Targeting HIV latency: resting memory T cells, hematopoietic progenitor cells, and future directions

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Abstract

Current therapy for HIV effectively suppresses viral replication and prolongs life, but the infection persists due, at least in part, to latent infection of long-lived cells. One favored strategy towards a cure targets latent virus in resting memory CD4$^+$ T cells by stimulating viral production. However, the existence of an additional reservoir in bone marrow hematopoietic progenitor cells has been detected in some treated HIV-infected people. This review describes approaches investigators have used to reactivate latent proviral genomes in resting CD4$^+$ T cells and hematopoietic progenitor cells. In addition, we review approaches for clearance of these reservoirs along with other important topics related to HIV eradication.

Keywords

HIV/AIDS; HIV latency; antiviral therapy; HDAC inhibitors; resting memory CD4$^+$ T cells; hematopoietic progenitor cells

Introduction

Over 34 million people around the world are living with HIV infection as of 2011 [1]. Without therapy, HIV infection leads to the development of AIDS and eventually death in the majority of infected people. Current therapeutic regimens effectively suppress viral replication but do not cure disease and lifelong therapy is required. Thus, treatment poses an economic burden for HIV-infected people and for health care systems. In middle and lower-income countries, over 8 million HIV-infected people received antiretroviral drugs in 2011, while another 7 million who were eligible for treatment still did not have access [1]. 2013 treatment guidelines recommended even earlier treatment, and so the number of people eligible globally jumped from 15.9 million to 28.6 million, increasing the treatment gap

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even further [2]. Thus, there is an urgent need for the development of a therapeutic regimen that will cure disease.

Currently, therapies prevent new infections by inhibiting viral enzymes, including reverse transcriptase, integrase, and protease or by blocking viral entry into a cell. When used in combinations for optimal treatment, referred to as combination antiretroviral therapy (cART), these highly potent drugs reduce plasma viral loads to levels below detection by sensitive clinical assays. However, more sensitive approaches still detect viral genomes in plasma samples after up to 7 years of optimal treatment in HIV-infected people [3]. Thus, despite years of viral suppression, disruption of treatment inevitably leads to a rebound in circulating virus.

The main mechanism through which HIV is believed to persist is through latent infection of long-lived cells. After viral entry, the HIV reverse transcriptase creates a DNA copy of the HIV RNA genome. The DNA provirus then integrates into the host genome within the nucleus. If the proviral genome remains latent, there is little to no transcription of viral genes due to host or viral blocks. Latent infection can be established and maintained as a result of multiple factors: host transcription factor availability, epigenetic modifications, defects in the HIV Tat protein, site and orientation of integration, and post-transcriptional regulatory mechanisms (reviewed in [4], [5], and [6]). Current cART regimens, which target entry, reverse transcription and integration, effectively prevent new viral infections, but they do not affect integrated provirus.

Resting memory CD4^{+} T cells are the best-studied long-lived cellular reservoir of latent HIV infection. However, recent studies implicate bone marrow hematopoietic stem and progenitors cells (HSPCs) as a potentially important latent long-lived reservoir detectable in some donors [7–9]. While other shorter-lived cell types, including monocytes/macrophages and astrocytes ([10–13], reviewed in [14,15]), have also been implicated, this review will focus primarily on cell types that are long-lived. Thus, we will compare potential therapeutic strategies for eventual clearance of latent HIV infection of memory CD4^{+} T cells and bone marrow HSPCs. Important questions for further investigation of HIV reservoirs and implications of the currently proposed model of therapy are also discussed.

Defining latent reservoirs

A clinically significant latent reservoir is one that has the potential to produce infectious virus that can cause rebound viremia when treatment is stopped. Thus, this reservoir should have the capacity to harbor provirus for long periods of time, given that residual virus has been detected after more than 7 years of treatment [3].

Resting CD4^{+} T cells

It is well established that resting memory CD4^{+} T cells are a stable reservoir of latent HIV infection [16,17]. One study that estimated the size of the T cell reservoir using a viral outgrowth assay found that the CD4^{+} T cell reservoir decays extremely slowly with a half-life of 44 months [17]. Another study examining resting memory T cells predicted no

significant loss of integrated HIV DNA over time, with a predicted half-life of roughly 25 years [18].

Resting $CD4^+$ T cells contain barriers to productive viral infection, including rigid cortical actin, which inhibits transport of the preintegration complex, expression of cellular restriction factors that inhibit reverse transcription and low transcriptional activation (reviewed in [19]). Because of these barriers to infection of resting T cells, most latent infection may occur when infected, activated T cells become quiescent. Alternatively, direct latent infection of resting T cells may be facilitated by cytokines, endothelial cells, or other environmental interactions ([20], reviewed in [21]).

The gold standard for the detection of latently infected cells utilizes an assay in which resting memory $CD4^+$ T cells are activated and viral outgrowth is measured. However, a recent study indicates that this technique potentially underestimates latent genomes in circulating resting T cells by up to 60-fold [22]. In this study, Ho *et al* found a significant subset of the non-induced proviruses did not contain lethal mutations indicating that these non-induced proviruses are capable of producing new infectious virions upon reactivation. Additionally, reconstructed non-induced proviruses produced virions with similar infectivity to those reconstructed from induced proviruses. Because these proviral genomes did not appear to be activated and cleared by standard T cell activation methods, there appear to be barriers to reactivation of functional proviruses in latently infected resting T cells that are not well understood [22].

Resting memory T cells have been divided into different subtypes, including central memory (T_{CM}), transitional memory (T_{TM}], effector memory (T_{EM}], and the recently-characterized stem cell memory T cells (T_{SCM}]. T_{CM} cells localize to lymph nodes and, upon stimulation, will become T_{EM} cells that can move into tissues to perform inflammatory and cytotoxic functions [23]. T_{TM} cells show an intermediate phenotype between T_{CM} and T_{EM} cells [24]. The contribution of each of these subtypes to the HIV-1 reservoir is variable [23–27]. A study by Chomont *et al*. implicated T_{CM} and T_{TM} cells as the major components of the $CD4^+$ T cell reservoir [25]. T_{CM} cells form a reservoir of reduced size that decays slowly in HIV-infected people with normal $CD4^+$ T cell counts who started treatment early after infection. T_{TM} cells, on the other hand, are the primary reservoir in HIV-infected people with lower CD4 counts at the time of cART initiation. Evidence was presented that these latently infected cells may be maintained over time by homeostatic proliferation due to continuous immune activation [25].

