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REVIEW ARTICLE

# Characterizing the Latent HIV-1 Reservoir in Patients with Viremia Suppressed on cART: Progress, Challenges, and Opportunities

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**Abstract:** Modern combination antiretroviral therapy (cART) can bring HIV-1 in blood plasma to level undetectable by standard tests, prevent the onset of acquired immune deficiency syndrome (AIDS), and allow a near-normal life expectancy for HIV-infected individuals. Unfortunately, cART is not curative, as within a few weeks of treatment cessation, HIV viremia in most patients rebounds to pre-cART levels. The primary source of this rebound, and the principal barrier to a cure, is the highly stable reservoir of latent yet replication-competent HIV-1 proviruses integrated into the genomic DNA of resting memory CD4+ T cells. In this review, prevailing models for how the latent reservoir is established and maintained, residual viremia and viremic rebound upon withdrawal of cART, and the types and characteristics of cells harboring latent HIV-1 will be discussed. Selected technologies currently being used to advance our understanding of HIV latency will also be presented, as will a perspective on which areas of advancement are most essential for producing the next generation of HIV-1 therapeutics.

**Keywords:** HIV, latency, provirus, integration, residual viremia, antiretroviral, T cell, CD4, TCR, clonal expansion, homeostatic maintenance, multiple displacement amplification, MDA.

## 1. INTRODUCTION

Combination antiretroviral therapy (cART) against HIV-1 infection is a truly remarkable achievement of modern medicine, capable of reducing viral load to <50 particles/mL and halting disease progression [1-3]. Moreover, individuals whose viral load is stably suppressed cannot sexually transmit the virus [4]. Typically comprised of two nucleoside reverse transcriptase inhibitors (NRTIs) and an integrase strand transfer inhibitor (INSTI), a non-nucleoside reverse transcriptase inhibitor (NNRTI), or a protease inhibitor (PI) with a pharmacokinetic (PK) enhancer such as cobicistat or ritonavir [5], modern cART is better tolerated than previous combinations of antiretroviral drugs. However, the cost of cART treatment is high, long term cardiac and hepatic toxicity remains a concern [6-9], and because low-level viremia persists on cART and quickly rebounds upon cART cessation [10, 11], lifelong treatment is required.

The principal barrier to a cure for HIV-1 infection is the stable reservoir of latent virus in resting memory CD4+ T cells [12-18] maintained even in patients whose viremia is suppressed on long-term cART [19-24]. With a t½ alternatively measured at 3.7 [21, 23] or 3.6 years [20], the slow decay rate of the latent reservoir is prohibitive to cure by lifelong cART alone. Hence, the next generation of antiretroviral therapeutics must target the latent viral reservoir for reduction and eventual elimination, and for this, a better understanding of HIV-1 latency and the processes that promote, maintain, and reverse it, is required.

Prolonged, reversibly quiescent infection is the essence of viral latency [25]. This requires that (i) the infecting virus does not elicit an immune response that kills the host cell, (ii) expression of viral genes is highly restricted yet can be activated under favorable intracellular conditions [25], and (iii) infected cells and their progeny can persist for an extended period of time. In the case of HIV-1, the first condition is met by integration of viral DNA into the genome of the infected cell [26, 27], creating a provirus essentially invisible to host antiviral surveillance. How HIV-1 meets the second condition is less clear, although evidence that the latent reservoir is maintained in CD4+ resting memory T cells that have an intracellular environment unfavorable for viral RNA transcription will be discussed. Finally, resting memory T cells are long lived and maintained by homeostatic proliferation, thus satisfying the third condition.

The reservoir of latent virus is established early in infection, even among those rare individuals who spontaneously control HIV-1 infection without cART [28, 29]. Hence, early administration of cART, while reducing the size and diversity of the latent reservoir relative to those treated during later stages of infection [17, 30], does not prevent its establishment [29, 31]. This result and conclusion are supported by experiments with rhesus macaques infected with SIV, which also establishes a latent reservoir in resting CD4+ T

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cells [32, 33]. Specifically, initiation of cART in these animals three days after infection effectively suppressed viremia but did not prevent establishment of the latent reservoir [34]. Taken together, these observations demonstrate that despite continuous cART, the latent reservoir of HIV-1 in resting T cells is established within a few days of infection and is large and stable enough to support continual low-level viremia and viral rebound if treatment is withdrawn.

## 2. A GENERALIZED MODEL OF HIV-1 LATENCY

There is ample evidence that the latent HIV-1 reservoir is comprised primarily of resting memory CD4+ T cells. For instance, by activating cells to reverse latency, and regardless of the duration of ART, replication-competent HIV-1 can be recovered from highly purified resting CD4+ T cells isolated from infected individuals [14, 19-22, 35]. Indeed, resting CD4+ T cells are the only undisputed source of persistent, replication-competent HIV-1 when cART is optimal [36]. Other cell types, including macrophages, may contribute to HIV persistence [37-48], although this is not easy to confirm, since tissue macrophages, especially those that are CNS resident, can be difficult to sample [42]. Latent HIV-1 has also been found in naïve CD4+ T cells [12-14, 16-18]; however, these cells constitute only a small fraction of the latent reservoir.

Any theory explaining the mechanisms of HIV-1 latency must address how HIV-1, while demonstrating a strong propensity to infect activated CD4+ T cells [49, 50], establishes a latent reservoir primarily in resting memory CD4+ T cells [12-14, 16-18]. Actively infected T cells are short lived (t½ of 1-2 days [51, 52]), yet each is capable of producing on the order of 2000 infectious particles during its lifespan [53]. In contrast, the latent reservoir of infected memory T cells is largely inert with respect to virus production and has a collective t½ of 3.6-3.7 years [20, 21, 23]. Arguably, the most straightforward explanation for this dichotomy is that HIV-1 primarily infects activated T cells, some fraction of which is then transformed into memory cells that enter a quiescent, resting state conducive to viral latency [54].

