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The Latent Reservoir for HIV-1: How Immunologic Memory and Clonal Expansion Contribute to HIV-1 Persistence

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Abstract

Combination antiretroviral therapy (ART) for HIV-1 infection reduces plasma virus levels to below the limit of detection of clinical assays. However, even with prolonged suppression of viral replication with ART, viremia rebounds rapidly after treatment interruption. Thus ART is not curative. The principal barrier to cure is a remarkably stable reservoir of latent HIV-1 in resting memory $CD4^+$ T cells. Here we consider explanations for the remarkable stability of the latent reservoir. Stability does not appear to reflect replenishment from new infection events but rather normal physiologic processes that provide for immunologic memory. Of particular importance are proliferative processes that drive clonal expansion of infected cells. Recent evidence suggests that in some infected cells, proliferation is a consequence of proviral integration into host genes associated with cell growth. Efforts to cure HIV-1 infection by targeting the latent reservoir may need to consider the potential of latently infected cells to proliferate.

Introduction

In 2014, ~37 million people were living with HIV-1 infection (www.unaids.org). Optimal patient outcomes are achieved by initiating combination antiretroviral therapy (ART) as soon infection is diagnosed, regardless of the $CD4^+$ T cell count (1–3). ART reduces plasma virus levels to below the clinical detection limit (20–50 copies of HIV-1 RNA/ml) and halts disease progression (4–6). Recommended initial regimens consist of two nucleoside analog reverse transcriptase inhibitors and a third drug, either an integrase inhibitor or the protease inhibitor darunavir (3). Although ART effectively suppresses viremia, it is not curative, and viremia rebounds upon ART cessation (7, 8). Therefore, lifelong treatment is required. Providing lifelong treatment for all infected individuals poses a major economic and logistical challenge. Only 15 million people currently receive ART. The tolerability of ART regimens has improved dramatically, but long term drug toxicity is also a concern. Other problems include the emergence of resistance with suboptimal treatment and the stigma associated with the infection. For these reasons, there is great current interest in a cure (9, 10).

The principal barrier to cure is a stable reservoir of latent HIV-1 in resting $CD4^+$ T cells (11, 12). The reservoir persists even in patients on long term ART who have no detectable

viremia (13–18). The cells comprising this reservoir have a memory phenotype (12, 19–23). Direct measurements of the latent reservoir in patients on ART show a very slow decay rate ($t_{1/2}$=3.7 years) (16, 17). At this rate, eradication of a reservoir of 10^6 cells would require 73 years, making cure unlikely even with lifelong ART. Thus, research towards a cure focuses on eliminating this reservoir. Recent reviews have discussed molecular mechanisms of HIV-1 latency (24–27) and approaches for eliminating the reservoir (10, 28–30). Here we consider explanations for its remarkable stability.

Why does HIV-1 establish latent infection?

Viral latency is a reversibly nonproductive state of infection of individual cells (31). Latently infected cells contain a stable form of the viral genome, either as a circular plasmid in the case of herpesviruses or as a linear provirus stably integrated into host cell DNA in the case of HIV-1. During latency, there is highly restricted expression of viral genes (31). For some herpesviruses, latency evolved as an essential mechanism of immune evasion and viral persistence (31, 32). For HIV-1, latency is not necessary for persistence as active viral replication occurs throughout the course of infection in untreated patients (33). Escape from immune responses is through rapid evolution of variants not recognized by cytolytic T lymphocytes (CTL) or neutralizing antibodies (34–41). Nevertheless, a latent reservoir is established rapidly in all HIV-1-infected individuals (42). Latently infected cells can be detected in the rare individuals who spontaneously control HIV-1 infection without ART (43). Early ART restricts the size of the reservoir (22, 44) but does not block its establishment (42). In rhesus macaques infected with simian immunodeficiency virus (SIV), which also establishes a latent reservoir in resting $CD4^+$ T cells (45, 46), initiation of ART on day 3 post infection prevents detectable viremia but not the establishment of a latent reservoir (47). Thus it is difficult to prevent the establishment of the latent reservoir.

A recent theory suggests that HIV-1 evolved a mechanism for rapid establishment of latent infection to facilitate transmission across mucosal barriers (48, 49). Latency is proposed to serve as a “bet-hedging strategy” that allows some infected cells to survive long enough to transit the mucosa. However, as is discussed below, infected cells can remain in a latent state for years, and a long time interval between mucosal exposure and viremia has never been documented.

