Folia Microbiol
DOI 10.1007/s12223-016-0474-7

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Current views on HIV-1 latency, persistence, and cure

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Received: 10 May 2016 / Accepted: 20 September 2016
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Abstract HIV-1 infection cannot be cured as it persists in latently infected cells that are targeted neither by the immune system nor by available therapeutic approaches. Consequently, a lifelong therapy suppressing only the actively replicating virus is necessary. The latent reservoir has been defined and characterized in various experimental models and in human patients, allowing research and development of approaches targeting individual steps critical for HIV-1 latency establishment, maintenance, and reactivation. However, additional mechanisms and processes driving the remaining low-level HIV-1 replication in the presence of the suppressive therapy still remain to be identified and targeted. Current approaches toward HIV-1 cure involve namely attempts to reactivate and purge HIV latently infected cells (so-called "shock and kill" strategy), as well as approaches involving gene therapy and/or gene editing and stem cell transplantation aiming at generation of cells resistant to HIV-1. This review summarizes current views and concepts underlying different approaches aiming at functional or sterilizing cure of HIV-1 infection.

Introduction

HIV/AIDS infection can be successfully treated and controlled with the combined antiretroviral therapy (cART) affecting different steps of HIV-1 replication cycle. Thus, HIV-1 infection could be viewed as a chronic disease with a relatively long life expectancy. However, cART, which is still not available to all in need, cannot cure HIV infection due to the presence of latently infected reservoir cells. The latent proviral DNA cannot be recognized by the immune system nor targeted by cART. Consequently, a lifelong therapy is necessary, which is expensive and leads to various complications and treatment failures. Therefore, new approaches toward functional or sterilizing cure are intensively explored – namely attempts to reactivate and purge HIV-1 latently infected cells (so-called "shock and kill" strategy), as well as approaches involving gene therapy and/or gene editing and stem cell transplantation aiming at generation of cells resistant to HIV-1. The ongoing research focuses especially on the mechanisms of establishment and maintenance of the latent reservoir, assessment of its size and composition, as well as on stimulation of the innate and specific immunity to promote HIV-1 clearance.

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HIV-1 latency

Retrovirus replication cycle is specific by a step of reverse transcription and a consequent stable integration of the proviral DNA into the host cell genome. Depending on the status of the host cell, either the HIV-1 provirus can be immediately expressed and the virus replication cycle can proceed further or the provirus can become dormant and wait until the latently infected cell encounters the right stimulus (often a specific, possibly rare antigen) and becomes activated. It is the very presence of the latently infected cells that makes HIV-1 infection incurable as these cells serve as a source of virus rebound after a discontinuation or failure of the antiretroviral therapy. After the activation of the host cell or specific

Published online: 05 October 2016

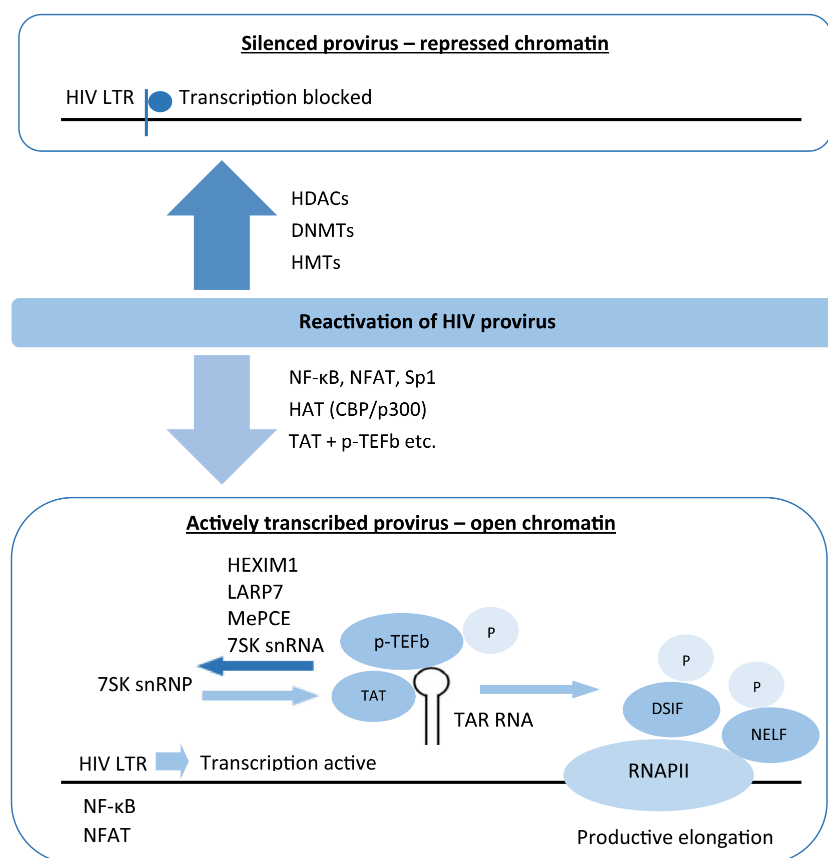

Fig. 1 Latency and reactivation (simplified scheme). HIV-1 latency is characterized by a repressed chromatin, presence of histone deacetylases (HDACs), histone methyltransferases (HMTs), and DNA methylases (DMTs), as well as lack of transcription factors, resulting in a transcription block. Reactivation is associated with epigenetic changes that lead to open chromatin structure, namely presence of histone acetyltransferases (HATs), nuclear translocation of transcription initiation factors NF-κB and NFAT, increased levels of Tat and formation of its complex with p-TEFb. Tat-p-TEFb complex binds to TAR RNA, resolving promoter-proximal pausing of RNAP II and allowing efficient transcription elongation. p-TEFb can be sequestered in the 7SK snRNP inhibitory complex

changes in the epigenetic regulation, the chromatin status and availability of transcription and other factors change and HIV-1 replication can restart (Fig. 1).

HIV-1 persistence during therapy

HIV-1 infects namely CD4+ T lymphocytes but also myeloid cells like macrophages, microglia, astrocytes, and dendritic cells, even though to a lesser extent. Acute infection of CD4+ T cells usually leads to cell death, but occasionally, these cells survive and revert back to a resting memory state. Alternatively, latency may be established directly in resting CD4+ T cells. Resting memory cells, including the latently infected ones, then persist and/or are replenished by homeostatic proliferation. In contrast, macrophages are resistant to cytopathic effects of HIV-1 infection and support virus persistence in various anatomical sanctuaries as tissue resident macrophages (Kim and Siliciano 2016; Kumar et al. 2015; McKinstry et al. 2010).

