Chapter 3

Molecular Mechanisms Controlling HIV Transcription and Latency – Implications for Therapeutic Viral Reactivation

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Additional information is available at the end of the chapter

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Abstract

Persistence of transcriptionally silent replication competent HIV-1 is a major barrier to clearance of the virus from patients; current combinatorial antiretroviral therapies are successful in abrogating active viral replication, but are unable to eradicate latent HIV-1. A "shock and kill" strategy has been proposed as a curative approach in which latent virus is activated and infected cells are removed by immune clearance, while new rounds of infection are prevented by antiretroviral therapy. Much effort has been put toward understanding the molecular mechanisms maintaining HIV latency and the nature of reservoirs, to provide novel therapeutic targets. This has led to the development of latency reversal agents (LRAs), some of which are undergoing clinical trials. Targeting multiple mechanisms underlying HIV latency via a combination of LRAs is likely to result in more potent activation of the latent reservoir. Therefore, novel as well as synergistic combinations of therapeutic molecules are required to accomplish more potent latency reversal.

Keywords: HIV-1 latency, Latency reversal agents (LRAs), Combinatorial antiretroviral therapy

1. Introduction

Human immunodeficiency virus-1 (HIV-1) is a lentivirus, a subgroup of Retroviridae. Like all retroviruses, HIV-1 virions consist of an RNA genome with viral proteins encapsulated in a viral envelope. The viral proteins execute key steps to establish a productive infection by stably integrating into the host genome. Unlike most retroviruses, HIV-1 can also directly infect nondividing cells. HIV-1 preferably infects a subset of T-lymphocytes (CD4+ T-cells) that play a crucial role in the immune response. HIV-1 infection causes exhaustion and ultimately

depletion of the host immune system, a syndrome termed acquired immuno-deficiency syndrome (AIDS). HIV-1 came into prominence with the outbreak of the AIDS epidemic in the 1980s. Major steps have been taken toward treating this viral infection. In particular, combinatorial antiretroviral therapy (cART) successfully abrogated HIV-1 replication. Thus, for compliant patients with access to c-ART, HIV infection has become a chronic rather than a lethal disease. However, cessation of antiretroviral therapy results in viral rebound in infected patients, even after years of cART. This is because in a small fraction of infected cells, HIV persists in a latent but replication-competent state. Latent HIV is unaffected by cART, but infection can rebound upon cART interruption. Therefore, HIV latency is the main challenge in developing a curative therapy for HIV.

The quest for an HIV-1 cure involves the development of either a sterilizing or a functional cure. A sterilizing cure would require complete removal of replication competent viral genetic material from the infected patient and thus the stable depletion of latently HIV-infected cells. A functional cure, on the other hand, requires the patient's immune system to suppress HIV-1 replication life-long in the absence of cART without disease progression, loss of CD4+ T cells and HIV transmission. The functional cure does not aim to eradicate the virus entirely from the patient. Both the sterilizing and functional cure strategies are currently the subject of major research efforts.

2. Clinical picture of HIV

The AIDS epidemic in the 1980s led to the identification of HIV as the causative agent. AIDS is a condition in which depletion of CD4+ T-cells overtime leads to the loss of the host immune system's ability to fight infections and cancers, eventually leading to death. As HIV was identified as the causative agent, cure efforts focused on disrupting the viral lifecycle. In the early 1990s, the first antiretroviral therapies – monotherapies – had limited success as they resulted in rebound of viremia due to the appearance of resistant viral strains. Resistant HIV required novel therapeutic strategies. Therefore, a combination of anti-retrovirals, targeting distinct steps of the viral life cycle was developed, so-called combinatorial antiretroviral therapy (cART). cART has proven to be extremely successful in lowering the amount of viral RNA in plasma below the limits of detection by standard laboratory techniques. Unfortunately, the therapy does not eradicate the virus as cessation of medication causes re-emergence of viral replication [1–3]. Thus, a fraction of the virus escapes the effects of cART. The source for this recurring viral replication is a small pool of latently infected cells that harbor integrated proviruses which, while silent, are not recognized by either the immune system nor are they subject to cART. Moreover, HIV can persist in the presence of cART in certain anatomical sites if drug penetrance is incomplete.

According to the World Health Organization (WHO), the number of HIV-infected individuals worldwide in late 2014 was estimated to be approximately 37 million [4]. The vast majority of infected people live in sub-Saharan Africa, where access to appropriate diagnostic centers and cART is limited. Estimates put new infections at 5,600 a day in 2014.

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2.1. HIV-1 replication cycle and state-of-the-art antiretroviral therapy

HIV-1, as all viruses, is a parasite of the host cell and hijacks key cellular processes to establish a productive infection. To produce new virions, the virus goes through a viral replication cycle. HIV's replication cycle consists of entering the cell by docking at the cell surface receptor CD4 and co-receptors CCR5/CXCR5 and fusing to the cell, un-packaging of the genome, reverse transcription of the viral RNA genome into double-stranded DNA, which is the main component of the pre-integration complex, followed by integration of the double-stranded DNA genome into the host genome, transcription of the provirus, translation of viral proteins, and ultimately virion biogenesis followed by budding from host cell and maturation. Modern cART targets most steps in the HIV viral replication cycle (Figure 1). There are currently 28 approved agents for the treatment of HIV infection [5]. They fall into six mechanistic major classes, which act at different stages in the HIV replication cycle:

1. Fusion inhibitors: enfuvirtide (ENF, T-20), the only currently available fusion inhibitor, binds to the gp41 receptor site, preventing the fusion of the virus with the target cell.

2. C-C chemokine receptor type 5 (CCR5) antagonists: maraviroc (MVC) is currently the only available CCR5 antagonist. This drug is an entry inhibitor, specifically blocking the human chemokine receptor CCR5.

3. Nucleoside (nucleotide) reverse transcriptase inhibitors (NRTIs) block the addition of nucleosides to the DNA chain during reverse transcription of RNA.

4. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) bind to and inhibit the enzyme reverse transcriptase (RT), preventing conversion of viral RNA to DNA during infection.

5. Integrase inhibitors (INIs): raltegravir (RAL), elvitegravir (EVG) and dolutegravir (DTG) are the only currently available drugs in this class. They target the HIV enzyme integrase (IN) that is required for insertion of viral genetic material into human DNA.

6. Protease inhibitors (PIs) bind to the catalytic site of HIV aspartic protease, blocking the processing of viral proteins (eg. Saquinavir).

These antivirals comprise the various current cART regimens that are used in the clinic. cART has proven to be extremely successful in suppressing viral replication in compliant patients. In fact, it has been argued that the theoretical potential of cART has already been reached [6]. Therefore, in the developed world with access to medication, HIV has become a chronic and not a lethal disease.

2.2. The burden of lifelong cART

Implementation of cART has provided long-term suppression of viral replication, improving the life expectancy and life quality of infected patients. Unfortunately, the economic burden of cART is debilitating. According to the Centers for Disease Control and Prevention (CDC), lifetime costs of treating HIV infection is estimated to be $379,668 per infected individual in the United States [7].

Moreover, patients on cART overtime can experience several side effects of cART such as: cardiovascular diseases (e.g., myocardial infarction); non-AIDS cancers (e.g., anal cancer, liver cancer, Hodgkin's disease); liver, kidney, and bone disease as well as neurologic complications, such as dementia [8]. Interestingly, most of these conditions are associated with the ageing process. Hence, it is thought, that HIV infection controlled by cART accelerates ageing. And importantly, HIV persists in a latent state that is not targeted by cART, rendering cART a therapeutic management of the disease as opposed to a curative treatment. Thus, there is much need to develop a curative therapy for HIV.

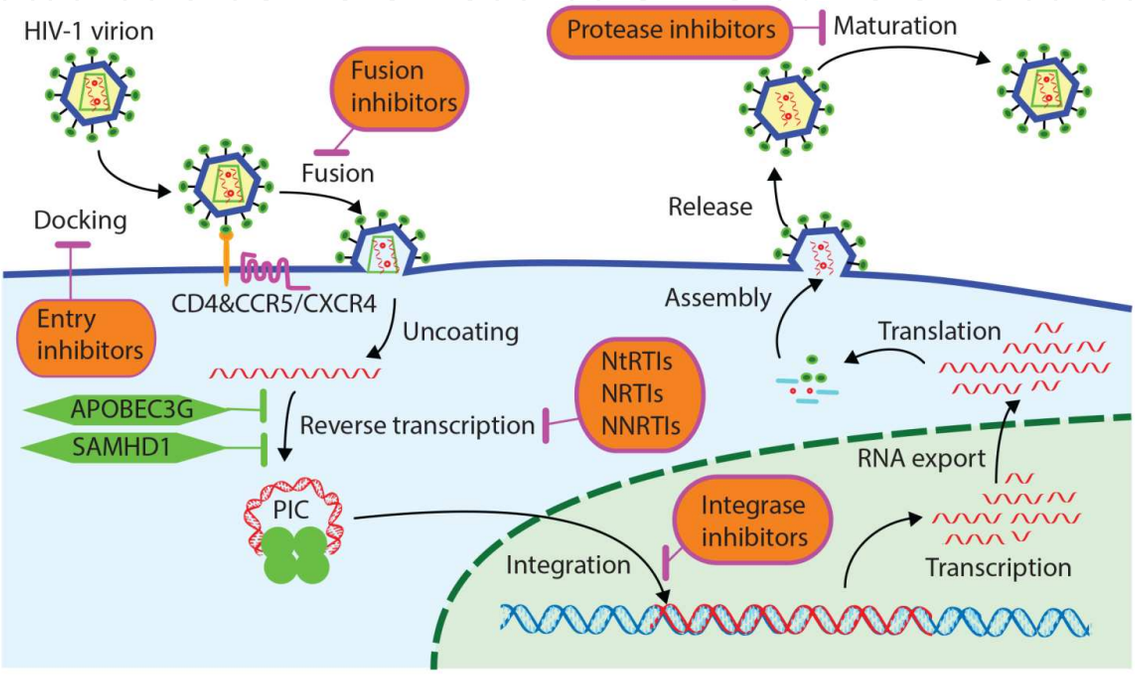

Figure 1. The viral replication cycle can be targeted pharmacologically at different stages

2.3. Clinical latency

The first step in finding a cure for HIV-1 infection is to identify the main source of cells that carry silenced, replication-competent HIV-1. Therefore, it is critical to define which cells or anatomical compartments constitute a reservoir of latent but replication-competent HIV-infected cells.

