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Barriers for HIV Cure: The Latent Reservoir

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Abstract

Thirty-five years after the identification of HIV-1 as the causative agent of AIDS, we are still in search of vaccines and treatments to eradicate this devastating infectious disease. Progress has been made in understanding the molecular pathogenesis of this infection, which has been crucial for the development of the current therapy regimens. However, despite their efficacy at limiting active viral replication, these drugs are unable to purge the latent reservoir: a pool of cells that harbor transcriptionally inactive, but replication-competent HIV-1 proviruses, and that represent the main barrier to eradicate HIV-1 from affected individuals. In this review, we discuss advances in the field that have allowed a better understanding of HIV-1 latency, including the diverse cell types that constitute the latent reservoir, factors influencing latency, tools to study HIV-1 latency, as well as current and prospective therapeutic approaches to target these latently infected cells, so a functional cure for HIV/AIDS can become a reality.

Keywords: HIV, viral reservoirs, persistent infection, latency, shock and kill, block and lock

Introduction: Classes of HIV-1 Latency and Cell Types Constituting the Latent Reservoir

According to the most recent data from UNAIDS, more than 36.7 million people are currently living with HIV-1 worldwide, with 1.8 million newly infected individuals reported in 2016. Although the introduction of combination antiretroviral therapy (cART) has allowed inhibition of HIV-1 replication by a combination of mechanisms, which effectively decrease viral loads to undetectable levels, delay disease progression, restore T cell immune function, and prevent transmission to uninfected individuals, the HIV-1 latent reservoir represents a major hurdle to completely eradicate this infection.[1–3]

Despite remarkable efforts to provide an accurate definition for the HIV-1 cellular reservoir, to date it still remains challenging for the scientific community. In an exercise to bring consensus, Eisele and Siliciano defined the HIV-1 reservoir as infected cells harboring full-length transcriptionally inactive, but replication-competent, HIV-1 proviruses in individuals on cART with sustained viremia suppression.[4] These viral reservoirs are long-lived, self-replenishing, refractory to ART, and, due to their resting phenotype, unrecognized by the immune system.[5,6] HIV-1 latency is associated with a quiescent viral state established in resting $CD4^+$ T cells and other long-lived immune cell populations, in which the HIV-1 proviral DNA is transcriptionally silenced with no (or minimal) production of infectious viral particles.[1,2,6,7] This viral latency can be achieved by two distinct mechanisms: (1) by preventing proviral integration in the cellular genome (preintegration latency), and (2) by enforcing proviral silencing at the transcriptional level (postintegration latency). Details on factors influencing postintegration latency are discussed in the next section.

Preintegration latency was first reported in $CD4^+$ T cells,[8] but there is also evidence for this type of dormant infection in monocytes. In this case, the HIV-1 complementary DNA (cDNA) is partly or completely synthesized during reverse transcription, but fails at integrating into the cellular genome, remaining as an episome.[8–10] This form of latency is relieved upon T cell activation, allowing the integration of the provirus and, in consequence, leading to a productive infection.[9] Unlike postintegration latency, latent infections in which the virus life cycle has been compromised at a preintegration step are rather unstable in resting $CD4^+$ T cells, where the amounts of HIV-1 cDNA decrease rapidly.[11,12] In the case of nondividing cells, such as those of the monocyte lineage, the presence of restriction factors seems to play a major role in

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preventing proviral integration. In these cells, SAMHD1, members of the APOBEC3 family, and MX2 strongly interfere with early steps of the HIV-1 replication cycle,[13–19] and thus, one could speculate a more relevant role of preintegration latency in these cell types than in $CD4^+$ T cells. Despite the evidence of preintegration latency, more studies are needed to assess its persistence and contribution to the size and landscape of the viral reservoir, since our current knowledge on preintegration latency is still limited.[20,21]

Postintegration latency (referred hereafter as latency), however, accounts for most of the HIV-1 persistent reservoir. Postintegration latency is established once the provirus is integrated into the host DNA, but fails at properly expressing its genome, having restricted viral expression—so viral antigens are inefficiently produced and, presumably, poorly presented (or not at all) to the immune system. Although latency is rather infrequent, this silent state is remarkably stable and reversible.[1,2] Indeed, latently infected cells harboring replication-competent dormant proviruses maintain the potential to spontaneously activate, or reactivate, in the presence of cytokines, antigens, latency-reversing agents (LRAs), or when ART is interrupted,[22–24] causing viral rebound and further replenishing the latent reservoir. It is widely recognized that latency is established within days of the initial infection. However, as mentioned above, this pool of cells remains undetected by the immune surveillance and is refractory to ART.[2,25,26] Despite the fact that latently infected cells can reseed viremia upon stimulation, only few of these proviruses have the potential to re-emerge from latency. Errors during proviral integration causing deletions, insertions, or introducing mutations in the HIV-1 cDNA seem to be the main cause for their defective phenotype.[27] In fact, according to a recent study by the Siliciano laboratory, only 10% of all integrated HIV-1 proviruses maintain an intact genome, and just one tenth of these replication-competent proviruses has the potential to reactivate *in vitro*[3] (able to generate infectious virions upon cellular activation).

The latent reservoir includes a varied pool of cells, and thus, its origins are also diverse, including: (1) replication-competent proviruses in long-lived latently infected cells[1,28]; (2) viral sanctuary tissues where cART has poor penetration, so virus replication is maintained, generating residual viremia (i.e., central nervous system [CNS] and gut-associated lymphoid tissues)[29–31]; (3) cells that promote cell-to-cell virus dissemination, in which a low-level but ongoing viral replication is sustained[32,33]; (4) homeostatic proliferation of replication-competent proviruses in latently infected cells[5,34,35]; and (5) intermittent viremia from spontaneous activation of a small fraction of resting, infected $CD4^+$ T cells during cART.[5,36,37] Despite its type and origins, the latent reservoir is normally referred to three cell groups: $CD4^+$ T cells, dendritic cells (DCs), and macrophages.

Since latency is primarily established when infected and activated $CD4^+$ T cells transition to a resting memory phenotype, the best-characterized latently HIV-infected cells are resting memory $CD4^+$ T cells, particularly the central memory (T_{CM}: $CD45RA^-$ $CCR7^+$ $CD27^+$) and transitional memory (T_{TM}: $CD45RA^-$ $CCR7^-$ $CD27^+$) subsets.[5,6,25,38,39] Because they can persist for long periods of time,[40] resting memory $CD4^+$ T cells carrying replication-competent proviruses represent an important long-term viral reservoir in patients on cART.[1,2,6,7] In fact, latently infected $CD4^+$ T cells are present in blood and different tissues, including lymphoid tissues and the gut.[10,29,41] Besides resting memory $CD4^+$ T cells, latency has also been reported in other T helper cell subsets such as naive,[5,42] follicular,[43] and memory stem cells.[44,45] In addition, recent studies have demonstrated the presence of latent, replication-competent HIV-1 proviruses in the unconventional T cell subset $V\delta2$.[46] Therefore, the contribution of other $CD4^+$ T cell populations to the reservoir may currently be underestimated.

In addition to $CD4^+$ T cells, HIV-1 can also remain dormant in non-T cells and thus, cause viral rebound upon cART is discontinued, as evidenced by early studies by Chun *et al.*[47] It is now well known that HIV-1 can persist latently in cells of the myeloid lineage, including monocytes, macrophages, and DCs.[48–50] Macrophages are susceptible to HIV-1 infection, and thus, they are active producers of HIV-1 virions in both ART-treated and untreated patients.[51,52] Therefore, similar to $CD4^+$ T cells, macrophages have the potential to harbor latent HIV-1 proviruses. Macrophages present longer persistence of unintegrated viral DNA and are less susceptible to the cytopathic effects caused by HIV-1 compared to $CD4^+$ T cells.[12,48,53] Moreover, these cells present an extended lifespan in the presence of infection, and greater resistance to apoptosis,[54–57] since HIV-1 potently triggers the NF-κB pathway, inducing antiapoptotic genes.[58–60] The fact that macrophages can engulf HIV-1-infected $CD4^+$ T cells increases the probability of macrophage infection and latency establishment.[61] Importantly, since macrophages are antigen-presenting cells, viral reactivation in these phagocytes may increase HIV-1 transmission to other immune cells through the virological synapse,[62] but also through the production of chemokines, which would facilitate the recruitment and activation of other immune cells.[63] Therefore, although the contribution of macrophages to the latent reservoir appears smaller than that of $CD4^+$ T cells, they may be important contributors to viral maintenance and rebound due to their inherent cellular and immune characteristics.