T_{SCM} cells are the least differentiated T cell subset with the greatest capacity for self-renewal [26]. Recently, it was reported that T_{SCM} cells are also infected by HIV [26–29]. Buzon *et al*. studied these long-lived cells in HIV-infected people with optimal viral suppression for a median of 7 years and found that latently infected $CD4^+$ T_{SCM} cells contribute a significant portion of the HIV DNA in resting memory T cells. The T_{SCM} contribution increased over the course of therapy as more differentiated T cell subsets that initially contributed to the reservoir were lost. The authors provided a longitudinal phylogenetic analysis of plasma and resting T cell viral sequences in 3 HIV-infected people, beginning pre-therapy and continuing at multiple time points up to 13 years post-diagnosis.

These data provide evidence that T_{SCM} cells may be infected early and continue to harbor viral genomes for an extended period [27]. Thus, eradication strategies should also target T_{SCM} cells.

Though it is widely accepted that resting CD4+ T cells are an important source of latent infection, it is not clear that this is the only reservoir contributing to HIV persistence. One study of two optimally treated HIV-infected people found that sub-genomic amplicons derived from plasma virus exactly matched the same sub-genomic amplicons derived from virus produced by reactivated resting CD4+ T cells [30]. However, other studies that have isolated residual plasma virus from optimally treated people with suppressed viral loads were not able to match viral genome sequences to any provirus found in circulating resting T cells [31–33]. The study by Brennan *et al*. compared provirus in resting CD4+ T cells with plasma virus, and found significant compartmentalization of sequences in circulating T cells versus the plasma in 12 out of 14 optimally treated HIV-infected people [31]. Buzon *et al*. reported close relationships between plasma viral sequences and provirus from T cell subsets. However they did not report any identical viral sequences that were found in both plasma and resting CD4+ T cells [27]. Thus, there may be additional cellular reservoirs besides resting CD4+ T cells that produce virus in optimally treated people.

Hematopoietic Progenitor Cells

A long-lived infected HSPC could also be an important contributor to residual HIV in treated HIV-infected people as HSPCs express HIV receptors [7,8]. HSPCs are a heterogeneous population of cells and include subsets with extensive capacity for self-renewal. Because some analyses of plasma virus found that certain identical sequences predominate in circulation over multiple time points, it was proposed that latently-infected stem cells, with the capacity for self-renewal, contributed clonal virus upon intermittent activation [3]. Indeed, a number of studies have provided evidence that HIV can infect CD34+ bone marrow progenitors [7–9,34–36]. A study of HIV-infected people in Africa revealed that HIV-1 subtype C could infect HSPCs *in vitro* and *in vivo*. Participants with HIV-infected bone marrow progenitors also had higher rates of anemia [34].

More recent studies have now shown that HIV-1 subtypes B, C, and D can all infect HSPCs *in vitro* [7]. Moreover, these studies demonstrate that HIV can infect multipotent progenitors that form colonies of multiple different lineages in methylcellulose assays. Notably, HIV can also infect bona fide stems cells *in vitro* based on engraftment and production of all major hematopoietic lineages in an irradiated immune-deficient mouse [7,8].

To study latent infection in HSPCs, Carter *et al*. utilized an HIV molecular clone that expresses viral proteins under the control of the viral promoter and GFP under a constitutively-active promoter [7]. Thus, it was possible to distinguish uninfected (GFP−Gag−), actively infected (GFP+Gag+) and latently infected (GFP+Gag−) cells. When latently infected HSPCs were treated with cytokines that stimulate myeloid lineage differentiation (granulocyte macrophage-colony stimulating factor [GM-CSF] and tumor necrosis factor [TNF]-α), viral gene expression was induced. These studies demonstrate that HIV can infect HSPCs and cause both active and latent infection *in vitro*.

In addition, HIV Gag$^+$ CD34$^+$ progenitors were detected in bone marrow aspirates from some HIV$^+$ donors with high viral loads. Progenitor cells from one donor that initially lacked detectable Gag expression, expressed Gag upon culture with GM-CSF and TNF-α. Examination of HIV-infected individuals on cART with undetectable viral loads revealed no detectable Gag expression in HSPCs, but HIV genomes were amplified with quantitative PCR from 4 out of 9 donors [7]. These initial studies provided evidence supporting the conclusion that latent HIV infection occurs in bone marrow HSPCs *in vivo*.

Two other groups have searched for latent HIV genomes in CD34$^+$ bone marrow cells from HIV$^+$ donors on long-term cART without success. Josefsson *et al.* did not detect HIV amplicons in CD4$^-$ CD34$^+$ HSPCs in a cohort of eight virally suppressed HIV-infected people: five who initiated cART during acute or early infection and three who started cART during chronic infection [37]. In this study, the authors removed CD4$^+$ cells to deplete the sample of T lymphocytes. However, a subset of HSPCs express CD4 and CD4 is required for HIV infection of HSPCs [8]. Thus, it is likely that the negative results from this study were due to the absence of susceptible cells in the samples. The study by Durand *et al.* tested HSPCs from a cohort of 11 optimally treated HIV-infected people, 10 of whom were diagnosed prior to 2001 [38]. These investigators were unable to detect HIV DNA in CD34$^+$ HSPCs by real-time PCR. Nor could they detect virus produced using a co-culture assay of HSPCs stimulated with GM-CSF and TNF-α plus activated CD4$^+$ lymphoblasts. Based on the latter study, some investigators suggested the possibility that CD4$^+$ T cell contamination confounded prior results [7]. However, because the Durand *et al.* study was not powered to detect DNA in HSPCs from donors diagnosed after 2001, an alternative explanation is that it is harder to detect HIV infection of HSPCs in people infected decades ago, before optimal therapy was available. Indeed, all donors who tested positive in the prior study were diagnosed more recently [7].