After initiation of cART, plasma viremia and the level of HIV-1-infected cells in peripheral blood decay with a well characterized kinetics based on populations with a different turnover contributing to plasma viremia (Hilldorfer et al. 2012) (Fig. 2). A rapid decline of plasma viremia during first two phases indicates the efficiency of antiretroviral drugs. Phase I represents the turnover of the free virus (half-life from minutes to hours) and mainly of productively infected CD4+ T cells (half-life of 1–2 days; Ho et al. 1995; Perelson et al. 1997; Perelson et al. 1996; Wei et al. 1995). Consequently, phase II corresponds to the decay of cells more resistant to HIV-induced cytopathic effect (partially activated T cells and cells of the monocyte-macrophage lineage with half-life of 2–4 weeks; Perelson et al. 1997; Shan and Siliciano 2013). The following phase III with a very low decay kinetics (half-life of 273 days) proceeds after several years into phase IV with a remaining stable low level plasma viremia that does not decrease any more (Hilldorfer et al. 2012; Maldarelli et al. 2007). Despite the very low plasma viremia below the limit of clinical assays (<50 copies/ml), which is detectable with only ultrasensitive assays (about 3 copies/ml), cell-associated proviral DNA (prDNA) and RNA (caRNA) are commonly detected by PCR-based assays in peripheral blood mononuclear cells (PBMCs) during this phase (Palmer et al. 2008; Palmer et al. 2003) (Fig. 2). The presence of the phase IV indicates the existence of additional cell populations that do not succumb to virus-induced cytopathic effects and/or either are refractory to cART (proviral DNA or transcription cannot be targeted by cART) or persist in anatomical sanctuaries that are not accessed by cART. Reactivation of the latently infected cells, which both persist and are replenished by homeostatic

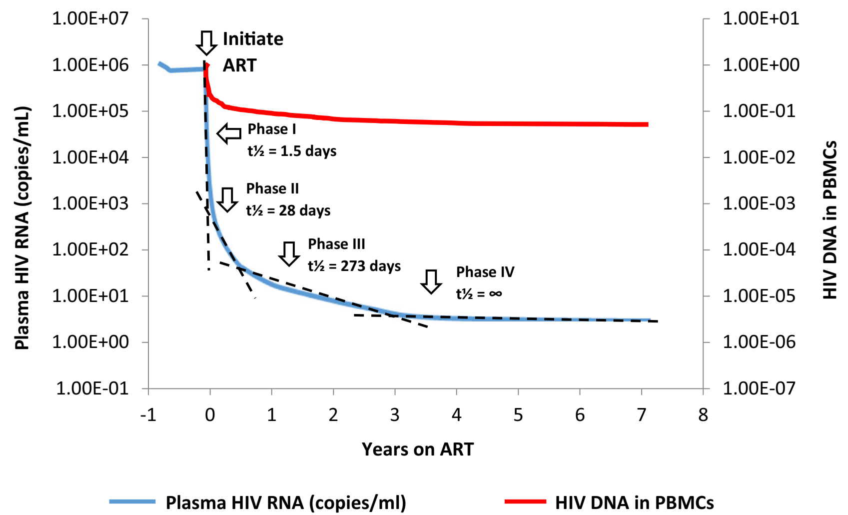

Fig. 2 Comparison of HIV-1 RNA and DNA decay curves upon introduction of cART. After initiation of cART, plasma viremia and the level of HIV-1 infected cells in peripheral blood decay with a well characterized kinetics based on populations with different turnovers contributing to plasma viremia (Phase I–IV). HIV-1 DNA reveals a relatively smaller decrease. Adapted from Hilldorfer et al. (2012) and Margolis et al. (2016)

proliferation (Chomont et al. 2009; Kim and Siliciano 2016), is viewed as the most relevant source of the new HIV RNA (Shan and Siliciano 2013). However, lower levels of antiretrovirals (ARV) found in anatomical/immunological sanctuaries like brain, genital tract, gut mucosa, or lymph nodes can support the ongoing HIV replication (Fletcher et al. 2014; Huang et al. 2016; Massanella et al. 2016). Based on sequence determination of HIV-1 present in lymph nodes and blood at several time-points after initiation of cART, the mathematical modeling of virus evolution and trafficking between the two compartments with low and high levels of ARV, respectively, supported the view that evolution of drug-sensitive viruses in lymph nodes can keep replenishing the viral reservoir despite the apparently efficient cART (Lorenzo-Redondo et al. 2016). Lately, it was described that SIV/HIV-1 may continue to replicate within B cell follicles due to follicular exclusion of CD8+ T cells and dysregulated responses of follicular regulatory T cells (TFR) and follicular T helper cells (TFH; Fukazawa et al. 2015; Miles et al. 2015; Saison et al. 2014; Tran et al. 2008). Furthermore, in addition to latently infected long-lived memory T cells and tissue resident macrophages (Avalos et al. 2016; Gludish et al. 2015), an evidence for existence of tissue resident memory T cells (TRM) emerged (Farber 2015; Farber et al. 2014).

Another cause of the residual low-level HIV replication under cART could involve the immune hyperactivation induced by various mechanisms. In addition to the residual productive infection that is likely to rise immune responses, there is a more frequent (95 %) abortive infection of resting nonpermissive CD4+ T cells. Due to the cytoplasmic DNA-sensing mechanisms like IFI-16, cGAS, and PQBP-1 (Gao et al. 2013; Thompson et al. 2014; Yoh et al. 2015), these cells might die by pyroptosis, a very inflammatory type of programmed cell death (Doitsh et al. 2014; Monroe et al. 2014). Furthermore, HIV-1 replication and disruption of the intestinal barrier lead to microbial translocation, stimulation of innate immune responses, and depletion of CD4 Th17 cells, a defect persisting even after initiation of cART (Chege et al. 2011; Schuetz et al. 2014). Finally, the activation and dysregulated function of regulatory T cells (Tregs), critical modulators of immune responses, apparently also contribute the immune hyperactivation (Mendez-Lagares et al. 2014; Saison et al. 2014). The residual low-level HIV replication that leads to an elevated immune activation thus further stimulates HIV replication, generating a vicious cycle.

In summary, HIV persistence during cART is supported by homeostatic and antigen-induced proliferation of latently infected cells, ongoing replication in the sanctuaries as well as by increased immune activation and inflammation (Chomont et al. 2009; Fukazawa et al. 2015; Kim and Siliciano 2016; Van Lint et al. 2013). Additionally, the latent reservoir is readily being replenished during episodes of viremia due to a treatment failure or other conditions of incomplete or missing pharmaceutical control.