Early work on HIV-1 latency already reported that proviral DNA was detected in monocytes/macrophages from infected individuals.[64] More recently, Yukl *et al.* demonstrated that gut tissue macrophages from ART-suppressed individuals contained viral DNA, although their studies did not directly address whether macrophages constitute part of the HIV-1 latent reservoir.[65] However, under ART conditions, $CD4^+$ T cells from blood and mucosal tissues decrease dramatically, while infected macrophages undergo death at a slower rate than that of T cells, consequently giving rise to the slower second phase of decay.[36] This, together with their intrinsic characteristics mentioned above, suggests that macrophages can sustain ongoing viral replication, and thereby, importantly contribute to the viral reservoir. In fact, both *in vitro* as well as *in vivo* assays revealed that latency can be established in monocyte-derived macrophages,[66] and that virus reemergence from latency can be observed from macrophage-tropic HIV-infected cells, in myeloid-only humanized mice on ART[67]—evidencing that tissue macrophages can persist as a viral reservoir. Furthermore, macrophages can be found as latently infected cells in tissue sanctuaries, where the access of drugs is limited, serving as a source of HIV-1 viral persistence. For example, macrophages residing in the CNS, together with microglial cells and astrocytes, are anatomical viral sanctuaries representing the principal HIV-1 reservoir in

the brain, and posing a major barrier for HIV-1 therapies.[68] However, it remains unclear to which extent these cells can be effectively reactivated to produce infectious particles. However, in the gastrointestinal tract macrophages represent a significant cellular reservoir for HIV-1, since they are extremely abundant in this tissue,[69] where HIV-1 intensively replicates and spreads.

DCs are antigen-presenting cells that act as a connection between the innate and the adaptive immune systems. Upon antigen recognition, they mature and migrate to lymph nodes for antigen presentation to T cells. DCs are classified into three major subtypes: myeloid DCs, plasmacytoid DCs, and Langerhans cells, all of them susceptible to HIV-1 infection, although inefficiently. DCs are also an important component of the HIV-1 reservoir, with follicular DCs (FDCs) being the major DC contributors.[48,70,71] In fact, work from Haase *et al*. in ART-treated patients demonstrated the presence of a relatively stable pool of virions on the surface of FDCs, but not on other mononuclear cells.[72] This was corroborated in later studies, suggesting that FDCs have the capacity to produce low-level viremia and to recruit resting $CD4^+$ T cells, which may aid in virus spread. Hence, since the trapped virions are replication competent, FDCs may contribute to pathogenesis and also serve as a significant HIV-1 reservoir for durable viral persistence.[71,73–76] Similar to macrophages, FDCs are in close contact with resting $CD4^+$ T cells within lymphoid tissues. Actually, early studies described the capacity of DCs to promote *trans* HIV-1 infection to $CD4^+$ T cells,[77,78] which requires the formation of infectious synapses.[79] Therefore, this DC-$CD4^+$ T cell interaction may be critical to drive and establish latency in resting memory $CD4^+$ T cells.[33]

Factors Influencing the Establishment and Maintenance of Latency

The establishment and maintenance of HIV-1 latency is a complex process modulated by many different factors[6,80–82]; some of which have the ability to suppress latency, such as cytokines involved in T cell activation,[6,83,84] Toll-like and Rig-I-like receptor agonists,[85–87] effectors of the NF-κB pathway,[82,88–90] inhibitors of histone deacetylases (HDACs),[91,92] or the pleiotropic effector mTOR[93]; whereas other factors promote proviral latency, for instance histone modifiers,[82,90,94,95] inhibitors of viral transcription,[96,97] cytokines that induce memory T cell differentiation,[98,99] or transcriptional repressors[82,90,100–102] (Fig. 1). Despite the vast number of elements that play a role in the modulation of viral gene expression and latency establishment, the mechanisms by which they ultimately achieve such regulatory effect *in vivo* are not fully understood. The elements presented above act at different molecular levels in the process of HIV-1 transcription, such as (1) integration site selection and epigenetic regulation, (2) transcription factor-mediated activation and repression, (3) RNA elongation, (4) transcription interference, (5) silencing mediated by microRNAs (miRNAs), (6) RNA splicing and transport, and (7) generation of antisense HIV-1 genomic transcripts, which downregulate gene expression.[103–105] The influence of the environmental factors presented above, as well as their synergistic/antagonistic effects on these molecular levels, has a profound impact on latency establishment and maintenance. Therefore, investigating how these molecular networks interact with each other is crucial to increase our fundamental understanding of HIV-1 latency.

Integration site selection and epigenetic regulation

The transcription of integrated HIV-1 proviruses, as well as the expression of host genes, is strongly regulated by variations in the condensation state of the chromatin. Genes located in high compacted DNA regions, or heterochromatin, remain silenced due to inaccessibility of transcription factors to their regulatory elements. By contrast, highly expressed genes are found in less condensed DNA areas (euchromatin).[106] Contrary to what it was previously thought, the process that leads to HIV-1 proviral DNA integration does not occur in a random manner, but in a more selective way: by targeting euchromatin regions through a direct interaction between the viral integrase and the host protein LEDGF/p75, which in turn interacts with RNA-binding proteins found in areas where DNA is being actively transcribed.[107,108] In addition to LEDGF/p75, other host proteins are involved in the selective integration of HIV-1 proviruses into actively transcribed chromatin areas, such as CPSF6 (through its direct interaction with capsid molecules found in the preintegration complex), and the host splicing machinery (in this case, through their association with LEDGF/p75).[107,109–111] Consistent with this, a high-throughput study of the integration landscape of HIV-1, in which primary cells from HIV-1 viremic progressors, viremic controllers, and patients under cART were analyzed, revealed that HIV-1 proviruses frequently integrate within intronic regions of highly expressed genes, irrespective of the type of patient (controllers or progressors).[112] However, in patients controlling viremia with cART, the majority of the proviruses analyzed corresponded to latent HIV-1, since actively infected cells are negatively selected by the antiretroviral drugs. In this context, these proviruses were rarely found within genes, and in cases where they integrated within coding regions, these genes were surrounded by heterochromatin.[112] This is in agreement with findings using *in vitro* T cell line models for latency, where HIV-1 proviruses are frequently located in/or near heterochromatin,[113] suggesting that the condensation state of the chromatin at the integration sites influences the outcome of the infection.[111] However, studies in primary cells showed that HIV-1 proviruses have an analogous integration pattern in both resting memory $CD4^+$ T cells and actively infected $CD4^+$ T cells,[114,115] reflecting that there must be factors other than chromatin condensation state and integration site selection involved in the establishment and maintenance of latency *in vivo*.

Although discrepancies have been found when analyzing the proviral integration sites in T cell lines and primary cells,[116,117] even when using the same cell line models, common features have been defined, such as the observation that integration favors *Alu* repeats both in genic and intergenic regions, implying that the integration sites may harbor some consensus sequence, although unknown yet.[112,115] Furthermore, the inconsistent results on the chromatin status surrounding latent proviruses may simply reflect versatile epigenetic modifications. As stated earlier, besides the HIV-1 integrase, cellular cofactors aid in the integration of the provirus, and these molecules preferentially bind to less condensed chromatin areas. Therefore, it is tempting to speculate that events occurring during the activated-to-resting transition of $CD4^+$ T cells may influence the overall chromatin structure, causing chromatin remodeling at the locations where HIV-1 proviruses are integrated.[118]