Latency is most simply explained as a consequence of viral tropism for activated $CD4^+$ T cells which can transition to a resting memory state that is non-permissive for replication (Fig. 1). HIV-1 has a strong propensity to infect activated $CD4^+$ T cells (50, 51). CCR5, a critical co-receptor for entry of the commonly transmitted forms of HIV-1 (52–57), is upregulated on $CD4^+$ T cell activation (58). Following entry, reverse transcription of the viral RNA genome into DNA and integration of the resulting provirus into host cell DNA occur within hours (59). Transcription of the integrated provirus then begins because active nuclear forms of key host factors needed for the initiation and elongation of viral transcription, including NFκB, NFAT, and pTEFb, are present in activated cells (60–67). In contrast, resting $CD4^+$ T cells mostly lack CCR5 expression (58), and other factors interfere with HIV-1 replication even when the virus has successfully entered. The cellular protein SAMHD1, a deoxynucleoside triphosphate triphosphohydrolase, depletes dNTP levels, thus

impeding reverse transcription (68–70). It is expressed at high levels in myeloid cells and resting $CD4^+$ T cells (52–55). Interestingly, SIV and HIV-2 encodes a protein, Vpx, that promotes SAMHD1 degradation (68, 71). However, HIV-1 lacks Vpx, and thus reverse transcription in resting $CD4^+$ T cells is inefficient, taking as long as 3 days (72–74). The static nature of the actin cytoskeleton in resting cells inhibits delivery of the reverse transcribed viral genome to the nucleus (75). These delays facilitate recognition of DNA intermediates generated during reverse transcription by a host innate DNA sensor, IFI16, leading to caspase-1 activation and a proinflammatory form of cell death known as pyroptosis (76–78). Additional barriers to replication in resting $CD4^+$ T cells include the lack of active forms of NFκB, NFAT, and pTEFb needed for transcription of the provirus (60–63, 65, 66).

Although activated $CD4^+$ T cells are the principle target for HIV-1, they die quickly after infection. Classic studies of viral dynamics revealed a rapid decay in viremia when new infection events are blocked with ART (6, 79–81). This decay reflects the short half-life of plasma virions ($t_{1/2}$ ~ minutes) and of the infected cells that produce most of the plasma virus ($t_{1/2}$ ~ 1 day). Activated T cells are prone to die in the contraction phase of immune responses due to activation-induced cell death (82). In addition, productively infected cells may die from other cell death pathways triggered by viral proteins or by integration of the provirus into the host cell genome (83, 84). Infected $CD4^+$ T lymphoblasts may also be lysed by $CD8^+$ CTL (34, 85–87). Surprisingly CTL do not appear to shorten the $t_{1/2}$ of productively infected cells (88, 89). Nevertheless, it appears that most productively infected $CD4^+$ T lymphoblasts are short-lived.

Given that resting $CD4^+$ T cells are resistant to infection and that activated $CD4^+$ T cells die quickly after infection, how is the latent reservoir established? Some infected $CD4^+$ T lymphoblasts may survive long enough to revert to a resting memory state that is non-permissive for viral gene expression (11), particularly if they are infected within a narrow time window when still permissive for steps in the life cycle up through integration, but not for high level gene expression (Fig. 1). Thus establishment of latent infection is a rare event, consistent with the low frequency of latently infected cells *in vivo* ($1/10^6$) (13, 16–18). Latency may be further enforced by silencing epigenetic modifications of the integrated provirus (90–92). In this latent state, the virus persists essentially as genetic information. When antigen or cytokines subsequently activate the cell, the provirus is transcribed, viral proteins are produced, and virus particles are released. Given the long $t_{1/2}$ of memory T cell responses and the fact that the latent proviruses in these cells are not detected by the immune system or targeted by ART, stable persistence of HIV-1 is not surprising. This simple model views latency in the context of the normal physiology of immunologic memory, thereby explaining all clinical observations regarding HIV-1 persistence without requiring the evolution of special viral mechanisms for latency.