HIV-1 infects cells expressing the cell surface CD4 receptor and either of the co-receptors CCR5 or CXCR4. These cells include T helper cells, monocytes, macrophages, and dendritic cells. *In vivo,* HIV infects mostly activated CD4+ T-cells as quiescent and resting CD4+ T-cells are less permissive to infection due to low expression of CD4 and CCR5, and minimal metabolism [9–12]. The low metabolism is characterized by low levels of available dNTPs for reverse transcription and lack of energy sources [13–17]. Additionally, the cortical actin barrier in resting cells is thought to inhibit virus entry, reverse transcription and nuclear import [18,19]. However, the biggest pool of latently infected cells comprises resting memory CD4+ T-cells.

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It is thought that these latent infections are predominantly generated while activated infected cells revert back to a resting memory state [20–22]. During this process, as the genome of the (partially) activated cell condenses and is silenced in transition to a memory state, so does the HIV genome [14,15]. There is also evidence for direct infection of resting cells by HIV, resulting in the generation of a latent infection [23]. Studying these cells in patients is challenging as the frequency of latently infected cells in suppressed patients is very low, estimated to be 1 latent cell per 1 million of uninfected cells [24,25]. Due to the long half-life of a latently infected resting memory CD4+ T-cells (estimated at 44 months), cART would take over 70 years in order to eradicate HIV from the infected patient [6,26,27].

Naive T-cells are also found to be latently infected; however, the frequency of such cells is even smaller than resting memory cells [28]. Interestingly, the naive T-cell reservoir may increase over time in suppressed individuals due to high proliferation of these cells compared to resting memory cells [29].

HIV is found also in cells of monocyte/macrophage lineage such as macrophages in brain and lung sections of infected individuals on anti-retroviral therapy [30,31]. However, proviral transcription occurs in these cells at low levels; therefore, it is debatable whether these cells are part of the latent reservoir [32,33].

Among the anatomical compartments affected by HIV-1, the central nervous system (CNS) and gut-associated lymphoid tissues (GALT) are two major sites [34–36]. The source of infection in the CNS is most likely infected monocytes, which are able to cross the blood–brain barrier as the virus itself cannot [37–39]. Approximately 5-10 times more HIV-1 RNA can be obtained from GALT than from blood cells in patients receiving cART [40,41], potentially indicative of lower penetrance of cART in cells within this anatomical site. However, the contribution of these compartments to rebound of viremia after cART cessation remains controversial [42,43].

2.4. Clinical proof-of-concepts for HIV-1 eradication

Thus far, only one patient, the so-called Berlin patient, was cured from HIV-1 after receiving treatment for acute myeloid leukemia [44,45]. HIV eradication in this patient was accomplished after several rounds of radio- and chemotherapy, total body irradiation, and two hematopoietic stem cell (HSC) transplantations from a donor bearing homozygous thirty-two base pair deletion in the CCR5 co-receptor gene (CCR5Δ32) were performed. The mutant CCR5 impedes viral entry of R5 tropic viruses in the first phase of the infection [46–49]. It is estimated that between 1% and 15% of the European Caucasian population harbor this mutation, while it occurs less frequently in African and Asian populations [47, 48]. In this patient, cART was ceased a day before the first transplant and after 7 years, no viremia or other indications of viral replication have been detectable [52].

Following the success of the case of the "Berlin patient", two HIV-1-positive patients, the "Boston patients", received HSCs transplants after developing Hodgkin's lymphoma [53]. Both patients carried heterozygous CCR5Δ32 mutation. While still under cART regimen, no viral production was observed which led to cessation of therapy. Unfortunately, after several

months, strong viral rebound occurred in these patients. Follow-up analysis pointed to the likely presence of a small refractory source of cells, which is thought to have seeded the viral rebound; phylogenic studies revealed that only a few latent proviruses contributed to the viral rebound [53]. Several other similar studies have been conducted with infected patients suffering from either leukemias or lymphomas who received autologous or allogenic HSC transplantation alongside cART as a strategy to deplete the latent pool of cells. However, in most of these studies, viral rebound was detected following therapy interruption [54].

In another case, the Mississippi baby, an infant presumably infected *in utero*, received cART 30 h after birth. As newborns do not have resting memory CD4+ T-cells, it was reasoned that cART will prevent establishment of the latent reservoir – the main impediment in eradication strategies. One month after therapy, viremia reached undetectable levels and cART was stopped after 18 months. Unfortunately, 2 years post therapy interruption, rebound of viremia was detected (52, http://www.niaid.nih.gov/news/newsreleases/2014/pages/mississippibaby-hiv.aspx).

The immune system of rare "elite controllers" maintains low HIV-1 plasma levels, without the need of medication for many years. Although the capability of these patient to control viral replication is not completely understood, their circulating myeloid dendritic cells and CD8+ T-cells are more effective in depletion of infected CD4 T-cells [56–61]. Interestingly, the ARNS VISCONTI cohort showed that cessation of long-term cART, started during the acute phase of HIV-1 infection, resulted in post-treatment control (PST) of infection. Fourteen of the studied individuals were able to keep or even further reduce the viral reservoir. Furthermore, these individuals were able to maintain long-lasting, low level of viremia [62]. Recently, a perinatally infected baby displayed more than 11 years of HIV-1 remission. At 3 months of age, plasma HIV-RNA reached 2.1 x 10^6 copies/ml, and cART was administered for about 5–6 years. At 6.8 years of age, no HIV-1 RNA was detectable and cART was discontinued. After more than 12 years, plasma viremia still remains undetectable [63]. Therefore, this case provides the first evidence that early initiated, long-term cART can result in stable and durable HIV-1 remission.

Data from the Berlin and Boston patients provided a rationale for the creation of HIV-resistant cells. Since the CCR5Δ32 homozygous mutation is not lethal and not associated with abnormal immune functions [52], many approaches to silence the CCR5 gene have been or are under investigation [64–67]. These studies all employ genome editing technologies such as transcription activator-like effector nuclease (TALEN), clustered regularly interspaced short palindromic repeats (CRISPRs) or zinc-finger nucleases (ZNFs), which target the genome with high specificity and introduce deletions in the sequence of interest, in this case in the DNA sequence of CCR5 or/and CXCR4 co-receptors [64,65,68]. The rationale for this approach is based on the notion that cells bearing mutated CCR5 protein are not permissive to infection with R5 HIV-1 viruses, while cells with a mutated CXCR4 are resistant to C4 viruses. The double knock-out of both CCR5 and CXCR4 would allow resistance to infection regardless of viral tropism. However, the safety of such an approach remains to be elucidated. Uninfected HSCs isolated from infected individuals are engineered with either technology and then transfused back into patients. The ZNF approach targeting CCR5 has shown some promising results, although the sizes of cohorts used have been small. Gene-modified cells persisted in

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patients over 9 months, and cells seemed to expand and undergo trafficking to other tissues [66]. An increase in CD4+ T-cell counts was observed in all individuals. Importantly HIV-1 DNA in the blood decreased. The encouraging outcome of this study has resulted in phase II clinical trials.

Another gene therapy-based approach is the introduction of HIV-1 expression-dependent suicide genes encoding either toxic or pro-apoptotic proteins such as members of the Bcl-2 protein family. Constructs that are responsive to Tat and Rev viral proteins were tested [69]. While obtaining encouraging results, activity of such suicide genes only affects cells that are actively producing viruses, thus the latent pool of cells would still be unaffected.

Despite many attempts at HIV-1 cure, thus far only two cases, the "Berlin patient" and the early treated infant have resulted in eradication [44,45,63]. Due to safety and economic issues associated with transplantation and gene therapy approaches, broad use of such a therapeutic approach is not feasible for HIV cure. Moreover, the gene therapy approach provides a functional rather than sterilizing cure. Nevertheless, all these studies provided valuable insights into the biology of the latent reservoirs. They constitute a proof-of-concept for HIV-1 cure. Moreover, it seems that immediate initiation of cART contributes to restricting the establishment of the latent pool.

These studies highlight the need for more robust, cheaper, and feasible treatments in order to achieve HIV-1 eradication among all infected individuals. In 2004, the concept of so-called "shock and kill" or "kick and kill" therapy was proposed [70–72]. The aim is to specifically reactivate proviruses in latently infected cells ("shock") and eliminate the infected cells via viral cytophatic effects or/and render the cells susceptible to immune clearance ("kill"). New rounds of infection would be prevented by cART. "Shock and kill" therapy relies on the identification of potent and specific latency reversal agents (LRAs) alongside induction of an effective immune response against the reactivated latent pool of cells. The LRAs currently under investigation do not result in sufficient reactivation of latent HIV *in vivo*. Therefore, novel molecules that specifically reactivate latent HIV-1 are urgently needed.

3. Model systems and assays to detect and study HIV-1

To study the complex nature of HIV-1 latency, reliable model systems are required that recapitulate the nature and dynamics of the latent reservoir in vivo. Several cell lines of lymphocytic or monocytic lineage, primary-cell models, as well as animal models, are used to study HIV latency [73].

3.1. Cell lines

Immortalized cell lines of T-cell and monocytic origin are cost-effective and easy to use in the study of latent HIV. They allow fast read-outs in large scale for mechanistic molecular characterization of HIV gene expression. Therefore, cell lines are an attractive platform for screening and mechanistic characterization of LRAs. To generate a latent cell line, cells must

first be latently infected with a HIV derived virus. Several different HIV derived viruses are used ranging from full length to minimal virus and can make use of a wide range of reporter constructs (e.g. GFP or luciferase). The viral Tat/TAR axis is of vital importance for the transcriptional regulation of HIV and can be included or excluded from the viral construct used. Latent infection of relevant cell lines derived from T-cells or monocytic lineage, depending on reservoir of interest generate cell lines that can be used to study the molecular mechanisms of HIV latency [74–78].

Ach-2 and U1 cells are characterized by low expression of HIV-1, which can be strongly upregulated upon TNFα or mitogens stimulation [74,79]. However, in these cell lines, latency results from mutations in Tat protein (U1 cell-line) or in RNA stem loop TAR (Ach-2) [76,77]. Therefore, these cell lines do not represent complexity of latency found *in vivo*, however, they do allow Tat/TAR-independent HIV-1 reactivation investigation.

A more appropriate system to study latency are J-Lat cell lines derived from Jurkat cells of T-lymphocytic origin [78, 80,81]. These cells have integrated replication-competent full-length or minimal proviral constructs with an intact promoter and Tat-TAR axis, a *GFP* reporter gene replaces the *Nef* sequence in full-length proviruses or is located downstream of *Tat* in minimal proviruses [78].

