HIV-1 latency in proliferating T cells
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Establishment and molecular mechanisms of HIV-1 latency in T cells.

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ABSTRACT
Treatment of an HIV infected individual with antiretroviral drugs is a successful way to suppress the plasma viral RNA load below the limit of detection (50 copies HIV RNA / ml plasma). This can provide lifelong protection against virus-induced pathogenesis in drug-adherent patients. Unfortunately, even after many years of continuous treatment, the virus persists and the plasma viral load will rebound rapidly when therapy is interrupted. The reason for this rapid rebound is the presence of a long-lived reservoir of latent HIV-1 proviruses that can be reactivated in resting memory T cells. Attempts to eliminate these proviruses have thus far not been successful and this long-lived latent reservoir is therefore considered a major obstacle towards a cure for HIV-1. A detailed understanding of the molecular mechanisms causing HIV latency and knowledge on the establishment of this reservoir may give us clues for future strategies aiming at the eradication of this reservoir.
INTRODUCTION ON HIV-1 LATENCY

In this review, we provide an overview on latency properties of the human immunodeficiency virus type 1 (HIV-1) in T lymphocytes and the underlying cellular and molecular mechanisms. Virus latency is described by Wikipedia as the ability of a pathogenic virus to lay dormant (latent) within a cell, which denotes the lysogenic part of the viral replication cycle. The most prominent example concerns the Herpesviridae that include Chickenpox virus and Herpes simplex viruses (HSV-1, HSV-2), which establish episomal latency in neurons and leave linear genetic material floating in the cytoplasm for years. The virus can reactivate later, denoted as the lytic part of the viral life cycle, to cause cold sores in case of HSV-1. As such, a latent viral infection is a type of persistent viral infection that provides a strategy to escape from immune pressure.

For the HIV-1 infected individual the situation is more complex because the creation and persistence of a latent reservoir coincides with ongoing virus replication in other cells. This creates a very dynamic situation in which new virus variants replenish the latent reservoir but, at the same time, re-activated latent viruses become part of the productive replication cycle. For retroviridae like HIV-1, the latent reservoir consists of inactive forms of the provirus, i.e. the DNA version of the viral genome that is integrated at a random position in the host genome. In the field of HIV research, proviral latency forms the basis for the concept of one or multiple viral reservoirs, referring to specific biological niches (cell types or tissues), that are characterized by persistence of latent virus. This reservoir became quite visible during antiviral therapy. Specifically, the presence of replication-competent, but latent HIV-1 in resting CD4-positive T cells allows this virus to persist for years despite prolonged exposure to antiretroviral drugs and immune surveillance, thus forming a stable archive of “early” virus (this is illustrated in the upper panel of Figure 1). This latent reservoir of HIV may explain the inability of antiretroviral treatment to cure HIV infection. In fact, one may even consider this reservoir a viral evolution strategy that allows for the generation of increased genetic variation in the progeny of the actively replicating viruses (this is illustrated by the change in color of the HIV-1 particles over time in Figure 1). Even when massive mutations tend to decrease the replication capacity of the progeny, there is always the archived early virus variant to come to the rescue upon reactivation from the reservoir. HIV-1 evolution within an infected individual leads to a quasispecies collection of variants that are adapted to the host (positive adaptation to cellular co-factors, escape from host restriction factors and the immune system). This specialized quasispecies may not be able to transmit to and/or to replicate in a new individual (lower panel Figure 1). The archived “early” virus (viral memory), which upon reactivation can reach small copy numbers, may be more fit for virus transmission because it is less adapted to the individual host. This may explain in part the reversion towards early genotypic markers described upon HIV-1 transmission¹.
CELLULAR RESERVOIRS FOR LATENT HIV-1

HIV infects cells expressing the CD4 receptor and a co-receptor, predominantly CCR5 and CXCR4 but others, such as CCR3, have also been implicated. These receptors are expressed on a wide range of cells that circulate in the blood, but the primary target for HIV-1 infection is the proliferating CD4+ T cell. After binding of the viral envelope to the appropriate receptors, the viral membrane fuses with the cellular membrane and the viral core with the viral RNA genome is released into the cytoplasm. The viral RNA genome is reverse transcribed into double-stranded DNA that can subsequently integrate at a random position in the host cell genome. The integrated viral DNA is termed the provirus, which is dependent on the host cell gene expression machinery (transcription, splicing, RNA nuclear export and translation) for the production of new infectious particles.