Residual viremia, the latent reservoir, and viral rebound

Trace levels of free virus (~1 copy/ml) are present in the plasma of most patients on ART (93–95). Sequence analysis of the residual viremia (RV) reveals that these viruses resemble viremia present earlier in infection, are sensitive to the patient's current ART regimen, and

generally do not show evidence of ongoing evolution (96–100). These features all suggest RV originates from a stable reservoir (101). In situations where evolution has been detected, suboptimal ART may be the cause (102). Importantly, intensification of standard three drug ART with additional antiretroviral drugs from a different class does not reduce RV (103–105), indicating that it originates from long-lived cells infected prior to ART. Latently infected resting $CD4^+$ T cells are at least one source of RV. The presence of RV suggests that multiple latently infected cells are become activated every day. While patients remain on ART, the viruses released do not infect additional cells. However, if ART is interrupted, viral rebound occurs. Rebound is typically seen within 2 weeks (7, 8), the time required for washout of antiretroviral drugs and growth of the recently released viruses to detectable levels. The rebound virus is archival in character, consistent with the conclusion that it originates from a stable latent reservoir (106). The limited variation in time to rebound, despite a two log variation in reservoir size, also suggests that multiple cells are activated per day (107). This conclusion is consistent with a recent analysis of viral rebound which detected multiple viral lineages emerging in multiple sites (lymph node, ileum, and rectum) (8).

Evidence for latent infection of resting memory $CD4^+$ T cells

This model for latent HIV-1 infection as a barrier to cure is supported by several lines of evidence:

1. Replication-competent HIV-1 can be readily recovered from highly purified resting $CD4^+$ T cells from essentially all infected individuals, regardless of the duration of ART (12, 13, 15, 16, 18, 108). Recovery requires activating the cells to reverse latency, as predicted by the model. As is discussed below, recovery only fails when the size of the latent reservoir is substantially reduced (44, 109–111). Controversy remains over the question of whether other cell types including macrophages serve as stable HIV-1 reservoirs (112–123). To date, long term persistence of replication-competent HIV-1 in the setting of optimal ART has only been demonstrated for resting $CD4^+$ T cells (124). This may in part reflect the difficulty of sampling tissue macrophages, particularly in sites such as the central nervous system. Persistence in tissue macrophages can in principle be studied in novel humanized mouse models (123) and in the SIV model, but only through the use of animals on fully suppressive, long term ART and with the caveat that restriction by SAMHD1 is counteracted by SIV Vpx (68, 69).

2. Latent HIV-1 is found in resting memory $CD4^+$ T cells but only to a limited extent in naïve $CD4^+$ T cells (12, 19–23).

3. The generation of latently infected cells can be reproduced *in vitro* in primary $CD4^+$ T cells that have been activated in some manner, infected, and then cultured to allow reversion to a resting state (125–129). Restimulation of these cells through the T cell receptor (TCR) leads to HIV-1 gene expression. Together, these results support persistence of latent HIV-1 in resting $CD4^+$ T cells that have been previously infected while in an activated state.

The general concept of HIV-1 latency is also strongly supported by the cure and "near cure" cases of the "Berlin patient" (109), the two "Boston patients" (110, 111), and the "Mississippi baby" (44). The Boston and Berlin patients were HIV-1-infected individuals who developed malignancies requiring hematopoietic stem cell transplantation (HSCT) resulting in immune reconstitution with donor cells. The Berlin patient received HSCT from a donor whose cells were homozygous for a deletion in CCR5, and was cured as the reconstituting T cells were not permissive for entry of R5-tropic HIV-1 (109). Attempts to reproduce this cure have thus far been unsuccessful due in large part to progression of the malignancy. In one case, the appearance of viral variants that utilize the alternative HIV-1 coreceptor, CXCR4, has been noted (130). The Boston patients received HSCT from donors with wild-type CCR5, and ART was continued throughout the transplant period to protect donor cells from infection. When apparently complete reconstitution with donor T cells had occurred, and HIV-1 was no longer detectable by standard assays, ART was interrupted. The patients maintained suppression of viremia for 3 and 8 months before sudden and dramatic rebounds.

The Mississippi baby, born to an infected mother who had no prenatal care, had a plasma HIV-1 RNA level of ~20,000 copies/ml shortly after birth and was immediately started on ART. Plasma HIV-1 RNA declined to below the limit of detection and remained there even after treatment was interrupted against medical advice between 15 and 18 months of age. Treatment was not restarted, and viremia remained undetectable for over 2 years before suddenly rebounding. Importantly, HIV-1-specific T cell responses were absent in all three subjects because of the transplant process or early treatment. Antibodies to HIV-1 were not detected in the Mississippi baby and were markedly decreased in the Boston patients. Because HIV-1 replication is exponential in the absence of immune responses and ART, HIV-1 persistence for months or years in these patients can be best explained by the non-replicating or latent form of the virus. In these cases, HSCT or early ART delayed rebound by reducing the number of latently infected cells to the point where stochastic reactivation was a rare event (107).