These cell lines have been extremely useful to delineate the molecular requirements of HIV transcription activation and silencing. Although useful for molecular analysis and screening platforms, the cell line model systems of HIV latency also present some limitations; first, clonal cell lines are derived from a single integration event, and therefore do not reflect the diverse distribution of integration sites in the host chromatin [82,83]. Consistently, results vary depending on the cell lines used, indicating possible clonal cell line effects [84]. Due to the above mentioned limitations and the considerable difference between cell line models and primary cells in terms of proliferative capacity, genomic stability and mechanisms involved in establishing and maintaining latency, generally latency models based on primary cells are preferable.

3.2. Primary cells

To more closely resemble infection *in vivo* and validate putative LRAs more accurately, several primary cell models have been developed. Depending on the cell status at infection, these models can be divided into two groups.

The first group relies on purification of CD4+ T-cells from healthy donors, that are then activated and subsequently infected. Depending on the method, CD4+T-cells are purified and stimulated with a-CD3/IL-2 [85], a-CD3/aCD-28 [86], a-CD3/aCD-28/IL-2 [87], or Ag-MDDC (antigen-loaded monocyte-derived dendritic cells; [88]), and infected with virus. Productively infected cells die due to virus-induced apoptosis or become latent by reverting back to a resting state. To limit infection to only one replication cycle, replication-defective viruses or antiretroviral drugs are also used. The rationale for these systems rely on the notion that a portion of activated, infected CD4+ T-cells transition to a quiescent state, shutting down general transcription and slowing down metabolism, resulting in latency [6,25,28,89–91]. Depending

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on the method used, different populations of latently infected cells are generated for use in reactivation studies. In the methods suggested by Sahu and Marini central memory T (T_{CM}) cells remain in culture, in Yang's protocol mainly effector memory T (T_{EM}) cells are produced, in Bosque and Planelles's method cells phenotype resembles central memory-like (T_{CM}). The main disadvantage of these methods is the time needed to obtain results, which varies from 1 to 4 months. Furthermore, they are labor-intensive and technically challenging.

The second group uses direct infection of resting memory CD4+ T-cells, which immediately after integration become latent. Cells are infected after purification and can be used after several days for reactivation studies [90, 91]. Stimulation of CCR7, CXCR3, or CCR6 receptors increases the susceptibility of resting memory CD4+ T-cells to infection without T-cell activation. In the methods of Swiggard and Lassen, central memory T (T_{CM}) and effector memory T (T_{EM}) cells are the source of latent HIV-1; in Saleh's method naïve resting memory T-cells, in addition to T_{CM} and T_{EM} cells, constitute the latent pool. The main advantage of these methods is the time needed to evaluate the potency of putative LRA, as results can be obtained within one week.

Depending on the protocol used, the amounts of cells that become latent differ from as little as 1% to up to 40%. In models where cells are activated, on average more latently infected cells are generated. Using these models, we can quantify the level of reactivation of HIV-1 in a reliable manner by measuring the production of the viral protein p24 by enzyme-linked immunosorbent assay (ELISA) or quantification of viral transcription by quantitative RT-PCR, or by detection of GFP/luciferase in case of reporter-based constructs.

A novel detection method distinguishes uninfected, productively infected, and latently infected cells using a dual reporter system. A modified HIV-1 derived genome containing GFP as a reporter of viral transcriptional activity and mCherry under an EF1a promoter as a reporter of infection (latent or productive) allows easy isolation of the different cell populations [23].

Ultimately, the golden standard for testing activity of LRAs are primary cells from infected individuals under cART obtained by leukophoresis, a process in which white blood cells are specifically isolated while other blood components are reverted back to the patients' circulatory system. The isolated cells are uninfected, latently infected, and infected with defective viruses. Large amounts of CD4+ T-cells are required and isolated from patients for testing LRAs.

The development of primary cell models greatly improved the quest for LRAs, yet results differ between each model system [84]. No *in vitro* models completely recapitulate the full range of latent cells *in vivo*; instead, only a small sub-fraction of latently infected cells is represented. Hence, the validation process of putative LRAs requires testing on cells derived from infected individuals [93].

3.3. Animal models of HIV-1 infection

The number of animal models available to study latency is limited. The toxicity of putative LRAs can be assessed with use of mouse and non-human primate (NHP) models [94]. Two mouse models have been developed and used in HIV latency studies: the humanized SCID

(SCID-hu) mouse, transplanted with human thymus and liver fragments, and the humanized blood, liver, and thymus (BLT) mouse which has a human immune system with full mucosal immunity [95–97]. Unfortunately, SCID-hu mice do not express human proteins involved in the viral replication cycle; therefore, the study of HIV-1 in these mice is restricted to events taking place within organs of human origin in this model. In addition, HIV-1 is not responsive to cART in these animals. BLT mice are a better model of HIV-1 infection, as they produce resting memory CD4+ T-cells of human origin. However, some components of cART do not repress replication in BLT mice [34].

NHP models employ the Simian immunodeficiency virus (SIV) infection in rhesus and pig tailed macaques to recapitulate HIV-1 infection in humans [98,99]. NHP models allow the monitoring of the spread of infection. Moreover, infection in this model can be controlled by antiretroviral therapy. NHP models are helpful in studying the first stages of latency establishment, as investigating this part of HIV-1 infection is extremely challenging in patients, as the pool of latently infected cells is established early during infection [100]. One caveat to the use of SIV-based NHP models of HIV latency is that the viral 5'LTR or promoter of SIV is considerably different in sequence from HIV-1 [101] and therefore latent SIV response to LRAs, which is a direct consequence of promoter-mediated transcription activation may vary substantially from latent HIV-1. In addition, animal models are far more expensive than cell-based systems. Nor do they fully reflect human infection or metabolism. Finally, ethical concerns are inherent to the use of NHP models of HIV latency.

3.4. Detection of the latent reservoir

The study of latent HIV infection requires accurate measurement of the size of the latent reservoir and the extent of reactivation following LRA treatment. Depending on the experimental aim, different detection methods can be employed. These methods generally rely on PCR, protein quantification, or reporter detection.

The quantitative viral outgrowth assay (QVOA) is a well-established method to estimate the latent pool. The assay relies on the use of serial dilutions of cells obtained from an infected individual in co-culture with uninfected cells that are permissive to infection. Viral proteins are detected by ELISA. Unfortunately, QVOA is time-consuming, costly, and might generate false-negative results as not all replication-competent proviruses are reactivated, and thus not detected [83].

The HIV reservoir can be approximated by detecting the number of viral DNA copies present in the cells. The recently introduced digital droplet PCR (ddPCR) improves on classic and nested qRT-PCR by simultaneously amplifying thousands of nanoliter reactions in combination with very sensitive detection system based on flow cytometry [94,102,103]. ddPCR is therefore superior to nested qRT-PCR in its ability to resolve rare events such as latent HIV-1. Although PCR based methods provide increased sensitivity for the detection of viral genetic material, these approaches also detect defective proviruses, which results in false-positive results.

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Another recent PCR-based method for reservoir detection evades false positive results from defective proviruses. The *Tat*/*rev* induced limiting diluting assay (TILDA) relies on PCR amplification of multiply spliced RNA (msRNA) of *tat*/*rev* transcripts that are present in productively infected cells and absent in latent infection [104]. Small amounts of cells isolated from patients are divided into two equal parts and distributed in limiting dilution. One half is left unstimulated while the other is activated with PMA/Ionomycin. After 12 hours, cells are lysed and subjected to ultrasensitive nested RT-PCR. By employing statistical modeling, the frequency of cells that are expressing msRNA in both groups is estimated and based on the unstimulated group a threshold of activation can be set. Using the TILDA assay, the size of the reservoir is estimated at 24 cells per million, which is more than measured by QVOA but less than measured by PCR methods [24,83,104]. The assay more accurately estimates the true size of the latent reservoir, is highly sensitive, reproducible, fast, relatively inexpensive, and requires only 10 mL of patients' blood. However, a limitation on the TILDA assay is that it detects the presence of viral transcripts but not the production or release of infectious viral particles; therefore, it may still overestimate the true size of the reservoir, yet to a smaller extent than other PCR-based methods. Additionally, signal detection relies on amplification of highly variable region of the HIV-1 DNA; therefore, detection of all subspecies of HIV-1 might be challenging and require extra optimization steps.

Unfortunately, all current methods to detect latent HIV-1 have limitations. First, the pool of latently infected cells in patients is extremely low, resulting in a high noise-to-signal ratio. Furthermore, defective or hyper-mutated proviruses are detectable by PCR-based techniques, yet irrelevant for eradication strategies. Moreover, not all replication-competent proviruses are inducible in the first round of treatment, yet get reactivated upon subsequent rounds of stimulation [83]. Thus, assays to measure latency reversal are overestimating – in the case of PCR-based methods – or underestimating – in the case of QVOA – the latent pool. This poses a main problem in measuring efficiency of the reactivation of HIV-1. A captivating approach employing the use of a biomarker (e.g., gene), which responds to treatment in the same way as HIV-1, would allow more easily quantifiable assessments as to whether latent HIV in patient cells would be responsive to a particular treatment.

4. Molecular mechanisms of latency

Although replication-competent, latent HIV is transcriptionally silenced but susceptible to reactivation upon certain stimuli. Following integration into the host genome, transcription from the HIV genome is controlled by key cellular host factors, and subject to host cell gene regulation similar to endogenous genes. Since viral transcription initiation, elongation, and termination are tightly regulated by host proteins, HIV is also widely used as a model system to study gene regulation.

4.1. Host antiretroviral mechanisms thwart infection

Host defense mechanisms impede HIV-1 infection. Upon entering the cell, HIV's RNA genome is reverse transcribed into double-stranded DNA (dsDNA). This process requires freely

available deoxynucleotide triphosphates (dNTPs). By limiting the pool of freely available dNTPs, the nucleotide scavenger SAMHD1 restricts viral replication in non-cycling myeloid cells and quiescent CD4+ T-cells [105–108]. Additionally, SAMHD1 has 3′–5′ exoribonucleases (RNAse) activity that specifically cleaves single-stranded RNA [109,110]. Interestingly, Vpx, encoded by HIV-2 and Simian immunodeficiency virus, is an accessory protein packaged into the virion, which induces SAMHD1 degradation [111].