To determine whether the year of diagnosis was indeed a determinant for detection of the HSPC reservoir, McNamara *et al.* recruited an additional 11 virally suppressed donors who had initiated cART during chronic infection [35]. For this study, CD133$^+$ cells, were isolated, which allowed purification of a population enriched for stem cells. HIV genomes were detected in 6 out of the 11 donors, including in two donors that had undetectable viremia for over eight years. Samples had high CD133$^+$ HSPC purity (<1% CD3$^+$ T cells) and for 5 out of the 6 donors positive for HIV DNA in HSPCs, the genomes detected were determined to not be due to contaminating T cells by statistical analysis in comparison to CD133-depleted bone marrow cells. Interestingly, donors with detectable HIV DNA in HSPCs received their diagnosis significantly more recently (after 2001) than the remaining donors, but had undetectable viral loads for similar periods. Further studies with larger numbers of donors are now needed to confirm that HIV-infected HSPCs are harder to detect in people who were diagnosed earlier in the pandemic before widespread use of cART. Moreover, additional studies are necessary to determine whether HSPCs harboring provirus are a clinically significant reservoir that contributes to residual plasma viremia.

Latency and Eradication

Targeting Latent Infection

As discussed above, latently infected cells do not produce viral proteins that would lead to cytopathic effects and eventual cell death. In addition, latently infected cells are not recognized and cleared by the immune system. Current anti-retroviral drugs, which target early stages of the HIV replication cycle, cannot inhibit this non-productive infection once established. Thus, to eradicate these infected cells, new latency-reversing agents (LRAs) are being developed to oppose latency and thus force the virus to reveal itself. With concurrent cART, this approach, termed 'shock and kill,' aims to eliminate the infected reservoir while blocking new infection events [39].

Multiple factors contribute to latent HIV infection, including host transcription factors that bind the viral promoter and epigenetic changes that affect chromatin and alter accessibility of the viral promoter to transcriptional machinery (reviewed in [4], [5], and [6]). Thus, current work has focused on strategies to counteract these factors in favor of 'shock' or reactivation of latent HIV. Reactivated infected cells then need to be 'killed,' preferably by activation of cellular death pathways or through the host immune response. Methods that have demonstrated *in vitro* efficacy at reactivation of latent CD4^{+} T cell infection have been employed in clinical trials with limited success (reviewed in [40]). Thus, more research is needed to better understand this approach. Here, we highlight a few of the major strategies for reversing HIV latency in resting CD4^{+} T cells, which have recently been reviewed in detail [41–44], and discuss our current understanding of the HSPC reservoir (Table 1).

Chromatin Accessibility—A major focus for reactivation studies *in vitro* and *in vivo* has been on compounds that affect the epigenetic regulation of the integrated HIV genome. Histone deacetylase complex inhibitors (HDACis), including suberoylanilide hydroxamic acid (SAHA; vorinostat), romidepsin, and panobinostat, have been at the forefront of these studies (reviewed in [43], [45]). SAHA, the best-studied HDACi, induces reactivation in both T cell lines containing integrated HIV and primary T cells [46,47]. However, a recent study using resting T cells from HIV-infected people found that SAHA primarily promotes read-through transcription from host gene promoters and only minimally activates HIV LTR-driven transcription. The result is low protein expression and little cytopathic effect [48]. Another *ex vivo* assay used to quantitate reactivation of latent proviruses determined that SAHA induced virion production from an average of 0.079% of the total proviruses in resting CD4^{+} T cells isolated from optimally treated HIV-infected people, indicating the need for stronger interventions for latency reversal [49].

Much less is known about the effect of HDACis on HIV latency in HSPCs (Table 1). In a primary cell model of HSPC latency that utilizes freshly isolated, infected and sorted cells, SAHA induced HIV gene expression, but at doses higher than 1 μM (2 to 10 μM) that are not physiologically achievable [9]. These levels of SAHA were also cytotoxic and generated less reactivation than TNF-α. Additional research is needed to determine how to enhance the efficacy and selectivity of LRAs.

DNA methylation — the de novo methylation of CpG islands in the viral genome post-integration — was thought to play an important role in the late establishment or maintenance of resting T cell latency, with many studies initially focusing on *in vitro* models of latency [50,51]. Studies with T cell line models of latency observed reactivation of latently infected cells with the DNA methylation inhibitor 5-aza-2'deoxycytidine (aza-CdR) and a synergistic effect of using this drug for reactivation in combination with an activator of NF-κB [50,51]. However, a recent study noted that there was little DNA methylation in latently infected resting CD4^{+} T cells from treated HIV-infected people with suppressed viral loads [52]. This was affirmed by a study that found only unmethylated CpG's when assessing over half the CpG islands in HIV genomes in peripheral blood mononuclear cell (PBMC) DNA samples from a diverse cohort of HIV-infected people [53]. Aza-CdR was also tested in an HSPC model of latency. Here, Aza-CdR by itself or combined with TNF-α did not detectably reactivate transcription of latent HIV genomes [9].

Activation of Host Transcription Factors—Another potential reactivation mechanism targets transcription factors in the host cell that are important for expression of the HIV genome. Immune modulating compounds discussed below, including TNF-a and toll-like receptor (TLR) agonists, can reactivate latent HIV in some cell systems through induction of NF-κB, which binds to specific sites in the HIV promoter region of the long terminal repeat (LTR) to promote transcription [54–57]. However, in primary CD4^{+} T cells, TNF-α is not sufficient to reactivate viral gene expression. In addition to NF-κB, positive transcription elongation factor b (P-TEFb) is needed for HIV transcription and resting memory cells have very low levels of this factor [58]. Hexamethylbisacetamide (HMBA) activates P-TEFb in CD4^{+} T cells by releasing it from an inhibitory cytoplasmic complex and allowing binding at the HIV LTR [59,60]. Resting CD4^{+} T cells isolated from HIV-infected people on antiretroviral therapy with undetectable viremia can produce virus upon HMBA treatment [60]. In contrast, TNF-α is sufficient for induction of HIV gene expression in latently infected HSPCs cultured *in vitro*, which have high baseline levels of P-TEFb [9]. Correspondingly, the addition of HMBA does not reverse latency in HSPCs [9]. Prostratin also activates the NF-κB pathway through protein kinase C (PKC) activation, and has been shown to reverse HIV latency in both primary T cells and Jurkat T cell line latency models [61,62]. In latently infected HSPCs, prostratin reactivated latent HIV at high doses (1 to 5 μM), but not to the same extent as TNF-α [9].