Explanations for the long $t_{1/2}$ of the latent reservoir

The decay rate of the latent reservoir was originally measured using a viral outgrowth assay (VOA), which quantifies viral outgrowth from limiting dilutions of mitogen-stimulated resting $CD4^+$ T cells from patients on ART (13, 131–133). The original VOA-based measurements of the reservoir decay, published in 1999 and 2003, indicated a $t_{1/2}$ of 3.7 years (16, 17). This value was confirmed in a more recent study ($t_{1/2}$ =3.6 years), indicating that despite the development of newer, less toxic, and more convenient ART regimens, the fundamental problem of the reservoir as a barrier to cure has not been overcome (18). A critical issue is whether the remarkable stability of the reservoir is the result of normal homeostatic mechanisms that maintain immunologic memory or other factors.

One controversial explanation for stability is that the reservoir is constantly replenished by a low level of *de novo* infection that continues despite ART (134). This replication may reflect inadequate drug levels in certain anatomical sites including the lymph nodes (135, 136) or cell-to-cell spread which is more difficult to block with ART (137). However, multiple lines

of evidence indicate that ART effectively curtails new infection of susceptible cells. Because HIV-1 replication is invariably accompanied by the progressive accumulation of mutations (138) reflecting the error prone nature of reverse transcriptase (139) and possibly hypermutation by the host restriction factor APOBEC3G (40, 140–143), the lack of sequence evolution in the viral reservoir (22, 97, 100, 144, 145) indicates that ART blocks ongoing cycles of viral replication. A recent report claiming viral evolution is confounded by sampling only in the first 6 months of ART, a period during which short-lived populations of infected cells not representative of the stable reservoir are dominant (146). Prior to the development of effective ART, a dominant clinical issue was the evolution of drug resistance (147–151), but the incidence of resistance is now decreasing (152–154). Indeed there are overwhelming clinical data that ART is effective, and treated patients can expect a near normal life expectancy (3, 155–158). As mentioned above, the failure of ART intensification to reduce RV indicates that current ART regimens stop new infection events (103–105). Finally, in the delayed rebound cases mentioned above, HIV-1 persistence during ART cannot be explained by ongoing replication as this would have led to immediate rebound. Therefore, the stability of the latent reservoir is most likely due to the long $t_{1/2}$ of memory T cells and their renewal through proliferation.

Functional studies have shown that memory $CD4^+$ T cell responses in humans can provide lifelong immunity. In individuals who received the smallpox vaccine or cleared Hepatitis C infection, virus-specific $CD4^+$ T cell responses persist for decades despite the absence of further antigen exposure (159, 160). Early studies of the memory cell lifespan in humans examined radiation-induced chromosomal abnormalities that preclude cell proliferation. This allowed estimates of the intermitotic $t_{1/2}$ of lymphocytes (161, 162). The measured $t_{1/2}$ of 22 weeks for memory T cells is roughly consistent with subsequent *in vivo* measurements using glucose or deuterium labeling which indicate a $t_{1/2}$ on the order of months for human memory $CD4^+$ T cells (163, 164; also reviewed in 165). This is substantially shorter than the $t_{1/2}$ of individual naïve T cells (1–8 years). Importantly, it is shorter than the $t_{1/2}$ of the HIV-1 reservoir (3.7 years) and of functional memory T cell responses (8–12 years). The discrepancy between the half-life of individual memory T cells and the overall memory immune response suggests that proliferation of memory cells must contribute to the stability of the latent reservoir. However, as discussed above, productively infected cells have a very short $t_{1/2}$, and therefore the concept that infected cells can proliferate is not well appreciated. The HIV-1 Vpr protein induces cell cycle arrest at G_2 by interacting with a host E3 ubiquitin ligase (166, 167) and stimulating the degradation of host proteins including the DNA replication factor MCM10 (168). For cells in a latent state of infection, this block to proliferation is not operative, and latently infected cells can, in principle, proliferate if the driving stimulus does not strongly upregulate HIV-1 gene expression (169–172).