Additionally, APOBEC3G limits viral replication by catalyzing the deamination of cytidine to uridine in the viral single-stranded DNA (ssDNA) genome during reverse transcription [112]. Interestingly, APOBEC3G is inactive in memory CD4+T-cells, which helps to explain why this cell type is more permissive to HIV-1 infection. Therefore, activated CD4+ T-cells are the main target cell type of HIV infection and of the main source of the latent reservoir.

4.2. Integration of HIV into the host genome required by host factors

The reverse-transcribed viral DNA genome is incorporated in the pre-integration complex (PIC). The PIC is imported into the nucleus. Host factors identified so far that affect viral integration are lens epithelium-derived growth factor (LEDGF/p75/PSIP1) and hepatoma-derived growth factor related protein 2 (HRP- 2/ HDGFRP2), through an integrase binding domain. In the absence of LEDGF, provirus integration is decreased 10-fold and HIV's pattern of integration is altered [113–115]. Simultaneous LEDGF and HRP-2 knockdown further decreases viral replication [116]. Nevertheless, knockdown of both factors does not completely abolish HIV-1 integration, indicating that IN alone and/or in cooperation with other host factors can still integrate the viral genome [117]. PIC nuclear import stimulates export to the cytoplasm of INI-1 and PML, disrupting this effect greatly improves integration efficiency [118–120]. Upon knockdown of transportin-3/TNPO3 and nuclear pore protein RanBP2/Nup35 HIV-1 integrates randomly [121]. Therefore, nuclear import affects the site of integration with a preference for open chromatin.

4.3. Pre-integration vs post-integration latency

Two states of latency can be defined based on the integration state of HIV: pre-integration latency and post-integration latency. Defects in integration or in a prior phase of the viral replication cycle (e.g., incomplete reverse transcription) might result in unintegrated viral DNA. The half-life of the linear pre-integration complex is approximately 1 day [122]. The linear unintegrated viral DNA can also be circularized, resulting in slightly extended half-life of the virus [123]. In quiescent cells, the pre-integrated virus can reside near the centromere for weeks [124]. Unintegrated virus can replicate, albeit very inefficiently [125]. The half-life of both forms of unintegrated virus is too short and replication inefficient to serve as the source required for the long-term persistence of latent HIV making pre-integration latency less clinically relevant.

Post-integration latency occurs when the HIV virus is stably integrated into the host genome, but a productive infection is not achieved. The site of integration and the abundance of transcription factors are crucial for determining whether an infection will be latent or produc-

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tive. The site of integration will determine the chromatin environment (such as histone modifications), relative position to other genes (intronic insertion vs gene desert) and position within the nucleus of the provirus.

4.4. Integration biases

The site of integration greatly determines the transcriptional activity of the provirus. HIV preferentially integrates into active genes both in patient material and transformed cell lines [82,126–128]. Moreover, HIV-1 integrates in regions of genome that are in close proximity to nuclear envelope [129]. Latent integrations are in or close to alphoid repeat elements in heterochromatin, whereas productive integrations avoid insertion in or near heterochromatin [78]. Integration is associated with transcription-inducing histone modifications (i.e., H3 & H4 acetylation and H3K4 methylation) but not transcription-inhibiting modifications (i.e., H3K27 trimethylation and DNA CpG methylation) [130]. A comparison of integration sites in resting and activated CD4+ T-cells showed that in both cell types HIV integrates in active genes. However, in activated cells, insertions were enriched for gene dense, CpG island-rich and high G/C-content regions [131]. Latency in infected Jurkat cell lines correlated with integrations in gene deserts, centromeric heterochromatin, and highly expressed cellular genes [128]. Within the nucleus, HIV-1 is located mostly in decondensed chromatin at the nuclear periphery, while it disfavors heterochromatic regions [132]. Interestingly, latent proviruses were found to interact with a pericentromeric region of chromosome 12 in quiescent cells [133]. In a study of viremic progressors and viremic controllers, integration was enriched into, or in close proximity to, Alu repeats, local hotspots, and silent regions of the genome [134]. In addition, close proximity of the provirus to PML bodies is associated with latency, an association that is lost upon reactivation [135].

4.5. Integration relative to host genes affects transcriptional state of the provirus

Sense and antisense integration relative to host genes can greatly affect the transcriptional state of HIV. Integration in sense orientation can lead to promoter occlusion, whereas integration in antisense orientation can lead to collision of the transcriptional machinery. Promoter occlusion occurs when the transcriptional machinery is depleted from the viral promoter by a dominant host promoter that is transcribed and negatively affects proviral expression.

Indeed, chimeric transcripts of the host gene and in sense viral integrations were observed [136,137]. Additionally, Han et al. compared the effect of sense and antisense insertions of HIV relative to the active HPRT gene [138]. In this setting, sense integration enhanced viral expression whereas antisense integration (transcriptional collision) led to suppression. Sense integrations were shown to be modestly preferred in latent cells, a preference that was not present in productively infected cells [139]. Transcriptional interference and transcriptional collision are examples of host genes interference with viral expression. On the other hand, reactivation of HIV may lead to suppression of host gene expression [136]. Indeed, in a cell model with a latent integration into the HMBOX1 gene, the host gene was repressed upon viral reactivation [140].

4.6. Viral transcription starts at the 5'LTR

The provirus is flanked by a 5′ and 3′ long terminal repeats (LTRs). While transcription can be initiated from both LTRs, the 5′ LTR is dominant and serves as the HIV promoter, although 3′ transcription is activated when the 5′ LTR is defective [141]. Transcriptional interference has been proposed as the mechanism by which the 5′ LTR exerts its dominance over the 3′ [142]. Interestingly, low-level antisense transcription takes place at the 3′ LTR, a mechanism by which latency can be maintained [143–147]. Sense transcription results in at least 40 coding transcripts due to alternative splicing of the HIV-1 genome [148]. Finally, both LTRs also act as a source of negative sense transcription, which could potentially affect the expression of neighboring genes [149,150].

4.7. The 5′ LTR contains numerous putative transcription factor binding sites

HIV-1 encodes a potent trans-activating protein – Tat – that drives viral expression during productive infection. However, initially, before sufficient levels of Tat are expressed, the provirus relies on host factors to initiate transcription. The 5′ LTR contains three regions – U3, R, and U5 (Figure 2) [151]. The R region, immediately next to the transcription start site (TSS), contains the trans-activation response (TAR) element, an important regulator of HIV expression. The U3 region contains the core promoter (nucleotides –78 to –1 upstream of TSS), a core enhancer (nucleotides –105 to –79), and a modulator region (nucleotides –454 to –104) [152,153]. The core promoter contains three Sp1 binding sites in tandem, a TATA box, and an initiator element at the transcription start site. The core enhancer contains two NF-kB-binding sites. The modulator region – so-called because early experiments with deletion upstream of the LTR caused increased activity of the LTR – was shown by later experiments to contain binding sites for both repressive and activating factors including nuclear factor of activated T-cells NFAT, STAT5, NF-kB p65/p50 heterodimers, lymphocyte enhancer factor (LEF-1), CCAAT/enhancer binding protein (C/EBP) factors, AP-1, and activating transcription factor/cyclic AMP response element binding (ATF/CREB) factors (Figure 2) [152,154–162]. It is well established that these transcription factors have binding sites within HIV-1 sequence. Moreover, they are strong activators of HIV-1 transcription of which NF-κB is considered the most critical [163–166]. In addition to the presence of these sites, bioinformatic tools indicate that this region of the HIV LTR contains a tightly clustered distribution of multiple transcription factor consensus binding elements [167].

4.8. Positive host factors bind to the 5′ LTR

Initial transcription of HIV-1 is entirely dependent on host factors. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) is a hetero dimer comprised of p50 and p65 subunits involved in T-cell activation. NF-kB acts as a transcription factor and is a potent activator of HIV-1 transcription initiation and elongation. It interacts and functions cooperatively with numerous proteins. Independent of Tat, NF-kB can reactivate HIV to high expression levels [168]. Mutated NF-kB-binding sites on the LTR inhibit basal transcription and Tat transactivation [169]. NF-kB, Sp1, and other factors (LEF-1, Ets1, and TFE-3) bind to sites near NF-kB sites and synergistically activate HIV transcription, even in the presence of repressive

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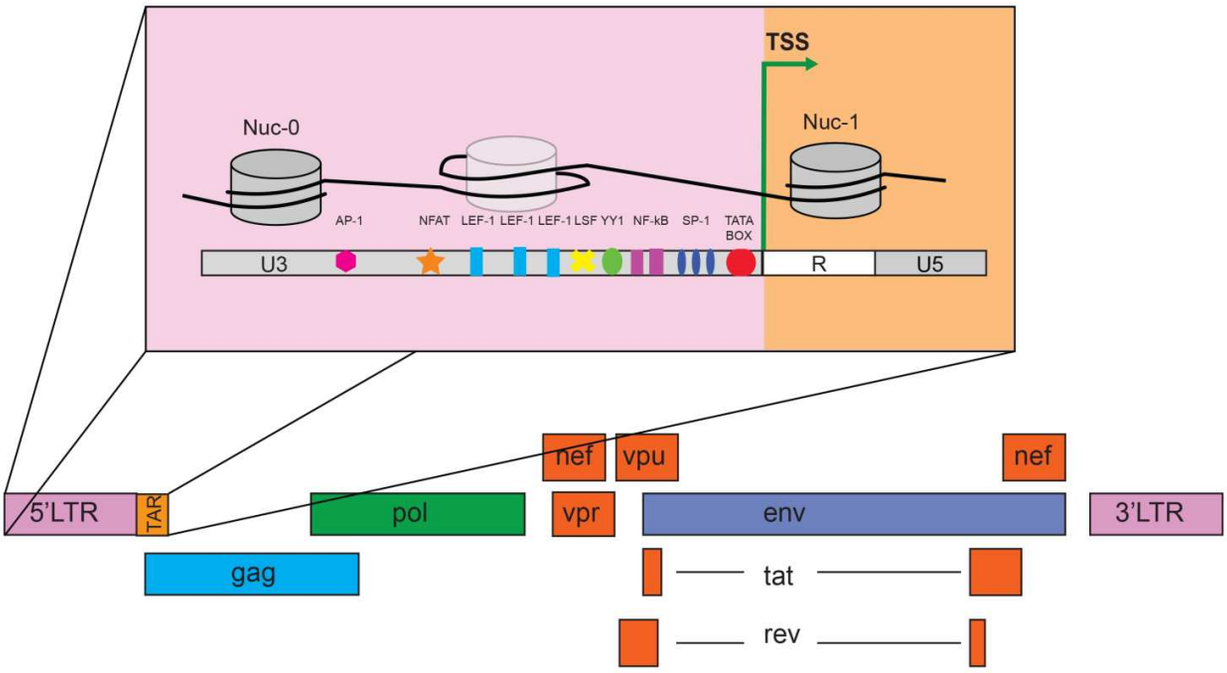

Figure 2. The genome of HIV-1

chromatin structures [170,171]. NF-kB and AP-1, a heterodimer of proteins from the c-Fos, c-Jun, ATF, and JDP families, cooperatively trans-activated LTR activity through the ERK1/ERK2 mitogen-activated protein kinase (MAPK) pathway [161]. Acetylation of Lys310 in NF-kB p65 subunit is an activating mark that is removed by NAD+-dependent protein deacetylases SIRT1 and SIRT2 [172]. Tat positively affects NF-kB by inhibiting SIRT1 and stimulating degradation of IkB, a protein that sequesters NF-kB in the cytoplasm [169,173]. The viral nucleocapsid (NC) protein enhances NF-kB-mediated activity by interacting with the LTR [174]. p65 recruits THIIH which is part of the preinitiation complex and its subunit CDK7 with kinase activity activates CDK9, resulting in increased HIV transcription [175,176]. The cell surface receptor OX40, bound by its ligand gp34, activates transcription from 5′ LTR, in a manner dependent on the presence of NF-kB-binding sites on the LTR [177]. The transcription factor E2F-1, a regulator of S-phase gene expression, inhibits LTR transcription through the recruitment of p50 at the NF-kB-binding sites on the LTR [178].