The contribution of a latently infected cell to the total viral reservoir in an infected individual depends on two characteristics. First, the ability of the latently infected

Fig. 1. The archived HIV-1 provirus provides viral memory. The viral reservoir of latent proviruses is established early during infection, providing an archive of the transmitted “early” viruses (upper panel). Virus evolution leads to many genetic variants (change in virus particle color) and this host-adapted quasispecies may not be able to transmit to and/or replicate in another individual. In the absence of this reservoir, transmission may be blocked (lower panel).
cell to preserve the reservoir by its long-term survival. Second, the ability of the latently integrated HIV provirus to reinitiate virus production at a later moment, e.g. upon cellular activation. Some integrated proviruses contain deleterious mutations that make them a non-reactivatable dead-end product of virus replication. These defective proviruses do obviously not contribute to the viral reservoir.

CD4+ T cells can be roughly divided into two subsets based on their activation status, the resting and proliferating T cells. Proliferating T cells are important for directing the immune system towards a humoral or cellular response, depending on the invading pathogen. Once the pathogenic threat is cleared, a fraction of the T cells will return to the resting phenotype that allows prolonged cell survival, thus providing immunological memory. Resting T cells are the dominant contributor to the long-lived HIV reservoir as latently infected resting T cells can survive for many years. It is however not yet fully understood how this reservoir is maintained. Persistence could be related to the long half-life of these cells, but there could also be homeostatic proliferation, e.g. a single cell division every 50 days in which the latent provirus is passed on to daughter cells. The resting cell as such does not contribute to virus production, but when the resting cell is activated, for example during immun system activation in response to a pathogen, virus production can be initiated.

It is also not yet fully understood how the HIV-1 reservoir in long-lived T cells is established. Figure 2 illustrates the current hypotheses on the establishment of this reservoir. De novo infection of resting T cells is very inefficient (illustrated by the dashed line) due to blocks in the processes of reverse transcription and integration3,4. Cytokine stimulation can alleviate these blocks without inducing cell proliferation or up-regulation of cellular activation markers5-8. Once the virus is able to establish an integrated provirus in the resting T cell, there will be no new rounds of virus production and the provirus remains dormant. Infection of resting T cells forms the “direct” route for establishment of the reservoir, but it is a very inefficient process compared to the infection of proliferating T cells, the primary target cell for HIV-1. Infection of these activated, proliferating T cells may contribute to the viral reservoir in an “indirect” way. These cells will support active virus production and will thus have a very short half-life in the infected individual because of the HIV-specific immune surveillance and HIV-induced pathogenicity. As such, proliferating T cells do not contribute to the viral reservoir. However, if the proliferating T cell returns to the resting state upon virus infection, it can become part of the long-lived reservoir. The “direct” and “indirect” routes for establishment of the long-lived HIV-1 reservoir are depicted in Figure 2.

This “indirect” route became apparent in studies on HIV-1 latency in T cell lines. Even though these cells are actively proliferating and many transcription factors are abundantly present, HIV was able to establish a latent provirus9,10. Additional
support for the “indirect” route was provided in subsequent studies with primary T cells. For instance, proliferating CD4+ T cells can be infected with HIV-1 and subsequently cultured with specific cytokines to induce the resting cell phenotype, and stimulation of these resting cells with anti-latency compounds or cellular activators reinitiated virus production\textsuperscript{11,12}.

It remained unclear whether the transcriptionally active provirus was silenced during the cell’s transition into a resting phenotype or whether the subset of productively infected cells was simply removed by apoptosis, thus leaving the cells with a latent provirus. Evidence supporting the “indirect” route was recently provided by two studies, including one from our own laboratory, showing that proliferating primary T cells frequently harbor latent HIV-1 provirus as early as 2 days post infection\textsuperscript{13,14}.

MOLECULAR MECHANISMS OF HIV-1 LATENCY

Once integrated the HIV-1 provirus is dependent on the host cell machinery for the production of new infectious particles. A blockade in any of the gene expression steps can prevent the production of viral RNA, proteins and eventually HIV-1 particles. Several proviral latency mechanisms have been described in literature and are illustrated in Figure 3. These mechanisms are arbitrarily split in three groups. The HIV-1 provirus can depend on cellular factors that influence latency (\textit{trans} effect, upper panel), there may be viral genetic defects that cause latency (\textit{cis} effect, middle panel), or viral latency is determined by viral and host cell effects (\textit{cis/trans} effect, lower panel). We will discuss these scenarios in more detail below.

