Heterogeneity of HIV-1 latent reservoirs

Jia-Cong Zhao1,2, Kai Deng1,2

1Institute of Human Virology, Key Laboratory of Tropical Disease Control of Ministry of Education, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, Guangdong 510080, China.
2Department of Immunology, Zhongshan School of Medicine, Sun Yat-Sen University, Guangzhou, Guangdong 510080, China.

Abstract
Antiretroviral therapy (ART) can effectively inhibit human immunodeficiency virus-1 (HIV-1) replication, but is not curative due to the existence of a stable viral latent reservoir harboring replication-competent proviruses. In order to reduce or eliminate the HIV-1 latent reservoir, characteristics of the latently infected cells need to be intensively studied, and a comprehensive understanding of the heterogenous nature of the latent reservoir will be critical to develop novel therapeutic strategies. Here, we discuss the different cell types and mechanisms contributing to the complexity and heterogeneity of HIV-1 latent reservoirs, and summarize the key challenges to the development of cure strategies for acquired immunodeficiency syndrome (AIDS).

Keywords: Clonal expansion; Heterogeneity; human immunodeficiency virus-1; HIV-1; Integration sites; Latent reservoirs

Introduction
As a critical step of the retroviral life cycle, human immunodeficiency virus-1 (HIV-1) integrates its viral genome into the host genome of the infected cells, a small fraction of which can survive and persist for a long time in a resting state, avoiding cytotoxic effects or immune clearance. Since antiretroviral therapy (ART) was designed to block new infections but cannot eliminate infected cells, these latently infected cells (HIV-1 latent reservoir) can produce infectious viruses and rekindle new infections once ART is interrupted.[1,2] These latently infected cells are extremely stable, as clinical data and mathematical modeling predicted that it might take more than 73 years for them to decay to zero under continuous ART.[3,4] Therefore, HIV-1 latent reservoir has been widely considered the major barrier to viral elimination and the focal point of acquired immunodeficiency syndrome (AIDS) cure research.

The successful cases of “Berlin Patient”[5] and “London Patient”[6,7] have ignited global enthusiasm of achieving AIDS functional cure for more HIV-1 infected individuals. However, no apparent reduction of HIV-1 latent reservoir has been observed in a series of clinical trials other than the bone marrow transplantations. One of the biggest challenges underlying the endeavors to reduce HIV-1 latent reservoir is the tremendous heterogeneity of these viral genome-harboring cells, thus a comprehensive understanding of the heterogenous characteristics of latently infected cells will be critical to develop novel strategies to eliminate these cells. In this review, we discuss the different cell types and mechanisms contributing to the complexity and heterogeneity of HIV-1 latent reservoirs and summarize the key challenges for developing effective therapeutic strategies for AIDS functional cure.

Distribution of HIV-1 latent reservoirs in various cell types and subpopulations

Many cell types including CD4+ T cells, macrophages, dendritic cells are susceptible to HIV-1 infection. However, only resting memory CD4+ T cells have been widely regarded and studied as the typical latent reservoirs for HIV-1, primarily due to their unique physiological state of active and resting,[8] and the dynamic process of effector-to-memory transition.[9] The earliest differentiation state of mature CD4+ T cells is called naive T cells (TN), which possess the greatest proliferative potential and a long half-life. After antigen exposure, these TN cells can further differentiate into central memory T (TCM), transitional memory T (T(TM)), effector memory T (TEM), and terminally differentiated T (T(D)) cells, as identified by the expression of cell surface markers including CD45RA, CD45RO, CCR7 or CD62L.[10] TCM cells are considered to be the primary component of HIV-1 latent reservoirs due to its abundance and long-life span.[11] TEM cells were also shown to harbor a
large portion of latent HIV-1.\textsuperscript{12} They have a shorter life span but higher proliferation rates, which may serve as an advantage for viral persistence. T\textsubscript{EM} cells have a very small proportion of CD4\textsuperscript{+} T cell reservoirs but integrated HIV-1 DNA can also be detected.\textsuperscript{13} Resident memory T cells (T\textsubscript{RM}) are widely distributed in tissues but only petrol in a limited area, expressing specific homing receptors and providing local surveillance and protection.\textsuperscript{14} It is recently reported that T\textsubscript{RM} cells are also targets of HIV-1 infection and sites of viral persistence, but their roles in HIV-1 latency still needs to be further elucidated.\textsuperscript{15}