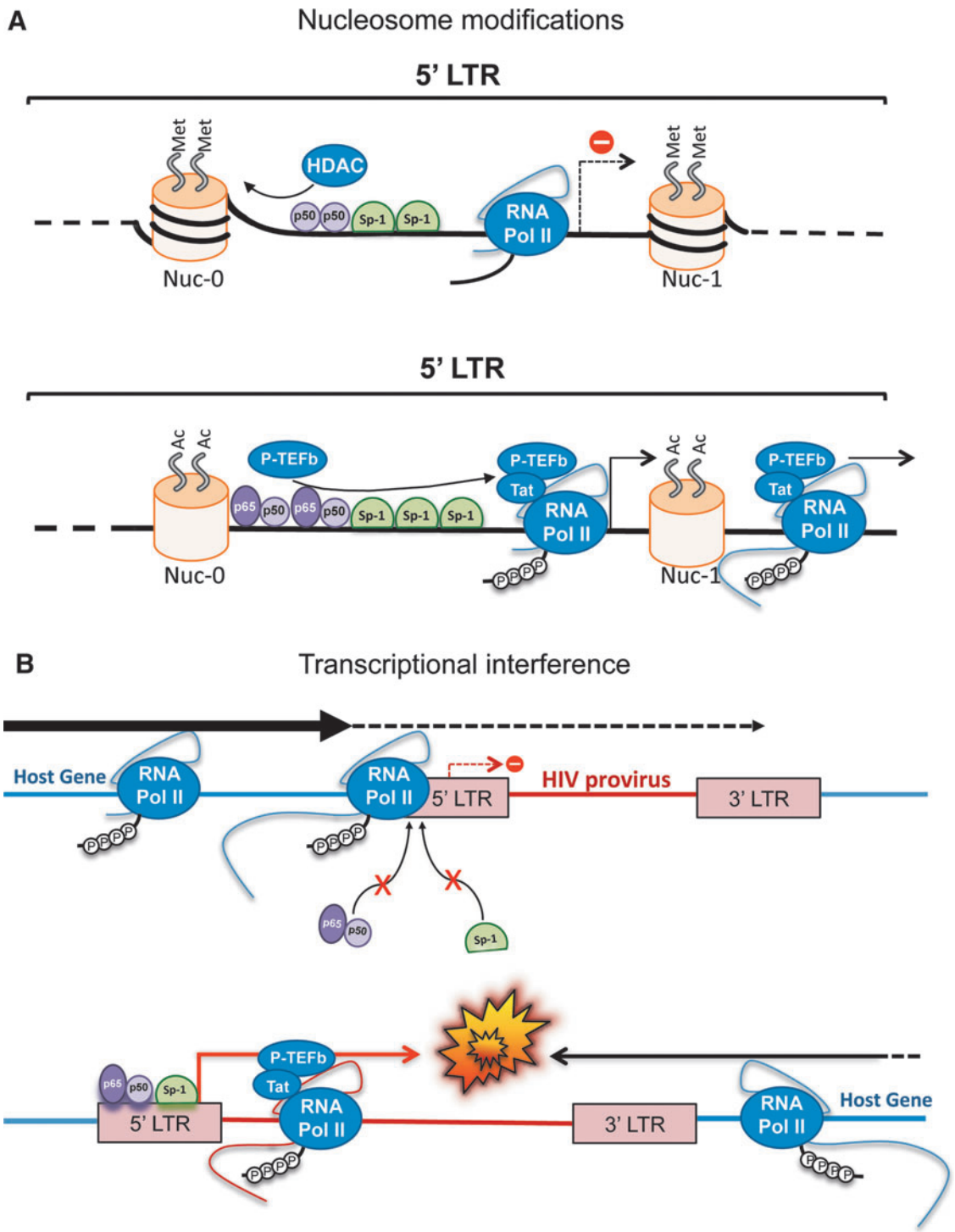

FIG. 1. Factors influencing the establishment and maintenance of latency. **(A)** Effects of chromatin condensation status and epigenetic modifications in the repression (*top*) or activation (*bottom*) of HIV-1 transcription. **(B)** Representation of scenarios for transcriptional interference. *Top*: when the HIV-1 provirus is in the same orientation as a highly expressed cellular gene. *Bottom*: when the provirus and the cellular host gene have opposite orientations. Color images are available online at www.liebertpub.com/aid

The condensation state of the chromatin, and therefore, its related transcriptional levels, is regulated by a highly dynamic process, which involves posttranslational modifications (PTMs) of different DNA-associated proteins. Chromatin is wrapped around histone proteins, forming nucleosomes. However, unlike euchromatin, heterochromatin is characterized by a highly condensed status, where the chromatin–histone interactions are more rigid. Differential combinations of PTMs on these histones will determine the condensation state of surrounding chromatin, since PTMs can alter the ability of histones to recruit Mi-2/NuRD and SWI/SNF complexes, which are directly

related to DNA condensation and unwinding processes, respectively. Histone acetyltransferases (HATs) promote histone acetylation and DNA relaxation, increasing transcriptional activity, whereas HDACs promote chromatin condensation, which represses gene expression.[106] Similar to cellular DNA, the 5′ long-terminal repeat (LTR) of HIV-1 proviruses contains two nucleosomes: (1) nuc-0, located at the 5′ end of the LTR (between nucleotides −405 and −245, from the transcription start site), and (2) nuc-1, located after the provirus promoter (between nucleotides +20 and +165)[119–121] (Fig. 1). These nucleosomes are present even when proviral DNA integration occurs within euchromatin regions.[122,123] Therefore, their condensation status will directly influence proviral transcription, mainly because both nuc-0 and nuc-1 partially overlap with the transcriptional control regions in the LTR, and thus, can alter the association of transcriptional regulators to the HIV-1 promoter as well as interfere with RNA elongation[123] (Fig. 1A). For instance, viral reactivation and nuc-1 remodeling was observed in different studies when HATs, such as CBP or GCN5, were recruited to the provirus integration site.[124–126] Similar results were obtained when resting $CD4^+$ T cells were exposed to HDAC inhibitors, which led to nuc-1 displacement and facilitated the recruitment of the RNA Pol II to the LTR.[6,127] These results indicate that the acetylation and, in turn, condensation state of the HIV-1 nucleosomes, play an important role in the establishment and maintenance of latency.

In addition to histone acetylation status, epigenetic control can also occur by directly modifying DNA through methylation, a reversible modification that has a profound impact on gene expression. The methylation of cytosines on CpG-rich regions causes transcriptional repression. This has also been observed for HIV-1 proviruses, particularly in areas of the proviral DNA containing the transcriptional control regions. Methylation of cytosines in this location causes proviral DNA silencing and promotes latency[6,82,102,128] (Fig. 1A, top). Several reports have shown a high methylation pattern on the 5′ LTRs of latent proviruses from latently infected $CD4^+$ T cells obtained *in vitro*.[128–131] These epigenetic modifications prevent transcription factors from binding to their cognate enhancers and thus, repress provirus transcription.[132] In agreement with these results, demethylation of the provirus LTRs has been associated with transcriptional reactivation and virus reemergence from latency *in vitro*.[128,133,134] However, DNA methylation does not seem to play a major role in HIV-1 latency *in vivo*, since no significant levels of CpG methylation are found within the 5′ LTR in resting $CD4^+$ T cells from HIV-1-infected individuals on suppressive ART.[135] These observations suggest that the contribution of CpG methylation in the establishment of the latent reservoir in patients is minimal compared with the other epigenetic factors mentioned above.

Transcription factors

As indicated earlier, the largest reservoir for HIV-1 is found in resting memory $CD4^+$ T cells, after they transition from an activated status to a resting memory phenotype. Because HIV-1 RNA synthesis relies on the presence of cellular transcription factors that are only temporarily available upon antigen exposure, HIV-1 transcription declines in this activated-to-resting transition.[6,118,136] Therefore, the availability of these transcription factors is a major factor governing the establishment of latency. The HIV-1 proviral DNA contains several regulatory regions on its 5′ LTR with responsive elements for different host transcription factors, which, upon binding, alter viral RNA synthesis by modulating RNA Pol II access and recruitment to the TATA box in the promoter.[102,137,138] Among these factors, NF-κB, NFAT, and Sp-1 play a key role in this transactivation process.[6,102,139–141] The presence of these three transcription factors in the nucleus, and their ability to interact with their enhancer elements at the LTR, is required to maintain basal HIV-1 transcriptional levels,[140,142] whereas their cytoplasmic sequestration, or inability to bind to the regulatory elements due to mutations, has been linked to latency in $CD4^+$ T cells.[6,102,140,142,143] In addition to NF-κB, NFAT, and Sp-1, there are other transcription regulators, AP-1,[144] C/EBPb,[145] USF-1, and Ets-1,[146] with the ability to cooperate in HIV-1 transcription.[6,147]

Above all, NF-κB seems to play a central role in the establishment and maintenance of latency. In response to pathogens and proinflammatory cytokines, NF-κB monomers, p50 and RelA (p65), dimerize and translocate into the nucleus, where they interact with the NF-κB regulatory elements in their target cellular genes, but also in the proviral LTR, strongly activating HIV-1 transcription.[139,148] In resting $CD4^+$ T cells, RelA is found sequestered in the cytoplasm by the inhibitory protein IκBα.[148–150] Moreover, while IκBα retains RelA, p50/p50 homodimers are able to translocate to the nucleus and further reinforce proviral latency by recruiting HDACs to the HIV-1 provirus, promoting its heterochromatinization (Fig. 1A, top).[102,127] Interestingly, a recent study uncovered that the cellular protein breast cancer associated-gene 2 (BCA2, also known as Rabring7) is an important negative effector of NF-κB, and in turn, detrimentally affects HIV-1 transcription and replication.[151] Specifically, BCA2 promotes the SUMOylation of IκBα, rendering this molecule resistant to degradation and a stronger NF-κB inhibitor.[151] Ongoing studies in our laboratory have revealed that BCA2 plays a pivotal role in fine-tuning NF-κB in primary $CD4^+$ T cells, and that its depletion from naive $CD4^+$ T cells makes HIV-1 less prone to undergo latency. In line with these observations, upregulation of BCA2 enforces and prolongs proviral silencing while the selective knockdown of this gene in latently infected cells results in spontaneous viral reactivation, highlighting the relevance of BCA2 and its NF-κB regulatory activity to proviral latency (Colomer-Lluch *et al.*, unpublished data).