Disulfiram, an inhibitor of acetaldehyde dehydrogenase used for treating alcoholism, reactivates latent HIV in a primary CD4^{+} T cell model of HIV latency [63] but did not reactivate latent infection in the HSPC latency model (McNamara, Ganesh and Collins, unpublished studies). Disulfiram activates the protein kinase b (Akt) pathway that eventually leads to activation of NF-κB [64]. A recently published clinical trial found that 14 days of disulfiram treatment in 15 HIV-infected people on antiretroviral therapy did not decrease the size of the latent reservoir in circulating PBMCs [65]. However, the drug was well tolerated and a short-lived increase in plasma viremia immediately after receipt of disulfiram was observed. It is possible that disulfiram will demonstrate greater efficacy at higher doses or in combination with other therapies [65].

Immunomodulators—Immune-modulating molecules have been investigated as potential LRAs for latently infected cells. Interleukin-7 (IL-7) is one of the top cytokine candidates for induction of latent provirus in memory CD4^{+} T cells [66,67]. However, a recent study found that IL-7 might actually contribute to viral persistence in HIV-infected people. Vandergeeten *et al.* found that IL-7 treatment induced proliferation of cells harboring latent virus and expanded the reservoir [68]. In other studies, homeostatic proliferation of central memory T cells did not activate latent HIV [69]. Thus, cytokine induction of proliferation may increase the amount of proviral DNA within an infected individual without any clearance.

TNF-α has long been known to induce expression of HIV in T cell line models of latency [70]. In HSPC latency models, reactivation of latent infection in HSPCs was induced by TNF-α treatment via an NF-κB-dependent mechanism [9]. However, TNF promotes differentiation of progenitors towards a myeloid lineage. Thus, it is not a good candidate for general administration given its non-specific effects on HSPCs and other immune cells (reviewed in [71,72]).

TLR agonists that specifically activate innate immune pathways have also been shown to activate latent HIV in resting memory CD4^{+} T cells, but have not yet been tested in HSPCs ([56,73–75], reviewed in [40]). A recent double-blind randomized controlled clinical trial examined the effects of administering three doses of a pneumococcal vaccine with a TLR9 agonist in 31 HIV-infected people compared with a placebo adjuvant in 37 HIV-infected people [76]. This treatment resulted in a small, but significant, decrease in PBMC proviral load within the group treated with the TLR9 agonist compared with the mainly unchanged control group. This decline in the experimental group was accompanied by an increase in HIV-specific CD8^{+} T cell immunity, which points toward the potential for these agonists to both reactivate latent infection and clear the latent reservoir. HSPCs express TLRs, and signaling through these receptors occurs in mouse hematopoietic stem cells (HSC) [77,78]. In mice, activation of these receptors induces HSC proliferation, biases to myeloid differentiation, and diminishes self-renewal and engraftment capacity [72,78–80].

In a recent study, evidence was provided that pegylated (Peg) interferon-α-2a can suppress HIV replication and reduce the numbers of T lymphocytes harboring provirus in treated people [81]. In this study, the authors recruited 23 optimally treated, HIV-positive people. All subjects received Peg-interferon-α-2a therapy in addition to cART for 5 weeks; then cART, but not Peg-interferon-α-2a therapy, was interrupted for 12–24 weeks. Intriguingly, a significant decrease in the number of proviruses per CD4^{+} T cell was detected in 7 subjects who maintained viral suppression at week 12. Based on these results, Peg-interferon-α-2a may assist in clearance of the viral reservoir. While these data are interesting, more studies with larger numbers of participants are needed to understand the significance as it is not clear by what mechanism Peg-interferon-α-2a could clear latent reservoirs [82].

Challenges with Latency-Reversing Agents

Although many of the LRAs discussed above show potential for antagonizing HIV latency, recent studies emphasize the need for further work to understand their clinical utility; there have been variable results when the same compound is tested side-by-side in different *in*

vitro latency models and limited success thus far as sole therapies in clinical trials. Spina *et al*. [83] measured the effect of a panel of LRAs on multiple widely used models of latency compared with the standard quantitative viral outgrowth assay (QVOA) that uses patient-derived latently infected resting CD4+ T cells. They found that no *in vitro* latency model recapitulates the *ex vivo* QVOA results, with many of the models seemingly biased towards reactivation by only specific classes of agents. PKC agonists generally induced latent HIV in the majority of models tested (Table 1). This paper underlines the potential difficulties of using a single *in vitro* model to identify the best clinical approach for 'shocking' latent HIV.

HDACis (SAHA, romidepsin and panobinostat) and disulfiram did not induce viral outgrowth in a newly developed *ex vivo* assay that may better reflect *in vivo* conditions because it uses cells from HIV-infected people and does not employ allogeneic T cells, which may confound results [48]. Using this assay, viral outgrowth was only observed from donor CD4+ T cells treated with T cell activating agents [48] (Table 1). T cell activation and bryostatin-1, a PKC agonist, significantly induced HIV mRNA expression whereas the HDACis and disulfiram did not.

Humanized mouse and primate models may be useful *in vivo* models for further trials of LRAs and other strategies for eradicating virus [84,85]. Kauffman *et al*. recently developed a Rhesus Macaque cART model [85]. This model recapitulates what has been observed in human studies with respect to plasma viral sequence diversity after suppression with antiretrovirals and treatment interruptions.