There are several differences in the cellular microenvironments of activated and resting memory CD4+ T cells that may account for their differing permissiveness with respect to HIV-1 fusion with the cell membrane, reverse transcription, integration, and gene expression. For instance, CCR5, a critical co-receptor for entry of the commonly transmitted forms of HIV-1 [55-60], is upregulated upon T cell activation [61], thus facilitating viral entry. Subsequent reverse transcription of viral RNA and integration of viral DNA into the infected cell genome occurs within hours [62], in part due to the relatively fluid cytoskeleton of the activated T cell and ready availability of cellular factors required for nuclear entry and chromatin binding. Active nuclear forms of key host factors such as NF-kB, NFAT, and pTEFb, which promote and facilitate transcription of viral RNA from the integrated provirus, are also present in abundance in activated cells [63-70]. Collectively, these cellular characteristics demonstrate why infection and virus replication are favored in activated T cells at the expense of latency.

Activated T cells are also short-lived, undergoing programmed cell death as part of the contraction phase of the T cell response [71], and their lifespan may be shortened further by infection with HIV-1. Studies of viral dynamics after initiation of cART demonstrate a rapid, almost immediate decline in viremia, a function of both the short life span of actively infected T cells (t½ of ~ 1 day) and the decay of plasma virions (t½ of minutes) [3, 52, 72, 73]. In addition to contraction-phase apoptosis, cell death can result from the production of toxic viral proteins, integration of the HIV-1 provirus into a metabolically critical segment of the host cell genome [74, 75], or a CTL response [76-79]. The contribution of the last of these possibilities is apparently minor however, as the CTL response does not appear to shorten the t½ of productively infected cells in aggregate [80, 81].

In contrast to activated CD4+ T cells, resting memory T cells generally do not express the CCR5 HIV-1 co-receptor [61], thus impeding cellular entry, and the static nature of the actin cytoskeleton inhibits delivery of the reverse transcription complex to the nucleus [82]. In addition, SAMHD1, a cellular restriction factor expressed at high levels in myeloid cells and resting CD4+ T cells [55-60], depletes cytoplasmic dNTP levels, thereby inhibiting reverse transcription directly [83-85]. As a consequence, reverse transcription of HIV-1 RNA in resting T cells can take as long as three days [86-88]. Prolonged residency of the reverse transcription complex in the cytoplasm increases the likelihood that viral DNA intermediates are recognized by the intracellular DNA sensor IFI16, which leads to activation of caspase-1 and a pro-inflammatory form of cell death known as pyroptosis [89-91]. Together, these aspects of the cellular microenvironment serve to inhibit all stages of the HIV-1 life cycle up to and including integration. However, if viral DNA integration is successful, transcription of viral RNA is hindered by reduced levels of the active forms of NF-kB, NFAT, and pTEFb characteristic of resting memory T cells [63-65, 67, 68, 70]. Epigenetic modifications to the host cell genome, which contributes to the quiescence of memory T cells in the resting state, may likewise contribute to longer-term and more complete silencing of transcription from the HIV-1 provirus [92-94]. Hence, both the dearth of important transcription factors and epigenetic modification of host cell DNA in resting memory T cells are likely to play important roles in establishing and maintaining the latency of an otherwise transcriptionally prolific virus.

## 3. DIFFERENTIATION OF CD4+ MEMORY T CELLS

At least three models have been proposed to explain transition and differentiation among naïve and memory T cell subsets, namely, linear differentiation (LD), decreasing potential/progressive differentiation (DP/PD), and divergent differentiation/disparate fate (DD/DF) [95]. In the LD model, naïve CD4+ T cells challenged with antigen become activated and progressively differentiate along a linear path to become effector cells, some of which then survive the contraction phase and persist as quiescent (resting) memory cells [71, 96-101]. Primary effector cells also become progressively differentiated into effector cells in the DP/PD model; however, their progression down this path, and their capacity for acquiring memory function, respectively, are directly and