Besides these memory T cell subsets, T\textsubscript{N} cells can also differentiate into the polarized, functional subsets, including Th1, Th2, Th17, follicular helper T (T\textsubscript{FH}), and regulatory T (T\textsubscript{reg}) cells, which are reported to also harbor replication-competent HIV-1 in patients under long time ART. Similar studies have found that CD4\textsuperscript{+} T helper cells with Th1/Th17 polarization have a preferential role as long-term HIV-1 reservoirs during ART.\textsuperscript{16-18} As T\textsubscript{reg} cells harbor high frequency of HIV-1 provirus and play an essential role in immune regulation, specific eradication of T\textsubscript{reg} as a HIV-1 reservoir will be an obstacle.\textsuperscript{19} A new ex \textit{vivo} method called QUECEL (quiescent effector cell latency) mimics the process of T cell differentiation by polarizing T\textsubscript{N} cells into Th1, Th2, Th17, and T\textsubscript{reg} cells, and then infects them with a reporter pseudovirus, which can serve as a powerful tool to study the mechanism underlying HIV-1 latency.\textsuperscript{20} T\textsubscript{FH} cells expressing the C-X-C chemokine receptor type 5 (CXCR5) also have been identified as a major component of the HIV-1 latent reservoirs, which are infected more frequently by both productive and latent forms of the virus than non-T\textsubscript{FH} cells.\textsuperscript{21-23} It is recently found that the ratio of X4-tropic provirus in peripheral T follicular helper (pT\textsubscript{FH}) cells reflects disease progression and treatment outcomes during ART.\textsuperscript{24}

According to the definition of HIV-1 latency, if a cell is integrated with a replication-competent provirus and can persist for a relatively long time under ART, it can be considered as a part of the reservoirs. Therefore, non-T cell reservoirs can also play a role in HIV-1 latency and has become a topic of attention lately. As a key member of the innate immune system, macrophages can express low-level genes without specific homing receptors and allowing for binding of NF-κB (NF-κB) and nuclear factor of activated T cells (NFAT).\textsuperscript{25} The transcriptionally silent state of HIV-1 provirus in ART-suppressed infected individuals is maintained through a variety of mechanisms.\textsuperscript{26} First, the key initiation factors for HIV-1 transcription are sequestered in cytoplasm, such as nuclear factor κB (NF-κB) and nuclear factor of activated T cells (NFAT).\textsuperscript{27} The active form of NF-κB is bound by inhibitor of NF-κB (IkB), while NFAT is phosphorylated in its inactive form. Second, the positive transcription elongation factor b (P-TEFb), a critical cofactor for HIV-1 transcriptional elongation through RNA polymerase II phosphorylation, is restricted in an inactive complex.\textsuperscript{28} Furthermore, epigenetic modifications also maintain the proviral latency.\textsuperscript{29} High levels of histone deacetylases (HDACs) and histone methylases (HMTs) accumulate at the long terminal repeat (LTR) of latent HIV-1, and DNA methyltransferases lead to DNA methylation on the Cpg islands, resulting in transcription silencing.

The “Shock and Kill” strategy has been considered as one of the more promising strategies for achieving potential HIV-1 cure.\textsuperscript{30} “Shock” refers to reactivate the latently infected cells to express viral mRNA and produce viral protein through latency reversal agents (LRAs). Then killing of cells with reactivated virus will be achieved by enhanced cytopathic effect and immune clearance, or other additional interventions. A series of LRAs have been developed from \textit{in vitro} and \textit{ex vivo} systems, and some of them have been tested in experimental clinical trials.\textsuperscript{31,32} HDAC inhibitors (HDACi), like vorinostat (SAHA) and panobinostat, increase acetylation of the promoter regions including HIV-1 LTR to activate the transcription of many genes without specificity and allow for binding of NF-κB and NFAT.\textsuperscript{33} Other kinds of chromatin-modifying enzyme inhibitors, such as histone methyltransferases inhibitors (HMTi) like BIX01294 and DNA methyltransferases inhibitors (DNMTi) like decitabine, also can activate viral gene expression but will be more effective in combination with other LRAs.\textsuperscript{34,35} Protein kinase C (PKC) agonists mainly activate NF-κB by releasing it from IkB, Which results in its translocation to the nucleus and binding at the HIV-1 LTR to promote transcription. Both prostratin and bryostatin-1 are categorized to PKC agonists\textsuperscript{36} P-TEFb activator like JQ-1 can activate HIV-1 transcription independent of NF-κB via releasing active form of P-TEFb, which subsequently can be
recruited to HIV-1 Tat and promote viral transcriptional elongation via RNA polymerase II. What is more, recent studies have presented a wide range of LRAs by other mechanisms, including toll-like receptor (TLR) agonists GS-9620, non-canonical NF-κB agonists and second mitochondrial-derived activator of caspases (SMAC) mimetic AZD5582, proteasome inhibitor thiorrepton, and so on.