Based on initially promising *in vitro* studies, SAHA, panobinostat, disulfiram, and IL-7 have been or are currently being tested in clinical trials with no clear success as yet (reviewed in [40]). The first study using the 'shock' strategy examined the effect of the HDACi valproic acid plus a viral entry inhibitor over a three-month period [86]. In this study, four HIV-infected individuals on cART had declines in numbers of infected CD4+ T cells ranging from 68% to over 84%. However, subsequent trials of valproic acid failed to replicate these results [87–90]. In a separate study, SAHA treatment was found to increase HIV RNA expression in resting CD4+ T cells, but had no detectable impact on residual plasma viremia [91]. As mentioned above, a pilot study of disulfiram treatment also demonstrated no effect on the size of the circulating latent reservoir [65]. While clinical trials with single agents have not yet been successful, combinations of LRAs may prove effective in further studies [42].

The HSPC reservoir is particularly difficult to assess with respect to understanding its response to treatment as infected cells are rare and there is no clear protocol for monitoring the size of the latent reservoir in this cell population. Previous studies have utilized bone marrow aspirations, which are more invasive than peripheral blood collection [7,35,37,38]. It is also unknown how representative a single bone marrow sample is of the HIV reservoir in marrow sites throughout the body and so it may be challenging to observe the effect of reactivation strategies on this reservoir.

Clearing Infection after Reversal of Latency

Reactivating reservoirs of latent HIV is only the first step of the 'shock and kill' approach. Strategies to eliminate cells after reversal of latency are an equally important consideration for a cure. The two main strategies for killing a cell with reactivated infection are activation of cell-death pathways and immune-mediated clearance.

Induction of Cell Death—In response to viral infection, cell death pathways become activated to prevent further spread of an infection [92]. However, HIV encodes strategies to delay death of the cell and favor the establishment of infection [93]. Further research should consider how well LRAs of interest can induce cell death in the various cell types implicated as reservoirs for latent HIV, as this effect may be cell-type dependent. One study found that *ex vivo* reactivation of latent virus with a 6-day treatment of the HDACi SAHA in PBMCs from cART-treated HIV-infected people did not reduce the number of latently-infected cells by a limiting dilution viral outgrowth assay [94]. Moreover, SAHA did not promote cell death of resting CD4+ T cells in an *in vitro* latency model, whereas T cell activation did [94].

Similar to what has been observed in T lymphocytes, active infection of an HSPC induces apoptosis based on annexin V staining and loss of infected cells in culture [7]. However, it is not yet known whether reactivating latent infection in HSPCs leads to cell death *in vitro* or *in vivo*. If infected HSPCs are able to divide and differentiate without reversal of latency and activation of cell death, mature myeloid or lymphoid cells could retain latent HIV contributing to viral persistence (Figure 1) and these cell types would also need to be effectively targeted. Thus, studies are needed to better understand HIV infection of HSPCs and their progeny.

The importance of targeting pathways for cell death to augment clearance of HIV-infected cells or reactivated latent infection was recently covered in an excellent review [93]. The authors promote the idea of 'prime, shock, and kill,' in which cells would be pre-treated in a way that induced death after reactivation [93]. Two drugs already approved for clinical use, the topical antifungal ciclopirox and the iron chelator deferiprone, are promising as agents to induce cell death [95]. These drugs preferentially kill infected cells by lowering the natural threshold to apoptosis in all cells, while opposing viral proteins that prevent the induction of apoptosis in response to viral infection. Thus, the development of strategies that promote the death of cells treated with LRAs may facilitate clearance of residual HIV reservoirs.

Immune Clearance—Another strategy for clearing latent infection utilizes immune defenses to target and kill reactivated cells. According to the common definition of latency, there is little to no production of viral proteins, which makes them poor targets for cytotoxic T lymphocytes (CTLs). Anti-HIV CTLs limit replication of the virus, but these cells often show functional defects in the context of HIV infection [96]. A small group of HIV-infected people, referred to as elite controllers, have low levels of HIV replication without therapy, and these HIV-infected people have HIV-specific CTLs that can kill autologous resting CD4+ T cells that reactivate latent infection *ex vivo* [94]. In cART-treated HIV-infected people, latently-infected resting CD4+ T cells reactivated with SAHA *ex vivo* are not cleared

by CTLs isolated from the same patient, unless those CTLs are pre-stimulated with HIV Gag peptides [94]. The susceptibility of infected bone marrow HSPCs to immune clearance has not yet been assessed, but is certainly an important consideration for targeting this potential reservoir.

Interestingly, in optimally suppressed HIV-infected people, there is evidence that a subset of resting CD4+ T cells, termed Gag-positive reservoir (GPR) cells, express Gag without supporting a spreading infection [97]. Though these cells are not truly latent given that transcription is occurring, Graf *et at*. found a correlation between the effectiveness of CTLs to clear GPR cells and lower levels of integrated HIV genomes per PBMC *in vivo* [98]. Thus, there is some evidence that CTLs can limit the reservoir size [98]. GPR cells are targeted more effectively by CTLs from elite controllers than non-controllers and could perhaps be killed, even without reactivating infection, by boosting the CTL function of HIV-infected people on therapy.

One problem with relying too heavily on the efficacy of CTL killing for reservoir clearance is that HIV has strategies to limit effective CTL recognition. In side-by-side assays, HIV-1-infected cells lacking Nef are more sensitive to CTL recognition and lysis than cells infected with wild type virus *in vitro* [99–102]. There is also evidence that the ability of Nef to promote immune evasion from CTLs by downmodulating major histocompatibility complex-class I molecules (MHC-I) is necessary for infection *in vivo*. Simian immunodeficiency virus (SIV) Nef alleles with difficult to revert mutations that specifically disrupt the ability of Nef to downmodulate MHC-I, rapidly evolve *in vivo* to acquire compensatory changes elsewhere in Nef that restore the ability of the recovered virus to downmodulate MHC-I [103]. Additionally, *ex vivo* analysis of Nef alleles provide evidence for the importance of this activity in HIV-1 infected people [104,105]. MHC-I downmodulation is one of the best-characterized functions of Nef and there is ample evidence that Nef enhances the ability of the virus to evade CTL clearance *in vitro* and *in vivo*.