Members of the SV40-promoter (Sp) specific transcription factor family regulate LTR activity. Sp1 and Sp4 are activators of HIV-1 [179]. Expression of Sp transcription factors changes during monocytic maturation, suggesting differences in susceptibility to LTR activation during differentiation [180].

Nuclear factor of activated T-cells (NFAT) can induce LTR activity in T-cells [155]. NFAT recruits HATs through CBP/p300, which results in reactivation of HIV-1 transcription [181]. The Janus kinase (JAK)/signal transducers and activators of transcription (STAT5) can stimulate or inhibit HIV transcription. STAT5 binds to its binding sites in the U3 enhancer region on the LTR where it promotes transcription [156]. In response to a broad range of cytokines (e.g., IL-2, IL-7, IL-15) and granulocyte-macrophage colony-stimulating factor (GM-

CSF) JAK-mediated phosphorylation of a C-terminal tyrosine residue activates STAT5A and STAT5B. Homodimers or heterodimers of activated STAT5A and STAT5B translocate to the nucleus to stimulate HIV expression [182,183]. Interestingly, STAT5Δ, an isoform of STAT5 truncated on the C-terminus, acts as a repressor of LTR activity [184]. Indeed, in the promonocytic cell line U1 high levels of STAT5Δ are present. Upon stimulation with GM-CSF, STAT5Δ blocks RNAPII from binding to LTR U3 region, inhibiting activity of HIV promoter [185]. STAT5Δ promotes p50 homodimers binding to the LTR, contributing to latency maintenance [186].

In monocytes and macrophages, CCAAT/enhancer binding protein (C/EBP) factors are crucial for activation of HIV-1 [160,187–189]. C/EBP, a member of the bZIP superfamily, contains a DNA-binding domain and a leucine zipper for homo- and heterodimerizations. Similar to Sp-1, levels of C/EBP vary during myeloid development [190]. Interestingly, the HIV-1 LTR contains several C/EBP binding sites [159].

Some studies employing mutagenesis of binding sites for activator protein-1 (AP-1) within proviral genome showed that AP-1 transcription factor is the crucial activator of proviral transcription, as proviruses with altered AP-1-binding sites were less prone to reactivation even if treated with strong activator such as phorbol 12-myristate 13-acetate – PMA [191]. Furthermore, the latent pool was bigger in cells infected with a virus carrying a deletion in AP-1 sites, implicating that the AP-1 protein is necessary for successful provirus transcription [192]. Heterodimeric protein AP-1 is formed upon phosphorylation od c-Jun N-terminal kinase (JNK) in JNK/MAPK pathway [193]. It is well established that activation of TLR signaling induces nuclear localization of NF-kB and AP-1 mediated via JNK pathway [194–196].

In addition to the already mentioned host factors, the potent viral trans-activating protein Tat and to a lesser extent the multifunctional viral protein, viral protein R (Vpr), positively affect viral transcription. Productive infection requires the presence of Tat. Exogenous expression of Tat rescues HIV from latency [197]. A defective Tat mutant (C22G) is incapable of full-length viral expression [198]. Additionally, the Tat mutant (H13L) is more prone to establish latency [197]. Tat recruits the positive transcription elongation factor b (P-TEFb), which shifts RNAPII promoter proximal pausing to transcriptional elongation leading to a productive infection [199,200]. P-TEFb consists of CDK9, a serine/threonine kinase, and CyclinT1. The N-terminal cystein-rich region of Tat (Cy22-Cy37) binds to CycT1 through Zn2+-mediated interactions [201–203].

Vpr is a multifunctional HIV-1 protein that plays a role in nuclear import of the PIC and cell cycle arrest in proliferating cells. Vpr also activates LTR activity through multiple mechanisms. Vpr recruits p300 to the 5′ LTR increasing acetylation, resulting in HIV-1 transcription [204]. Moreover, Vpr interacts with Sp1 and TFIIB, part of the transcription initiation complex, stimulating proviral transcription [204–206].

4.9. Repressive host factors at the 5′ LTR

Not all host transcription factors have an activating effect on LTR activity (Figure 3). YY1 and LSF recognize binding sequences in the LTR and repress transcription through epigenetic

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modification [207]. C-promoter binding factor-1 (CBF-1) also represses HIV through epigenetic silencing [208,209]. c-Myc recruits an epigenetic silencing factor to repress HIV-1 [210].

Transcription factors initiate LTR activity, but full-length transcripts are not produced because transcription elongation is inhibited. DRB sensitivity-inducing factor (DSIF), a heterodimer composed of hSpt4 and hSpt5 proteins, induces capping of RNA from newly initiated transcription complexes [211]. The subunit hSpt5 interacts directly with nascent RNA as it appears from the RNAPII exit site and recruits negative elongation factor (NELF) (Figure 3) [212–214]. Escape of transcripts from the promoter proximal pause site is prevented by NELF, which induces termination of transcription over several hundred bases [215]. Moreover, the binding sequence of NELF subunit E recognizes a homologous sequence on TAR, increasing association of NELF with the LTR, which results in transcription silencing. Indeed, experiments where NELF is knocked down show higher basal HIV transcription and reactivation from latency [216–218].

A novel, RNA interference independent, mechanism mediated by microprocessor and termination factors causes transcriptional silencing and chromatin remodeling at the HIV-1 promoter [219]. Microprocessor binds to TAR, which is then cleaved by Drosha into two RNAs, a 5′-end and 3′-end product. The 5′ is further processed in an Rrp6-dependent manner into a transcription repressing RNA species. The 3′ RNA recruits termination factor Xrn2 and Setx, which induces RNAPII pausing and premature termination of transcription [219].

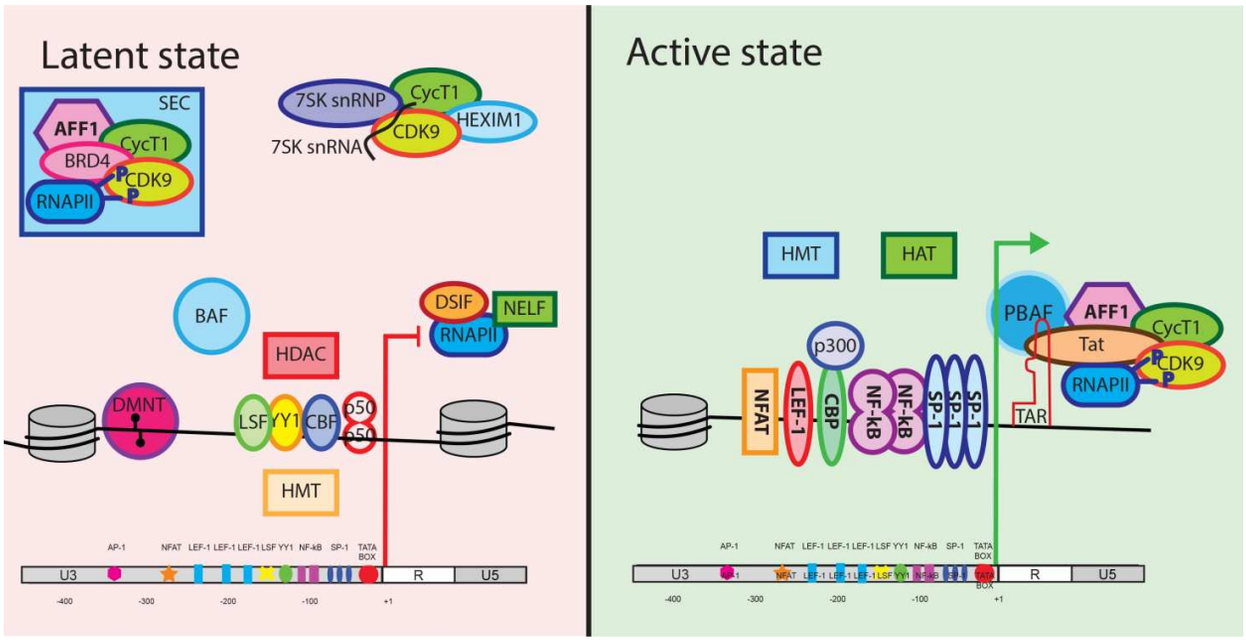

Figure 3. Molecular mechanisms in latent and productive HIV-1 infection

4.10. Host factors induce transcriptional initiation, but not elongation

While some host transcription factors recruit RNAPII, in the absence of Tat, transcription elongation does not occur resulting in the generation of short abortive transcripts by promoter

proximal pausing [220,221]. These ~60nt transcripts include TAR, which has a stem-loop structure and binds near the HIV 5′LTR, inhibiting RNA-polymerase. TAR directly binds Tat, which recruits transcriptional elongation complex to the LTR [222].

4.11. Tat-dependent transcription leads to productive infection

If cells become activated or due to leaky transcription, Tat can be produced. Tat binding to P-TEFb induces significant conformational changes in P-TEFb, allowing Tat and CycT1 to cooperatively recognize and stably bind TAR [200,223].