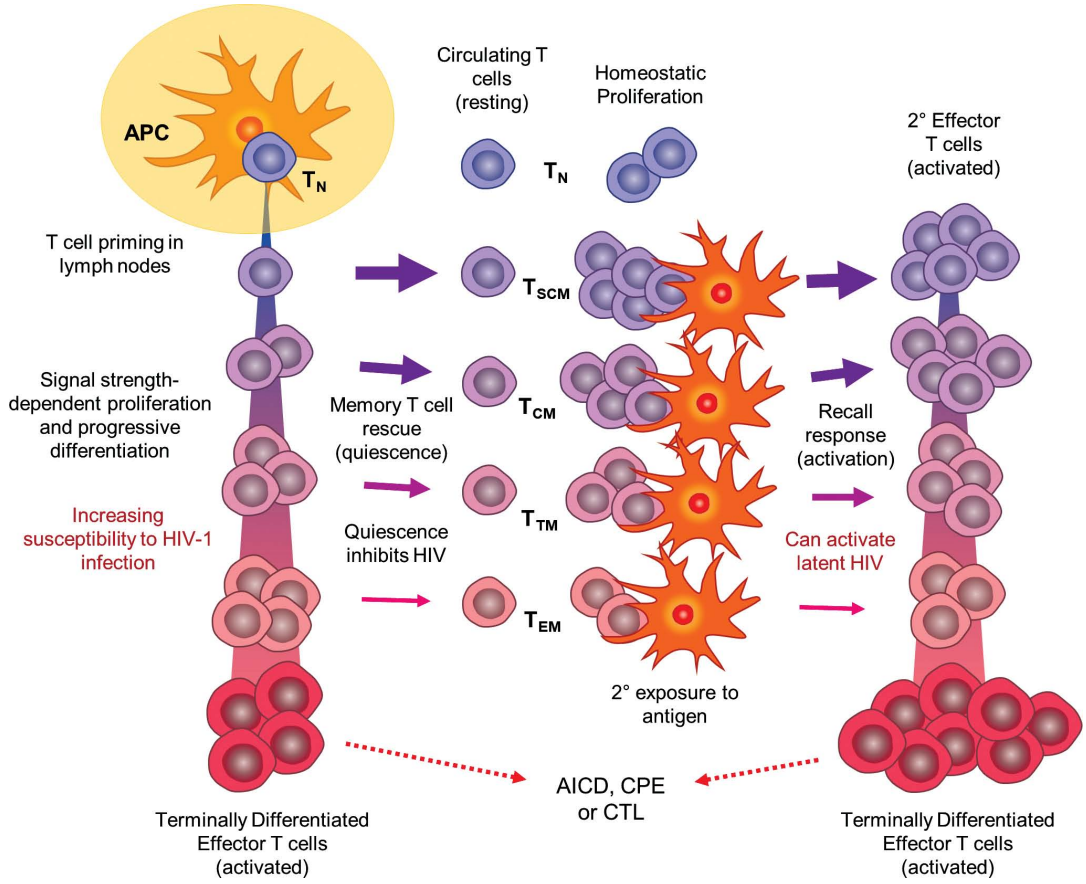


**Fig. (1)**. Depiction of the DP/PD model of CD4+ T cell differentiation and immune response as a framework for HIV-1 infection, latency, and re-activation (see text for details). Upon initial antigen exposure, naïve T cells ($T_N$) are primed by antigen-presenting cells (APCs) in secondary lymphoid organs. Depending on the strength and quality of stimulatory signals, CD4+ T cells progress along an activation and differentiation pathway, losing naïve and acquiring effector properties. It is postulated that less differentiated cells are rescued into memory with greater frequency, while highly differentiated effectors are more susceptible to HIV-1 infection and support virus replication. All terminally differentiated and most other effector cells are not rescued into memory, but are instead subject to programmed, activation-induced cell death (AICD), are killed by cytotoxic T-lymphcytes (CTL), or if infected with HIV-1, are killed by the cytopathic effects of active virus. Latency is established when infected effector cells are rescued into memory T cells in the resting state after the stimulating antigen is removed. Differentiation of memory cell subsets ($T_{SCM}$, $T_{CM}$, $T_{TM}$, $T_{EM}$) parallels effector cell differentiation, with less specialized cells tending to be longer-lived and having a greater capacity for homeostatic proliferation. Response to subsequent antigen exposure is faster and more potent as memory cells re-enter the effector differentiation pathway; however, this process may also cause quiescent HIV-1 to emerge from latency. As with initial exposure, some secondary effector cells are rescued into memory after the antigen has been removed, thus perpetuating the cycle of T-cell maintenance and response, and potentially HIV-1 latency.

inversely related to the strength and duration of TCR and costimulatory signaling [97, 102]. In other words, in the DP/PD model, early CD4+ effector intermediates can ultimately transition into memory cells, but the most activated, terminally differentiated effector T cells cannot. Finally, according to the DD/DF model, initial proliferation of activated cells during the expansion phase yields heterogenous progeny with different capacities for differentiation to either effector or memory cells. This fascinating model invokes asymmetric cell division, in which the distribution of activation-associated signaling receptors between progeny is uneven [103, 104]. The DD/DF model also supports the existence of terminal effector and memory precursor CD4+ T cells subsets homologous to subsets in the CD8+ T cell lineage [98]. Since none of these models alone can fully explain the observations of CD4+ T cell differentiation reported in the literature, it is likely that memory is generated through multiple pathways, and the range of phenotypes and capacity for plasticity among effector and memory cells is extensive.

In Fig. (**1**), proposed mechanisms for HIV-1 infection, latency, and emergence from latency are superimposed on the DP/PD model of CD4+ T-cell differentiation and memory response. However, regardless of which specific T cell differentiation pathways are applicable, the simplest explanation for establishing HIV-1 latency involves infection of T cells of sufficient activated character to permit progression through stages of the virus life cycle up to and including integration, but which assume a resting state quickly thereafter so as to be non-permissive to high-level transcription of viral RNA and virus replication [15]. Such a mechanism would not only explain most physiological and clinical findings related to HIV persistence, but would do so without

requiring the evolution of special viral mechanisms of latency. One notable consequence of the stringent timing requirement suggested by this model is that establishment of latent infection would be expected to be rare, and indeed, only approximately 1 in $10^6$ resting CD4+ T cells are latently infected in HIV-infected individuals on cART [14, 20-23]. Moreover, the feasibility of this model is supported by experiments in which induction of and emergence from HIV-1 latency have been successfully recapitulated in primary T cells *in vitro*; *i.e.*, isolated primary T cells are activated, infected, and then cultured under conditions that promote reversion to a resting state [105-109]. Re-stimulation of these cells through the T cell receptor (TCR) leads to HIV-1 gene expression.