The capacity of CTLs to recognize targets is also influenced by the availability of antigenic peptides. While there are examples in which CTLs require just a single epitope to kill a target [106], CTLs kill HIV-1 infected primary T lymphocytes more effectively when more viral antigen is available for presentation [107]. If safe and effective Nef inhibitors could be developed, combined approaches with latency antagonists and Nef inhibitors would be expected to optimize CTL recognition and clearance.

Strategies to boost immune recognition and clearance of HIV-infected cells are of interest. In addition, anti-HIV vaccinations and broadly neutralizing monoclonal antibodies as therapy to enhance the host immune response have had some promising results and these could be combined with LRAs to increase clearance of latent virus from T cell and HSPC reservoirs (reviewed in [108–110]). Recent studies using rhesus CMV-based vaccine vectors in macaque models are encouraging in this regard [111]. Another approach to immunotherapy includes stem cell-derived HIV-specific CTLs. Kitchen *et al*. genetically engineered and delivered a T cell receptor specific for a Gag epitope into human HSCs [112]. The transduced HSCs developed into mature T cells in human thymus implants in

immunodeficient mice. These engineered CTLs could significantly suppress HIV replication in a humanized mouse HIV model, but have not yet been tested in humans.

Expert Commentary and Five-Year View

Hope for a global cure of HIV infection has been stimulated by the documented cure of an HIV-infected man following bone marrow transplantation in Berlin and the transient 'functional cure' of an infected baby from Mississippi [113,114]. However, there remain important questions that need to be addressed in the expedition toward a cure.

What are the important reservoirs in viral persistence?

The current focus on the well-studied reservoir of latently infected resting memory CD4^{+} T cells will need to be supplemented by investigations of other sources of persistent infection. Thus far, clinical trials with agents shown to reactivate infection in T cells have not been successful in eradicating the virus from HIV-infected people. This review discusses the possible contribution of bone marrow HSPCs to persistence, but additional studies may identify other reservoirs.

There is evidence that shorter-lived myeloid cells, including monocytes, macrophages, and dendritic cells are able to harbor integrated HIV and contribute to persistence (reviewed in [14,15]). Though infrequent, monocytes with integrated genomes have been recovered from HIV-infected people after many years of optimal viral suppression. Proviral genomes from these cells closely match residual plasma virus in a study of 7 HIV-infected people [115,116]. Monocyte-derived cells, including perivascular macrophages, microglial cells, and astrocytes have been implicated as reservoirs in the central nervous system ([11–13], reviewed in [15]). Because these cells are shorter-lived, their persistence may play a role in settings in which therapy is not optimal such that low level active infection can occur.

Although not discussed in depth here, current antiretroviral therapy may not completely block virus spread directly between cells and may also allow ongoing replication in anatomic sites with decreased drug penetration. Emerging evidence indicates that low level active infection can continue to occur in some people on effective antiretroviral treatment [108,117–125]. Studies in animal models have detected viral RNA in lymphoid tissue from the gastrointestinal tract, draining lymph node, spleen and in some cases, bone marrow [126]. Studies in human subjects have also revealed evidence of persistent active infection in CD14^{+} monocytes [124]. In addition, some intensification studies have detected unspliced HIV RNA in the ileum, suggesting ongoing productive infection in some HIV-infected people on ART [125]. Thus, infected cells in lymphoid tissue can potentially produce low levels of HIV that could re-seed the reservoir of persistent HIV. Continued virus production and infection could also lead to inflammation [117,127], which may play a role in maintaining the persistent reservoir of HIV. These additional issues may also need to be addressed for effective clearance of persistent virus.

Can a functional cure be achieved and is it enough?

In discussion of a cure, two categories have been proposed: sterilizing and functional [128]. With a sterilizing cure, there is complete eradication of all replication-competent HIV from

a patient. On the other hand, with a functional cure, there is suppression of viral replication and maintenance of CD4^{+} T cell function without anti-retroviral therapy indefinitely. Thus far, there have been a few instances of functional cures when treatment was initiated early after initial infection. In one case, an HIV-infected woman from Mississippi who did not receive prenatal HIV treatment gave birth to a baby that immediately received cART [114]. The infant's initial viral load decayed after treatment began, and, after treatment was stopped at 18 months of age, circulating virus remained undetectable for about 2 years without any therapy. Eventually however, the child developed detectable viremia and needed to resume treatment [129]. The extended period of virological control that occurred after therapy cessation offers hope that proviral reservoirs can be reduced with early treatment. A complementary study of infants infected perinatally found lower levels and higher decay rates of PBMC provirus in four children that began cART sooner (age 0.5–2.6 years) compared with four that began cART later (age 6–14.7 years) [130].

In adults, recent studies suggest that early treatment can lead to a higher than expected rate of post treatment controllers (PTCs). PTCs refer to treated individuals who are found to have very low levels of viral replication after interrupting therapy. A group of 14 adult PTCs were recently identified from a cohort that started treatment early during primary HIV infection, and were able to maintain viral control at least 24 months after treatment interruption [131]. These HIV-infected people generally had small HIV reservoirs in PBMCs and less infection of long-lived subsets of resting T cells. While complete eradication of HIV-infected cells would be ideal, it is practical to consider the goal of a functional cure, which could theoretically involve viral suppression without therapy after clearance of just a fraction of reservoirs. Additionally, treatments to boost immune function or prevent viral immune evasion, as with a Nef inhibitor, may be the most helpful to allow a patient's own immune defenses to effectively control HIV replication.

What approaches besides 'shock and kill' should be considered?

Additional strategies towards a cure that are being considered include stem cell transplants, potentially augmented genetically to make cells resistant to infection. Gene therapy approaches that target latently infected cells are also being tested.

