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Review

Residual Proviral Reservoirs: A High Risk for HIV Persistence and Driving Forces for Viral Rebound after Analytical Treatment Interruption

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Abstract: Antiretroviral therapy (ART) has dramatically suppressed human immunodeficiency virus (HIV) replication and become undetectable viremia. However, a small number of residual replication-competent HIV proviruses can still persist in a latent state even with lifelong ART, fueling viral rebound in HIV-infected patient subjects after treatment interruption. Therefore, the proviral reservoirs distributed in tissues in the body represent a major obstacle to a cure for HIV infection. Given unavailable HIV vaccine and a failure to eradicate HIV proviral reservoirs by current treatment, it is crucial to develop new therapeutic strategies to eliminate proviral reservoirs for ART-free HIV remission (functional cure), including a sterilizing cure (eradication of HIV reservoirs). This review highlights recent advances in the establishment and persistence of HIV proviral reservoirs, their detection, and potential eradication strategies.

Keywords: HIV; SIV; cell-associated viral reservoir; proviral reservoir

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1. Introduction

In human immunodeficiency virus (HIV) life cycle, HIV RNA genome post-viral entry is reverse-transcribed into a double-stranded DNA, followed by transportation into the nucleus. Such a viral DNA/integrase complex preferentially integrates into the transcriptionally active sites of host chromosomes [1]. Notably, HIV DNA integration is an essential hallmark of viral production as integrated proviral DNA serves as a dominant template in the process of HIV replication, compared with unintegrated viral DNA forms [1–3]. By contrast, nonintegrated linear viral DNAs are unstable and accompanied by limited transcription to transiently express early viral elements [4–7], while nonintegrated long terminal repeat (LTR) circles, representing extrachromosomal bystander products that failed upon viral integration [8], basically lose the capacity of viral replication and are ultimately diluted due to cell proliferation [9]. However, the sporadic emergence of linear viral DNA and 2-LTR circles observed under ART is perhaps indicative of ongoing viral replication at low levels [6]. In the context of HIV replication from provirus transcription to virion assembling, unspliced RNA (~9-Kb) and more than 100 differentially spliced transcripts (predominant two-class sizes of early ~2-Kb and late ~4-Kb RNA) are generated [10,11]: the unspliced transcript is a template for gag/pol translation and viral RNA genome packaging; 4Kb incompletely spliced transcripts encode viral proteins env, vif, vpr, and vpu following export into the cytoplasm; and multiply spliced viral RNAs (~2 Kb) express regulatory tat, rev, and nef for transactivation and nuclear export of the viral RNAs [12–20] (Figure 1). Therefore, various cell-associated HIV RNA/DNA forms may reflect the different viral replication status and clinical significance [8,21–26]. For example, HIV gag RNA transcripts represent bona fide genomic HIV RNA or gag and gag-pol polyproteins in viral replication [27,28]; spliced HIV tat/rev RNAs are functional for viral replication and production [29]; and integrated proviral DNA is a marker to estimate the proviral

reservoirs [22,24,30–34]. A small number of HIV-infected cells harboring proviral DNA may replenish proviral reservoirs through clonal expansion or cell division, maintaining viral persistence [9,35–39].