Models of HIV-1 latency

Our knowledge of the mechanisms, maintenance, and reactivation of the latent reservoir is based namely on various in vitro and in vivo models as the presence of latently infected cells in human body is very low, around 1–10 millions in the whole organism, and difficult to study (Crooks et al. 2015; Finzi et al. 1999; Massanella and Richman 2016; Siliciano et al. 2003).

Individual models of HIV latency reproduce to some extent certain aspects of the complex situation in vivo and allow for studies of certain latently infected populations or specific aspects of latently infected cells. Models used to study HIV-1 latency in vitro include HIV-infected cell lines (immortalized lymphocytic or monocytic cells like J-Lat, ACH-2, U1;

Clouse et al. 1989; Folks et al. 1989; Folks et al. 1987; Jordan et al. 2003), primary cell models (derived from HIV-1 negative donor PBMCs by infection with a particular HIV-1 isolate or a recombinant), and resting CD4 cells (derived from HIV-1-infected patients; Spina et al. 2013). While the cell lines are commonly available and easy to handle, they reveal several aspects that often make them behave differently from the situation in vivo (namely, clonality based on the integration site or mutations of specific HIV-1 or host cell sequences).

Primary cell models might seem to be closer to the situation in vivo, while the percentage of latently infected cells available for further studies is much higher than in vivo. Different primary cell models developed by different laboratories use either resting or activated CD4+ T cells that are infected with wild-type (typically NL4-3) or recombinant HIV-1 (often expressing a fluorescent reporter gene like EGFP) and treated with combinations of several cytokines or chemokines. After establishment of the infection, latency is induced by ARVs followed by reactivation by different means. The individual models differ in the target cell population, percentage of latently infected cells generated, type and time of readout (Bosque and Planelles 2009; Gondois-Rey et al. 2006; Lassen et al. 2012; Saleh et al. 2007; Spina et al. 2013; Tyagi et al. 2010; Yang et al. 2009b). Nevertheless, it is questionable how closely these models reflect the situation in vivo, and how much they are biased due to the experimental setup and mode of readout.

Resting CD4 cells derived from HIV-1-infected patients cultured and stimulated ex vivo thus seem to be the most relevant in vitro model. However, their use is limited by a very low presence of latently infected cells, high background rate of defective integrated proviruses, and difficulties of any analysis due to a very high background of uninfected cells (problems with sensitivity and specificity).

The in vivo models of HIV latency include namely different types of humanized mice and macaques infected with HIV, SIV, or various recombinants. The advantage of humanized mice consists in their relative affordability and ease of handling; on the other hand, their preparation is tedious while graft-versus-host disease (GvHD) and other differences, namely due to their genetic background, the way of immune reconstitution with human tissues, and lineage precursors, may limit their use and relevance of the results.

Humanized mice used for HIV research were originally based on SCID mice (severe combined immunodeficiency mice) that were irradiated and then transplanted with fetal human thymus and liver (SCID-hu Thy/Liv). In this model, latent infection is established during thymopoiesis (deactivation phase), leading to generation of latently infected naïve CD4+ T cells. Among other limitations, this model does not provide an efficient peripheral reconstitution and human cells are found in relatively small numbers (Brooks et al. 2001; Marsden et al. 2012).

Lately, BLT mice (bone marrow-liver-thymus humanized mice) are considered as a better model for complex studies of HIV reservoirs and latency reversing agents (LRA) as they provide a robust peripheral reconstitution. These mice are most commonly based on irradiated NSG mice (NOD/SCID-gamma chain null mice) transplanted with fetal human thymus and liver and then reconstituted with bone marrow or purified CD34+ stem cells (Donahue and Wainberg 2013). This particular combination results in high-level systemic reconstitution of all human leukocyte lineages with improved T cell maturation and selection in a thymic environment and in generation of latently infected naïve and resting CD4+ T cells (Denton et al. 2012). There are also modifications of these mice using reconstitution with only discrete cell types like T cells or macrophages (T cell-only mice (ToM); myeloid-only mice (MoM); Honeycutt et al. 2013; Honeycutt et al. 2016), allowing studies of the role of the individual cell types in the establishment of latent reservoir or reactivation.

Although the use of BLT mouse model is valuable for the HIV studies, its important limitation consists in the development of GvHD, typically around 6 months after engraftment (Karpel et al. 2015). Further, these mice are unable to develop proper HIV-specific adaptive immune responses consisting in high levels of hyper-mutated, class-switched IgG antibodies as human cells of non-hematopoietic origin, namely those giving rise to stromal cells, are not transplanted, and secondary lymphoid organs do not properly develop (Malhotra et al. 2013; Wang et al. 2011).

Yet another BLT model, based on human $Rag2^{-/-}\gamma c^{-/-}$ mice (Choudhary et al. 2012; Traggiai et al. 2004; Zhang et al. 2007), was recently developed and led to identification of central memory CD4+ T cells (TCM) as the main latently infected population after suppressive cART similarly as in human (Choudhary et al. 2012; Donahue and Wainberg 2013). This model was further improved by generation of humanized TKO-BLT mice (triple knockout-BLT mice) on a C57Bl/6 background (C57BL/6 $Rag2^{-/-}\gamma c^{-/-}CD47^{-/-}$) in which the GvDH was much reduced, complement was functional, and secondary lymphoid organs with a well organized architecture and virus-specific immune responses were developed (Lavender et al. 2013).

A model closest to HIV-1 infection in human is infection of non-human primates (NHP), namely rhesus macaques, with various SIV strains. It is believed that different SIVs crossed the species barrier into humans many times, namely SIVsmm naturally infecting sooty mangabeys and SIVcpz infecting chimpanzees resulted in HIV-2 and certain clades of HIV-1 (Hirsch et al. 1989; Huet et al. 1990; Sharp and Hahn 2011).

The NHP models reveal many important features comparable to HIV-1-infected humans like anatomy, physiology, immune system, infectious agent itself, and susceptibility to antiretroviral treatment (Gardner and Luciw 2008; Policicchio et al. 2016). The use of NHP models has been essential for

understanding pathogenesis of HIV/AIDS as well as for studies of different therapeutic and vaccination approaches. Many conclusions could be also inferred by comparing immune responses to SIV infection in the natural and unnatural hosts, sooty mangabeys and rhesus macaques, respectively, in which the SIV infection results in non-pathogenic or pathogenic conditions. There is a high viremia in both hosts but a very little loss of CD4+ T cells and preservation of architecture and function of lymph nodes in the natural host. In the gut of the natural host, CD4+ T cells are only moderately depleted and numbers and functions of Th17 cells remain preserved, while microbial translocation is lacking. Further, TFH and TCM are only weakly infected (Ploquin et al. 2016).