Memory $CD4^+$ T cell proliferation can be driven by antigen, cross-reactive recognition of other self or foreign peptides presented with MHC class II, or cytokines. In the murine system, neither antigen nor class II MHC is required for memory T cell persistence (173), although memory $CD4^+$ T cells that persist in the absence of MHC class II are functionally impaired (174). The requirements for maintenance of human memory $CD4^+$ T cell responses is less clear, and could include interactions with cognate signals or cytokines. Little is currently known about the antigen specificity of cells harboring latent HIV-1, although a

small subset of them may be HIV-1-specific (175). However, human memory $CD4^+$ T cells exhibit cross-reactivity, and specificities for antigens never encountered can be detected among memory $CD4^+$ T cells in peripheral blood (176). As discussed above, it is expected that stimuli acting through the TCR will upregulate expression of latent HIV-1, but this may not be the case for cytokine-driven proliferation. The cytokines implicated in memory T cell homeostasis and survival are IL-7 and IL-15 (reviewed in 177). IL-7 is required for stimulating homeostatic proliferation of memory $CD4^+$ T cells. Mice deficient in IL-7 (or IL-7R) have severely reduced total T lymphocyte levels, and reduced splenic size and cellularity (178). IL-15 also plays a role in homeostatic proliferation of memory $CD4^+$ T cells (179). Early *in vitro* studies indicated that IL-7 can actually induce expression of latent HIV-1 (180, 181). However, in patients on ART, infusion of IL-7 leads to the proliferation of memory $CD4^+$ T cells, including latently infected cells, with little or no induction of HIV-1 gene expression (182, 183). *In vitro* studies in a primary cell model of HIV-1 latency confirm that latently infected cells can proliferate in response to IL-7 (plus IL-2) without upregulation of HIV-1 gene expression (169). These studies suggest that the latent reservoir can be maintained within memory T cells undergoing homeostatic turnover. Analysis of memory T cell subsets has provided additional insight into this issue.

Memory $CD4^+$ T cell subsets

Memory T cells can be divided into two main subsets, central memory (T_{CM}) and effector memory cells (T_{EM}), based on expression of homing and chemokine receptors involved in preferential trafficking to secondary lymphoid organs or peripheral sites, respectively (184). HIV-1 DNA is preferentially harbored in T_{CM} and another subset of memory T cells, transitional memory T cells (T_{TM}) (21). T_{TM} have a phenotype ($CD45RA^-$, $CD27^+$, $CCR7^-$) intermediate between T_{CM} and T_{EM}. The two main subsets of $CD4^+$ memory T cells that harbor latent HIV-1, T_{CM} and T_{TM}, may provide a more stable reservoir for HIV-1 than T_{EM} cells, which have a higher proliferative index and are more susceptible to programmed cell death (21, 185). A recent study using the viral outgrowth assay rather than PCR demonstrated replication-competent HIV-1 persisting in T_{CM} but to a much lesser extent in T_{TM}, indicating that T_{CM} may represent the major source of persistent HIV-1 in most patients (186).

Another recently defined subset of memory $CD4^+$ T cells that may contribute to HIV-1 persistence is the stem cell-like memory T cell subset (T_{SCM}) (187). T_{SCM} are phenotypically similar to naïve T cells (T_N) in that they are $CD45RO^-$, $CD45RA^+$, and $CCR7^+$. However, they also express surface markers characteristic of memory cells, such as CD95 and IL-2Rβ (187). T_{SCM} rapidly respond to antigen and secrete IFN-γ, IL-2, and TNF. They are also stimulated to proliferate by IL-7. A stepwise progression from T_N to T_{SCM} to T_{CM} to T_{EM} has been proposed, with T_{SCM} potentially able to give rise to other types of memory T cells and self-renew upon stimulation. T_{SCM} can be infected with HIV-1 *in vitro*, and in patients on ART, HIV-1 DNA is present in T_{SCM} at higher levels than in other memory subsets (188). Although latently infected T_{SCM} represent only a small fraction of the total reservoir, they may be of particular importance because of their stability and capacity for self-renewal (23, 188).

In summary, analysis of memory subsets reveals HIV-1 genomes distributed in multiple memory cell subsets, with higher frequencies in subsets with greater potential to survive. Several issues remain. One concern is that many studies of the distribution of HIV-1 genomes in T cell subsets rely primarily on PCR-based measures of the proviral DNA. This is problematic in that the vast majority of proviruses in resting $CD4^+$ T cells from treated patients are highly defective (189). There is also substantial patient-to-patient variability in the distribution of viral genomes within these subsets. Finally, these subsets are not static and can interconvert in ways that are not yet fully understood, and it is therefore unclear whether latent HIV-1 stably persists in a given subset.

Anatomical distribution of the latent reservoir

Most studies of the latent reservoir sample $CD4^+$ T cells from peripheral blood. Given the continuous recirculation and wide tissue distribution of memory T cells, it is generally presumed that latently infected resting $CD4^+$ T cells will be present in most secondary lymphoid organs and in non–lymphoid tissues (190–192). Early studies demonstrated latently infected cells at roughly equal frequency in blood and lymph nodes (12). In the SIV model, latently infected resting $CD4^+$ T cells were demonstrated in blood, lymph node, and spleen (45, 46). Interestingly, as is discussed below, some recently described memory cell populations that are not present in the blood may also contribute to HIV-1 persistence.