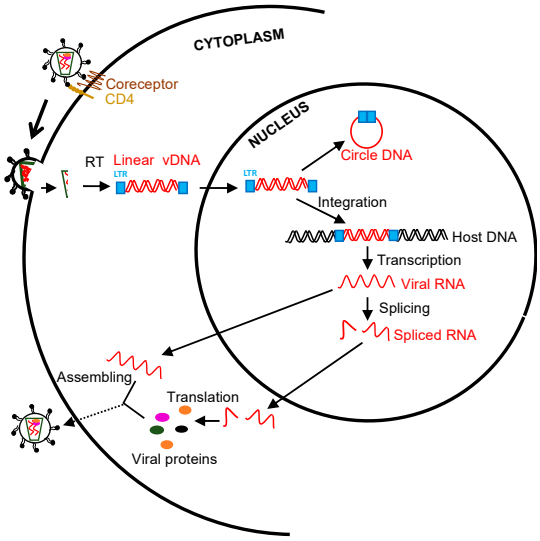

Figure 1. Schematic overview of human immunodeficiency virus (HIV)/ simian immunodeficiency virus (SIV) life cycle and measurable viral parameters. Viral particles enter target cells, followed by reverse transcription, integration, transcription, splicing, translation, and virion packaging. The unspliced viral RNAs are transcribed from the integrated provirus. The transported single 5′ capped genomic viral RNAs (~9 Kb) are assembled to the nascent virions. Two or three guanosines 5′ full-length viral RNAs directly translate viral proteins (gag and pol). Rev-dependent export of incompletely spliced RNAs (~4 Kb) to cytoplasm contributes to env, vif, vpr, and vpu expression, whereas rev-independent multiply spliced species (~2 Kb) constitutively express accessory and regulatory proteins (tat, rev, and nef). Cell-associated viral RNA transcripts and viral DNA can be directly measured [18–20].

2. Establishment and Anatomical Distribution of Proviral Reservoirs

Given numerous, substantial differences in the immunology of humans and mice [40], nonhuman primate models (NHPs) provide an excellent model of human immunology, diseases, and therapies due to their remarkably similar genomes, physiology, and immune systems [41–43]. In addition, it is challenging to access sufficient tissues for analysis in humans because of sample collection limitations. It is especially difficult to evaluate the proviral reservoir seeding that may lead to viral dormancy, "occult" infection, or viral rebound after treatment interruption [44]. Simian immunodeficiency viruses (SIVs) and chimeric simian–human immunodeficiency viruses (SHIVs) that carry HIV envelope from transmitted founder (T/F) viruses are widely used in nonhuman primate models to recapitulate HIV infection in humans, including HIV transmission, pathogenesis, viral latency, and curative strategies [45–49]. Early six-month ART is initiated at 6h, or day 1, 2, and 3 post-SIV infection in rhesus macaques. Once treatment is discontinued, counterpart viral rebound is respectively shown at 0%, 20%, 60%, and 100% of animals, which correlates with levels of integrated viral DNA in LN CD4+ T cells [50], suggesting that the proviral reservoirs are rapidly seeded post-HIV/SIV infection, resistant to ART. In lymphoid tissues that represent typical sanctuary sites for HIV, anti-HIV drugs maintain suboptimal levels in the follicle and germinal center niches [51–54], and virus-infected cells are shielded from specific CD8+ T cell responses [55]. All of these may maintain residual proviral reservoirs in lymphoid tissues in HIV+ subjects, even on lifelong ART. The size of the HIV-1 reservoir differs in tissues, with the frequency of infection generally higher on a per-cell basis in the tonsils, lymph nodes (LN), gut-associated lymphoid tissues (GALT), and spleen, which basically contain abundant organized lymphoid structures [56–58]. Our recent study shows

that the levels of cell-associated SIV RNA/DNA in PBMCs and lymph nodes increase in rhesus macaques on prolonged ART (>20 months) after ATI [59]. Further examination of anatomical distribution and size of the viral reservoir (multiple cell-associated viral parameters) after ATI indicated that all cell-associated RNA/DNAs were recovered to the levels prior to treatment in various tissues (blood, spleen, axillary and mesenteric lymph node, jejunum, and rectum), except bone marrow, in which viral nucleic acid was undetectable, yet jejunum and rectum had very low levels of MS SIV RNA, probably attributing to massive depletion of CD4+ T cells in GALTs. Lymphoid tissues maintained higher US SIV transcript levels and stable MS SIV RNA (Figure 2A,B), while levels of total SIV DNA and 2-LTR DNA were equivalent in all tissues examined (Figure 2C,D). Most notably, integrated proviral DNA was detected in all tissues (detectable in bone marrow from only 1 of 4 animals), yet also higher in peripheral and lymphoid tissues (Figure 2E,F), consistent with converging evidence that systemic and lymphoid tissue compartments may serve as important sites for viral reservoirs [54,60–63]. These findings also support the conception that viral reservoirs persist in multiple tissues, highlighting the rapid recovery and replenishment of HIV reservoirs in tissues once anti-HIV treatment is discontinued.