Additionally, recombinant viruses like RT-SHIV or SHIV containing different parts of HIV-1 genome can be used to overcome certain differences between HIV-1 and SIVs, e.g., in studies involving inhibitors specific for HIV reverse transcriptase (Jiang et al. 2009; Ndung'u et al. 2001). One important difference in comparison with HIV-1 is that most SIVs (and HIV-2) encode a vpx protein that allows virus infection also in non-dividing cells as it targets for degradation cellular SAMHD-1, an enzyme that would hydrolyze dNTPs and thus decrease reverse transcription (Hofmann et al. 2012). Despite many advantages, a major drawback of using macaques consists in their cost and ethical and legal regulations of their use.

Obviously, the most relevant approach to study HIV latency and reactivation is in HIV-infected human patients in clinical studies. However, performance of clinical studies is strictly regulated, expensive, and must be preceded by extensive pre-clinical testing (including animal models). Additionally, human studies never provide really homogeneous and reproducible experimental settings with all necessary controls.

In summary, the results obtained in various in vitro and in vivo models and in human patients indicate that the latent HIV-1 reservoir is represented mainly by latently infected resting CD4+ T cells, long-lived central memory CD4$^+$ T cells (TCM cells), and transitional memory CD4$^+$ T cells (TTM cells). In addition to these cells, latent reservoir may comprise also other cell populations like CD34+ hematopoietic progenitor cells, naïve CD4+ T cells, CD4+ memory stem cells (TSCM cells) or $\gamma\delta$ T cells, as well as myeloid cells like macrophages and dendritic cells (Buzon et al. 2014; Carter et al. 2010; Chomont et al. 2009; Honeycutt et al. 2016; Soriano-Sarabia et al. 2015; Wightman et al. 2010). The importance of these cells constituting the latent reservoir consists in their different survivals and stabilities as well as in different requirements for signaling and activation and thus HIV-1 provirus induction (Archin et al. 2014b).

Assessment of the size of the latent reservoir

Under suppressive cART, plasma viremia (virion-associated unspliced RNA) is undetectable or below the detection limit of common commercial assays (<50 copies/ml). However, markers of immune hyperactivation persist and cell-associated HIV RNA can be readily detected both in peripheral blood and tissues like lymph nodes, tonsils, gut, or testes. Thus, in order to determine the effect of treatment intensification strategies and namely efficiency of LRA explored for therapeutic reactivation, availability of specific and sensitive methods allowing an accurate assessment of the size of the latent reservoir is crucial (Fig. 3).

The latently infected cells can be defined as cells harboring quiescent, replication-competent provirus. A gold standard in determination of latently infected cells is a quantitative virus outgrowth assay (qVOA) that is very material-, labor-, and cost-demanding. It requires large volumes of patients' blood to isolate sufficient numbers of highly purified latently infected resting CD4+ T cells and plate them in serial dilutions along with activated donor PBMCs (CD8 negative) or with a MOLT4/CCR5 cell line. It requires a 2–3-week incubation with changes of medium and other additives (Finzi et al. 1997; Massanella and Richman 2016; Siliciano and Siliciano 2005).

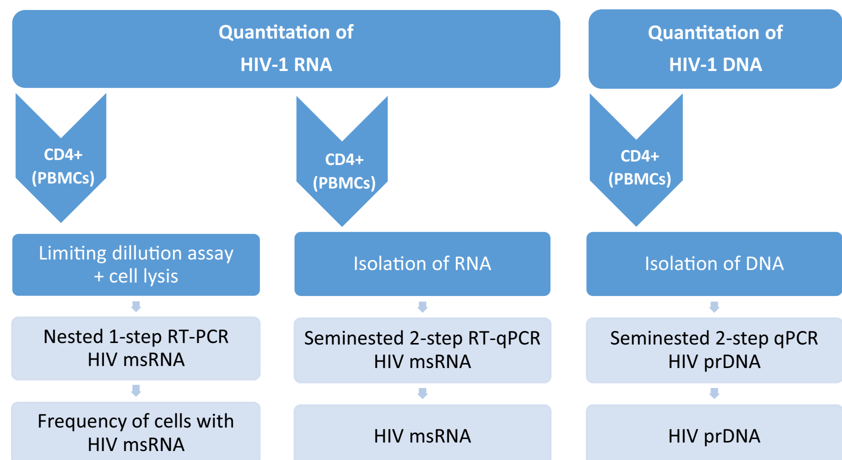

Fig. 3 PCR-based detection of HIV-1 RNA and DNA. Determination of cell-associated multiply spliced RNA (msRNA) and proviral DNA (prDNA) using two-step seminested (RT-) qPCR or using a combination of a limiting dilution assay and one-step nested RT-PCR (e.g., TILDA; Bullen et al. 2014; Kiselinova et al. 2014; Procopio et al. 2015)

However, this assay apparently underestimates the size of the latent pool, as it has been already demonstrated that consecutive rounds of stimulation/reactivation of the isolated cells could lead to increased proportion of the reactivated proviruses (Bruner et al. 2015; Ho et al. 2013). Further, only 1–2 % of the body's lymphocytes are present in peripheral blood, while most of them are found in tissues and the distribution of latently infected cells might not be proportional (Blum and Pabst 2007; Massanella and Richman 2016). The real size of the latent reservoir thus might be at least 60× higher than observed in a common qVOA, complicating the effort to cure HIV using latency reversal (Bruner et al. 2015; Ho et al. 2013). Importantly, about 10 % of HIV-1 proviruses that remain silent after the maximal stimulation were found to be fully replication competent, suggesting that they might be reactivated in vivo upon different conditions than those used ex vivo during qVOA. Also, the induction of intact proviruses was proposed to be stochastic, dependent on the levels of Tat, but independent of the cellular activation status (Ho et al. 2013; Weinberger et al. 2005; Weinberger et al. 2008).

An analog of qVOA, mouse virus outgrowth assay (MVOA), allows to determine virus outgrowth in humanized mice. It has been recently described to detect latently infected cells with higher sensitivity than the standard qVOA as a large number of cells can be used and a GvHD promotes HIV-1 reactivation. Nevertheless, it provides only qualitative results (Metcalf Pate et al. 2015).