The multifactorial mechanisms underlying HIV-1 latency and the diverse profile of latency reversing agents again highlight the complex heterogeneity of HIV-1 latent reservoir. Although a series of LRAs have shown robust activities in reactivating latent viruses in vitro or ex vivo, no apparent reduction of latent reservoir has been reported in clinical trials. Even the unspecific global T cell activator phytohemagglutinin (PHA) was not sufficient to reactivate all proviruses after a single dose. Therefore, other factors may also play a role in controlling viral latency and reactivation.

**Diverse HIV-1 integration sites add to the heterogeneity of latent reservoir**

After integration into the host genome, HIV-1 relies on the host transcriptional machinery to replicate itself, so the microenvironments around the viral integration site largely determine the transcriptional outcome of the provirus. As a result, it is entirely possible that HIV-1 integration sites significantly contribute to the heterogeneity of HIV-1 latent reservoir. Transportation and integration of HIV-1 genome are achieved by a nucleoprotein structure called pre-integration complex (PIC), which contains viral integrase, HIV-1 DNA, and other components. PIC interacts with the host chromatin-binding protein Lens epithelium-derived growth factor (LEDGF)/p75 to target the host genome. The process of integration is not completely random, because the LEDGF/p75 promotes HIV-1 DNA to preferentially integrate into transcriptional unit of active genes. But in fact, the latent and replication-competent provirus can also be found at regions with low levels of transcription. The heterogeneity of the integration sites is also illustrated through that they are distributed not only in structurally relaxed euchromatin, but also in heterochromatin with great difference in chromatin accessibility. After all, integration of provirus at actively transcribed sites promotes an efficient transcription of viral proteins, while integration in the site with low level of transcription would delay viral expression or result in latency. Besides its location, another important factor is the orientation of the provirus relative to the host gene. Generally, when HIV-1 provirus is integrated into active genes, parallel orientations could increase the HIV-1 gene expression by 10-fold, while antiparallel orientations reduce the viral transcriptional level by 4-fold.

**Figure 1:** The evaluation of latency reversal activities of LRAs should consider both efficacy and functional range targeting latently infected cells with diverse integration sites. (A) A schematic diagram of a latently infected cell being reactivated to produce HIV-1 transcripts and viral proteins. (B) After HIV-1 latent reservoirs are “shocked” by LRAs, some LRAs show wide functional range with lower efficacy (LRA a), while some show higher efficacy but more limited functional range (LRA b). LRA: latency reversal agent; HIV-1: human immunodeficiency virus-1.
Maldarelli et al.\(^{(59)}\) developed a method to detect HIV-1 integration sites in latently infected cells from five patients under long-term ART, and a total of 2410 different integration sites were recovered. Surprisingly, approximately 70% of the locations targeted more than once were known to be directly related to cell growth or mitosis. Another research conducted by Wagner et al.\(^{(60)}\) also found that HIV-1 integration sites were enriched in cancer-associated genes. Both studies have found many identical integration sites existing in multiple cells within each patient, suggesting that a large population of latently infected cells were generated and maintained through clonal expansion. Also, these observations indicated that the effect of integration sites in these cells could contribute to their long-term survival.