HIV-1 can infect follicular helper T cells (T_{FH}) (193–196), and this population has received considerable attention because $CD8^+$ CTL lack chemokine receptors needed for migrating into B cell follicles (196), thus making the follicles a site of “immune privilege”. In the subset of rhesus macaques that spontaneously control SIV, viral replication is restricted to T_{FH}, presumably because $CD8^+$ CTL lyse infected cells elsewhere in the node (196). 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. If latently infected T_{FH} persist, HIV-1 eradication strategies may need to include not only latency reversing agents and stimuli to enhance the $CD8^+$ CTL response (197), but also interventions to disrupt B cell follicles to permit access by CTL (196).

Another population of memory T cells that could potentially harbor latent HIV-1 is the tissue resident memory T cell (T_{RM}) populations (198, 199). Pioneering studies in the murine system demonstrated wide distribution of memory $CD4^+$ T cells, including in non-lymphoid tissues such as liver and lung (190). Subpopulations of memory cells may be generated in or recruited to particular non- lymphoid tissues where they reside for long periods of time (192, 199). These T_{RM} lack expression of CCR7 and share phenotypic and functional properties with T_{EM}. However, unlike other memory subsets, they express CD69, a cell surface lectin that is upregulated at early times following T cell activation. In humans, the majority of T_{EM} cells in lymphoid and mucosal tissues, including lungs and intestines, express CD69 and therefore may be retained in these sites as T_{RM} (191, 200). Human skin also contains significant T_{RM} populations (198). Thus far, T_{RM} have not been directly examined for the presence of latent HIV-1. However, persistent HIV-1 has been detected in gut associated lymphoid tissue of individuals on ART (201, 202). T_{RM} are prominent in the lamina propria and among intraepithelial lymphocytes, and it is possible that T_{RM} harbor HIV-1. Many

$CD4^+$ T_{RM} exhibit activated phenotypes, with reduced surface expression of CD28 (191), and therefore it is unclear whether latent infection can be established in these cells. Further characterization of tissue-specific reservoirs for HIV-1 is an important research priority.

Evidence for clonal expansion of infected cells

Consideration of the mechanism of memory cell homeostasis suggests that the stability of the latent reservoir is at least partially dependent upon the ability of infected cells to proliferate. Several studies have provided direct evidence for clonal expansion of infected cells, beginning with studies of RV (100, 102). Although patients starting ART during chronic infection harbor diverse viral quasispecies (138), the RV is often dominated by identical sequences detected on independent sampling over months to years. The origin of these sequences is unknown, but may reflect infection of cells that then proliferate giving rise to multiple progeny cells carrying identical proviruses (100, 102, 203). The fraction of identical HIV-1 sequences within samples from patients on ART increases with time, consistent with proliferation of infected cells (145). More recent studies have used integration site analysis to provide definitive evidence for the proliferation of infected cells. The sites of integration in different cells are generally different and are distributed widely throughout the human genome. Early studies in cell lines infected *in vitro* with HIV-1 (204) and in resting $CD4^+$ T cells from patients on ART (205) revealed a strong preference for integration within active transcriptional units. However, integration occurs in either orientation with respect to the host gene, and there is no consensus sequence at the integration site. Therefore, the precise human sequence at the junction between host and HIV-1 DNA uniquely identifies individual infection events and thus all the clonal progeny of a single infected cell. In addition, novel deep sequencing analysis allows enumeration of the clonal progeny of a single infected cell within a sample by detection of differences in the random break points in fragments of sheared DNA containing the same integration site (170, 206). Application of this and related approaches to $CD4^+$ T cells from patients on ART has provided dramatic evidence for clonal expansion (170, 171, 207). Maldarelli et al. showed that 43% of 2410 integration sites in $CD4^+$ T cells from 5 patients were in clonally expanded cells (170). The finding that multiple cells with the same integration site can be captured in a single blood sample reflects dramatic clonal expansion *in vivo*.