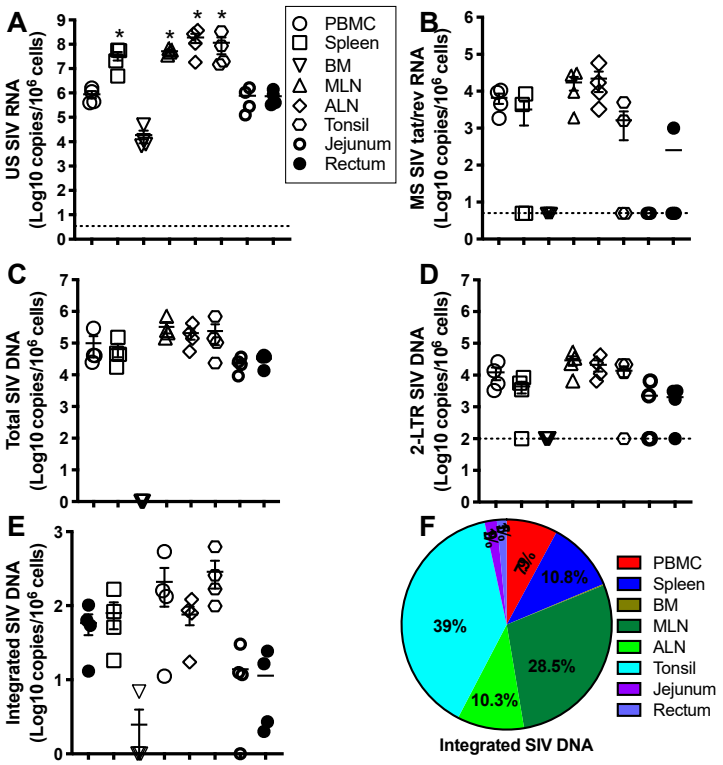

Figure 2. Representative levels and anatomical tissue distribution of cell-associated SIV RNA/DNA in SIV-infected macaques. Adult Indian-origin rhesus macaques (Macaca mulatta) were intravenously inoculated with 100 TCID50 SIVmac251. After 8 weeks, these animals received three anti-HIV drugs (TFV 20 mg/kg/day; FTC 30 mg/kg/day and DTG 2.5 mg/kg/day) for 20 months. The levels of cell-associated unspliced (US) SIV RNA (**A**), multiply spliced (MS) SIV tat/rev RNA (**B**), total SIV DNA (**C**), circular SIV 2-long terminal repeat (LTR) (**D**), and integrated proviral DNA (**E**), in blood, spleen, mesenteric lymph node, axillary lymph node, jejunum, and rectum from SIV-infected animals 3 months after ATI, reaching the levels prior to treatment. (**F**) Distribution of proviral reservoir in tissues examined. Note that integrated proviral DNA was predominantly distributed in peripheral blood and lymphoid compartments, and rapidly increased to pre-treatment levels after ATI. Cell-associated SIV RNA/DNA are expressed as copies per one-million cells. * $p < 0.01$, compared with PBMCs.