Stem Cell Transplants

The first person to be cured of HIV infection was treated with a bone marrow transplant for acute myeloid leukemia [113]. Often referred to as the Berlin patient, this 40-year-old man received an allogeneic bone marrow transplant (BMT) from a donor with a homozygous deletion in the *CCR5* gene. Thus, the donor cells were inherently resistant to HIV infection because they lacked expression of an HIV co-receptor. At the time of the transplant, the patient stopped anti-retroviral therapy, and had no detectable viremia without antiretroviral therapy for over 5 years [113,132]. Whether the donor stem cells or the bone marrow ablation strategy, or a combination of the two, led to this cure is unknown. However, this case renewed interest in stem cell therapy as a potential cure, though with no additional successes yet. Indeed, recent studies that have examined the impact of bone marrow transplant have not replicated the conditions that led to a cure of the Berlin patient. Cillo *et al*. detected plasma virus and HIV DNA in 10 HIV-infected people after they had received

autologous BMTs [133]. Two other HIV[+] men experienced a decline of peripheral blood HIV reservoir after allogeneic transplants from wild type-CCR5^{+} donors [134]. After a treatment interruption, they had undetectable viral levels for a prolonged period, but eventually both experienced viral rebound [134,135].

Despite the failure of BMT as a therapy so far, an alternative approach is to transplant genetically modified hematopoietic stem cells to allow continued production of immune cells that are resistant to infection. Some studies have used genetic approaches that delete CCR5 or insert restriction factors into stem cells to prevent infection ([136], reviewed in [137]). Gene therapy has also been used to modify T cells. In a preliminary trial, re-infusion of autologous T cells that had been edited by zinc-finger nucleases to eliminate CCR5 gene expression was well tolerated in 12 HIV-infected people [138].

Gene Therapy

Additionally, gene therapy approaches have been utilized as a strategy to directly target latently infected cells. A recent study of interest utilized the clustered regularly interspaced short palindromic repeat (CRISPR)/ CRISPR-associated protein (CAS) 9 system to edit an integrated HIV genome and prevent transcription. This unique strategy aims to cure infection by permanently silencing proviral genomes [139].

Alternative Strategies

Other approaches to directly target latently infected cells include therapies specific to infected cells. For example, treatment with an HIV-targeted immunotoxin in combination with anti-retroviral therapy effectively kills cells with productive infection in a humanized mouse model [140]. Another approach utilized radiolabeled antibodies recognizing the HIV envelope protein to selectively clear HIV-infected cells in mouse models without severe toxicity [141]. If proven safe and effective, these therapies could be used to specifically target latently-infected cells, assuming a marker can be found that is uniquely expressed on cells with transcriptionally silent infection. One study found that CD2 expression is usually high on resting memory T cells harboring latent HIV [142]. However, this marker is also commonly found on uninfected cells and many infected cells were CD2^{−} Further characterization of which subsets of cells are infected within the resting T cell and HSPC reservoirs could reveal a targetable characteristic for cell-directed therapies.

Five-Year View

Our increased understanding of HIV pathogenesis, effective anti-retroviral drugs, and viral reservoirs has transformed HIV treatment and research over the past decades. Looking forward, we can see how this knowledge may be applied to future goals and priorities of HIV clinicians, researchers, and policy-makers. Clinicians, acting on the new World Health Organization treatment guidelines proposed in 2013, will be working to begin treatment during initial stages of infection and help HIV-infected people adhere to treatment to decrease deaths and prevent new infections. A longitudinal population study in Canada and a randomized controlled trial performed in 9 countries are the most recent evidence that earlier and expanded coverage with cART can significantly decrease HIV morbidity, mortality, and transmission [143,144]. As discussed above, the case of the Mississippi baby

and PTCs illustrate the possibilities that initiating treatment earlier may allow at least a portion of HIV-infected people to attain a functional cure. Thus, treatment will be expanded by the push to implement cART earlier and apply therapies targeting latent reservoirs. A major obstacle to the expansion of treatment will be the large burden on governments and health care systems to supply medications for all the HIV-infected people who should be treated, especially the majority that live in low or middle income countries. For the millions of HIV-infected people, in whom resting CD4^{+} T cells and other reservoirs are already established, researchers will strive to narrow the pool of plausible agents for 'shock and kill' or other strategies for targeting latent infection. As a deeper understanding of HIV persistence directs clinical trials, new and more economical treatment regimens could emerge. The universal goal remains to provide a cure and end the prospect of a life of illness and arduous treatment for all HIV[+] people.

Acknowledgments

Abbreviations

HIV	human immunodeficiency virus
AIDS	acquired immune deficiency syndrome
cART	combination antiretroviral therapy
HSPCs	hematopoietic stem and progenitor cells
GM-CSF	granulocyte macrophage-colony stimulating factor
TNF-alpha	tumor necrosis factor-alpha
LRAs	latency-reversing agents
HDACi	histone deacetylase complex inhibitor
LTR	long terminal repeat
IL-7	interleukin-7
TLR	toll-like receptor
PBMC	peripheral blood mononuclear cell
PKC	protein kinase C
SAHA	suberoylanilide hydroxamic acid
MHC-I	major histocompatibility complex-class I molecules
PTC	post treatment controller

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Reference annotations:

* = of interest

** = of considerable interest

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Key Issues

- HIV forms a latent infection that allows the virus to persist despite therapy.

- The best-studied latent reservoir of HIV is in resting memory CD4$^+$ T cells, including central memory, transitional memory, and stem central memory T cell subsets.

- Bone marrow HSPCs can be latently infected and viral genomes have been detected in these cells in a subset of HIV-infected people.

- HDAC and DNA methylation inhibitors have been tested to reverse latent HIV in CD4$^+$ T cells and HSPCs, but more strategies are needed to boost the efficacy of these compounds.

- Agents that result in increased availability of host factors, such as NF-κB or pTEFb, can increase transcription of the latent genome and contribute to reactivation of latent infection.

- Immune-modulating compounds may antagonize latent infection, but are not ideal as treatments due to their non-specific effects on HSPCs and immune cells.

- Two major strategies for clearance of reactivated latent infection are activation of cell death pathways or the patient's own immune system.