## 4. T CELL SUBTYPES OF THE LATENT RESERVOIR

CD4+ memory T cell pools possess vast functional and phenotypic heterogeneity. Hence, identifying and characterizing the subsets that host long-term viral persistence during cART represents an area of active investigation and may have consequences for designing next-generation strategies to reduce and eliminate the latent reservoir. CD4+ memory T cells populate a differentiation hierarchy that includes naïve, T stem cell memory ($T_{SCM}$), T central memory ($T_{CM}$), T transitional-memory ($T_{TM}$), T effector-memory ($T_{EM}$) and terminally differentiated helper T cells (Fig. **1**) [110]. The more primitive T cells in this progression express a mix of naïve and memory cell markers, tend to be longer lived, and are more capable of homeostatic self-renewal, while the more differentiated cells are increasingly committed to specialized effector functions, are short lived, and are programmed to perish soon after the activating antigen is no longer present [111, 112]. Memory T cells may also be classified into subpopulations by function, each defined by specific antimicrobial properties, cytokine secretion patterns, and expression of signature transcription factors [113]. These include Th1 (antibacterial immune defense), Th2 (protection against helminths and other parasites), Th17 (protection against extracellular bacterial and fungal infections), Th9 (regulation of allergic inflammation, anti-tumor and anti-parasitic immunity), regulator T cells (regulation of antimicrobial immunity), and follicular helper cells ($T_{FH}$; support of humoral immunity). Tissue-resident memory cells represent another distinct subset, localizing to barrier tissues at interfaces with the environment, but also within the brain, kidney, and joints rather than the secondary lymphoid organs [114], as do γδ T cells, which expressing a T cell receptors (TCR) produced from recombinant γ and δ genes instead of the typical α and β [115]. The γδ cells play important functional roles in recognition of lipid antigens, and do not require antigen presentation by MHC complexes [116].

$T_{CM}$ cells generally constitute the largest fraction of the memory CD4+ T cell pool. In one study, among the cell subsets classified, $T_{CM}$ cells made the greatest fractional contribution to the viral reservoir in individuals on cART (*i.e.*, $T_{CM}$, $T_{TM}$, $T_{EM}$, terminally differentiated, and naïve CD4+ cells contributed an average of 51.7, 34.3, 13.9, 1.9 and 0.3% to the infected cell populations, respectively) [13]. Persistence of HIV-1 infection in this cell subset is likely facilitated by the relatively long half-life of these cells, greatly exceeding that of EM cells and approximating that of quiescent naïve T cells [117]. The preferential persistence of HIV-1 in $T_{CM}$ cells is consistent with the increased propensity of these cells toward homeostatic proliferation relative to $T_{TM}$ and $T_{EM}$ [118, 119]. When driven by IL-7, this maintenance mechanism is not associated with viral reactivation or expression of viral antigens, and therefore is unlikely to be restricted by antiviral immune mechanisms [120]. Some rare individuals, who after an initial period of suppressive therapy spontaneously maintained undetectable levels of HIV-1 replication in the absence of cART, were demonstrated to have very low residual levels of HIV-1 infected $T_{CM}$ cells [121], thus emphasizing the importance of these cells in maintaining the latent reservoir and contributing to viral rebound.

$T_{SCM}$ cells likewise make a significant contribution to the latent reservoir of patients on cART. Although these cells represent a minor fraction of total memory CD4+ T cells, and their contribution to the proviral reservoir has been measured as ~8%, the stem cell-like properties of $T_{SCM}$ cells confer greater capacity to proliferate and survive than even $T_{CM}$ cells [122]. $T_{SCM}$ cells appear to occupy a stage of differentiation between naïve cells and $T_{CM}$ cells, having a t½ of 277 months (compared to 144, 133, and 88 months for $T_{CM}$, $T_{TM}$, and $T_{EM}$ cells, respectively) and a strong propensity for homeostatic proliferation [16, 122]. Among patients on cART, the longitudinal decline in HIV-1 DNA associated with $T_{SCM}$ cells was less steep than in the $T_{EM}$ and terminally differentiated subsets [122]. Hence, HIV-1 infected $T_{SCM}$ cells are likely to be overrepresented in the latent viral reservoirs of many patients on long term cART [16, 122], and this fractional contribution is likely to increase with the duration of therapy.

Functional polarization of T cell subsets is associated with developmental and maturational properties that influence cellular longevity and long-term survival [123], and the process is regulated by specialized transcription factors that determine characteristic gene expression changes in each polarized cell population. These properties are also likely to influence the capacity of functionally polarized T cells to host latent HIV-1 infection. For example, while Bcl-6 [124], FoxP3 [125] and Gata3 [126] transcription factors have been shown to contribute to functional polarization, they can also bind to the HIV-1 promoter, thus potentially activating HIV-1 gene expression and reversing latency. Th17 cells in particular exhibit common characteristics among cells known to contribute to the latent reservoir, including longevity and propensity for homeostatic proliferation [123, 127, 128], and were found to harbor high levels of HIV-1 DNA with greater longitudinal stability than Th1 cells in individuals on long term cART [129]. Moreover, some Th17-specific gene expression signatures produce a cellular microenvironment favorable for HIV-1 replication [130], and cells of this subset also seem to be intrinsically susceptible to HIV-1 infection in *ex vivo* assays [131].