Tat-P-TEFb phosphorylates NELF-E resulting in the dissociation of NELF from TAR and the paused RNAPII complex [214,216,218,224]. CDK9 phosphorylates RNAPII at the carboxyl terminal domain (CTD) at Ser2 and Ser5 residues of the 52 heptad repeats, which regulates progression to the elongation phase of transcription [225–227]. The phosphorylation status determines regular and alternative RNA splicing and the 3′ end recruitment of polyadenylation factors [228,229]. Ser2 phosphorylation of the RNAPII CTD recruits splicing-associated c-Ski-interacting protein, SKIP, and stimulates elongation transcription and alternative splicing of the Tat-specific splice site through interactions with U5snRNP proteins and tri-snRNP110K [230].

Phosphorylation of hSpt5, a subunit of DSIF, by CDK9 converts it into a positive elongation factor that prevents nascent RNA from breaking of from the transcription complex prematurely and inhibits pausing of RNAPII at arrest sites [231,232]. By removing several blocks Tat-P-TEFb induces transcriptional elongation as well as co-transcriptional processing. During active transcription elongation, increased recruitment of RNAPII to TSS maintains a stable level of RNAPII at the promoter proximal region [218]. Throughout transcription, Tat-P-TEFb remains associated with the elongating transcription machinery [231,233,234].

4.12. P-TEFb can be recruited in active and inactive form in the nucleus by Tat

In activated T-cells, inactive P-TEFb predominantly resides in the 7SK small nuclear ribonucleoprotein (snRNP) complex (Figure 3) [235–237]. The 7SK snRNP complex consists of 7SK snRNA, HEXIM1 (or its homolog HEXIM2), the La-related protein 7 (LARP7), and the 7SK-specific 5′ methylphosphate capping enzyme (MePCE). The snRNA functions as a scaffold: it binds two units of P-TEFb and one HEXIM1/2 homo-/heterodimers [238,239]. MePCE and LARP7 protect the 7SK RNA from nuclease degradation, MePCE binds the 5′ end, LARP7 the polyuridine 3′ end [240–242]. Tat disrupts the interaction between pTEFb and HEXIM1/7SK snRNA and recruits P-TEFb to 5′ LTR, resulting in active transcription [226].

BRD4 can also recruit P-TEFb from 7SK snRNP [241,243], to promote transcription. Due to similarities in their C-terminal P-TEFb interacting domains [244], Tat and BRD4 compete for P-TEFb [245,246]. In a latent model, knockdown of BRD4 results in Tat-dependent reactivation of HIV-1 [247].

Bromodomain and extra-terminal domain family of proteins (BET) play an important role in repression of the HIV-1 transcription. BET proteins are responsible for the recruitment of P-

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TEFb to transcribed genes [246,248]. BRD4 competes with viral protein Tat for binding site on pTEFb, and it represses HIV-1 transcription [245,246]. Knockdown of BRD2 indicates this protein contributes to the maintenance of latency. These results are consistent with the notion that BRD2 is binding to remodeling factors such as HDACs [249,250].

P-TEFb can be recruited to transcription complexes by other factors. CTIP2 recruits P-TEFb by binding HEXIM1 and negatively regulates the complex by repressing the CDK9 kinase activity of P-TEFb [251]. Phosphorylation of HEXIM1 at Tyr271 and Tyr 274 decreases retention of P-TEFb in the 7SK RNP [252]. Additionally, through the binding of nascent RNA, SRSF2 and P-TEFb are released from the 7SK complex and induce transcription elongation in a manner similar to TAR/Tat-mediated recruitment of P-TEFb [253].

4.13. P-TEFb is a subunit of the super elongation complex

P-TEFb is required for activation of HIV transcription but does not explain the maximum observed viral expression; therefore, additional factors are necessary [254,255]. P-TEFb is an integral part of the super elongation complex (SEC) (Figure 3), which is a potent activator of transcriptional elongation of host genes [234,256]. It is composed of one of two scaffold proteins, AF4/FMR2 proteins AFF1 or AFF4. Translocations of AFF1 and AFF4 resulting in fusion proteins are commonly found in mixed lineage leukemia (MLL) [257–259]. The resultant fusion proteins cause aberrant recruitment of SEC to MML-specific genes [260]. AFF1 and AFF4 recruit many other proteins to the SEC [261], such as ELL family of elongation stimulatory factors ELL1 and ELL2, which inhibit RNAPII pausing and synergistically improve Tat-transactivation with P-TEFb [256]. Moreover, knockdown of ELL2 strongly suppresses viral expression. [203,210,230,252]. Tat and AFF4 inhibit the polyubiquitination-mediated degradation of ELL2, increasing available levels of SEC. [256,262].

4.14. Tat can be extensively post-translationally modified – "Tat code"

Modifications on numerous amino residues of Tat regulate the interaction with a wide variety of host proteins. In comparison to the histone code which is used to explain the multiple modification on histone tails and their function, a "Tat-code" has been proposed [34]. Tat is phosphorylated on Ser16 and Ser 46 by CDK2, modifications which result in transcription inhibition [263]. Acetylation of Lys28 increases affinity for P-TEFb binding and is removed by HDAC6 [264–266]. Tat dissociates from TAR and binds acetyltransferase PCAF which acetylates Tat at Lys50 and Lys51 [264,265,267–270]. Acetylated Lys50 allows recruitment of the PBAF (SWI/SNF B) chromatin remodeling complex to the LTR [267,271–273]. SIRT1 deacetylates Tat at Lys50 as part of a late phase of transcriptional regulation, striping Tat of acetyl groups allowing its reuse in subsequent rounds of transcriptional cycles [274]. Monomethyl-transferase Set7/9 and LSD1, respectively, methylate and demethylate Lys51. Demethylated Lys51 of Tat enhances HIV-1 transcription [275,276]. Hdm2 polyubiquitinates Lys71, activating Tat [277].

4.15. Nucleosome positioning at the 5′ LTR controls viral expression

Regardless of integration position, the latent 5′ LTR typically contains two nucleosomes, Nuc-0 and Nuc-1, at fixed positions [278]. Nuc-1 blocks transcription elongation as it is positioned just downstream of the TSS. Nuc-1 is displaced upon virus reactivation [278–280]. Nucleosomes can be altered by chromatin remodeling complexes. A third unstable or loosely positioned nucleosome is located in between nuc-0 and nuc-1 [281] (Figures 2 and 3A).

BCL11B, together with the chromatin remodeling complex NuRD, strongly represses HIV-1 transcription [282]. BCL11B is specifically expressed in T-cells and neurons. Interestingly, the NuRD complex consists of several proteins with histone deacetylase activities – i.e., HDAC1 and HDAC2 [283,284].

The ATP-dependent chromatin remodeler BAF (SWI/SNF-A) was discovered by our group to be essential to both the establishment and maintenance of HIV latency (Figure 3). The BAF complex utilizes energy from ATP to push Nuc-1 from an energetically favorable position upstream of the TSS to a suboptimal region, downstream of TSS, resulting in a transcriptional block [281]. siRNA depletion of the BAF complex de-repressed proviral transcription. Furthermore, in siRNA-mediated BAF knockdown, latency establishment occurred less frequently than in the presence of the functional complex. The PIC through LEDGF interacts with INI-1 a subunit of BAF, allowing nucleosomes to be deposited at the provirus, contributing to latency establishment [118].

4.16. Epigenetic modifications regulate latency

Epigenetic modifications of nucleosomes such as histone-acetylation and -methylation and of DNA such as DNA-methylation play an important role in regulating the proviral transcription. Nucleosomes are the basic units of organization of chromatin and consist of a combination of histone subunits. Histones have an amino acids tail that can be extensively modified. Two broadly studied modifications that regulate expression effects are histone-acetylation and histone-methylation

Histone-acetylation by histone acetyl transferases (HATs) induces chromatin loosening, while histone deacetylases (HDACs) reverse the effect by removing the acetyl group (Figure 3). HATs such as p300/CREB-binding protein (p300/CBP) and p300/CBP-associated factor (P/CAF) can be recruited to activate the HIV LTR [158,285]. HDAC1, HDAC2, HDAC3, and HDAC6 repress HIV [286–289]. Numerous host factors recruit HDACs to the LTR. A negative regulator of P-TEFb, CTIP2 in cooperation with COUP-TF and Sp1 also recruits HDAC1 and HDAC2 to the HIV LTR in microglial cells [290,291]. Host factors LSF and YY1 co-operatively bind to the LTR, where YY1 recruits HDAC1 to deacetylate Nuc-1[207]. CBF-1 and c-Myc also repress HIV through the recruitment of HDAC1 [208–210].

Methylation of histones by histone methyltransferases (HMT) can act as an activating or repressing mark depending on the histone tail residue modified (e.g., methylation of lysine 4 on histone 3 (H3K4) is activating whereas H3K9, H3K27, and H4K20 methylation is repressive). HMTs modify specific histone residues, e.g., EZH2 (H3K27me3), SUV39H1 (H3K9me3), G9a (H3K9me2), and G9a like protein, GLP (H3K9me2). The repressive methyl groups deposited

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by these HMTs contribute to the maintenance of latency [292–295]. Moreover, EZH2 is suspected to recruit additional repressive proteins such as HDACs and other HMTs [294].

DNA methylation at CpG dinucleotides represses transcription by disrupting the binding of transcription activators to their binding sites or indirectly through the binding methyl-CpG binding proteins (MeCPs). In cell line models of latency, the HIV-1 LTR contains two CpG islands that are hypermethylated (Figure 3) [296]. Methyl-CpG binding domain protein 2 (MDB2) and HDAC-2 bind to the second CpG island on the HIV LTR and are displaced from there when cells are treated with cytosine-methylation inihibitor 5-aza-2′deoxycytidine [296]. In memory CD4+ T-cells from long-term aviremic and viremic patients, an increase in HIV LTR DNA methylation was observed in the aviremic patients [297]. The methylation of the HIV LTR in long-term non-progressors and elite controllers is increased compared to the LTR of aviremic patients on cART [298]. In contrast, this difference was not found in the first CpG island of resting memory CD4+ T-cells from aviremic patients, indicating that the mechanism by which DNA-methylation regulates latency deserves further exploration.

4.17. Viral and host non-coding RNAs regulate viral expression

Non-coding RNAs exert post transcriptional control on gene expression. Small non-coding RNAs (<200 nt) and in particular microRNAs (miRNAs) are well established to have regulatory function. The study of long non-coding RNAs (lncRNA, >200 nt) is an emerging field because of their epigenetic regulatory potential. Both viral and host miRNAs and lncRNAs affect replication of HIV-1 [146,299–301].