At least four HIV-1 latency mechanisms are controlled by trans-acting factors of the host cell. HIV-1 transcription initiation depends on transcription factors such as NF-κB and SP-1. These factors may not be present in a sufficient nuclear concentration to support HIV-1 transcription from the 5’ LTR promoter, as is the case for resting T cells\textsuperscript{15}. Alternatively, transcriptional repressor proteins may bind and silence the LTR promoter (YY1 and LSF) or simply prevent transcription factors from binding to the LTR (AP1 and TFII-I)\textsuperscript{16-20}. Even if HIV-1 transcription is initiated, efficient elongation requires the presence of sufficient amounts of elongation factors such as the positive elongation factor-b (P-TEFb) that restricts HIV-1 gene expression in resting T cells\textsuperscript{21}. Additionally, host and/or virus derived microRNAs (miRNAs) can direct the degradation of viral mRNAs by the RNA interference (RNAi) pathway\textsuperscript{22,23}.

The chromatin structure surrounding and within the integrated provirus can affect viral gene expression via epigenetic silencing. A more closed conformation of the chromatin structure via nucleosome modification can limit the access of transcription factors to the viral LTR\textsuperscript{24}. Additionally, the viral promoter sequences can be modified by methylation via DNA methyltransferases, thus preventing transcription factor binding to the affected TFBS in the LTR. It has recently been
proposed that this latter mechanism is not necessarily involved in the establishment of latency, but rather may play an important role in the maintenance of latency\textsuperscript{25,26}.

HIV-1 may control latency (in cis) by genome mutations. Low level transcription of the HIV-1 promoter is triggered by host cell transcription factors, but high level transcription depends on the viral Tat protein that initiates a positive autoregulatory loop via binding to the TAR RNA hairpin motif in the nascent transcript encoded by the viral LTR. Mutations in the Tat gene or TAR motif can impair transcription and proviruses with such mutations do accumulate in resting T cells\textsuperscript{27}. In fact, two HIV-1
Latency models use cells infected with a mutationally attenuated HIV-1 provirus\textsuperscript{28,29}. It is obvious that a completely crippled provirus cannot be reactivated, and thus is not representative of the viral reservoir.

Several molecular latency mechanisms are established by a combination of viral (\textit{cis}) and cellular (\textit{trans}) determinants. This includes the mechanism of transcriptional interference of the 5’LTR promoter by upstream cellular promoters. HIV-1 preferably integrates in introns of actively transcribed genes\textsuperscript{30}. As a consequence, (strong) transcription of that upstream host gene can interfere with transcription initiation at the 5’LTR promoter when both transcription units face the same direction as illustrated in Figure 3\textsuperscript{31,32}. The HIV-1 provirus may also be influenced by a (strong) cellular promoter from a downstream position when transcription runs into the provirus. This anti-sense cell-HIV-fusion transcript can anneal with regular sense HIV-1 transcripts to form perfectly basepaired double-stranded RNA. These molecules will be recognized by the RNAi machinery, e.g. the Dicer endonuclease, to produce HIV-specific siRNA inhibitors\textsuperscript{33,34}. There may also be an impact on the HIV-1 LTR promoter via RNA-induced transcriptional gene silencing (TGS)\textsuperscript{35-37}.

Most studies concerning the molecular mechanism of HIV-1 latency were performed with resting T cells and consequently less is known about the latency mechanisms in proliferating T lymphocytes. We and others suggested that HIV-1 proviral latency
can occur in proliferating T cells at the level of transcription\textsuperscript{9,16,26}. Besides host cell determinants (e.g. availability of transcription factors), there are also important viral determinants of latency (e.g. the configuration of transcription factor binding sites (TFBSs) in the viral LTR promoter). Interestingly, there may be significant differences in the latency properties between HIV-1 isolates and especially among HIV-1 subtypes as each of them have a specific TFBS architecture of the LTR promoter\textsuperscript{38,39}. Changes in this TFBS setting can affect the virus replication rate, but it can also affect viral latency\textsuperscript{40,41}. For instance, HIV-1 subtypes A and C have an AP1 site that in the LTR domain that was recently termed the latency establishment element (LEE) as it makes the provirus more prone to become latent\textsuperscript{18}. Three recent studies suggested that HIV-1 subtype AE is less vulnerable to become latent than the other subtypes\textsuperscript{14,18,41}. This AE-specific latency profile could be linked to a point mutation that inactivates the upstream NF-κB site, but at the same time creates a unique GABP site\textsuperscript{41,42}. These results may imply that future clinical trials, which try to purge the latent virus in an attempt to eradicate it and to reach a functional cure, should focus on the HIV-1 subtype AE.