HIV-1 can also infect $T_{FH}$ cells [132-135], which represent a major site for HIV-1 replication and production during untreated HIV-1 infection [135]. These cells are characterized by surface expression of CXCR5 and PD-1, reside in lymph node follicles in immediate anatomical proximity to B

cells, and support the germinal center reaction essential for generation of effective humoral immunity [136]. This tissue residency may render HIV-infected $T_{FH}$ cells especially difficult to eradicate by immunotherapy since CD8+ CTL lack chemokine receptors needed for migrating into B cell follicles [133]. This problem is exemplified in rhesus macaques that spontaneously control SIV, and where viral replication is restricted to $T_{FH}$, presumably because CD8+ CTL lyse infected cells elsewhere in the lymph nodes [133]. Hence, if latently infected $T_{FH}$ cells persist, HIV-1 eradication strategies that disrupt B cell follicles to permit access by CTL may need to be considered [133]. Currently, however, the extent to which $T_{FH}$ serve as a long-term reservoir for HIV-1 in the setting of optimal ART remains to be determined, although one study revealed a sharp decline of HIV-1 DNA levels in $T_{FH}$ within 1-2 years of suppressive cART [135]. Relatedly, HIV-1 DNA has been detected in PD-1 and CXCR5-expressing $T_{FH}$-like cells circulating in peripheral blood in individuals on cART [129], although the contribution of these cells to HIV-1 persistence is unknown.

The contribution of regulatory T cells to the latent reservoir has likewise not been determined. These cells express FoxP3 as a master transcription factor and play a dual role in HIV-1 pathogenesis, both reducing pathologic immune activation and inhibiting beneficial antiviral immunity. Regulatory CD4+ T cells from patients on cART have been shown to harbor abundant levels of HIV-1 DNA, with an infected cell t½ of 20 months [137].

Constitutively expressing low levels of CD4 receptor, γδ T cells may be thought to represent less preferred target cells for HIV-1 infection. However, HIV-1 DNA has previously not only been detected in γδ T cells in patents on cART at levels exceeding those in resting CD4 T cells, but in most such instances, virus retrieved from these samples was found by the virus outgrowth assay to be replication competent [138]. Finally, tissue-resident memory cells represent a subset of CD4+ T cells that includes lymphocyte populations in peripheral mucosal tissues, barrier surfaces, and in other non-lymphoid and lymphoid sites, and, at least in adipose tissues, may support HIV-1 infection [139, 140]. However, whether either γδ T cells or tissue resident memory cells contribute significantly to HIV-1 reservoirs in individuals on cART remains unclear.

## 5. RESIDUAL VIREMIA AND HIV PERSISTENCE

Analyzing the evolution of viremia and HIV-1 DNA associated with peripheral blood mononuclear cells (PBMC) throughout the course of cART can provide insight into the mechanisms of HIV-1 persistence. For instance, levels of plasma HIV-1 RNA have been found to decrease by 4-5 orders of magnitude during of the first year of therapy, suggesting that pre-treatment viremia is almost exclusively produced by short-lived infected cells [141-143]. In contrast, levels of PBMC-associated HIV-1 DNA in these individuals was reduced no more than 10-fold over the same period; hence, infected cells that persist despite cART harbor HIV-1 DNA but do not produce virus at high levels.

Viremia declines steadily for approximately four years after treatment initiation [144-146], after which trace levels of free virus on the order of 1 copy/mL of blood plasma can be detected in individuals on effective cART. Such residual viremia reflects the nature of cART, which inhibits attachment and fusion, reverse transcription, integration, and/or particle maturation after release but does not prevent virus production or release from the infected cell. Hence, virus generated despite effective cART is unlikely to be fully mature, and new infections originating from these viruses should be strongly inhibited at multiple stages. These postulates are supported by data indicating that residual viremia in individuals on effective cART does not produce new infections [147-149]. Moreover, when administered early, cART seems to be effective in arresting expansion and diversification of the HIV-1 reservoir, as evidenced by the strong correlation among effectively treated patients between levels of PBMC-associated HIV-1 DNA and the state of infection at the time therapy was initiated [142, 143, 150]. These data also demonstrate that early treatment is critical to minimizing the size and diversity of the latent reservoir.

Residual viremia in well controlled patients does not appear to be affected by treatment intensification, suggesting that there is little or no background virus replication to suppress by supplementing cART with additional classes of antiviral drugs [147-149, 151]. Longitudinal studies of HIV-1 sequence diversity in individuals on cART support this postulate as well. Specifically, HIV-1 RNA sequences obtained from patient blood samples after prolonged cART resemble those present earlier in infection and generally do not show evidence of ongoing evolution [152-156]. In fact, HIV-1 sequences obtained at different times during treatment are often identical, and the fraction of identical sequences within samples increases with time [152, 157, 158]. Together, these observations controvert the notion of HIV-1 persistence being driven by ongoing replication and multiple rounds of infection, and instead support the idea of rare, sporadic viral activation from a reservoir of latently infected resting memory T cells maintained by clonal expansion [159].

When cART is interrupted, viral rebound is usually observed within two weeks [10, 11]. It is postulated that this is the time required for systemic clearance of antiviral drugs and stochastic activation of sufficient latent virus to seed new infections systemically. Despite an estimated 2 log variation in reservoir size, variation in the interval between treatment withdrawal and viral rebound is limited [160], and there is evidence to suggest that rebound is seeded by spontaneous activation of multiple cells per day from multiple anatomical sites (*e.g.*, lymph node, ileum, and rectum) [11]. Moreover, as with residual viremia, sequences from early rebound viruses resemble those of viruses before treatment withdrawal, suggesting that rebound, like residual viremia, originates from a stable latent reservoir established before initiation of cART rather than an immune-privileged pool of perpetually replicating virus [161].