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism. miRNAs post-transcriptionally suppress or silence gene expression as part of the RNA-induced silencing complex (RISC) forming a protein–RNA complex. Pri-miRNAs are generated by RNAPII and are subsequently processed by microprocessor into pre-miRNAs in the nucleus. Following export to the cytoplasm, they are cleaved by Dicer and incorporated into RISC. RISC generally binds in the 3′-untranslated region (3′UTR) of a target mRNA. The bound transcript is degraded or transcription is impeded depending on the level of homology, resulting in translational repression. The RNAi affects the infectivity of monocytes and macrophages [302]. Comparisons of productively infected, suppressed, and uninfected patients found difference in miRNA profiles, but it is very unlikely that the observed effects are due to viral activity because the number of infected cells is low in elite controllers or under cART [303–305]. Knockdown of Dicer or Drosha, a component of microprocessor, stimulates HIV-1 replication, indicating that miRNA generally are responsible for suppression of proviral transcription [299,300]. However, phenotypic effects are hard to interpret due to the pleiotropic side effects of microprocessor depletion. RNAi affects infectivity by targeting transcripts of key host factors and viral proteins involved in HIV-1 repression. In resting T-cells, the polycistronic miRNA cluster miR-17/92 is suppressed by HIV, resulting in PCAF upregulation [299]. Additionally, CycT1 is negatively regulated by miR27b [306]. Moreover, during differentiation from monocytes to macrophages, expression of miRNA198 and miR27b decreases relieving suppression of CycT1 [307,308]. In infected cells Tat, and possibly Vpr, inhibit RNAi [309–311]. In resting, but not activated, CD4+ T-cells a cluster of five miRNAs (miR-28, miR-125b,

miR-150, miR-223, and miR-382) were found to be upregulated. They all target viral mRNAs for degradation; therefore, these miRNAs are contributing to latency maintenance [312]. However, further studies are required as results thus far are inconsistent [313–319].

The viral protein Nef is targeted by miR29a which interferes with HIV replication [300,320]. TRIM32 activates HIV-1 expression through the NF-kB pathway and is downregulated by miRNA-155 [321]. Tat-induced upregulation of miR34a and miR217 inhibits SIRT1 expression, which in turn results in high abundance of NF-kB, enhancing proviral transcription [322,323]. miRNA-182 has a positive effect on LTR activation by Tat [324]. miR-1236 restricts viral replication by repressing Vpr (HIV-1)-binding protein expression, VprBP [325].

HIV-1-derived miRNAs (vmiRNAs) were predicted *in silico* [326]. Applying deep sequencing technologies vmiRNAs were observed in cell line model systems of latency [327,328]. The TAR-derived miRNA-TAR5p and miR-TAR3p are asymmetrically processed and both repress LTR activity [329]. The *Nef*-derived miR-N367 inhibits viral promotor activity [330]. Nevertheless, relevance of vmiRNAs is debatable as no vmiRNAs were detected in PBMCs or macrophages of infected patients [331].

lncRNAs can modulate gene expression through different proposed mechanisms: (1) affecting mRNAs through sequence recognition, (2) recruiting proteins to DNA, (3) blocking host factors by assuming a secondary structure, (4) functioning as a scaffold for protein complexes. An anti-sense lncRNA of HIV-1 inhibits viral replication[146]. The non-coding repressor of NFAT (NRON) inhibits LTR activity in a NFAT-dependent manner [301].

4.18. Stochastic gene expression

The current model of HIV latency proposes that resting memory CD4+ T-cells are deprived of host factors that are necessary for viral expression. An alternative model proposes that expression is highly stochastic. Due to fluctuations in chromatin state and availability of the transcription factors, the latent and productive state co-exist [332]. In support, clonal lines (containing the same integration) showed binominal distributions of viral expression [333]. Transcriptional bursts of 2–10 mRNA transcripts were estimated to be the source of HIV-1 gene expression [334]. Tat-controlled positive feedback extends the expression reactivation [335]. The sensitivity to reactivation is also stochastic, as cells derived from patients remained latent during a first round of activation and were reactivatable in the next round of activation [83]. Moreover, molecules that increase gene expression fluctuations synergistically enhance HIV-1 reactivation [336].

5. HIV cure

Mechanistic insight into the complex nature of latent HIV-1 infection provides a rationale for eradication strategies. Therefore, identification of molecules that inhibit activity of repressors or potentiate HIV-1 activators alongside with immune system boosting are important objectives in eradication strategies.

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5.1. Shocking the virus: screening for Latency Reversal Agents (LRAs)

The initial step of LRA discovery is screening drug libraries with cell-line-based models. Positive hits are evaluated further using primary-cell-based models as they better recapitulate the nature of latent reservoirs. If effective and not toxic, putative LRAs should undergo reactivation studies using primary cells derived from HIV-1-positive individuals that are on cART as well as toxicology studies in animal models, in case of novel molecules. It is advantageous to include molecules that are already approved drugs in such putative LRAs libraries, employing them into clinical practice would be time and resources effective. Moreover, in order to easily diffuse through cell membranes, ideal LRAs are small molecules, with molecular weight below 900 daltons, although clinical practice shows that most effective compounds do not exceed 500 daltons [337,338].

The first attempts to reactivate proviral DNA failed, due to the use of agents (e.g., IL-2 or a monoclonal antibody against CD3 receptor) which resulted in global T-cell activation. Indeed, viral p24 and plasma HIV-1 RNA levels increased, but the toxicity of such treatment left this approach useless [339–341]. Therefore, there is a need for more specific agents, which are able to reactivate proviral transcription without T-cell activation.

5.2. HDAC inhibitors (HDACis)

Histone deacetylase inhibitors (HDACis) are a very promising class of LRAs which include valporic acid (VPA), Vorinostat (SAHA), Romidepsin, Panobinostat, Givinostat, Droxinostat, or Entinostat. Some (Vorinostat (SAHA), Romidepsin, Panobinostat) are undergoing clinical trials [94,342–344].

The focus on HDACis is due to their ability to loosen up the compact chromatin structure at the latent proviral promoter. Inhibition of HDACs results in an increase of histone acetylation level by HATs. HDACs 1, 2, and 3 are of particular interest as they considerably contribute to HIV-1 repression [287]. Fortunately, HDACis are already used in clinical therapies, e.g., VPA is used in epilepsy and bipolar disorders, Vorinostat and Romidepsin are used to treat cutaneous T-cell lymphoma (CTCL) while Panabinostat is used in patients with multiple myeloma. In a very promising study by Archin et al., a single treatment with Vorinostat resulted in an increase in proviral RNA [345]. Unfortunately, the follow-up study with additional, multiple-dose rounds of treatment showed that increase on HIV-1 transcription is neither sustained nor elevated [346]. It is possible that other mechanisms maintaining latency compensate histone acetylation, in order to restrain proviral transcription. Alternatively such low concentrations of Vorinostat result in activation of pTEF-b instead of HDAC inhibition [347]. Since HDACs are involved in general regulation of gene expression; they have pleiotropic effects causing toxicities. Therefore, their use must be strictly controlled and monitored in order to provide maximal safety [348]. Nevertheless, HDACis are still under much interest. Especially, finding more specific HDAC inhibitors is very appealing, as current drugs are inhibiting a wide range of different HDACs, contributing to high toxicity [349].

5.3. BET inhibitors (BETi's)

Since BET proteins repress the HIV-1 promoter, it is worth to use their inhibitors in latency reversal strategies. Treatment with BET protein inhibitor JQ1 reactivates HIV-1 transcription in Tat-independent fashion [247]. Furthermore, BET inhibitor activity was positively tested in more relevant primary model system of latency [249]. Unfortunately, JQ-1 is not clinically available, due to its short half-life.

5.4. HMT inhibitors (HMTis)

Several histone methyltransferases (HMTs) such as EZH2, SUV39H1, and G9a interact with 5′ LTR contributing to maintenance of latency by deposition of repressive methyl groups on nucleosomal proteins [292–295]. Moreover, EZH2 recruits additional repressive proteins such as HDACs and other HMTs [294]. Several inhibitors of these proteins were tested in cell lines or primary cells from HIV-1 positive patients. Among which, Chaetocin (SUV39H1 inhibitor) and BIX-01294 (G9a inhibitor) were most potent [292,350]. However, high toxicity, due to pleiotropic effects, makes them unsuitable for clinical practice. Therefore, identification of novel compounds that are able to inhibit the activity of HMTs is needed.

5.5. DNMT inhibitors (DNMTis)

Inhibition of DNA methyltransferases (DNMTs) with 5-aza-2′ deoxycytidine (aza-CdR or Decitabine) leads to modest reactivation of latent HIV-1. This activity can be further enhanced with PKC agonists [351]. However, 5′ LTR methylation in patients material remains controversial [352]. Thus, further investigation of provirus methylation *in vivo* is needed.

5.6. Toll-like receptors (TLRs) stimulation

TLRs recently gained more attention, as theirs agonists are strong reactivators of HIV-1 [353–357]. The main role of these receptors is to activate an immune response against bacterial or viral infections [358]. Stimulating TLRs (as adjuvants in immunization) as well as opportunistic bacterial infections elevate plasma HIV-RNA and improve immune function [359–363].

Vaccine adjuvant – CPG 7909 (TLR 9 agonist) is able to decrease plasma HIV-1 RNA via activation of HIV-specific CD8+ T-cells in peripheral blood [359]. More recently, in SIV-positive rhesus monkeys undergoing cART were treated with GS-9620, a TLR7 agonist, reversible CD8 cytotoxic T-cells activation alongside with modest CD4 T-cell activation were observed. Moreover, elevated plasma viremia was observed as well as decrease in HIV-1 DNA in blood, colon, and lymph nodes. Interestingly, viral load returned back to undetectable levels when GS-9620 was no longer administrated. More strikingly, when cART was stopped, GS-9620-treated monkeys had 0.5 log lower viral set-point than untreated, infected animals. Additionally, in cells isolated from HIV-positive individuals transcription of HIV-1 was observed. However, some variability between samples was noticed. Clinical trials with the use of this compound are planned [364,365].