CONCLUSIONS
This review provides a brief overview on the topic of HIV-1 latency in T cells. Latent proviruses are established early during infection, but - upon activation - these archived “early” viruses may provide an advantage over host-adapted “late” viruses, for instance in virus transmission to a new individual. The latent HIV-1 reservoir consists predominantly of resting T cells, but it is not yet fully understood how this reservoir is established. We discussed two scenarios. Most well-known is the “direct” route in which the resting T cell is infected with HIV-1. However, recent evidence supports an alternative “indirect” route, in which HIV-1 infects a proliferating T cell, resulting either in a productive infection or the establishment of a latent provirus. This latently infected proliferating T cell can turn into a resting T cell, thereby contributing to the long-lived reservoir. We described the molecular mechanisms that may contribute to HIV-1 proviral latency, including a role for the cellular environment (e.g. availability of active transcription factors) and intrinsic viral properties (e.g. status of the Tat-TAR axis that controls transcriptional activation).

The most widely discussed approach to eliminate the long-lived HIV-1 reservoir is the so-called ‘shock and kill’ approach that purges the provirus from latency with anti-latency drugs while the patient continues anti-retroviral therapy\textsuperscript{43,44}. The anti-latency drug induces viral gene expression form the latently infected cell (‘shock’), and the anti-retroviral drugs prevent new productive infections of the released viruses. The virus-producing cell will die due to virus-induced cytopathic effects or via host CD8\textsuperscript{+} T lymphocytes-induced apoptosis (‘kill’). The ideal anti-latency drug reaches all cellular reservoirs, has low toxicity and is a specific inducer of transcription from the viral LTR promoter. Most anti-latency drug
candidates have been tested previously for other diseases, predominantly cancer, such that toxicity information is available\textsuperscript{45}. As HIV-1 transcription uses the host cell machinery, the identification of truly specific and non-toxic anti-latency drugs remains a major challenge. Targeting of all HIV-1 reservoirs remains another challenge because this should ideally not leave a single latently infected cell, as this may trigger repopulation of the virus pool in the future. Of note, the currently available anti-latency drugs are effective in purging the latent provirus in resting T cells, but not in proliferating T cells\textsuperscript{13}. Thus, it may be necessary to develop drug combinations for such purging purposes.

Our understanding of the establishment and maintenance of the HIV-1 latent reservoir has increased significantly over the years, but several important research questions remain. These will likely be addressed given the new programs that have been launched such as “Cure for AIDS” by the International AIDS Society and “Beyond HAART” by the National Institutes of Health\textsuperscript{46}. Given the major challenges ahead of us, it may be advisable to start eradication attempts with a virus that is relatively “easy” to purge, in particular the HIV-1 subtype AE.

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   •• Description of HIV-1 latency upon infection of activated T lymphocytes, and natural ways to overcome such latency by dendritic cell contact.
   •• Development of an HIV-1 reporter assay in which latently infected cells can be separated from productively infected cells without long-term culturing or purging. This is the first assay that allows for the specific detection and/or selection of latently infected cells because infection is monitored separately from LTR activity.
   •• First description of a latency-control domain in the HIV-1 LTR promoter.
  • This study shows that HIV-1 infected T lymphocytes express HIV-derived miRNAs and siRNAs. A new mechanism for anti-HIV siRNA production was proposed.
  • This study demonstrates that a molecular mechanism known to purge HIV from latency in vitro can be used to therapeutically target latently infected cells isolated from HIV-1 infected individuals on anti-retroviral therapy.
  • This review discusses the strategies for complete eradication of the latent reservoir and for the alternative approach, stringent control of viral replication without anti-retroviral therapy.
  • This reference summarizes the data of current pharmacological approaches towards HIV eradication from the infected patient.