There have been at least three notable cases of "near cure" in which the interval between treatment withdrawal and HIV-1 rebound greatly exceeded two weeks. Specifically, virus rebound in the two "Boston patients" did not occur until 3 and 8 months after cART withdrawal [162, 163]. Prior to cART cessation, both individuals received stem cell bone marrow transplants to treat lymphomas not

responding to chemotherapy, and this was preceded by "conditioning" therapy in which the patients' existing T cells were destroyed by chemotherapy and radiation. In the case of the "Mississippi baby", an infant was born to a mother known to be HIV-1 positive and was treated with cART almost immediately after birth [30]. Treatment was withdrawn after 15 to 18 months against medical advice, after which the child had undetectable levels of viremia for approximately 3 years prior to virus rebound. An important shared feature of these cases is that the reservoir of infected T cells was probably extremely small when antiviral treatment was withdrawn, being either diminished by pre-transplant conditioning or prevented from becoming established by early administration of cART. This marked reduction in reservoir size might, in turn, be expected to delay virus rebound, especially if rebound requires rare, spontaneous activation of latent HIV-1 harbored in quiescent CD4+ T cells. Conversely, it is difficult to imagine why rebound seeded by even a few T cells perpetually generating large amounts of virus would be delayed at all, since infection would be expected to expand exponentially in this case. Such exponential growth is actually typical of new infections, as indicated in phylogenetic studies of HIV-1 demonstrating that nascent infections are most often seeded by a single "founder" virus yet produce extreme levels of viremia over the course of a few weeks [164, 165].

Collectively, these data support the idea that residual viremia and viral rebound are seeded by a stable reservoir of latently infected cells that infrequently become activated to produce low levels of virus despite cART [166]. Nevertheless, there remains a contention that residual viremia and the persistent HIV-1 reservoir are in part maintained by ongoing virus replication in sanctuary sites such as lymph nodes [167-171] with subsequent trafficking of recently infected cells into the blood [171, 172]. Proponents of this argument cite evidence of HIV-1 evolution in lymph node and blood samples taken from three patients on cART [171], as well as reports of poor drug penetration in lymph nodes and mucosa-associated lymphoid tissue (MALT) [169]. However, it must be noted that these findings have not been reproduced by other investigators applying different models of evolution to the same set of HIV-1 sequences [173], and most other studies of patients on long-term suppressive cART have not found evidence of sequence diversification from pre-therapy in blood or tissues [17, 152, 159, 174-176].

## 6. MAINTAINING THE HIV-1 RESERVOIR BY CLONAL EXPANSION OF CD4+ MEMORY T CELLS

Numerous lines of evidence indicate that HIV-1 persists in individuals on effective cART within a reservoir of long-lived, latently infected CD4+ resting memory T cells. As depicted in (Fig. **1**), such cells are natively produced by differentiation from naïve T cells primed by their cognate antigen for the purpose of establishing a capacity for memory response to a subsequent exposure. Memory responses can provide lifelong immunity, as exemplified in individuals who received the smallpox vaccine or have cleared hepatitis C infection [177, 178]. However, this persistence cannot be explained solely on the basis of memory T cell longevity, as the average t½ of memory T cells (approximately 22 weeks; [179-182]) is substantially shorter than that of naïve T cells (1-8 years), the functional memory T cells response (8-12 years), and even the HIV-1 reservoir (3.7 years). Hence, it stands to reason that homeostatic proliferation of memory T cells must also contribute to persistence of both the functional memory T cell response and the reservoir of HIV-infected T cells in patients on effective cART.

The cytokines thought to drive homeostatic clonal expansion of CD4+ memory T cells are IL-7 and IL-15 [183]. Indeed, *in vitro* studies in a primary cell model of HIV-1 latency confirm that latently infected cells can proliferate in response to IL-7 (and IL-2) without upregulation of HIV-1 gene expression [120], which would be detrimental to cell survival [120, 184-186]. These results are supported by clinical studies of patients on cART, where infusion of IL-7 leads to the proliferation of latently infected memory CD4+ T cells with almost no induction of HIV-1 gene expression [119, 187]. Like IL-7, IL-15 has also been shown to be important for homeostatic proliferation of CD4+ memory T cells [188].

Clonal expansion of CD4+ memory T cells can also be driven by recognition of cognate antigens presented on MHC class II receptors by antigen-presenting cells, as well as by cytokines produced as part of the normal course of an immune response. The antigen specificity of HIV-1 infected T cells has not been well classified, although some may be HIV-1 specific [189]. Unlike homeostatic proliferation, antigen-driven proliferation acting through the T cell receptor (TCR) is likely to both activate infected memory T cells and upregulate expression of HIV-1. Such activation, coupled with proliferation and subsequent reversion of a small number of activated cells to the resting state, may contribute to the maintenance of the HIV-1 reservoir, persistence of residual viremia of individuals on effective cART, and virus rebound upon cessation of treatment.

Precise identification of HIV-1 integration sites within T cell genomic DNA has become an important tool for characterizing and quantifying clonally expanded infected T cell populations. Although HIV-1 integration is not random, demonstrating a local sequence selectivity and favoring active transcriptional units [190, 191], sites of integration are of sufficient diversity that the probability of independent infections producing proviruses integrated at precisely the same location in the respective host cell genomes is exceedingly low. Instead, infected cells harboring proviruses that share a common integration site must almost certainly be the product of clonal expansion from a common parent cell. Such determinations can now be made relatively routinely, due to development of novel integration site mapping techniques reliant on semi-nested PCR and next-generation sequencing [184, 192]. Moreover, the application of such methods to DNA extracted from PBMC patient samples has provided convincing evidence for the clonal expansion of HIV-1 infected cells [184, 186, 193].