At the other side is a PCR-based determination of the cell-associated DNA, which apparently overestimates the size of the latent pool, as most of the integrated proviruses are mutated or incapable of reactivation for unknown reasons (Ho et al. 2013; Sanchez et al. 1997). Depending on the primer/probe sequences, total, integrated, or 2-long terminal repeat (2-LTR) circular DNA can be commonly detected (Murray et al. 2014; Pasternak et al. 2013). Finally, determination of cell-associated RNA (caRNA) using various combinations of PCR-based techniques like seminested RT-qPCR or ddPCR (Bullen et al. 2014; Kiselinova et al. 2014) seems to provide a more relevant marker of viral persistence and/or reactivation. However, it is important to use approaches distinguishing between commonly present prematurely terminated short gag transcripts, multiply spliced transcripts (msRNAs), and unspliced RNA (usRNA). Levels of usRNA are generally higher and therefore better detectable than msRNA. On the other hand, determination of msRNA (e.g., tat/rev; Pasternak et al. 2008) or correctly terminated viral transcripts (using primers/probe detecting the polyadenylated tail; Shan et al. 2013) better correlates with the ability of the cell to produce infectious viruses (Bullen et al. 2014; Pasternak et al. 2013).

Based on the PCR detection of tat/rev spliced transcripts, a new quantitative assay allowing single-cell based determination of the inducible viral reservoir called TILDA (Tat/rev-induced limiting dilution assay) was recently presented. The advantage of this assay is use of only 10 ml of blood, serial limiting dilutions allowing detection of even single positive cell, and a good correlation with qVOA (Procopio et al. 2015).

A disadvantage of all PCR-based assays is their inability to differentiate between RNA that might remain retained in the nucleus and for this or other reasons not to be translated into a protein and RNA giving rise to HIV proteins that could be presented on the cell surface or constitute new virions (Lassen et al. 2006). Therefore, determination of cell-free RNA (cfRNA) or p24 Ag in culture supernatant better estimates virion production. On the other hand, proportion of released virions and detectable cfRNA is much lower in comparison with caRNA (1.5 and 7 %, respectively; Cillo et al. 2014).

This problem can be partially solved also by a new assay termed Prime Flow RNA that combines cell-based detection of proteins with antibodies and detection of intracellular RNA with specific probes. It was reported to detect one infected cell in 10^4–10^5 cells (comparable numbers are found in peripheral blood of patients on cART) (Romerio and Zapata 2015).

Apparently, highly sensitive assays for determination of HIV proteins in culture supernatant are necessary for a better assessment of the efficiency of LRA. One such fully automated assay based on a Quanterix Simoa technology could detect low levels of p24 Ag in cell lysates (3 pg/ml; Howell et al. 2015). Another approach might employ TaqMan chemistry-based protein assays (https://www.thermofisher.com/cz/en/home/life-science/pcr/real-time-pcr/real-time-pcr-applications/real-time-pcr-protein-analysis/protein-expression-taqman-assays.html#workflow).

In summary, assays based on virus outgrowth are very laborious and generally underestimate the size of the latent pool as their efficiency can be increased by repeated cycles of induction of reactivation. On the other hand, technically easier PCR-based detection of HIV-1 prDNA, or caRNA, overestimates the size of the latent reservoir, as most of viral DNA is mutated or not transcribed for unknown reasons, while not all RNA transcripts yield functional viral proteins and/or virions. Detection of virion RNA, cfRNA, or viral proteins in culture supernatant is more accurate but less sensitive due to lower levels of these products. Highly sensitive assays for determination of viral proteins are therefore needed.

Mechanisms of establishment and maintenance of the latent reservoir

There are two types of HIV latency. A pre-integration latency that occurs after infection of non-permissive cells, which is short-lived as unintegrated viral DNA is recognized by cytoplasmic DNA sensors like cGAS or IFI-16 leading to activation of interferon and inflammasome responses (Gao et al. 2013; Thompson et al. 2014; Yoh et al. 2015). On the other hand, the post-integration latency occurring in cells that

become quiescent after the infection is the cause of virus persistence. The latent reservoir is established very early during the HIV-1 infection and its size can be limited by an early introduction of cART. It consists of both T cells and myeloid cells (Marban et al. 2007; Wires et al. 2012), and it is very stable with half-life determined by qVOA of about 44 months (Crooks et al. 2015; Siliciano et al. 2003).

A key determinant of the future fate of the HIV-1 provirus is the site of its integration. Most commonly, sites of HIV-1 insertion are found in intragenic regions of actively transcribed genes (Lewinski et al. 2006; Serrao and Engelman 2016). Perhaps for this reason, the majority of repressed but inducible proviruses are also located within the introns of the expressed genes (Lewinski et al. 2005; Shan et al. 2011). The establishment of HIV latency is thus regulated independently of the control of expression of the target host genes (Mbonye and Karn 2014).

Insertion of the HIV-1 provirus in the actively transcribed genes may result in transcriptional interference, contributing to the regulation of HIV-1 latency. Divergent orientation of the cellular promoter and viral LTR can lead to the lack of recruitment of transcription factors, while convergent promoters may lead to a collision of the transcription machinery and premature termination of HIV-1 transcription. Parallel orientation of the HIV LTR located downstream of the cellular promoter can lead to the viral promoter occlusion by a read-through transcription from the cellular gene, displacing key transcription factors on the HIV LTR (Han et al. 2008; Lenasi et al. 2011). In latently infected cells, a preference for a parallel orientation of the promoters was observed, while there was no preference in acutely or persistently infected cells, suggesting that transcriptional interference may be one of the important factors in the establishment and maintenance of HIV-1 latency (Shan et al. 2011).

HIV-1 itself does not encode any specific transcription repressors, but high levels of HIV-1 transcription activator Tat and its interactions with a cellular cofactor p-TEFb, resolving promoter-proximal pausing of RNA polymerase II (RNAP II) are absolutely critical for the provirus expression (Fig. 1; Yamada et al. 2006). In the absence of Tat-pTEFb complex, transcription efficiency decreases drastically and only short Gag transcripts are generated (Kao et al. 1987; Lassen et al. 2004; Price 2000). Transcriptional silencing of the proviruses thus results from a series of epigenetic and non-epigenetic changes occurring at the promoter region and from processes during the transcription initiation and elongation phases that decrease levels of Tat and availability and/or binding of cellular factors (Mbonye and Karn 2014).