CD4+ T cells are primary target cells of HIV/SIV, constituting the predominant productive and latent reservoirs [36,64]. CD4+ T cells harboring proviral DNA may mostly contribute to persistent HIV/SIV infection [65]. The HIV latently infected cells are resting memory CD4+ T cells with regenerative potential, including T memory stem cells (Tscm) and central memory cells (Tcm) [36,64], and also include macrophages and dendritic cells in various tissues [66–69]. Our previous studies indicate that organized lymphoid tissues are major sites for persistent SIV reservoirs, in which follicular T helper (Tfh) cells are differentiated and accumulated in chronic stage and contain high levels of both SIV RNA and proviral DNA [53,70]. Of these, elevated pro-inflammatory cytokines and continuous antigen persistence may drive Tfh precursor differentiation toward Tfh cells, resulting in abnormal accumulation of productively and latently infected Tfh cells [53,71–73]. In primary infection, we also identify that Th17 cells are the initial foci of SIV infection in vaginal tissues [74]. In addition to CD4+ T cells, recent advances reveal that the tissue-resident myeloid cells (e.g., monocytes and macrophages) in the brain and other tissues might be a resource of HIV reservoir throughout ART [75–79]. However, myeloid cells in the blood and colon likely contain HIV transcripts, but few proviruses are detected in a large fraction of HIV+ patients under ART, compared with CD4+ T cells with readily detectable proviral DNA [80,81]. In terms of myeloid cells, circulating monocytes might not be considered reservoirs due to their infrequent HIV infection, low levels of proviral DNA, and short life span [81], while macrophages are probably another long-lived cellular reservoir for viral persistence, viral rebound, and reestablishment of productive HIV infection when treatment is interrupted [82–88].

The markers of HIV reservoirs are desperately required to determine whether ART should resume [89,90]. Early indicators that can predict resurgence of viremia after ATI may aid treatment decisions in people living with HIV. Ideally, a predictor of viral rebound would be measured prior to ART interruption to gauge whether an HIV-infected individual could safely stop ART. It is reported that T-cell exhaustion markers, including PD–1, Tim–3, and Lag–3, strongly predict time to the return of viraemia [91], yet this is questionable because functional T-cell response is not the only determinant in the viral containment of aviremic patients [92]. A number of markers for productively or latently HIV-infected cells with enrichment of HIV DNA, such as CD2 [93], CD20 [94], CD30 [95], and CD32a [96], are still controversial and require further confirmation [97–100]. Notably, it is the small HIV-integrated proviral reservoirs that are distributed in various lymphocyte subsets of anatomical sites for persistence of replication-competent viruses. These reservoirs become a driving force for viral rebound after ATI. However, markers of proviral reservoirs are multifactorial and complex. For example, heterogeneous proviral reservoirs exist in various anatomic tissues but not merely in blood, and their measurement also lies in ultrasensitive assay. The proviral reservoirs also laboriously analyze the intact viral genome from tissues in the body of patients and perform limited proviral assessment by integration site analysis [101,102]. Despite viral DNA levels being a potential cause of viral rebound, some patients with low-viral DNA levels do not show delayed viremia rebound [22,103]. Our recent study indicating an increased ratio of proviral DNA after ATI likely better predicts the emergence and degree of viral rebound [59], yet more reliable markers are still needed toward developing a cure for HIV.

3. Impact of Early Antiretroviral Therapy on the Proviral Reservoir Elimination and Sustained Virologic Remission

Early ART, even initiated as early as one day of infection in adult subjects or within 30 min after birth in infants, usually fails to achieve a sustained state of ART-free virologic remission, leading to HIV rebound after months or years of treatment interruption [50,104–113]. Although the Berlin, London, and Düsseldorf adult patients seemingly appear to have a "sterilizing HIV cure" [108,114–117], the treatment of these three cases, which involved toxic chemotherapy due to hematological malignancies followed by hematopoietic stem cell transplantation, is not feasible in others because of its complexity and risk. Rapid establishment of proviral reservoir harboring replication-competent virus remains the major