Results from a seminal study in this area showed that of 2410 integration sites identified in CD4+ T cells from five HIV-infected individuals, 43% were from clonally expanded populations [184], thus conclusively demonstrating that populations of infected cells are maintained by clonal expansion. Intriguingly, some of the integration sites identified in

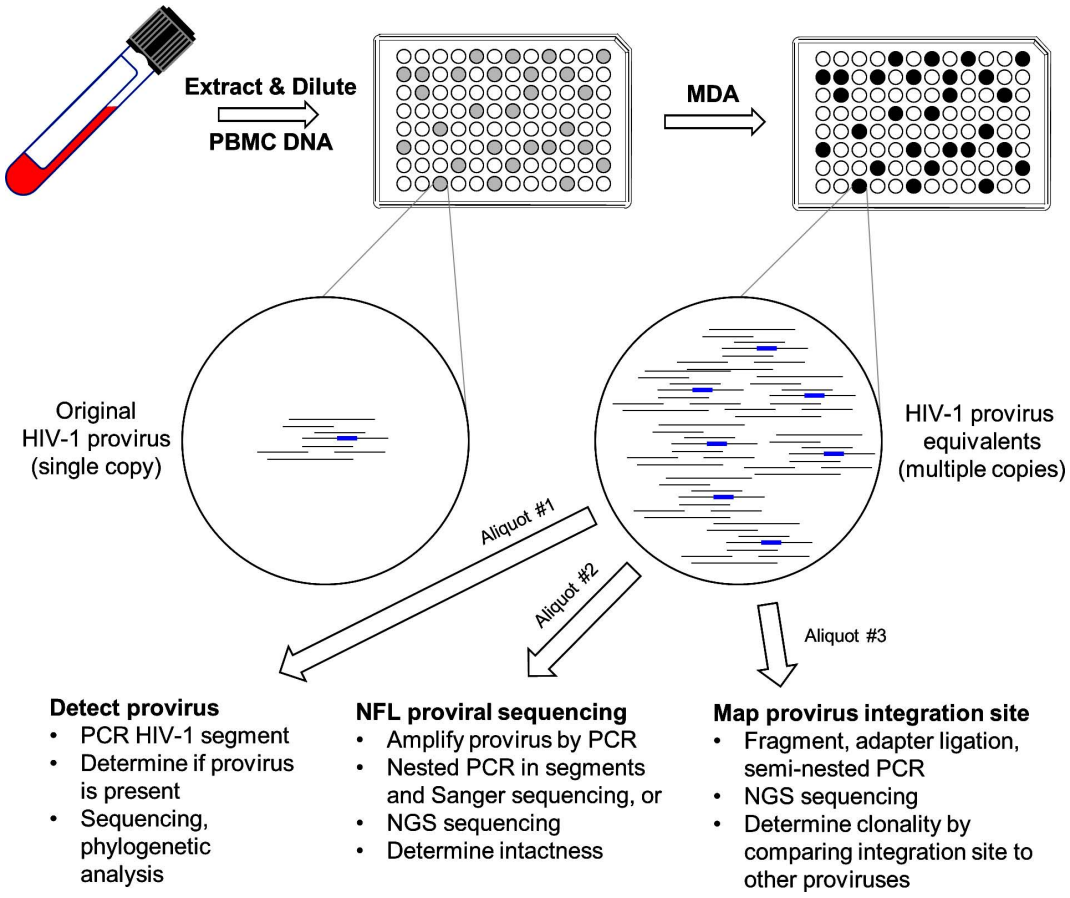


**Fig. (2).** Multiple displacement amplification (MDA) – single HIV-1 genome sequencing (SGS) workflow. Genomic PBMC DNA from an HIV-infected individual is diluted and dispensed into 96-well plates to maximize the number of wells containing exactly one provirus. MDA is then used to amplify DNA in each of these wells on the order of 1000-fold. Reaction products from wells originally containing a single HIV-1 provirus may thus be partitioned for downstream assays, including PCR-amplification and sequencing of segments of the virus genome (*e.g.*, the RT gene), amplification and sequencing of near full length (NFL) provirus, and integration site determination. Further and more complete characterization of the latent HIV-1 reservoir will depend upon continued development of techniques such as these that permit examination of multiple facets of individual HIV-infected cells.

the clonally expanded populations were within known human proto-oncogenes associated with cell growth [194-196] and demonstrated in other *in vivo* and *in vitro* studies to harbor HIV-1 integrants [184, 186, 190, 193, 197]. These genes include (i) myocardin-like protein 2 (MKL2), a transcription factor, and (ii) basic leucine zipper transcription factor 2 (BACH2), a transcription regulator affecting lymphocyte growth, activation, senescence, and cytokine homeostasis. Of integration sites in these genes detected in patient samples, all were localized to specific intronic regions and found to be in the same transcriptional orientation as the gene in question, although a similar pattern was not observed in DNA acquired from *in vitro* infections [184, 186, 191]. Insertion of the HIV-1 promoter into a non-coding region of a growth-related gene might be expected to increase transcription levels, thereby conferring a selective advantage favoring proliferation and/or survival of these T cell clones *in vivo*. Indeed, enrichment for BACH2 integrants was confirmed in a recent study of HIV-1 integration sites in T cells both *in vitro* and from patient samples [198]. The same study also shows disproportional integration within FOXP1 and STAT5B, two genes involved in T cell differentiation and activity. Together, these data suggest that HIV-1 integration at specific sites and in a favorable orientation within select host genes may increase the propensity of the infected cells toward homeostatic proliferation, prolonged survival, or both.