In the activated CD4+ T cells, which are productively infected most often, the intracellular milieu with high levels of cellular transcription factors, namely transcription initiation factors NF-κB, nuclear factor of activated T cells (NFAT), and AP-1, drives HIV expression (Mbonye and Karn 2014). However, as the host cell returns to the resting memory phenotype, cytoplasmic sequestration of these factors causes a significant decrease of transcription initiation at the HIV LTR and allows transcriptional silencing of the provirus, possibly with help of transcriptional inhibitors (Bodor 2006; He and Margolis 2002; Tyagi and Karn 2007).

The chromatin structure of the proviral 5′ LTR is a critical parameter in control of HIV expression. Regardless of the site of insertion, 5′ HIV LTR is occupied by nucleosomes Nuc-0 and Nuc-1 in specific positions at the start site, imposing a block on RNAP II initiation (Verdin et al. 1993). Several negative DNA-binding factors (e.g., CBF1, YY1, LSF, BRD2, p50 homodimer; He and Margolis 2002; Karn 2013; Tyagi and Karn 2007; Williams et al. 2006) then facilitate recruiting of other repressor complexes and histone- and DNA-modifying enzymes at both core promoter and enhancer regions. Histones of the nucleosomes at the 5′ LTR of silent proviruses are deacetylated and trimethylated, which are features of the repressive heterochromatin. Specifically, a trimethyl mark on histone H3 lysine 27 (H3K27me3), generated through the action of the polycomb repressive complex PRC2, is a mark of the facultative heterochromatin responsible for reversible silencing of various inducible genes and contributes to viral quiescence, including HIV-1. On the other hand, H3K9me2/3 is linked with the formation of the constitutive heterochromatin during development as well as with the establishment of HIV-1 latency (Friedman et al. 2011; Maricato et al. 2015; Matsuda et al. 2015). Further, histone methyltransferases (HMTs) can be found associated with the latent proviral LTR (du Chene et al. 2007; Friedman et al. 2011; Imai et al. 2010; Keedy et al. 2009; Lusic et al. 2013). Namely, a dominant HMT EZH2 constitutes part of the PRC2 complex which serves as a binding platform for additional chromatin-modifying enzymes, histone deacetylases (HDACs), and DNA methyltransferases (DNMTs)(Cheng et al. 2011; Friedman et al. 2011; Tae et al. 2011; Vire et al. 2006). Methylation of DNA (CpG islands; Bednarik et al. 1990) at transcription start site has been suggested to be the most stable modification of the latent provirus LTR. It might stabilize DNA and prevent provirus reactivation (Blazkova et al. 2009; Kauder et al. 2009). Lately, relative frequency of proviruses with a higher LTR DNA methylation was suggested to be increased by a prolonged ARV treatment or multiple rounds of reactivation (Trejbalova et al. 2016).

An additional block in HIV expression may consist in post-transcriptional processes inhibiting HIV-1 protein expression. Namely, both unspliced and spliced HIV-1 transcripts may be retained in nuclei. The export of HIV-1 transcripts is supported by binding of Rev to Rev response element (RRE) present in partially spliced and unspliced genomic HIV-1 RNAs and by the interactions with exportin 1 (Crm-1), a nuclear export factor. Further association with polypyrimidine tract-binding protein (PTB) and related factors seems to affect the export efficiency. Thus, unavailability of either factor may promote the

retention of HIV-1 RNAs in the nucleus (Kula et al. 2013; Lassen et al. 2004; Lassen et al. 2006; Zolotukhin et al. 2003).

Further, several cellular miRNAs have been reported to modulate HIV-1 expression by targeting essential cellular cofactors involved in HIV-1 transcription like PCAF and cyclin T1. The former is targeted by miR175p and miR-20a while the latter by miR27b and miR198 (Sung and Rice 2009; Triboulet et al. 2007). There are also several cellular miRNAs (e.g., miR-28, miR-125b, miR-150, miR-223, and miR-382) that recognize the 3′-end of HIV-1 mRNAs, repressing their expression in resting CD4+ T cells (Huang et al. 2007). Finally, there are miRNAs derived from viral sequences (vmiRNAs), namely from TAR and Nef (miRTAR5p/3p and miRN367, respectively; Bennasser et al. 2004; Klase et al. 2007; Omoto et al. 2004; Schopman et al. 2012). Both cellular and viral miRNAs may cause HIV-1 RNA degradation or inefficient expression of HIV-1 proteins.

Mechanisms of reactivation

The reactivation of the functional, inducible latent provirus depends on the chromatin status and availability of the cellular transcription factors (Fig. 1). Upon appropriate stimulation and nuclear translocation, NF-κB and NFAT can bind to HIV LTR if the chromatin landscape of the promoter region is favorable (Bhatt and Ghosh 2014; Lusic et al. 2013; Ott and Verdin 2013). The accessibility of the HIV LTR can be affected by methylation of CpG islands (Bednarik et al. 1990) or binding of other transcription factors like Sp1 that are able to promote the chromatin configuration favorable for binding of the main transcription factors. In fact, Sp1 is required for the formation of the pre-initiation complex and interacts with NF-κB (Perkins et al. 1993). NF-κB and NFAT probably bind in a mutually exclusive, possibly sequential way, as their HIV-1 LTR-binding sites overlap (Giffin et al. 2003; Mbonye and Karn 2014). NF-κB is found in cell lines and primary naïve T cells, while NFAT is typically present in memory T cells (Dienz et al. 2007). In primary memory T cells, NFAT and Lck are required for optimal latent virus reactivation and HIV-1 can be activated in an NF-kB-independent way by transcription factor DVII-Ets-1, without causing significant T cell activation (Bosque et al. 2011; Bosque and Planelles 2009; Yang et al. 2009a). On the other hand, NFAT is dispensable in Jurkat cell models. NF-κB is commonly activated by PMA or TNF-α, while NFAT is activated by calcium/calcineurin signaling (Bosque and Planelles 2009; Chan et al. 2013; Kim et al. 2011).