obstacle to HIV cure or remission. Early ART in adults infected with HIV, even if initiated within days of infection, has no curative effects [104–106]. Additionally, ART initiation in adult animals, from 4 to 14 dpi, fails to prevent the virus from spreading and reservoir seeding, leading to viral rebound within four months ATI [118–120]. Distinct from those in adults, the immune system in newborn infants is compartmentalized by the "immature" systemic immune system and the "functional/mature" mucosal system [121,122]. Thus, viral susceptibility, viral reservoir seeding, and immune responses in developing neonates exposed to HIV might differ from those in adults [123,124]. Immediate initiation of ART, ideally within hours after birth, may restrict viral reservoir size, maintain normal neonatal immune development, and possibly provide opportunities for being drug-free after going off ART [44,110,125–130]. However, in the pediatric AIDS clinical trials and cases, there is no precedent to achieve HIV remission in the HIV+ infants treated by early ART; eventual viral rebound is observed once treatment is discontinued [107,108,124,126,130–135]. Conceivably, integrated proviral reservoirs established in anatomic tissues cannot be completely eliminated by ART [22,129,136–138], explaining why they contribute to viral rebound after ATI. Together, these findings highlight that a small number of integrated proviral reservoirs seeded could be a key hurdle to advance HIV treatment towards a cure.

4. Measurement of HIV Reservoirs

As described in HIV life cycles, the existence and abundance of HIV RNA/DNA represent different infectious status and clinical significance [8,21–26]. The proviral reservoir could be a potential indicator to produce infectious progeny for the viral rebound after ATI. The advantages and disadvantages of the HIV reservoir measurements have been recently reviewed and discussed [139]. Current scalable assays to measure viable proviral reservoirs may underestimate the bona fide replication-competent reservoir [31,140–147], including Quantitative Viral Outgrowth Assays (QVOA) that assess the size of the replication-competent HIV latency in resting CD4+ T cells under conditions with latency reactivation [26,144,148,149], and Tat/rev-Induced limiting Dilution Assays (TILDA) that measure the frequency of productively HIV-infected cells with inducible multiply-spliced HIV transcripts [140,142,150], in which a proportion of intact proviral DNA may be non-reactivable in the host for a lifetime [144,151,152]. They may even maintaining repressive silence when intact proviral DNA integrates into the "gene desert" sites of chromosomes enriched in repressive chromatin marks [153]. In comparison, quantitative PCR [31,140–146] and near-Full-Length Individual Proviral sequencing (FLIPS) [154] may overestimate the proviral reservoir. More than 90% of proviruses in ART-treated patients might be replicated defective because of internal deletions, mutations, premature stop codons, or defects in splicing and packaging signals [146,155–157]. However, these defective proviruses may partially express viral proteins, eliciting host responses [145,158,159]. Proviral "quasispecies" may be distributed on distinct chromosomal sites in single cells of various tissues [160], and intact proviruses may not necessarily produce replication-competent virions at both transcription and translation levels [146,161–163], weakening the interpretation of the results from assays above. Although sensitive and practical assays are well developed, there is generally no single "gold-standard" approach to reliably evaluate proviral reservoirs in systemic and lymphoid tissues [140,150,163,164]. Biomarkers are still desperately needed to assess viral persistence, effective therapy, and treatment resumption.

5. Strategies to Eradicate Viral Reservoirs

The persistence of latent HIV-infected cellular proviral reservoirs represents the major hurdle to virus eradication in patients treated with ART. Since the transcription of HIV genes depends on cell activation state, integrated HIV DNA is transcriptionally silent in these cells and therefore unaffected by ART [165]. Therefore, various cure strategies are proposed toward HIV cure (Table 1) [114,116,117,166–175]. Of these, "shock and kill" and "block and lock" strategies are attempted to reactivate HIV-1 latency or to create a deep latent state. In "shock and kill", combined with ART, cells harboring latent HIV provirus are