In addition to BCA2, there are other transcriptional regulators that negatively impact HIV-1 transactivation and thus, enhance the establishment of latency. Among these molecules we find CYLD and Naf1, which also inhibit NF-κB-driven viral transcription,[152,153] LSF-1 and YY-1, which recruit HDAC1[154]; CBF-1, which reduces the amount of RNA Pol II in the LTR[155]; Blimp-1, which interferes with the Tat-trans-activation response (TAR) association[156]; or Bcl-11b, which represses HIV-1 transcription by promoting heterochromatinization.[100] Therefore, besides the epigenetic elements considered above, the relative abundance of cellular transcription activators, such as NF-κB, and transcriptional repressors will dictate the fate of the HIV-1 infection in terms of latency.

Tat and RNA elongation

One of the main obstacles during HIV-1 transcription is the inefficient synthesis of genome-length transcripts due to RNA Pol II pausing, an event caused by secondary structures in the nascent RNA that halts RNA elongation.[157,158] To overcome this block, HIV-1 uses the viral transactivation protein Tat, which increases the elongation efficiency during transcription by interacting with a secondary structure in the nascent HIV-1 RNA (TAR element), allowing Tat to associate with positive and negative host regulatory protein complexes. To release the RNA Pol II pause, HIV-1 requires the presence of the transcription elongation factor (P-TEFb).[159,160] P-TEFb is a protein complex containing cyclin T1 and CDK9 kinase, which phosphorylates the carboxyl-terminal domain of the RNA Pol II recruited at the 5′ LTR, enhancing in turn its transcription elongation activity.[159,161] However, most P-TEFb molecules are held catalytically inactive through their association with the 7SK small nuclear ribonucleoprotein (snRNP) complex. Tat promotes P-TEFb dissociation from 7SK, allowing P-TEFb to become catalytically active, hyperphosphorylate RNA Pol II, and release the transcriptional pause.[162,163] By contrast, the negative elongation factor (NELF) complex plays the opposite role in the regulation of RNA Pol II activity.[160,164] Specifically, NELF associates with RNA pol II, limiting its escape from the HIV-1 promoter region.[165] Besides this primary role, NELF may also influence the overall structure of the chromatin. In latent proviruses, RNA pol II complexes accumulate near the transcription start site at the 5′ LTR (nucleotide +11), and at nucleotide +50[165,166]—the latter is analogous to the major pause observed in host genes.[167–169] However, in the absence of NELF, the +50 pause site is shifted 20 nucleotides downstream, reflecting repositioning of nucleosomes.[165] Therefore, NELF seems to impact HIV-1 transcription at several levels. In productive infections, however, P-TEFb counteracts this repressing effect by phosphorylating NELF and rescinding NELF's association with the nascent HIV-1 RNA.[170] Consistent with this, *in vitro* as well as *in vivo* studies have associated low levels of P-TEFb with HIV-1 latency in resting $CD4^+$ T cells.[118,136]

Despite the fact that Tat significantly enhances the efficiency of HIV-1 RNA synthesis, the first round(s) of viral transcription after the integration of the proviral DNA need to occur in a Tat-independent manner, since Tat is a nonstructural protein. Although these initial Tat-independent transcriptional events are very inefficient, some genome-length HIV-1 transcripts are successfully synthesized, mainly due to the action of NF-κB. Similar to Tat, RelA (a monomer of the NF-κB heterodimer) also has the ability to recruit P-TEFb to the 5′ LTR[171] (Fig. 1A, bottom), increasing the efficiency of RNA elongation and generating in turn full-length transcripts that will subsequently be translated into viral proteins, among which, we find Tat. From this point on, and due to a positive feedback, a highly efficient Tat-dependent HIV-1 transcription phase begins by the mechanisms described above.[82,159,161,162,172] Additionally, Tat recruits chromatin-remodeling factors, such as SWI/SNF, which facilitate transcription by promoting DNA relaxation and consequently nuc-1 remodeling.[173]

Tat's role in latency has been extensively documented.[159,174] For instance, Tat protein alone was found to be sufficient to successfully reactivate HIV-1 transcription in latently infected $CD4^+$ T cells isolated from patients undergoing cART.[159,175,176] This was demonstrated by delivering *tat* either by transduction with non-HIV vectors,[176] or by ectopically delivering recombinant Tat protein to these cells.[175] In this particular case, a soluble Tat-GFP chimeric protein was produced from pancreatic cells of transgenic mice, and delivered to primary, latently infected $CD4^+$ T cells of patients undergoing cART. Remarkably, ectopic delivery of Tat or T cell receptor (TCR)-mediated cell activation caused similar levels of reemergence from latency.[175] Likewise, the ectopic expression of this chimeric Tat protein, or *tat*'s stable expression through transduction, in Jurkat and THP-1 cells prevented the establishment of latency and reactivated latent proviruses.[175,177–179] Consistent with these findings, HIV-1 variants encoding attenuated *tat* genes are more prone to undergo latency, as evidenced by studies by the Karn laboratory using primary $CD4^+$ T cells infected either with wild-type HIV-1 NL4-3 or a NL4-3 variant carrying the attenuating H13L mutation in Tat.[118,178,180] Therefore, despite the presence of cellular transcription factors that aid in HIV-1 RNA synthesis, defects in Tat will make HIV-1 more prone to latency due to the inability to relieve the RNA Pol II pause block.

Interference

Not only does the integration site of the HIV-1 DNA influence virus transcription (due to the local chromatin state), but also the presence of active host genes surrounding the provirus, and their relative orientation, have a significant impact on HIV-1 replication.[181–184] As mentioned earlier, HIV-1 shows a preference of integration within transcriptionally active regions in the latently infected J-Lat cell line[181] as well as in resting $CD4^+$ T cells isolated from patients on cART.[114] Despite the fact that these proviruses can easily gain access to transcription factors and RNA Pol II, the presence of actively transcribed genes nearby the proviral DNA drastically affects HIV-1 RNA synthesis. In fact, the elongation of RNAs from neighboring host genes can have both a repressive and activating effect on HIV-1 transcription through different mechanisms: (1) when the integrated provirus and the nearby host gene share the same polarity, RNA Pol II elongation may prevent transcription factors from binding to their cognate sequences on the 5′ LTR, and thus, promote proviral silencing and latency[181,183] (Fig. 1B, top); (2) alternatively, the activation of host genes upstream the HIV-1 5′ LTR can cause read-through transcription of the provirus, which is an important limitation to a productive infection. This phenomenon consists of adjacent promoters initiating transcription of a host gene and continuing through the HIV-1 coding sequence, therefore impacting HIV-1 gene expression.[114,181,185] Although these HIV-1 transcripts are generally nonproductive, the effect of host gene read-through transcription might prevent latency and up-regulate proviral expression if the provirus is integrated in the same orientation as the host gene. (3) On the other hand, proviral integration in the opposite orientation of an actively expressed host gene leads to (i) a premature termination of transcription due to the collision between RNA Pol II complexes moving in different directions, which inevitably reduces the amounts of HIV-1 transcripts[181,184] (Fig. 1B, bottom), or (ii) the generation of

antisense HIV-1 RNAs. Although this event has mainly been observed from the HIV-1 3′ LTR,[103–105] one can envision a similar phenomenon occurring from host genes that are located downstream, and in the opposite direction of the HIV-1 provirus, leading to antisense HIV-1 genomic transcripts. Nevertheless, upon cellular activation, the repressive effect of these interfering mechanisms is diminished by the strong binding affinity that NF-κB exhibits for the HIV-1 5′ LTR, enhancing transcription from the virus promoter rather than from the host gene.[6,186] Although these observations indicate that the proviral integration site can affect HIV-1 transcription due to (1) the chromatin condensation status, which may hinder the recruitment of the cellular transcriptional machinery, and (2) the interference of cellular genes surrounding the HIV-1 provirus, most of these studies examined only few clones of latently infected cells. Therefore, a comprehensive interrogation of more clones is needed to ascertain how the orientation of HIV-1 proviruses relative to host genes affects the outcome of the infection.