- Important questions to be considered in future studies include: Which HIV reservoirs should we target? How can we attain a functional cure? What are the alternative approaches to a cure?
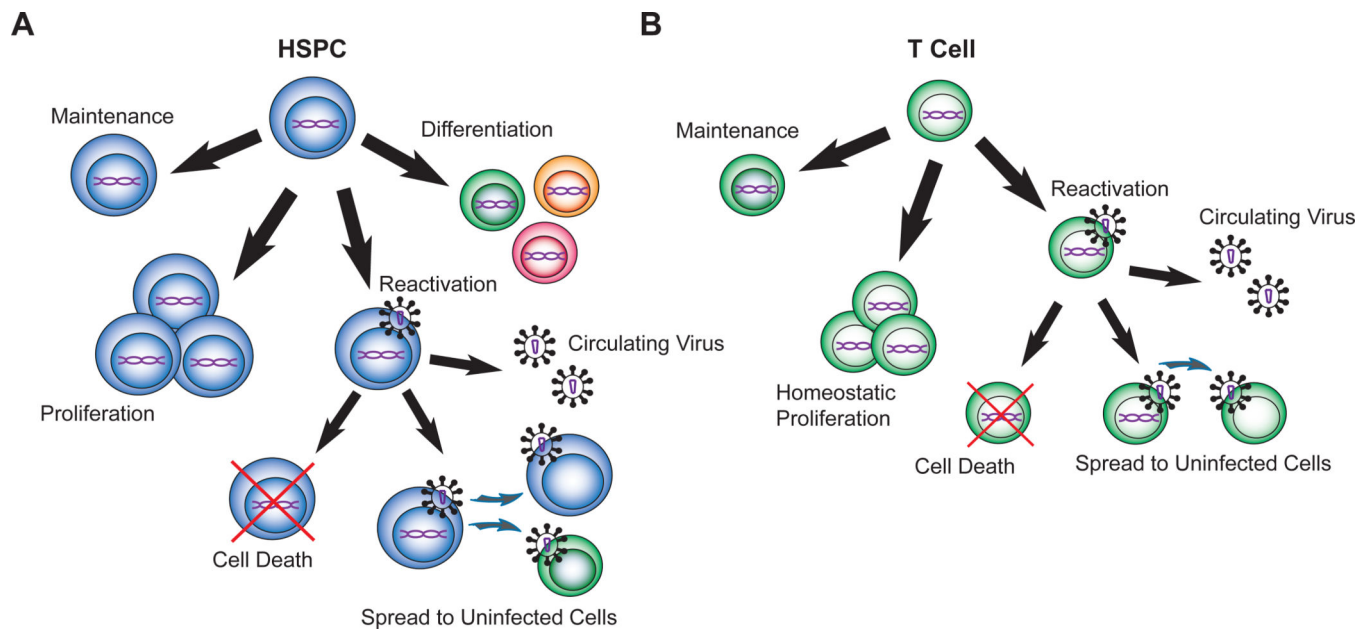
Figure 1. Potential outcomes of latent infection in a hematopoietic stem and progenitor cell versus a T cell

A. Diagram representing conceivable fates of a hematopoietic progenitor with an integrated viral genome (purple). An infected HSPC can maintain or expand the pool of latently infected cells through remaining quiescent or proliferating without differentiation. With stimulation by cytokines or reactivation agents, the HSPC could go from a latent to an actively infected state, where cell death could be induced, virus could be produced to infect other cells, and new virions could contribute to plasma virus. An HSPC could theoretically differentiate into a mature hematopoietic cell such as a T cell and retain viral DNA. **B.** A latently infected T cell could persist through maintenance or homeostatic proliferation. With reversal of latency, the actively infected T cell could die, infect additional cells, and release virus into the periphery. HSPC: hematopoietic stem and progenitor cell

Table 1

Summary of Latency-Reversing Agents

Category	Agent	Mechanism *	Reactivation in HPCs	Viral outgrowth in *ex vivo* T cell models ***	Other Major Latency Models Tested ****	Clinical effect on T cell reservoir
Chromatin Accessibility	SAHA	HDAC inhibition	Yes [9]	Yes [83], No [48]	U1,ACH-2, J89[42]	No reduction of proviral DNA, increase in HIV transcription [91]
	Romidepsin	HDAC inhibition	Not tested	No [48], Yes[45]	Primary T cell [45]	Not tested
	Panobinostat, Givinostat, Belinostat	HDAC inhibition	Not tested	No [48] (Panobinostat)	U1, ACH-2, J89 (Givinostat, Belinostat) [42]	Not tested
	Aza-CdR	DNA methylation inhibition	No [9]	Not tested	J-Lat, ACH-2, U1 [42]	Not tested
Activation of Transcription Factors	HMBA	P-TEFb activation	No [9]	Yes [83]	U1, ACH-2 [42]	Not tested
	Prostratin, Bryostratin-1	PKC activation	Weak [9] (Prostratin)	Yes [83] (Prostratin), No [83] (Bryostatin), No [48] (Bryostratin)	J-Lat, SCID-humanized mouse, and Bcl-2 (Prostratin) [42], THP-p89 and J1.1 (Bryostratin) [42]	Not tested
	Disulfiram	AKT activation	No **	No [48]	Bcl-2 [42]	No reduction of proviral DNA [65]
Cytokines	IL-7	Cellular activation and cell cycling	Not tested	No [83] (IL-7+IL-2), Yes [66,67]	SCID-humanized mouse [145]	Not tested
	TNF-alpha	NF-κB activation and histone remodeling	Yes [7,9]	Yes [83]	ACH-2, J-Lat [57]	Not tested
	TLR agonists	NF-κB activation	Not tested	Not tested	Primary T cell, primary monocyte, U1 [40]	Not tested
	Interferon alpha	Unknown	Not tested	Not tested	None	Reduction in proviral DNA [81]

*
See references in text.

**
McNamara, Ganesh, and Collins, unpublished.

***
All *ex vivo* models test the effect of latency-reversing agents on latently infected resting CD4^{+} T cells isolated from optimally suppressed HIV^{+} donors. Study [48] added a T-lymphoblast cell line, MOLT4/CCR5, while others added allogeneic PBMCs.

****
Direct comparison of ex vivo versus multiple *in vitro* models for many of these latency-reversing agents can be found in reference [83].

Bcl-2 = model using primary CD4+ T cells transduced with Bcl-2

SCID-humanized mouse = immunodeficient mouse with human immune cells after bone marrow engraftment