Research update

On the way to find a cure: Purging latent HIV-1 reservoirs

Christian Schwartz^{a,b,*}, Sophie Bouchat^{c,1}, Céline Marban^e, Virginie Gautier^{d,1}, Carine Van Lint^{c,1}, Olivier Rohr^{a,b,1}, Valentin Le Douce^{d,1}

^a University of Strasbourg, EA7292, DHPI, Institute of Parasitology and Tropical Pathology, Strasbourg, France

^b University of Strasbourg, IUT Louis Pasteur, Schiltigheim, France

^c Université Libre de Bruxelles (ULB), Service of Molecular Virology, Institute for Molecular Biology and Medicine (IBMM), 12 rue des Profs Jeener et Brachet, 6041 Gosselies, Belgium

^d UCD, Centre for Research in Infectious Diseases (CRID), School of Medicine University College Dublin, Belfield, Dublin 4, Ireland

^e University of Strasbourg, Inserm UMR 1121 Faculté de Chirurgie Dentaire Pavillon Leriche 1, place de l'Hôpital Strasbourg, France

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ABSTRACT

Introduction of cART in 1996 has drastically increased the life expectancy of people living with HIV-1. However, this treatment has not allowed cure as cessation of cART is associated with a rapid viral rebound. The main barrier to the eradication of the virus is related to the persistence of latent HIV reservoirs. Evidence is now accumulating that purging the HIV-1 reservoir might lead to a cure or a remission. The most studied strategy is the so called “shock and kill” therapy. This strategy is based on reactivation of dormant viruses from the latently-infected reservoirs (the shock) followed by the eradication of the reservoirs (the kill). This review focuses mainly on the recent advances made in the “shock and kill” therapy. We believe that a cure or a remission will come from combinatorial approaches i.e. combination of drugs to reactivate the dormant virus from all the reservoirs including the one located in sanctuaries, and combination of strategies boosting the immune system. Alternative strategies based on cell and gene therapy or based in inducing deep latency, which are evoked in this review reinforce the idea that at least a remission is attainable.

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* Corresponding author at: University of Strasbourg, EA7292, DHPI, Institute of Parasitology and Tropical Pathology, Strasbourg, France.

E-mail address: schwartz.christian@unistra.fr (C. Schwartz).

¹ Members of the EU4HIVCURE Consortium (H2020-MSC-RISE-2015).

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1. Introduction

Since its discovery in 1983, HIV-1 has killed about 38 million people [1]. Today there are still more than 37 million infected people worldwide, the majority in developing countries. The introduction of the combination antiretroviral therapy (cART) in 1996 [2,3] which increased drastically life expectancy [4] was a key event in the management of the epidemic. However the treatment does not lead to a complete cure as cessation of the therapy results in rapid viral rebound [5]. For this reason, a lifelong adherence to cART is required, which is associated with drug toxicities and the development of drug resistance [6–8]. The increase of disease events and mortality unrelated to AIDS has been noticed in patients under cART and in some HIV non-progressors [9–11]. Indeed, well described co-morbidities such as cardiovascular [12] and renal diseases [13] led to a decrease of life expectancy. It is believed that these events are in relation with chronic inflammation due to the persistence of HIV-1 reservoirs [14]. Of note, some protective effects of cART have been described in cases of cardiovascular and renal-diseases in a cohort of newly diagnosed patients [15,16]. However, the persistence of latently-infected reservoirs is a serious obstacle to HIV eradication. Latently-infected reservoirs are defined as cells that comprise the integrated HIV-1 genome and are transcriptionally silent but competent in terms of replication [17]. This capacity of replication of the integrated virus to produce infectious viruses upon activation explains the viral rebound after cART cessation. The main cellular reservoirs of HIV-1 are resting CD4+ T cells including central memory T cells, transitional T cells, effector memory T cells, effector T cells and naïve T cells [18]. There are some other types of reservoirs [19], e.g. peripheral blood monocytes, dendritic cells, macrophages including the microglial cells which are the Central Nervous System (CNS) resident macrophages, the astrocytes and the hematopoietic stem cells. Several of these viral reservoirs are located in immune privileged sanctuaries with a poor drug penetration index and thus opposing to HIV cure [18]. A number of these sanctuaries are now well documented e.g. male genital tract [20,21], fat tissue [22,23], lymph nodes [24,25] and the CNS [26,27]. One of today's main goals is to eliminate the virus (referred as a cure) from the body or to reduce the pool of HIV-1 reservoirs leading to a long-term control of HIV in the absence of cART and disease progression (referred as a remission). This is a top research priority of the action of the international AIDS association "Towards an HIV Cure" [28]. It is believed that reduction of the HIV-1 latent pool would mimic what happens naturally in Elite Controllers, who are capable to control HIV-1 without any treatment.

In this review, we first discuss how the reduction of the pool of reservoirs is a *sine qua non* condition to achieve a cure or more likely a remission. Next, we outline the technical aspects of the purge of reservoirs. Recently developed cell and animal models appear to be well suited for the evaluation of new treatments. Finally, we focus on the most studied strategy: the "shock and kill" strategy. Other alternative strategies e.g. cell and gene therapy, are briefly discussed here. Cell therapy is based on stem cell transplantation (SCT) of cells homozygous for the mutated form of the co-