MicroRNAs

It is well established that the presence of cellular miRNAs can regulate gene expression through the RNA-induced silencing complex (RISC), either by impairing translation or by promoting the degradation of transcripts.[187] This is also true for HIV-1, since it has been reported that cellular miRNAs present in memory $CD4^+$ T cells can directly affect HIV-1 RNA synthesis.[188–191] Using computational analysis, miR-29a, miR-29b, miR-149, miR-324-5p, and miR-378 were identified as miRNAs with a regulatory effect on HIV-1 transcription.[189] Ahluwalia *et al.* confirmed that miR-29a inhibits HIV-1 transcription in Jurkat cells by targeting the *nef* region on HIV-1 transcripts, although Frattari *et al.* reported that this miRNA affects HIV-1 RNA levels by targeting the 3′ untranslated region (UTR).[192,193] miR-28, miR-125b, miR-150, miR-223, and miR-382 have also been reported to reduce HIV-1 gene expression and infectivity by targeting the 3′ UTR of the HIV-1 RNA.[190] In addition, miRNAs can also affect the overall expression levels of HIV-1 dependency factors, playing a role in HIV-1 latency.[194] This has been observed for cyclin T1[195] and Pur-α, a co-factor of Tat.[196]

The study of miRNAs is still an emerging field, and so is the investigation of their effects on HIV-1 infection. Since the amount and species of these small RNA molecules depend on multiple intracellular and extracellular factors, research on how miRNAs affecting HIV-1 are regulated, as well as their mechanisms of action, will not only increase our understanding on their role in HIV-1 latency, but also could open new avenues to neutralize this pool of cells.

RNA export

Even if the epigenetic signature, cellular environment, and the presence of cellular transcription factors is optimal to support HIV-1 RNA synthesis, HIV-1 might, in theory, still establish a latent infection if viral transcripts are not exported, translated, or packaged into nascent virions. An important mechanism directly involved in the regulation of HIV-1 gene expression is the transport of viral transcripts from the nucleus to the cytoplasm.[197,198] The efficiency of this process relies on the presence and activity of both host and viral proteins. Rev is an auxiliary HIV-1 protein that binds to a specific nucleotide region (rev-responsive element or RRE) within viral transcripts (nonspliced and singly spliced), facilitating their cytosolic translocation.[199–202] In fact, the presence of low levels of Rev is required for a productive HIV-1 infection, whereas its absence appears to play a role in proviral latency.[203] In addition, Rev-independent events related to RNA transport also impact the establishment of latency. For instance, nuclear retention of HIV-1 transcripts, particularly multiply spliced HIV-1 RNAs, was observed in primary, latently infected $CD4^+$ T cells obtained from patients undergoing cART.[176] Although the mechanism by which this retention only occurs in resting, latently infected cells is unknown, the overexpression of the RNA-binding protein PTB circumvented this posttranscriptional block, allowing for the production of virions without requiring $CD4^+$ T cell activation.[176] Hence, factors that affect HIV-1 gene expression at a posttranscriptional level also influence the outcome of the infection, and can enforce latency.

Implication of these mechanisms in the establishment of latency in vivo

The relevance of each of the mechanisms presented above in the formation of latent reservoirs is widely accepted. However, how their combination affects the outcome of the HIV-1 infection is not well understood. In addition, the fact that many of the studies discussed in this review were performed in the context of cell lines or *ex vivo* models may not accurately reflect how latency is established *in vivo*, which complicates our understanding of the molecular bases influencing latency in patients. Despite these limitations, few aspects stand out for their convergence *in vitro*, *ex vivo* and *in vivo*, such as the roles of Tat and host transcription factors. In particular, Tat and NF-κB displace the effect of regulatory elements, such as epigenetic modulation and transcriptional interference, allowing HIV-1 RNA synthesis to proceed.[138,150,159,162,163,171,173,175] Nevertheless, we need to stress that our knowledge of the roles of less investigated elements, such as miRNAs and HIV-1 RNA export in the outcome of HIV-1 infection is incomplete. Therefore, additional studies on physiologically relevant systems are needed to better comprehend their influence over the other mechanisms, and their overall contribution to HIV-1 latency.

Current Therapeutic Strategies to Eradicate the HIV-1 Latent Reservoirs

Although current cART regimens efficiently limit HIV-1 replication, reducing the viremia to undetectable levels in $HIV\text{-}1^+$ individuals, they fail at clearing the cellular HIV-1 reservoir, posing a major challenge to achieving a sterilizing cure. As mentioned earlier, these dormant long-lived cells are resistant to ART and persist undetected by the immune system, having the capacity to emerge and support fully replicating and infectious viral particles spontaneously or if ART is discontinued.[1,2,6,7] In addition, the high cost of cART, the need for lifelong adherence, and the associated toxicities to prolonged treatment are important limitations. Thus, there is an agreement among the scientific community to develop new therapies that go beyond continuous cART treatments to finally achieve the end of the HIV/AIDS pandemic. For this,

numerous efforts are being devoted to explore novel strategies to either clear or permanently suppress the HIV-1 latent reservoir[204–207] (Fig. 2).

Shock and kill strategies

Multiple therapeutic strategies aimed at achieving a functional cure of the HIV-1 infection are focused on the ''shock and kill'' strategy, using LRAs which, in combination to cART, disrupt HIV-1 latently infected cells, so they can be eliminated by immune effectors (cytotoxic T cells or CTLs) or cleared by the cytopathic effect of the reactivated virus.[208–211] Optimally, effective and safe LRAs should reactivate the latent provirus and promote viral transcription followed by viral protein production. This approach should enhance HIV-1-specific immune responses (CTL-mediated responses) to recognize the presentation of viral antigens and clear the reactivated cells. So far, several assays in *ex vivo* latency models, as well as in clinical trials, have been performed to evaluate the feasibility of small-molecule LRA candidates to effectively reverse HIV-1 latency, which have recently been reviewed elsewhere[210,211] (Fig. 2, left panel). These include:

(1) Protein kinase C (PKC) agonists (e.g., PMA, prostratin, bryostatin, and ingenol molecules). PKC agonists activate PKC cellular isoforms, which then initiate NF-κB signaling to induce HIV-1 transcription, thereby, potently reversing latency.[88,212,213] According to recent studies, only bryostatin-1 has shown efficacy when tested in cells derived from patients on suppressive cART.[214,215] However, one of the main concerns when applying PKC agonists relates to the associated side effects resulting from continuous activation and inflammation.[214] To address this, alternative approaches have been proposed to reduce the PKC agonist-mediated off-target effects of T cell activation. For example, the use of PKC agonists in combination with adjuvant agents, such as the JAK inhibitor ruxolitinib, has shown a decrease in proinflammatory cytokine production while maintaining the capacity to reverse latency.[210] Also, an attractive option to restrict PKC agonist-derived toxicities is the recently proposed oral therapy based on the PKC agonist ingenol, a plant-derived natural compound, which reactivates latent HIV-1 and when combined with other LRAs shows synergistic effects, facilitating a reduction in the dosing of both agents.[216] Another possibility to reduce toxic effects associated with polyclonal T cell activation might be the combination of PKC agonists with Rapamycin. In a recent study by the Siliciano laboratory, the combination of LRAs with Rapamycin, a drug that triggers autophagy but also works as an immunosuppressant, showed