Interestingly, some expanded clones had proviruses integrated in human genes associated with cell growth, and some of these genes have been observed to contain integrated proviruses in multiple independent studies (170, 171, 205, 207, 208). These include myocardin-like protein 2 (*MKL2*), a transcription factor, and basic leucine zipper transcription factor 2 (*BACH2*), a transcription regulator affecting lymphocyte growth, activation, senescence, and cytokine homeostasis. For these genes, integration events were found in a specific regions of the gene and in the same transcriptional orientation as the host gene. This skewed pattern reflects a post-integration selection process that favors the *in vivo* growth and survival of cells with those integration events since these patterns were not seen in *in vitro* infections (170, 171, 204). These results raise the interesting possibility that integration into certain host genes contributes to HIV-1 persistence by stimulating infected cells to proliferate in a manner distinct from homeostatic proliferation. The molecular mechanisms are currently unclear.

A caveat to these studies is that the methods used do not capture the full sequence of the integrated provirus. Some methods capture only the junction between host and HIV-1 DNA. Given that the vast majority of proviruses are defective, as a result of large internal deletions or APOBEC3G-mediated hypermutation (189, 207), it must be assumed that most expanded clones carry defective proviruses. There is no selective pressure against cells carrying defective proviruses that do not produce viral proteins. Previous studies have described expanded clones carrying defective proviruses, some of which persist for many years (22, 209). However, a recent report has described dramatic *in vivo* expansion of an infected $CD4^+$ T cell clone in a treated patient who also had squamous cell carcinoma (210). Importantly, this clone was capable of producing replication-competent virus. The integration site could not be precisely localized because it was in a region of repetitive sequence. The clone was found widely distributed in sites of metastatic tumor throughout the body, raising the possibility that the clonal expansion occurred in response to tumor antigen. A current issue of great importance is the extent to which expanded cellular clones harbor replication-competent HIV-1.

Implications

The stability of the latent reservoir is the principal reason that HIV-1 infection cannot be cured. The normal mechanisms that maintain immunologic memory provide a simple explanation for this stability. However, the pool of latently infected cells is not static. While the total pool size decreases only very slowly, cells in the reservoir are continually being activated to produce virus that is evident as residual viremia. These cells may die, but homeostatic proliferation of memory cells helps to balance the loss. In addition, a more cell autonomous process of proliferation driven by integration-site dependent alterations in host gene expression may allow some infected cells to undergo dramatic clonal expansion. Efforts to target the latent reservoir have generally assumed that intervention-dependent reductions in the frequency of latently infected cells will be stable so that repeated interventions will ultimately allow cure. The possibility that subpopulations of infected cells can continue to proliferate may further complicate eradication efforts.

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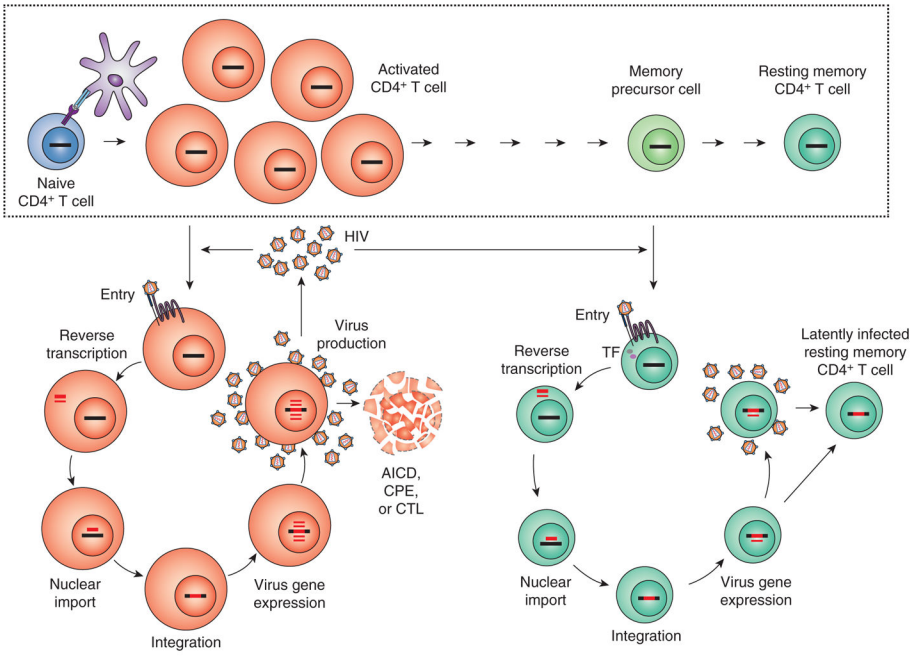

Figure 1.