activated by cytokines (e.g., IL-2), lipopolysaccharides, bacterial superantigens, anti-T cell antibodies (OKT3), histone deacetylase inhibitors/HDACi (SAHA), or protein kinase agonists. Once activated, these cells could be eliminated through viral cytopathic effects or host cytolytic T lymphocytes (CTL) responses [176,177]. However, most, if not all of these agents, are not effective in fully reactivating HIV latency in cells from patients on ART or reducing the size of latent reservoirs [178–182]. It remains uncertain whether ART combined with immune activation strategies could eventually (or ever) eliminate all productively infected cells through viral cytopathic effects or other immune mechanisms [166,179,183–185]. In the alternative "block and lock" approach, latency-promoting agents are applied to permanently prevent latency reactivation and replenishment [172,186]. However, this approach essentially lacks specificity of cells containing residual viral genome and efficiency in deep tissues, likely leading to lifelong treatment-induced adverse outcomes and complications. Given that extracellular HIV envelope glycoprotein (Env) could be presented on the productively HIV-infected cells, these cells could be recognized by anti-HIV antibody drug (e.g., toxin or radionuclides) conjugates [187,188] or broadly neutralizing antibodies (bnAbs) [173,189,190], likely leading to specifically and selectively killing of residual HIV-infected cells. However, current passive therapy by bnAbs does not fully eradicate proviral reservoir with potential in selecting escape variants and eventual viral rebound in ART-treated subjects after treatment cessation [191–195]. Meanwhile, although novel genome-editing technology is promising to disrupt or ablate proviral genome, it requires more advances in vivo delivery and specificity of target cells [196–198].

Table 1. General HIV curative strategies.

HIV Cure Strategy	Goal	Efficacy	Limitations
ART	HIV cure	Suppression of HIV replication	Failure to cure HIV
Vaccine	Prophylactic and therapeutic effects against HIV infection	Protective immune responses	No successful vaccine to date
Shock and Kill	HIV latency reactivation, cytolytic effects of target cells	Partial latency reactivation	Failure or limited reservoir perturbation
Lock and block	Permanent silencing of HIV latency	Suppression of HIV reactivation	Delivery difficulty and safety
bnAb therapy	Long-term sustained HIV remission	Suppression of sensitive HIV strain	Emergence of resistant viruses, likely limited effect on viral reservoirs
Gene editing	Disruption of viral genome or HIV coreceptors	Ablation of viral genome	Difficulties of delivery and specificity in vivo, antigenicity, and genotoxicity
Stem cell transplantation	Immune reconstitution and CCR5 mutation	Cases of "sterilizing HIV cure"	Combinations of toxic chemotherapy with transplantation are not feasible in others

6. Perspective

HIV cure remains the greatest challenge for therapeutic strategies. The existence of small cellular reservoirs containing an integrated intact viral genome is a major obstacle in finding a cure for HIV infection. Lifelong treatment by antiretroviral drugs or latency-reversing or promoting agents predisposes one to a high risk of physiological function and

complications. The other challenges may also lie in discrepant latent status in individual HIV+ subjects and full evaluation of the proviral reservoir, not only in peripheral blood, owing to limitations in human sample collection. Among cure strategies, novel gene-editing applications in vivo might be able to potentially ablate HIV genome from cellular proviral reservoirs, albeit far more studies are still needed.

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Institutional Review Board Statement: All animals in this study were housed at the Tulane National Primate Research Center in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care International standards. All studies were reviewed and approved by the Tulane University Institutional Animal Care and Use Committee under protocol number P0401. Animal housing and studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH, AAALAC #000594) and with the recommendations of the Weather all report; "The use of non-human primates in research". All clinical procedures were carried out under the direction of a laboratory animal veterinarian. All procedures were performed under anesthesia using ketamine, and all efforts were made to minimize stress, improve housing conditions, and to provide enrichment opportunities (e.g., objects to manipulate in cage, varied food supplements, foraging and task-oriented feeding methods, interaction with caregivers and research staff).

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