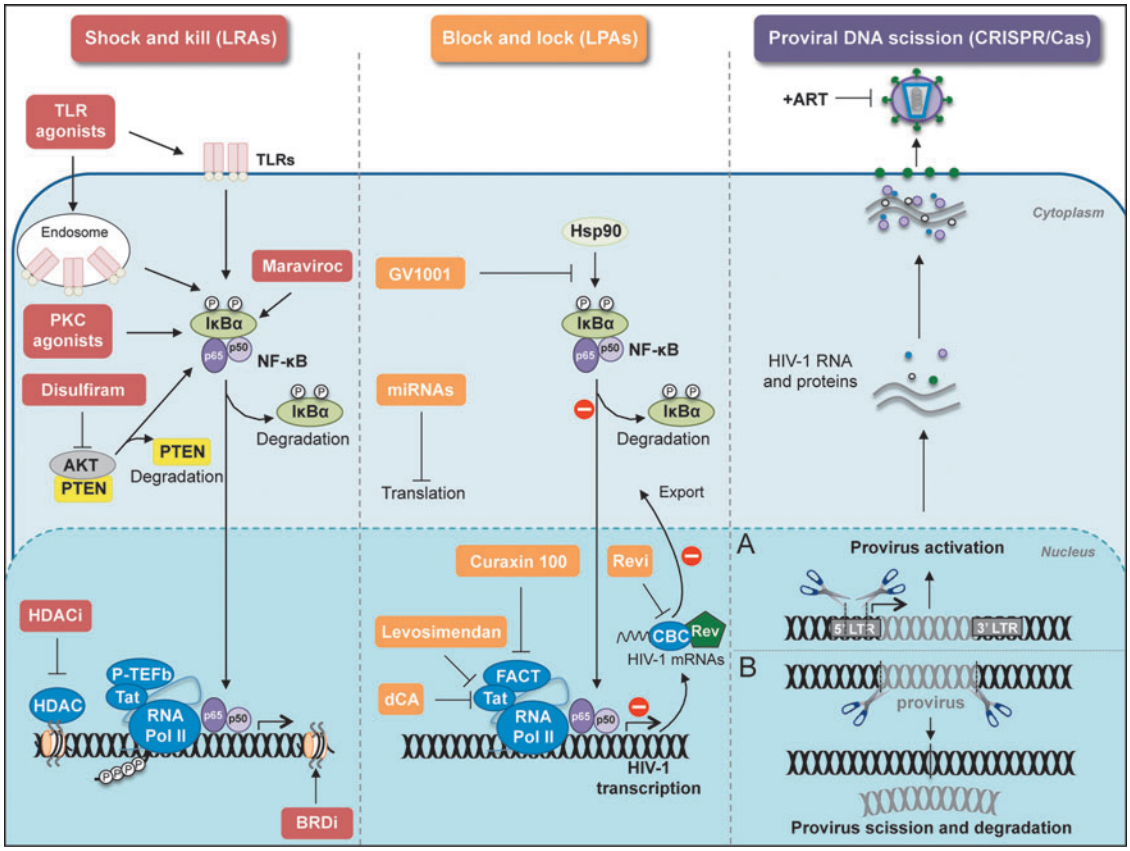

FIG. 2. Current therapeutic strategies to eradicate the HIV-1 latent reservoirs. Schematic representation of the molecules targeted by (1) latency-reversing agents in shock and kill approaches (*left panel*), (2) latency-promoting agents in block and lock approaches (*central panel*), and (3) CRISPR-mediated DNA scission techniques (*right panel*). **(A)** Gene editing tools to reactivate proviruses. **(B)** Gene editing tools to excise HIV-1 proviruses. CRISPR, clustered, regularly interspaced short palindromic repeat. Color images are available online at www.liebertpub.com/aid

efficient viral reactivation with no toxic effects from the systemic release of proinflammatory cytokines.[217]

(2) Histone deacetylase inhibitors (HDACi; e.g., vorinostat/SAHA, panobinostat, and romidepsin). As previously stated, histone deacetylation (HDAC) is an epigenetic modification of chromatin that results in the repression of HIV-1 RNA synthesis.[218] Small-molecule inhibitors targeting HDAC enzymes have shown to safely reverse proviral latency by promoting HIV-1 transcription both *in vitro* and *in vivo*, although with different success. For example, the HDACi vorinostat/SAHA modestly reactivates latent HIV-1 in resting $CD4^+$ T cells.[208,209,219–221] However, this proviral reactivation is not associated with an effective killing of latently infected cells *in vivo*,[221] suggesting that CTL activation is a prerequisite for HDACi treatment success. By contrast, treatment with panobinostat not only reactivates proviruses, but also increases innate immune activity of natural killer (NK) cells, suggesting that innate immune responses can critically modulate the efficacy of LRAs.[222] In the recent past, HDACi have been the pillars of latency reversal in clinical trials, evidencing that after HDACi treatment an enhancement in HIV-1 RNA and subsequent increase in viral production was observed.[208,223–225] Unfortunately, to date, none of the HDACi-based treatments tested in clinical trials has succeeded at significantly reducing the size of the reservoir (reviewed in Spivak and Planelles[210]).

(3) Disulfiram, an FDA-approved drug, reactivates latent HIV-1 in a primary $CD4^+$ T cell model by decreasing the levels of the PTEN cytoplasmic protein with the subsequent activation of the Akt signaling pathway.[90,226] Two clinical trials have recently assessed disulfiram as a LRA, with no significant reduction in the size of the latent reservoir being observed.[227,228]

(4) Bromo and extraterminal (BET) bromodomain inhibitors, together with toll-like receptor agonists, have shown promising results as LRAs, but the efficacy of many of these compounds *in vivo* remains to be examined.

(5) Remarkably, in a search for novel LRAs, maraviroc, a drug currently used in cART as a CCR5 antagonist, was found to reverse HIV-1 latency in infected individuals under cART in a phase II clinical trial.

(6) The authors of this study suggested that the interaction of maraviroc with CCR5 induces the activation of NF-κB and the subsequent enhancement of HIV-1 transcription in resting $CD4^+$ T cells. Therefore, maraviroc shows potential as a LRA to reduce the HIV-1 viral reservoir in ART-treated patients.[235]

Despite all this progress, the shock and kill strategy is beset by significant limitations before effective elimination of the reservoir can be achieved. Although LRAs may disrupt latency in $CD4^+$ T cells, LRAs are poorly efficient in other HIV-1 target cell populations, such as macrophages and microglial cells. Thus, their potential to reactivate all the cell types that constitute the latent reservoir is partial at best. Even the most effective LRAs have been reported to fail at reactivating all replication-competent proviruses.[3] An important caveat to LRAs is that they modulate gene expression in a general manner; thus, their potential for off-target or toxic effects is a major concern. For example, HDACi, such as SAHA act by opening the chromatin structure around the promoter of the HIV-1 integrated provirus, and therefore, may lead to the expression of other host genes, and potentially induce reactivation of other integrated proviruses alongside HIV-1. Additionally, some LRAs may activate uninfected HIV-1 target cells, which then may become susceptible to HIV-1 infection. In some cases, despite their combination with ART, LRA implementation might result in reinfection of target cells in anatomical viral sanctuary tissues, where ART has less penetration, including the CNS.[236]

Another main challenge of this approach is that LRA compounds fail at inducing sufficient viral cytopathic effects and potent HIV-1-specific CTL responses to purge HIV-1 reservoirs, showing so far a moderate impact on the reservoir size. Obstacles, such as the presence of $CD8^+$ T cell escape mutations in the proviral genome of latently infected $CD4^+$ T cells,[237] LRAs limited success at reactivating latent proviruses to allow antigen presentation for CTL-mediated elimination,[238] or the presence of HIV-1 reservoirs in lymph nodes and the CNS, where CTL infiltration is more difficult, seem to prevent the CTL-mediated clearance of latently infected cells, enhancing even further the establishment of HIV-1 reservoirs.

Additional considerations include the window opportunity of the LRAs and the fact that these compounds could alter the host immune function. In this regard, a recent study explored the effect of LRA combinations in HIV-1-specific $CD8^+$ T cell responses and found that although the administration of romidepsin (HDACi) and bryostatin-1 (PKC agonist) effectively results in the reversal of the latent sate, this dual treatment inhibits HIV-1-specific $CD8^+$ T cell function to recognize and eliminate HIV-1-reactivated cells.[239] Interestingly, another study suggests that natural latent HIV-1 reservoirs in *ex vivo* $CD4^+$ T cells from ART-treated patients present intrinsic resistance to $CD8^+$ T cell-mediated clearance, which may complicate even further their eradication.[240] The authors speculate that the observed resilience may be attributed to (i) HIV-1 Nef downmodulation of MHC-I on the surface of HIV-1-infected cells, resulting in a lack of recognition by $CD8^+$ T cells, and/or (ii) HIV-1 latently infected cells with inherent resistance may be selected to conform a large fraction of the reservoir. These limitations underscore the need for more effective and specific LRAs for viral reactivation accompanied by potent immunotherapeutic interventions to ensure stronger HIV-1-specific immune responses, and urge the design of alternative therapeutic strategies to successfully eliminate the latent reservoir.