Both NF-κB and NFAT recruit CBP/p300 and other histone acetyltransferases (HATs) (Garcia-Rodriguez and Rao 1998) to further acetylate Nuc-1 and to attract SWI/SNF chromatin remodeling complex. After the minimal initiation and transcription through the TAR element, RNAP II pauses. If Tat and p-TEFb, composed of CDK9 and cyclin T1 (Herrmann and Rice 1995; Wei et al. 1998) are available to bind and form a complex with TAR RNA hairpin and the transcription machinery, kinase activity of p-TEFb mediates phosphorylation of negative elongation factors (DSIF and NELF) and of RNAP II and allows formation of a superelongation complex and continuation of transcription further into the elongation phase (Fig. 1). Thus, Tat transactivation and its interaction with p-TEFb is absolutely necessary for the efficient transcription elongation of the HIV provirus. When not complexed with Tat, pTEFb is sequestered and held inactive in the transcriptionally inactive 7SK RNP complex containing 7SK small nuclear RNA (7SK snRNA), inhibitory factor HEXIM1 and RNA binding proteins LARP7 and MePCE. Yet, Tat may be out-competed by BRD4 in binding to p-TEFb, leading to p-TEFb targeting to transcription of cellular genes (Mbonye and Karn 2014).

Besides this conventional view of events underlying the HIV-1 reactivation, it has been suggested that the decision between HIV-1 replication and latency can be determined by a stochastic noise in gene expression similarly as in other cell-fate decisions. In this view, the probability of HIV-1 reactivation is increased by a higher rate and variation of fluctuations in HIV-1 gene expression and further stimulated by Tat-mediated positive feedback mechanism (Dar et al. 2014; Ho et al. 2013; Weinberger 2015; Weinberger et al. 2005; Weinberger et al. 2008).

Latency reversal and purging HIV-1

Latently infected cells represent the major barrier to HIV-1 cure (Donahue and Wainberg 2013). The major reservoir is considered to reside in resting memory T cells, and therefore, this population is in the focus of most efforts to decrease the size of the latent reservoir (Bruner et al. 2015).

The initial attempts to eradicate HIV-1 latently infected cells were first described by Fauci et al. (Chun et al. 1998; Chun and Fauci 1999). Later, the term shock and kill strategy was introduced (Archin et al. 2012; Deeks 2012). In short, it consists in the attempts to reactivate a dormant provirus silently present in latently infected cells, namely long-lived memory CD4 T cells, that would lead to death of the cells harboring the latent provirus and decrease the size of the latent pool in the presence of cART. Originally, it was assumed that virus reactivation could be achieved with a single agent, namely HDAC inhibitors (HDACi) or PKC inducers, and that the replication of the reactivated virus and the virus-induced cytopathic effects would be sufficient to kill the host cells and thus decrease the size of the latent reservoir. Today, it is largely accepted that combinations of two or more agents with different mechanism of action together with an additional stimulation of anti-HIV immune responses would be necessary. Namely, improvement

of cytotoxic responses using engineered dual-affinity (DART) and broadly specific antibodies (bNAbs) toward HIV-1 are intensively investigated (Caskey et al. 2015; Sloan et al. 2015). Still, there are doubts if the HIV cure can be ever achieved because of unknown fraction of the cells remaining refractory to the reactivation strategies. Further, it has been suggested that a prolonged antiretroviral treatment together with random or intentional cycles of reactivation might lead to an increased methylation of HIV-LTR and thus stabilization of the latent provirus (Trejbalova et al. 2016). Therefore, another logical approach would be to inhibit the spontaneous reactivation of latent HIV-1 (Mousseau et al. 2015).

Activity of LRA is usually identified in vitro in tissue culture screens involving various cell lines and primary cells. The efficiency of the selected compounds must then be verified in vivo. Most intensively investigated LRA, namely those in clinical trials, involve compounds in use or under development for treatment of various cancers and other diseases. These are especially HDACi and PKC agonists (NF-κB inducers). There are 11 HDACs subdivided in four classes (Mottamal et al. 2015; Wang et al. 2009). Particularly, HDACs 1–3 (class I) seem to be important in maintaining HIV latency (Keedy et al. 2009). HDACi non-specifically activate transcription of many genes by increasing acetylation of the promoter regions, including HIV LTR. Increased acetylation should modify chromatin status and allow for binding of transcription factors, namely NF-κB and NFAT. Among these, vorinostat (SAHA) has been the most extensively explored, but newer compounds with higher potency like givinostat, panobinostat, or romidepsin seem more promising (Banga et al. 2016). The ability of individual HDACi alone to induce HIV-1 reactivation ex vivo, in cells isolated from HIV-1-infected patients on cART, apparently depends on specific experimental conditions as well as on patients' history, as not all attempts ex vivo were successful (Blazkova et al. 2012; Bullen et al. 2014; Rasmussen et al. 2013). However, lately, vorinostat, panobinostat, romidepsin, and namely givinostat were found effective in a modified VOA employing a prolonged or repeated treatment with individual HDACi (Banga et al. 2016), repeating the results of clinical studies, in which vorinostat and some other HDACi were able to increase levels of caRNA (Archin et al. 2014a; Rasmussen et al. 2014; Sogaard et al. 2015). Nevertheless in these studies, the size of the latent reservoir was not found decreased in vivo, suggesting that combination with other agents or strategies increasing immunological killing of the infected cells would be necessary (Rasmussen et al. 2013). Other classes of chromatin-modifying enzyme inhibitors include inhibitors of histone methyltransferases (HMTi) like chaetocin, BIX-01294 or GSK343, or inhibitors of DNA methyltransferases (DNMTi) like 5-aza-2′deoxycytidine. However, they are more likely to be effective in combination with other LRA (Kumar et al. 2015).

The other important classes of LRA represent PKC agonists that induce activation and nuclear translocation NF-κB and p-TEFb. They can also trigger activation of MAPK and nuclear translocation of AP1. The natural PKC inducer effective in HIV-1 reactivation is TNF-α, a cytokine found increased in untreated HIV-1 infection. However, due to its pro-inflammatory pleiotropic effects, its use as a LRA in vivo cannot be considered. Similarly, the other very effective agent of this class, phorbol myristate acetate (PMA), cannot be considered for therapy as it reveals a strong tumorigenic potential (Kumar et al. 2015). However, newer PKC inducers include non-tumorigenic phorbol ester prostratin (Biancotto et al. 2004; Kulkosky et al. 2001), macrolide lactone bryostatin-1 (Mehla et al. 2010), or diterpenoids ingenol B and ingenol-3-angelate (Jiang et al. 2014; Jiang et al. 2015). PKC inducers downregulate expression of cell surface receptors CD4, CXCR4, or CCR5 in uninfected cells, thus limiting the spread of the newly released virus (Hezareh et al. 2004; Jiang et al. 2014; Mehla et al. 2010). Further, PKC was reported to phosphorylate HEXIM1, suggesting that PKC inducers might affect also this inhibitory protein (Fujinaga et al. 2012). Hexim phosphorylation is commonly mediated by AKT kinase that can be stimulated by HMBA (Contreras et al. 2007), leading to the release of p-TEFb from the inhibitory complex 7SK RNP. Yet another compound affecting availability of p-TEFb is JQ1, bromodomain inhibitor affecting factors BRD2 and BRD4 (Boehm et al. 2013).