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5.7. Super elongation complex stimulation

Treatment of cell lines and cells isolated from aviremic patients on cART with hexamethylene bisacetamide (HMBA), an anticancer drug that transiently activates PI3K/Akt pathway, results in phosphorylation of HEXIM1. P-TEFb is subsequently released and interacts with RNAP II, resulting in latency reversal [366–368]. Moreover, HMBA provides CDK9 recruitment to the viral promoter by interaction with SP1, which enhances transcription from proviral DNA. Furthermore, Klichko et al. showed that treatment with HMBA resulted in a decrease of CD4 receptor expression without affecting transcription of CCR5 and CXCR4 co-receptors [369]. Moreover, HMBA does not trigger activation of T-cells. Studies on P-TEFb's role in HIV-1 latency indicate that this heterocomplex might be an interesting target for inclusion in "shock and kill" therapies.

5.8. PKC pathway activation

Another interesting approach is the use of molecules that are able to selectively activate the protein kinase C (PKC) pathway. PKC pathway agonists trigger nuclear localization of NF-kB, NFAT, and AP-1 transcription factors. Therefore, PKC agonists are one of the most potent activators of HIV-1 transcription. Currently, two PKC agonists are being scrutinized clinically: prostratin and bryostatin, due to their safety and specificity toward HIV-1 reactivation. The latter is a clinically available drug [370]. Moreover, these two compounds prevent *de novo* infections, as they downregulate viral receptor and co-receptors CD4, CCR5 and CXCR4 in PBMCs [371]. A rather controversial molecule that reactivates HIV-1 transcription via NF-κB pathway is arsenic trioxide (As_2O_3). In the Jurkat model system of latency, As_2O_3 activates NF-κB leading to HIV-1 replication. Moreover, it synergizes with prostratin, tumor necrosis factor alpha (TNFα), and VPA [372]. Interestingly, arsenic is already used in clinical practice to treat acute promyelocytic leukemia (APL). Therefore, it would be interesting to test this compound in more relevant models of HIV-1 latency such as primary cells infected *ex vivo* and in cells derived from aviremic patients.

The use of PKC agonists raises concerns about their safety in a clinical setting. The protein kinase enzyme family consists of several isoenzymes that play important roles in signal transduction cascades [373]. As activation of latent HIV-1 is mediated via PKCα and PKCθ, the identification of more specific agonists of PKCα and PKCθ is needed. Alternatively, lowering the concentration of a specific agonists might decrease toxicity and contribute to latency reversal [374].

5.9. JNK/MAPK pathway activation

Studies employing mutagenesis of binding sites for activator protein-1 (AP-1) within the proviral genome showed that the AP-1 transcription factor is a crucial activator of proviral transcription, as proviruses with altered AP-1 binding sites were less prone to reactivation even if treated with a strong activator such as phorbol 12-myristate 13-acetate – PMA [191]. Furthermore, the latent pool of cells infected by virus with deletion in AP-1 sites was bigger, implicating that AP-1 is necessary for provirus transcription [192]. Heterodimeric protein AP-1

is formed upon phosphorylation of c-Jun N-terminal kinase (JNK) in JNK/MAPK pathway [193]. It is well established that activation of TLR signaling induces nuclear localization of NF-kB and AP-1 mediated via JNK pathway [194,196,376,377].

Virtual screening followed by validation of positive hits in cell line model systems for HIV-1 latency discovered 8-methoxy-6-methylquinolin-4-ol (MMQO) as a specific activator of the JNK-AP-1 pathway, which is able to reactivate HIV-1 from its latent state. Interestingly, MMQO inhibits IL-2 and TNFa expression, contributing to maintenance of resting state of CD4+ T-cells [378]. The recently synthetized panel of inhibitors of farnesyl transferase (FTase) are able to moderately reactivate HIV-1 transcription via JNK pathway. Interestingly, strong synergy with other LRAs, such as Vorinostat or TNF-a, was observed for these molecules in latency reversal [379,380].

5.10. Canonical Wnt signaling pathway activation

Recently, our group showed that treatment with Wnt3A/Rsp (natural stimulators of Wnt pathway) and lithium (inhibitor of Wnt repressor protein GSK3) leads to latency reversal in latent cell lines and enhances the latency reversal potential of HDAC inhibitors in CD4+ T primary cells obtained from patient volunteers when co-treated [381]. This observation shows a functional role for three LEF1 binding sites in the 5′ LTR contains, which are downstream targets of the classical Wnt pathway [381,382]. It would be very interesting to find more potent and selective inducers of Wnt pathway, as lithium exhibits many pleiotropic, toxic effects [383,384].

5.11. Chromatin loosening

It was discovered by our group that a main player in the establishment and maintenance of latency is the BAF complex (SWI/SNF-A), which belongs to ATP-dependent chromatin remodelers' family. Interestingly, Dykhuizen et al. [378] screened a library of compounds that would be able to mimic BRG-1 knock out. In their study, they found 20 compounds that were transcriptionally mimicking BAF complex disruption. We showed that several of those molecules were able to decrease the frequency of latency establishment and reactivate HIV-1 in cell line and primary cells models of latency [386, in press]. Moreover, they synergize with other LRAs – SAHA and prostratin. Two most potent inhibitors – caffeic acid phenethyl ester (CAPE) and pyrimethamine (PYR) did not activate T-cells derived from healthy donors and cells obtained from aviremic patients. Moreover, PYR is a registered drug used in malaria treatment. Therefore, these inhibitors are promising molecules to include in eradication strategies.

5.12. Multifunctional LRAs

In vitro treatment with cocaine leads to increase in HIV- replication in PBMCs as well as increased viral load in mouse models of HIV infection [387–389]. Interestingly, in *ex vivo* infected primary CD4+ cocaine treatment resulted in downregulation of miR125-b expression, which led to enhanced replication of HIV-1 [314]. In primary human macrophages and myeloid

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cell systems of latency, cocaine increased replication of HIV-1. Cocaine treatment activates NF-κB and leads to phosphorylation of mitogen- and stress-activated kinase 1 (MSK1). Furthermore, pMSK1 phosphorylates RELA (p65), a subunit of NF-κB promoting the interaction of NF-κB with p300 and recruitment of P-TEFb to the proviral 5′ LTR [390]. Moreover, treatment with cocaine results in histone H3 phosphorylation, thus increasing accessibility of HIV-1 promoter for transcription factors [390]. Therefore, cocaine not only reverses latency via NF-κB pathway but also causes epigenetic changes on 5′ LTR as well as blocks repressive miRNA.

Oral bacteria secrete short-chain fatty acids (SCFAs) including butyric acid, propionic acid, isovaleric acid, and isobutyric acid that are capable of HIV-1 and herpesviruses latency reversal [384,385]. Some of these molecules are known HDACis (e.g. Butyric acid) [393]. Moreover, SCFAs not only promotes histone acetylation, but also inhibit repressive histone formation and DNA methylation. Furthermore, they activate P-TEFb resulting in increased elongation of transcription from 5′ LTR. [345,385,386].

5.13. Immune clearance of reactivated cells – "Kill"

The majority of chronic patients are facing immune exhaustion, characterized by low cytokine secretion, smaller proliferative capacity, and low cytopathic potential of CD8+ T-cells [394,395]. Therefore, the first line of action would be reviving normal immune activity. Indeed, inhibition of programmed cell death protein 1 (PD-1) leads to restoration of immune functions in mouse models of HIV-1 infection [396]. However, these results were obtained in viremic animals. Nevertheless, an IgG4 antibody targeting PD-1 receptor is undergoing clinical trials to assess safety, immunotherapeutic activity, and the ability of treatment to reduce pool of latently infected cells [397].

In so-called "elite controllers", CD8+ T-cells effectively restrain infection without intervention of cART, by killing CD4+ T-cells that are actively producing HIV-1 particles [398,399]. The immune system can be boosted by specific amplification of HIV-1-specific CD8+ T-cells. These observations again aroused the idea of developing a vaccine. Indeed, rhesus monkeys vaccinated with CMV vectors resulted in broad cellular immune response to SIV [400–402]. However, safety issues related to the use of such vectors remain to be elucidated. Another platform being investigated to increase immune response against HIV-1 are Ad26 vectors, as it was shown that vaccinated rhesus monkeys were protected against infection with SIV as well as viral loads were lowered after vaccination [403,404].

A very interesting group of immunoglobulins to include in eradication strategies are broadly neutralizing monoclonal antibodies (mAbs or bNAbs) isolated from chronically infected patients. New generations of bNAbs exert higher potency and wider range of activity against many HIV-1 subtypes. It was shown that a combination of bNAbs is potent enough to transiently suppress viremia in rhesus monkeys as well as to reduce the amount of HIV-1 DNA in the blood, lymph nodes, and gastrointestinal mucosa [403,405,406].

6. Future perspectives and challenges

A reservoir of latent HIV is the main obstacle in finding a functional and sterilizing cure. Several challenges need to be addressed in order to overcome this obstacle. Defining the latent reservoir is impeded by the rare occurrence of a latent infection in a high background of defective proviral integration. Although HIV prefers integration in or near transcriptionally active genes which leaves ample room for variation in chromatin environment and available host transcription factors. This puts considerable demands on LRAs. LRAs should be effective, yet specific, without being toxic. As LRAs act via pathways involved in distinct cellular processes, pleiotropic effects are to be expected. Furthermore, recent studies on material obtained from HIV-1-positive suppressed patients revealed that currently available LRAs are not strong enough to reactivate the whole pool of latent proviruses, even after multiple rounds of stimulation. One of the concerns arising from "shock and kill" therapy is whether putative LRAs are strong enough to drive virus production to a level at which the immune system will be able to recognize and destroy HIV-1-producing cells. Indeed, trials aiming at testing HDAC inhibitors are inconsistent in showing depletion of latently infected cells while showing increased proviral transcription [407–412]. A complementary strategy would be to use multiple LRAs in combination to broadly and potentially synergistically reactivate the diversely integrated latent proviruses. Synergism between LRAs was already identified, e.g., Vorinostat and Prostratin [84]. Therefore, the quest for identification and characterization of novel compounds which are able to reactivate HIV-1 transcription as well as identifying combinations of drugs that can synergize to reverse latency is needed. Currently, no cell model is able to recapitulate the complexities of latency *in vivo*. A better system that more closely resembles the *in vivo* situation would greatly aid the understanding of molecular mechanisms underlying latency and the screening of new LRA. Moreover, as HIV-1 persists in a silent state, it contributes to a low level of inflammation, which over time leads to immune exhaustion. Furthermore, depletion of cells harboring latent provirus requires antigen-specific CTLs stimulation [399]. Most likely successful eradication therapies will be based on the combination of LRAs coupled with boosting HIV-1-specific immune response. A "shock and kill" approach in combination with immune therapies provides hope for reversing HIV-1 infection.

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