Block and lock strategy

The block and lock approach has recently emerged as an alternative to the shock and kill strategy to functionally cure HIV-1. This approach consists of the use of small-molecule inhibitors, HIV-1 latency-promoting agents (LPAs), to effectively silence (block) the HIV-1 promoter region in latently infected cells, and therefore, restrict (lock) viral transcription of HIV-1 integrated proviruses, impairing in turn viral rebound, and further intensifying their latent state. Several compounds are being investigated for their LPA

potential, some of them with promising results (Fig. 2, central panel). These include:

(1) Levosimendan, which specifically blocks the Tat-enhanced HIV-1 transcription in a PI3K pathway-dependent manner.[241]
(2) The small-molecule Curaxin 100 (CBL0100), which impairs the access of the RNA Pol II and FACT at the HIV-1 promoter region, blocking the elongation process.[242]
(3) The G1V001 telomerase-derived peptide, which inhibits HIV-1 transcription and viral reactivation by blocking the NF-κB pathway in an Hsp90-dependent fashion.[243]
(4) Didehydro-Cortistatin A (dCA), a Tat inhibitor characterized by Mousseau *et al.*[96,244] that potently silences HIV-1 transcription.

Altogether, with this study, other compunds affecting Tat biogenesis have already been reported to cause proviral silencing.[245] However, to our knowledge, dCA is the only compound used *in vivo* that provided the first proof of concept for the potential use of a Tat inhibitor to durably suppress viral transcription.[246] With the exception of dCA, the effectiveness of the other compounds needs further investigation, particularly *in vivo*, before their ultimate therapeutic use as anti-HIV-1 agents.

Despite some limitations, the encouraging results obtained in these studies indicate that the block and lock strategy is feasible and thus, urge to explore the potential of other molecules as LPAs. One promising locking approach is to target the HIV-1 Rev protein. Rev is critical for viral replication, since it regulates the export of nonspliced and singly spliced viral transcripts from the nucleus to the cytoplasm and, as mentioned above, defects in RNA export are thought to play an important role in HIV-1 latency. Campos and collaborators recently characterized ABX464, a Rev inhibitor that blocks viral replication *in vitro* as well as in humanized mice, showing long-lasting effects and no associated adverse effects in normal cellular splicing.[247] Specifically, ABX464 binds to the Cap-Binding Complex (CBC) and impairs Rev-dependent export of nonspliced and singly spliced HIV-1 messenger RNAs (mRNAs), preventing their translation and neutralizing the expression of the viral genome. This molecule holds promise to affect the viral reservoir and encourages future studies to move further into clinical trials. Other attractive molecules as LPAs are miRNAs. In the past decade, more studies have revealed that miRNAs play a role in modulating latency in HIV-1-infected, quiescent cells.[248] As mentioned above, miR-29a, which is one of the best-characterized miRNAs affecting HIV-1, efficiently suppresses the expression of viral transcripts. This observation led the authors to consider miR-29a as a potential tool for suppressing the HIV-1 reservoir by potentiating the endogenous levels of miR-29a, but also through miR-29a agonists. Either strategy could lead to a permanently silent state of HIV-1 proviruses as part of the block and lock strategy.[193]

Although still an emerging line of investigation, permanently repressing these latently infected cells appears a promising approach to keep dormant proviruses in check, given the limited success of the shock and kill strategy. A major caveat to this approach is the need to identify cellular markers that will allow to specifically target LPAs into cells harboring replication-competent proviruses and minimize off-target effects.

Proviral DNA scission

In the past few years, the use of gene-editing technologies, such as CRISPR/Cas9 (clustered, regularly interspaced short palindromic repeats—CRISPR-associated proteins) has gained momentum, including in the HIV-1 field. Genetic modulation by CRISPR/Cas9 is based on the use of a guide RNA (gRNA) that recognizes and mutates target DNA in the HIV-1 genome, offering sequence-dependent specificity and high cleavage efficiency.[249,250] These properties make CRISPR/Cas9 a very attractive tool to permanently inactivate or even remove the proviral DNA from HIV-1 latently infected cells, and also to protect cells from future HIV-1 infections due to the continuous expression of the CRISPR/Cas9 system, thus, minimizing any potential harm to the host. To date, this technique has been tested in several cell types targeting critical HIV-1 genes as well as the LTR regions, since cleavage of both LTRs will result in the complete excision of the proviral DNA[249] (Fig. 2, right panel, B). For example, Ebina *et al*. reported the successful removal of HIV-1 proviruses in infected Jurkat T cells with CRISPR/Cas9 by targeting the HIV-1 LTRs.[251] In another study, by targeting the HIV-1 Rev region in the provirus, latently HIV-1-infected Jurkat cells were efficiently inactivated.[252] The CRISPR/Cas9 system has also been successfully implemented in human pluripotent stem cell-derived HIV-1 reservoir cell types, where this technique was used not only to excise latent proviruses, but also to neutralize incoming infections by specifically targeting the incoming viral cDNA.[253] Hu and collaborators further analyzed the capacity of the CRISPR system to eradicate the HIV-1 proviral DNA from myeloid lineage cells, the main HIV-1 cell reservoir in the brain. By targeting the U3 region in the HIV-1 LTRs, they effectively blocked viral gene expression, replication, and completely removed the proviral integrated genome that was flanked by the two LTRs, with no detectable off-target effects.[254] Despite these encouraging results, some studies have reported undesired effects when using CRISPR/Cas9 to edit the human genome.[255] This is mainly due to the cellular machinery that naturally repairs the cleaved DNA: nonhomologous end joining (NHEJ) or double-strand break (DSB) homologous recombination. Whereas homologous recombination has high fidelity, NHEJ is highly error prone, and the choice of one over the other is not controllable. To overcome this limitation, other cutting-edge gene-editing tools have been proposed to safely excise HIV-1 proviruses, such as a modified version of the Cre-Lox recombinase.[256] Although an attractive alternative, the recent advances and improvement of the CRISPR/Cas9 system, together with the fact that errors introduced by NHEJ will mainly affect HIV-1 proviruses rather than the host genome, has allowed to reliably clear integrated HIV-1 proviral DNA. For instance, the study developed by Kaminski *et al.* demonstrated efficient cleavage and removal of the HIV-1 genome in latently HIV-1-infected $CD4^+$ T cells by targeting the LTR with no associated harm to the DNA of host cells, and no undesired effects. Remarkably, in their *ex vivo* assays they found that their lentivirus-delivered CRISPR/Cas9 significantly reduced HIV-1 DNA

and protein levels in $CD4^+$ T cells from HIV-1-infected patients.[257] The same team also provided proof of concept in an *in vivo* study that the CRISPR/Cas9 system can cleave HIV-1 DNA in transgenic mice and rats.[258] Overall, these data support the solid potential of the CRISPR/Cas9-based tools to efficiently and safely cleave the HIV-1 integrated genome in latently infected cells, and therefore, block viral gene expression and replication as well as immunize against future HIV-1 infections.

Besides its use to inactivate proviruses, CRISPR/Cas9 can also be exploited to activate the transcriptional activity of the HIV-1 integrated provirus in latently infected cells in a site-specific manner, in line with the shock and kill strategy (Fig. 2, right panel, A). There is increasing evidence supporting the use of CRISPR-based transcriptional activation systems, alone or in combination with LRAs, to reverse HIV-1 latency and achieve the complete eradication of the latent infection. These CRISPR-based activation systems present substantial advantages over current LRA treatments, such as enhanced LTR activity compared with traditional shock and kill strategies, and minimal toxic and off-target effects,[259–261] triggering fully infectious viral production.[262]

One of the main concerns when using CRISPR/Cas9 to edit DNA in quiescent cells, such as the HIV-1 reservoir, is the reliance on the cellular enzymatic machinery to express the gRNA and the Cas9 protein. Most CRISPR/Cas9 tools involve the use of lentiviral or adenoviral vectors that encode for these components. Therefore, it is easy to envision that the success rate for efficiently editing the HIV-1 proviral genome would significantly increase by preactivating these latently infected cells with LRAs, so the cells are metabolically active by the time they are transduced with CRISPR/Cas9. However, this does not seem to be the case. According to a recent publication by Zhu *et al*., latently infected cells can be efficiently edited without preactivation.[252] Nevertheless, aspects such as the cell cycling status may interfere with the success of this technique, and should be investigated in detail.

There is no doubt that CRISPR/Cas9, as well as other gene-modifying techniques, has revolutionized the landscape of editing tools. Although very promising, there are some limitations and concerns that should be addressed before its general implementation as anti-HIV-1 treatments. HIV-1 viral escape is one of the main hurdles for a broad application of CRISPR/Cas9, and several studies have recently addressed whether HIV-1 could evade the CRISPR/Cas9-mediated suppression. Interestingly, Deng and collaborators found that the latent reservoir can also contain escape mutants for CRISPR/Cas9.[237] However, the use of multiple gRNAs targeting different HIV-1 conserved regions could circumvent HIV-1 escape. In fact, recent reports proved that HIV-1-infected cells can be cured by a combination of two gRNAs targeting various sites in the proviral DNA, effectively blocking HIV-1 replication and preventing viral escape.[263,264] Unfortunately, other studies evidenced HIV-1 evasion of the CRISPR/Cas9 suppression when mutations arose in the gRNA target sequence.[265,266] In addition, due to the limited success in the delivery of these CRISPR/Cas9 systems, alternative delivery methods such as nanoparticles should be tested to enhance the applicability of this approach. Another caveat is that most of the data we have on CRISPR/Cas9 and HIV-1 is derived from *in vitro* studies. CRISPR/Cas9 effectiveness and safety should be analyzed in actual resting, latently infected $CD4^+$ T cells obtained from HIV-1-infected patients to design customized CRISPR/Cas9 pairs, and this can be costly and time consuming.