Disulfiram, the inhibitor of acetaldehyde dehydrogenase used in therapy of alcohol abuse, induces degradation of PTEN, again allowing AKT-mediated phosphorylation of HEXIM1 (Doyon et al. 2013; Xing et al. 2011). This compound has been described as an effective LRA in vitro in a relatively artificial model of primary CD4+ T cells immortalized with the Bcl-2 protooncogene. However, the expectations of its potency were not fulfilled as there was no significant effect on the size of the latent reservoir found in vivo (Spivak et al. 2014).

As mentioned above, combinations of several LRA are likely to act more efficiently and cause fewer negative side effects. The examples of such combinations are chromatin-remodeling compounds, namely HDACi, together with different inducers of transcription. Chromatin remodeling compounds can be apparently considered as noise enhancers that are relatively ineffective in HIV-1 reactivation alone, while they reveal a synergism with real transcriptional activators that are able to increase HIV-1 expression alone (e.g., PKC inducers) (Dar et al. 2014; Kumar et al. 2015).

Apart from the mainstream studies, there are many other approaches toward latency reversal and HIV cure. Of these, use of a pro-oxidant gold-based drug Auranofin is of interest as it was shown to induce a partially selective killing of TCM and TTM CD4+ T cells, the main reservoir cells containing the latent HIV-1 (Lewis et al. 2011). Consequently, it was demonstrated that TCM and TTM CD4+ T lymphocytes are

more susceptible to the redox stress and apoptosis (Chirullo et al. 2013).

Our laboratory has demonstrated independently that Normosang, a hemin containing compound used to treat acute hepatic porphyria, could strongly potentiate reactivation of the latent provirus induced by PKC inducers like PMA, TNF-α, prostratin or bryostatin-1, while it inhibits HIV-1 replication during the acute infection through its effect on reverse transcription. The stimulatory effects of Normosang are mediated by a heme/iron-mediated Fenton reaction resulting in the increased redox stress, while there was no effect on the activation status of the cells (Melkova et al. 2015a; Melkova et al. 2015b; Shankaran et al. 2011). Based on our results, we propose a model in which redox-modulating agents induce chromatin remodeling, affect binding of specific transcription factors to HIV-LTR, and potentiate HIV-1 expression induced by PKC or other inducers (Melkova et al. 2015a; Melkova et al. 2015b).

Historically, there is a case of one HIV+ patient that was administered Normosang and consequently remained p24-negative for several months (Pavel Martasek, Faculty General Hospital Prague, personal communication). We suggest that the outcome in this particular patient resulted from the inhibition of reverse transcription by heme (Levere et al. 1991) together with a short-term reactivation and death of the infected cells due to heme/iron-mediated redox stress. Consequently, a stable heme degradation product, antioxidant bilirubin, might have mediated prolonged inhibitory effects on HIV-1 reactivation (Melkova et al. 2015a; Melkova et al. 2015b). This scenario would be compatible with the hypothesis of Chirullo et al. (2013) that auranofin decreases numbers of latently infected TCM and TTM CD4+ T cells by its pro-oxidant effects and thus reduces size of the HIV latent pool.

The idea of possible involvement and use of redox stress to purge the latent reservoir is further supported by work of (Iordanskiy et al. 2015) showing that ionizing radiation alone was sufficient to activate the HIV-1 LTR and to effectively kill the infected T cells. Consequently, this group proposed that a low-dose ionizing radiation could be used therapeutically to reactivate and kill latently infected reservoir cells (Iordanskiy and Kashanchi 2016). Additionally, in the Berlin patient, the only known case of HIV-1 cure, high-dose irradiation with bone marrow transplantation from a donor harboring CCR5Δ32 mutation with missing CCR5 expression was used, while irradiation was omitted in Boston patients in which virus rebound occurred (Henrich et al. 2013; Hutter et al. 2015).

Finally, any common infection involves increased generation of reactive oxygen and nitrogen species. Co-infections are well-known to increase HIV-1 replication and promote its spread (Modjarrad and Vermund 2010), while decreased levels of GSH, indicator of an increased redox stress, have been described early in HIV infection (Pace and Leaf 1995). Reactive oxygen and nitrogen species were shown to stimulate activation of the redox-sensitive transcription factor NF-κB and LTR-driven expression of reporter genes (Jimenez et al. 2001; Melkova et al. 2000; Pyo et al. 2008). Thus, increased generation of free radicals is likely to be helpful in the attempts to eliminate the latent HIV-1 in the presence of cART.

A major concern when considering testing of LRA in vivo is the induction of immune hyperstimulation upon reactivation of the latent HIV-1 and development of a harmful cytokine storm. On the other hand, it is not clear if any significant latency reversal is achievable without increased cytokine levels (Marsden et al. 2015). However, specific anti-HIV immune responses are generally hampered or dysfunctional. Further, it was shown that both HDACi (romidepsin, panobinostat) and PKC agonists (prostratin and bryostatin-1, but not ingenol B) inhibited HIV-specific T cell proliferation (Clutton et al. 2015).

Based on clinical studies, it has been widely accepted that stimulation or improved function of immune responses in addition to LRA would be necessary in order to achieve death of reactivated cells and thus decrease the size of the latent reservoir. Synergistic effects of different agents should also decrease the doses and/or negative side effects of individual compounds.

Summary/conclusions

In conclusion, in view of the currently described sources of the remaining low-level HIV-1 replication in the presence of suppressive cART, additional mechanisms and processes promoting HIV-1 persistence remain to be identified. Any attempt to achieve HIV-1 cure by latency reversal should involve combination of several LRA with different mechanism of action together with stimulation or improvement of immune responses toward infected cells. To validate the efficiency of individual approaches, development of sufficiently sensitive and specific methods allowing accurate determination of the size of the latent reservoir and its changes is necessary.

Acknowledgments We are grateful to Dr. Ivan Hirsch for helpful discussions and comments. The work of P.S. and M.M. was performed in partial fulfillment of the requirements for Ph.D. degree at the First Medical Faculty, Charles University. The work was supported by the Grant Agency of the Ministry of Health of the Czech Republic – project No. NT/14135-3.

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