While the notion that HIV-1 integration can confer a selective advantage to latently infected cells is intriguing, it is important to note that all MKL2 and BACH2 proviruses in clinical isolates analyzed to date have been defective [199], and thus are not truly part of the latent HIV-1 reservoir. This is consistent with findings that among individuals on long term cART, approximately 98% of proviruses in long-lived cells are not intact [200, 201]. This fraction is significantly higher than has been observed with SIV infection [202], and may even constitute an underrepresentation, as HIV-1 integrants having extensive deletions or hypermutations mediated by host cytidine deaminases of the APOBEC3 family [203] are difficult to detect by PCR. Moreover, there is probably selection against integration of a replication-competent provirus into a growth-related gene, since it is hard to imagine how HIV-1 promoter activity could increase expression of the host gene without also activating viral gene

synthesis and bringing the quiescent infection out of latency. Nevertheless, with respect to a general characterization of the latent reservoir, the question of whether cells with a high propensity for clonal expansion are capable of both harboring and maintaining the latency of a replication-competent provirus is highly salient.

This question was answered in studies involving an HIV-infected individual who also had squamous cell carcinoma [184, 185]. A single, highly-expanded clone was identified in samples obtained from this individual, comprising more than 50% of the patient's HIV-infected cells and approximately $10^8$ cells in total. The integrant was designated AMBI-1 (ambiguous) to indicate that the integration site of the provirus could not be uniquely determined. Cells harboring the AMBI-1 integrant were found to be widely anatomically distributed but enriched in cancer metastases, suggesting that the clone expanded in response to cancer antigen. More importantly, the AMBI-1 provirus was found to be intact and replication-competent, although only a small fraction of the highly expanded clone produced detectable levels of HIV-1 RNA at any given time. This highly expanded clone is the first shown to be capable of harboring a largely latent, replication-competent HIV-1 provirus despite cART.

## CONCLUSION AND PERSPECTIVE

The principle barrier to a cure for HIV-infection in individuals on effective cART is the reservoir consisting primarily of long-lived, latently-infected, CD4+ resting memory T cells maintained by clonal expansion. Residual viremia in these patients is supported by infrequent, stochastic activation of a few quiescent cells from this reservoir each day, as is virus rebound if cART is discontinued. The capacity to rapidly and thoroughly characterize these viral reservoirs is of paramount importance for developing the next generation of antiretroviral therapeutics. However, progress in this area has been slow, in large part, because the methodologies used for this purpose are often labor intensive, of low throughput, or incompletely characterize HIV-1 proviruses and infected cells.

Fortunately, methods are improving. Tens of thousands of viral RNA molecules can now be independently sequenced in a single experiment [204], and sequences of proviruses can be obtained almost in their entirety by nested PCR combined with Sanger or next-generation sequencing (NGS) [205]. Moreover, using NGS, hundreds of integration sites can be sequenced in parallel after implementing a multistep preparatory method involving DNA fragmentation, adapter ligation, and PCR [184]. More recently, an exciting technology was developed that combines the properties of the last two of these assays [206, 207]. In brief, PBMC DNA samples are diluted and partitioned into aliquots of which approximately 30% contain a single HIV-1 provirus. Each aliquot is then subjected to multiple displacement amplification (MDA; [208]), which uniformly amplifies both host and proviral DNA on the order of 1000-fold. Finally, by applying different assays to aliquots of each MDA reaction, both the sequences and integration sites of individual proviruses can be determined, thus significantly streamlining the characterization of proviral intactness and clonality (Fig. **2**).

Future efforts to characterize the latent HIV-1 reservoir will benefit broadly from continued development of methodologies that combine the advantages of existing approaches. Taken to the extreme, this could entail combining several assays into one and reducing reaction volumes to the nanoliter and single-cell scale. This might allow researchers to sequence and determine the integration sites of thousands of individual replication-competent proviruses in a single experiment, as well as examine the transcriptional profiles and sequence the TCR mRNA of infected cells.

The last of these possibilities is particularly intriguing, given the importance of clonal expansion in maintaining the latent reservoir. For instance, one of the greatest limitations of the otherwise promising 'shock and kill' strategy for eradicating the latent reservoir is that it is not selective; *i.e.*, there are no obvious means of activating only the latently infected resting memory T cells. Consequently, clinicians must either use an approach that activates only a small fraction of an individual's CD4+ T cells, leaving most of the reservoir unaffected, or activate them all, the immunological consequences of which would likely be severe. However, because a recombinant TCR is unique, yet common to all cells in an expanded clone [209], a TCR sequence determined for a single infected cell will be shared by all cells in the clonal population. Moreover, if the TCR sequences of multiple latently infected clones are known, this information could theoretically be used to segregate, activate, or even eliminate the respective clonal populations while leaving the unspecified T cells unaffected. Based on the promise of ideas such as these, the continued development of methods that combine the strengths of established approaches will advance our understanding of the latent HIV-1 reservoir and further the development of the next generation of antiretroviral therapeutics.

## LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| cART | = | Combination Antiretroviral Therapy |
| MDA | = | Multiple Displacement Amplification |
| PCR | = | Polymerase Chain Reaction |
| TCR | = | T Cell Receptor |
| $T_{EM}$ | = | Effector Memory T cell |
| $T_{FH}$ | = | Follicular Helper T cell |
| $T_{SCM}$ | = | Stem Cell-Like Memory T Cell |
| $T_{TM}$ | = | Transitional Memory T Cell |
| $T_{CM}$ | = | Central Memory T Cell |

## CONSENT FOR PUBLICATION

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## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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