Tools to Identify and Study the Latent Reservoir

One of the reasons why latency poses such a hurdle for HIV-1 eradication is the absence of effective methods to accurately detect latent reservoirs *in vivo*. The low frequency of infected resting $CD4^+$ T cells present in patients undergoing cART, together with the low levels of transcribed viral RNA, greatly limit our ability to recognize, quantify, and thus assess the size of the latent reservoir in infected individuals.[267–272] To date, the identification of unique cellular biomarkers that would correctly distinguish latently infected $CD4^+$ T cells from healthy, uninfected memory $CD4^+$ T cells still remains a challenge.[267] Different studies have attempted to address this with relative success, since the proposed molecules are either not entirely exclusive for latently infected cells or are present only in a small fraction of the latent reservoir.[273–276] Several molecules have been proposed as latency markers, including the membrane proteins CD2, PD-1, TIGIT, LAG-3, or HLA-DR.[274,275,277,278] However, the actual proportion of latently infected cells that express these surface receptors is still undetermined. Although challenging, the investigation of biomarkers exclusively found in latently infected cells is a high priority, and several groups are investigating this through different approaches, for instance by combining transcriptomics and bioinformatics datasets.[279]

If the current unavailability of specific biomarkers to detect latently infected cells is a major stumbling block to purge the latent reservoir, another important limitation is the need for better techniques to accurately measure the amount of integrated HIV-1 proviruses.[271,280]. Current methods to detect replication-competent proviruses include polymerase chain reaction (PCR)-based assays, and quantitative virus outgrowth assays (Q-VOA).[281,282]

Quantitative PCR-based methods

Different quantitative PCR (qPCR)-based methods are currently used to measure the relative amount of HIV-1 cDNA or HIV-1 RNA present in a certain pool of peripheral blood mononuclear cells or plasma obtained from infected patients.[283–285] For the DNA-based qPCR methods, the most widely used is the *Alu*PCR, which measures the total integrated proviral DNA. By combining primers that target *gag* or the LTR together with the highly repetitive elements *Alu* in the human genome, this technique exclusively detects integrated proviral DNA. However, it does not discriminate between actively or latently infected cells, nor if the detected proviruses are replication competent or defective.[271,286] For the RNA-based qPCR, the most common methods aim at measuring the relative levels of extracellular RNA (plasma viremia) over cell-associated RNA.[271,280,283,287] However, these methods also fail at pinpointing latently infected cells. Although PCR-based assays are among the most sensitive and simple available tools, their main caveat is the unwarranted detection of defective integrated proviruses.[271,280,288] In an attempt to address this limitation, Procopio *et al.* developed the Tat/Rev-induced limiting dilution assay (TILDA), which measures by qPCR the relative amount of cell-associated multiply-spliced HIV-1 RNA upon T cell

stimulation, which in turns correlates with the presence of replication-competent proviruses.[280]

Quantitative virus outgrowth assays

The Q-VOA, unlike PCR-based methods, can single out resting $CD4^+$ T cells capable of supporting a productive infection by measuring the relative release of infectious virions upon cellular stimulation. Hence, it is considered the gold standard method to quantify drug-resistant infected cells from resting $CD4^+$ T cells isolated from patients on cART.[271,281,282] However, compared with the qPCR assays, Q-VOA is more demanding, since this method is lengthy, requires large amounts of samples from donors, and seems to underestimate the actual size of the reservoir.[3,27,35,281] Unfortunately, comparative experiments using both Q-VOA and PCR-based assays have shown clear discrepancies between these two techniques. In addition, they are limited to the quantitative nature of the reservoirs and they are incapable of identifying which subpopulation of the pooled cells has the potential to sustain a productive infection.[288] Therefore, new improved methodologies to measure the latent reservoir in a simple, reproducible and effective manner are urgently needed. For instance, approaches based on proviral DNA sequencing are now gaining more attention, since they can accurately elucidate the composition of the proviral landscape within an individual, and discriminate between defective and replication-competent proviruses. Although they can be extremely expensive and labor intensive, the use of next-generation sequencing seems to streamline this approach significantly.[35]

In addition to the methods described above, several *in vitro*, *ex vivo*, as well as animal models have been developed to facilitate the study of HIV-1 latency. The *in vitro* systems include immortalized cell lines such as the T lymphocyte cell models, ACH2, J1.1, and Ca5, or the premonocytic U1 clone.[83,289–292] These cell lines remain chronically and nonproductively infected, but retain the potential to support a productive infection upon stimulation with mitogens and cytokines such as αCD3/CD28 antibodies or the tumor necrosis factor alpha (TNFα), respectively.[290,291] Jordan *et al.* generated the widely used J-Lat cell line, which was produced by inducing the stable and latent infection of Jurkat cells with a Δ*env*/Δ*nef* HIV-1 NL4-3 carrying a GFP reporter.[113] One of the weaknesses of these cell lines is that, unlike resting $CD4^+$ T cells, they remain in a constant proliferation state and thus, they may not entirely reflect latency *in vivo*. To overcome this, several groups have developed *ex vivo* systems of latency using primary $CD4^+$ T cells.[118,293–297] Among these studies, the method developed by Bosque and Planelles is characterized by the isolation of naive $CD4^+$ T cells to near purity, and inducing their differentiation through TCR engagement into a central memory-like phenotype. While activated, the cells are infected with a molecular clone of HIV-1, and are subsequently allowed to transition to this resting phenotype in a nonpolarized manner,[296,298] which closely resembles the characteristics of T_{CM} *in vivo*. *Ex vivo* systems have been extensively used to dissect signaling pathways that are involved in HIV-1 latency in $CD4^+$ T cells, but fail at addressing other aspects of proviral latency, such as the role of non-T cells and the contribution of viral sanctuary tissues to the size of the reservoir. In this regard, humanized mouse models represent a more physiological scenario for the study of latency. These animal models are generated by injecting HIV-1 into immunodeficient recipient mice transplanted with either (1) fetal human thymus and liver, which procures human T cell lymphopoiesis (Thy/Liv mice model),[299–301] (2) human bone marrow $CD34^+$ hematopoietic stem cells (HSCs; huCD4 mice model),[302] or (3) a combination of both, which generates the commonly used BLT mice model.[303] However, many investigators are skeptical to use humanized mice as a model for the study of HIV-1 latency. Although they virtually have a human immune system, the roles of other organs and tissues, such as viral sanctuaries, may not accurately reflect the overall picture of the HIV-1 reservoir in humans. Animal models that better resemble human physiology and lentiviral latency are nonhuman primates infected with SIV (simian immunodeficiency virus) or recombinant viruses of SIV and HIV (SHIV), such as rhesus macaques or pig-tailed macaques.[38,234,304–308] These models allow for (1) a more accurate study of latency and the roles of organs such as the CNS and the gut in the size and distribution of the reservoir,[307–309] and (2) testing the efficiency of antiretrovirals,[310] and possibly LRAs and LPAs. Despite these advantages, the use of nonhuman primates in AIDS research is expensive and requires the use of specialized facilities. Moreover, unlike the humanized mice models, this system requires long periods of experimental preparation.[234,308] Therefore, in spite of the improvement of the experimental systems in the study of latency[234,280,311,312] much work is still needed to develop cost and time-efficient models that would closely resemble HIV-1 latency *in vivo*.[313]

Concluding Remarks

To date, much effort has been devoted in eradicating HIV-1 from latently infected cells. However, to achieve a complete purge of the latent reservoir, a better understanding of the factors influencing the establishment and maintenance of latency, the contribution of T cells, as well as non-T cells to the latent reservoir—including HSCs[314,315]—as well as the identification of cellular biomarkers that will allow us to discriminate between healthy, resting cells and cells harboring replication-competent proviruses is needed. These technical advances will not only allow us to specifically target latently infected cells with the goal of clearing or silencing them, but also to assess the exact size and distribution of the reservoir in a more customized manner.

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Author Disclosure Statement

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