Model for the establishment of latent HIV-1 infection in resting memory $CD4^+$ T cells. The normal process of memory cell generation (boxed) involves the exposure of a resting $CD4^+$ T cells to antigen which leads to blast transformation, proliferation, and differentiation into effector cells. Many effector cells die during the contraction phase of the immune response, but a fraction survive and gradually return to a quiescent state as long-lived resting memory cells. Most resting $CD4^+$ T cells lack expression of CCR5, a critical coreceptor for HIV-1 entry. Activation of resting cells by antigen (Ag) upregulates CCR5 expression and reverses other blocks to HIV-1 replication in resting $CD4^+$ T cells, allowing productive infection of these cells. Most productively infected $CD4^+$ T lymphoblasts die rapidly from activation-induced cells death (AICD), viral cytopathic effects (CPE), or lysis by CTL. As activated cells transition back to a resting state, active forms of key host transcription factors needed for HIV-1 gene expression are sequestered. Infection at this stage may lead to latent infection rather than cell death. Other models posit direct infection of resting cells. Please see text for references.

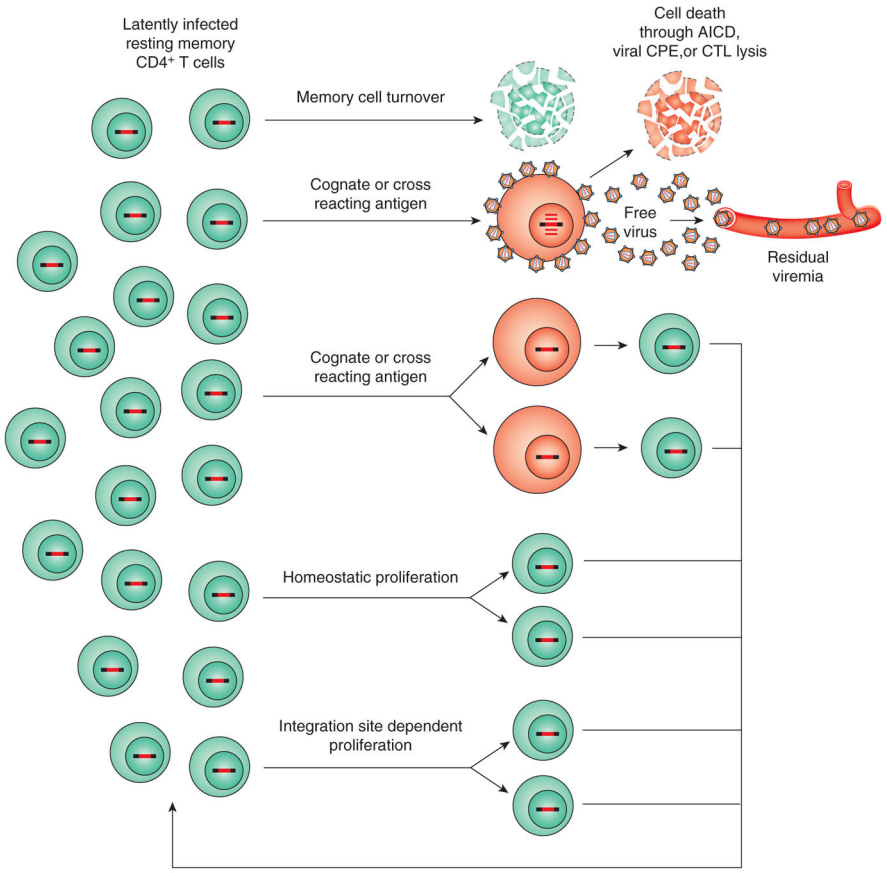

Figure 2.

Dynamics of the latent reservoir. ART largely blocks new infection of susceptible cells. In patients on long term ART, the pool of latently infected cells is extremely stable (t½ = 3.7 years) so that memory cell turnover must be largely balanced by proliferation of previously infected cells. Latently infected resting memory $CD4^+$ T cells occasionally encounter the relevant cognate antigen (or a cross-reacting antigen) and become activated. Activation reverses latency, allowing viral gene expression and virus production. In patients on ART, the released viruses do not successfully infect new cells, but may be detected at very low levels in the plasma where they constitute the residual viremia (RV). Most productively infected cells die quickly from AICD, CPE, or lysis by CTL. It is possible that some degree of antigen-driven proliferation may occur without activation of viral gene expression. Homeostatic proliferation of memory cells may also occur without reactivating viral gene expression. For some infected cells, integration of the provirus into genes associated with cell growth may also stimulate proliferation. See text for references.