize that their immune privilege and the blood-testis barrier may allow them to serve as a viral sanctuary. Miller et al., 2019[52], analyzed the genetics of proviruses sampled from blood and testes of 10 individuals finding that 60% exhibited a degree of genetic compartmentalization but that none had unique sequences in either.	Jenabien et al. provided no evidence of viral replication taking place in these tissues during the period of ART. Lack of genetic diversity does not necessarily indicate a lack of replication[35], and there is the possibility that unique sequences formed in the testes and migrated into the blood[40].
Urethra	Ganor et al., 2019[53], reported HIV-1 DNA, RNA, and virions in urethral macrophages of aviremic patients on ART. They demonstrate that reactivation of these macrophages results in productive infection.	Although compelling, this is not definitive evidence of active and productive viral replication. The virions may represent stored virions formed before the initiation of ART rather than an active infection. There is no evidence that any RNA transcribed or that any virions produced are going on to infect other cells. They do not perform any sequencing or integration site analyses that might have demonstrated the hallmarks of ongoing replication.
Gut	Yukl et al., 2010[54], reported that ART intensification led to a reduction in levels of HIV-1 RNA in the terminal ileum in patients who were already aviremic, suggesting that under some ART regimens, there may still be pockets of continuing replication.	There are no controls for the intensification; although the RNA levels fall in comparison to previous results from the same patients, this does not account for the possibility that inclusion in the trial may have altered patients' outcomes, for example, by improving adherence to treatment. Small sample size (7 patients), not double blind.
Brain	Gama et al., 2017[55], reported that the most abundant SIV genotype in the CSF of a monkey following reactivation with a latency reversing agent was genetically independent from those in the periphery, which suggests that viral replication and evolution may be able to occur in this compartment without being influencing in the peripheral blood samples.	Gama et al.'s findings appear to be unique to the monkeys given latency reversing agents and this is not seen in monkeys where viral rebound is caused by ART withdrawal. This is not consistent with the idea that the reservoirs are functionally separate under normal circumstances.
	Dahl et al., 2014[56], reported the findings of genetically distinct plasma and CSF sequences in one aviremic patient on suppressive therapy. Genetically distinct lineages are expected only with compartmentalized replication[38]. Oliveira et al., 2017[57], detected genetic compartmentalization in 7/8 patients from which they had paired samples. They measured no longitudinal evolution but this may be because they were only able to gather follow-up samples from two patients.	Dahl et al.'s findings may indicate that while compartmentalization occurs in some patients, but it may be a rare occurrence – they only found this phenomenon in 1/17 participants – and may, therefore, be rare or a result of patient specific factors such as non-adherence. The more sensitive sampling carried out by Oliveira et al., provided strong evidence of compartmentalization, however, this may have been established prior to ART initiation, and so itself is not evidence of ongoing replication under ART.
	Anderson et al., 2017[58], found that lower ART central nervous system penetration was associated with higher concentrations of HIV RNA in the cerebrospinal fluid in samples taken from 220 aviremic patients.	Initially designed as a cross-sectional study, with a relatively small longitudinal follow-up (55 patients). Requires corroboration from an independent study. RNA alone does not necessarily indicate production of infection capable virions.

which must be considered in further studies in this field.

There is a question as to whether immune privilege in certain sanctuary sites may contribute to the any ongoing viral replication in these tissues[62]. SHIV RNA-positive cells are compartmentalized in secondary lymphoid tissues during chronic SHIV infection, but not at <14 days infection or in SAIDS, and SHIV RNA-positive cells are inversely distributed to SIV-specific cytotoxic T lymphocytes (CTLs) during chronic infection[63]. The authors argue that the acute infection is before the induction of a strong CTL response, and that this response is weakened in SAIDs, and that, therefore, CTLs suppress viral transcription throughout most of the body but are unable to do so in immune privileged sites. There is a paucity of evidence regarding the significance of immune privileged sites in ART-treated subjects.

Could replication be occurring without contributing to the longevity of the viral reservoir?

Other infectious agents can cause activation of the immune system resulting in both the proliferation of latently infected cells[22] and viral replication resulting in transient viremia in patients who have otherwise achieved viral suppression. In one model, reactivated cells undergo a few rounds of replication before becoming extinct, meaning that there is no evolution and drug resistance is unlikely, as observed clinically[64]. They conclude based on this model that replication may occur but would not contribute to the development of a LI reservoir. This model, however, does not consider the possibility of newly infected cells returning to quiescence, which is believed to be how the reservoir is established initially[65], and to occur throughout infection[66], and may allow them to contribute to reservoir.

What novel techniques might help us answer this question?

Recently, it has been reported that performing ddPCR (PCR at multiple selected points on the genome of individual proviruses which are kept together in droplets) allowed separate quantification of intact and defective integrated provirus[67], and therefore a more methodologically robust way of determining replication competency of the virus . ddPCR has already led to the suggestion that the viral dynamics of infected cells are significantly different *in vitro* and *in vivo*[30], and there are calls for its incorporation into clinical HIV monitoring[68], and may prove an invaluable tool in future research. Immuno-PET – in which monoclonal antibodies are conjugated to PET tracers – is one such method, allowing for real-time *in vivo* imaging of SIV[43]. It is likely to be adaptable for HIV imaging in humans[45,69] and would help characterize ongoing replication in much of the body. Although immuno-PET is promising, antibodies do not readily pass the blood–brain or blood-testis barriers. Rapid postmortem studies have recently been pioneered to increase our understanding of tissue HIV reservoirs in humans[70,71] , however, using novel particle sorting techniques[72] , we are within reach of being able to take large postmortem samples of each putative sanctuary from patients who remained on ART until death and process them rapidly to look for evidence of ongoing replication.

Conclusion

While there is sufficient evidence to conclude that the proliferation of HIV-infected cells takes place in individuals on ART, we are yet to fully elucidate the mechanisms which drive this proliferation, a step which will be necessary for therapeutic development. Whether HIV replication continues under ART remains controversial and however with recent technological advances, we are coming closer to answering the question.

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