Due to the numerous diversities of HIV-1 integration sites, evaluation of latency reversal agents should not be limited to their efficacy to reactivate viral transcription, but should also consider their abilities to target proviruses with diverse integration sites [Figure 1]. The potential mechanism of HIV-1 integration sites selection still needs further exploration, and whether certain integration sites can promote latency establishment or clonal expansion of the

Figure 2: Principle of HIV-1 integration sites detection. (A) Genomic DNA of resting CD4\(^+\) T cells were extracted. (B) DNA fragments were randomly interrupted, and fragments possess both HIV-1 genome and human genome are targeted. Different cells with the same integration sites have diverse break point. (C) End repair and LTR digestion were conducted on the fragments, and several rounds of PCR were applied for targeted amplification. (D) Fragments from former step were sequenced and mapped to human genome to identify various integration sites. For the same integration sites, the variety of fragments with different length represents the number of clonally expanded HIV-1-infected cells. LTR: long terminal repeat; PCR: Polymerase chain reaction.
infected cells remain a critical question that need to be solved in the future.

**Potential mechanisms contributing to the clonal expansion of HIV-1 latent reservoirs**

Since the phenomenon of reservoir clonal expansion was first reported in 2014,[59,60] it has been confirmed by a series of studies[61,62] and widely accepted as an important mechanism maintaining the long-term stability of viral reservoirs. It has been estimated that more than half of the latently infected cells undergo clonal expansion.[62] Our recent study identified CD161+ CD4+ T cells as an important compartment of the HIV-1 latent reservoir containing a significant number of proviruses with identical sequences, also confirming the existence of clonal expansion.[8,13] Therefore, deciphering the elements driving clonal expansion will be significant to counteract the maintenance of the viral reservoir, and to develop novel strategies for AIDS functional cure.

So far, three possible mechanisms of the clonal expansion have been proposed: (1) antigen-driven proliferation. Evidence from infected individuals suggests that the reservoir cells can expand under the induction of tumor antigens[64] or co-infected viral antigens like cytomegalovirus, Epstein Barr virus.[65] In this situation, HIV-1-infected, antigen-specific CD4+ T cells are likely to undergo expansion, but they also might be reactivated to express HIV-1 protein, resulting in cytotoxic effect or immune clearance. Therefore, the expanded clones would fluctuate over time.[66] (2) Homeostatic proliferation mediated by cytokines. Studies have found that signals from IL-7 and IL-15 induce the homeostatic proliferation of memory CD4+ T cells with latent HIV-1 provirus.[13] The effect of cytokines driving homeostatic proliferation is relatively steady, because IL-7 and IL-15 does not induce viral antigen expression and thus expanded cells can evade immune surveillance.[67] (3) Integration site-dependent proliferation. The methods of integration sites detection identify both the integration site and the number of clonally expanded HIV-1-infected cells [Figure 2]. As reported by Maldarelli et al.[59] and Wagner et al.,[60] repeated integration sites tend to be localized to regions that related to cell growth or cancer-associated genes. Integration site-dependent clonal expansion of HIV-1-infected cells is likely driven by increasing transcription of a pro-proliferation gene or losing function of a tumor suppressor gene.[68,69] Integration site-dependent proliferation will gradually increase over time for it is out of immune control, but in a relatively low rate that may take years to be observed. Finally, all these mechanisms could work at the same time to drive the proliferation of HIV-1-infected cells.

**Conclusion and Perspective**

The tremendous heterogeneity of HIV-1 latent reservoir has certainly posted huge challenges to the AIDS functional cure efforts. However, understanding and acknowledging this challenge will help us develop novel strategies to target the HIV-1 latent reservoir. This review has discussed several layers of heterogeneity of HIV-1 latent reservoir, including cell types, latency and reactivation, integration sites, and clonal expansion, all of which play important roles in the establishment and maintenance of HIV-1 latent reservoirs. The study of these characteristics also gives us some inspirations: when we aim to reactivate or silence the HIV-1 provirus, we should consider the effectiveness of the method we use on all or most latently infected cells in various types and subpopulations, and whether the effort of intervention can reach different tissues or anatomical compartments (sanctuary sites); at the same time, we can target the mechanism of HIV-1 integration and clonal expansion to destabilize or reduce the latent reservoir.

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**Conflicts of interest**

None.

**References**


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