receptor CCR5 (delta 32 CCR5). As discussed later, SCT has allowed the cure of only one person in the world of HIV known as the Berlin Patient. However, many questions are still unresolved which might explain the lack of sterilizing cure in many clinical trials using SCT. For example, it is not clear whether or not the depletion of myeloid cells is a prerequisite for the elimination of the viral reservoir. Although it is important to eliminate the CD4+ reservoir other cell types harboring HIV should also be targeted. Moreover, there is a great demand for strategies, that facilitate drug penetration into reservoirs located in sanctuaries such as the brain and the lymph nodes [29]. Gene therapy is based on gene editing with the recent advance of the CRISPR/Cas9 system being the most attractive technology to target both viral genes and cellular genes such as the CCR5 co-receptor. This system uses a guide RNA coupled to a Cas9 nuclease, which targets specifically the provirus and mediates the excision of the integrated viral genome [30]. This promising approach is still in early development and will need further improvement, e.g. targeting all the potential reservoirs located in the peripheral blood or in sanctuaries. Another concern is related to the ability of the virus to subvert the DNA repair machinery making it resistant to CRISPR/Cas9 gene editing [31]. Readers are referred to some excellent reviews to get more information on these strategies [32–37].

2. Reducing the reservoir size: a way to get a cure or a remission

Latent reservoirs are established at an early stage of acute infections and limit the efficiency of cART even if it is introduced at the onset of the HIV-1 infection. Mounting evidence suggests that exhaustion of the latent viral reservoirs leads to either a complete cure (sterilizing cure) or a remission (functional cure). The first line of evidence of the importance for depleting latent reservoirs comes from the observation that some patients, i.e. the Elite Controllers, who control HIV-1 without cART, have a very low latent reservoir size. The second line of evidence comes from the observation that estimated 5–15% of patients, who benefited cART very early after infection, are able to control the disease. These patients are known as the Post-Treatment Controllers. Importantly, they also show a small latent reservoir size. Another case study argues also in favor of the importance of the reservoir purge. In this unique case, known as The Berlin patient study, a patient was completely cured from HIV-1.

2.1. The Elite Controllers

The importance of reducing the pool of reservoirs to achieve a remission is highlighted by the existence of a small population of infected people, the Elite Controllers. These patients (less than 1%) show a rare phenotype that enables them to control naturally the virus to below detection level for years [38,39]. The underlying mechanisms have been extensively studied [40,41]. Of great importance a low latent reservoir size is a common feature in Elite Controllers [42–44]. It is suggested to further reduce the pool of reservoirs since Elite Controllers have often higher levels of viremia compared to patients on cART [42,45]. It seems that activation of

the immune system and the consequent chronic inflammation helps Elite Controllers to contain their viremia but also to develop non-AIDS events such as cardiovascular diseases [46,47]. It was suggested that further decreasing the reservoir size in Elite Controllers by cART might reduce the occurrence of non-AIDS related diseases [48]. Whether or not the elite control of HIV [45] is the right model for a functional cure the observation that a lower reservoir size results in a better control of HIV infection is in favor for the development of strategies targeting latent reservoirs.

2.2. Post-Treatment Controllers

A Post-Treatment Control is defined as a control of the plasma viremia following cessation of cART. Post-Treatment Controllers (PTC) have been reported in several cohorts [49]. In the VISCONTI cohort, PTC were diagnosed with acute HIV-1 infection and who benefited cART very early after the infection (within 3–6 months). They have been maintained on cART for several years (at least 2 years) but for various reasons, at one moment they stopped therapy. Interestingly, an estimated 5–15% of these people were able to control their plasma viremia similarly as Elite Controllers [50–52]. However, the PTC and the Elite Controllers are distinct populations with different genotypes and phenotypes [49]. For example, the genomes of Elite Controllers are enriched in class I HLA type alleles (e.g. HLA B27 and HLA B57), which play a critical role in the immune control of HIV-1 infection. T cell response is also stronger in Elite Controllers and they show higher level of CD8+ T cell activation. Nevertheless, the PTC and the Elite Controllers share a common feature: they have low levels of circulating HIV-1 reservoirs [53]. Increasing evidence shows that initiating cART during acute HIV-1 infection results in smaller reservoir size and a better control of infection. Studies of non-human primates (NHP) showed that the latent reservoirs form very soon after infection and that the reservoir size is reduced if cART is initiated within 3 days of infection [54]. In humans, cART initiated early after acute infection seems to reduce the reservoir size also and is now recommended following diagnosis [55,56]. Post-treatment control of the viral load below detection limit might effectively be a functional cure. A definitive conclusion requires monitoring the PTC for an additional time since we do not know whether a viral rebound will occur in the coming years or not. Mechanisms underlying post-treatment control are different from those working for Elite Controllers, but they are clearly related to a drastic reduction of the latent reservoir size with an early introduction of cART.

2.3. Allogeneic transplantation

The Berlin patient is the only case report to date that describes a sterilizing cure [57]. The patient received hematopoietic stem cell transplantations in multiple rounds from a donor who was homozygous for the CCR5-delta32 mutation conferring resistance to HIV infection [58,59]. HIV became undetectable and the CD4+ T cell counts returned to normal [57,60,61] following the allogeneic transplantation and cessation of cART. It was postulated that the combination of radiotherapy and chemotherapy eradicated long-lived reservoirs preventing HIV rebound during the immune reconstitution following stem cell transplantation. Two other patients (known as the Boston patients), heterozygous for CCR5-delta32, who received stem cell transplants to treat their lymphoma from donors with the wild-type CCR5 gene had a short remission phase in the absence of cART. However, a viral rebound occurred after a delay of several months [62]. This observation strengthens the idea that stem cell transplants may reduce the reservoir size and allow partial control of HIV. Since an estimated 97% of new infections arise from the CCR5-tropic virus [63] allogeneic transplantation of stem cells acquired from donors whose

CCR5 gene is mutated might be a viable solution to eliminate the HIV reservoir [35]. Since the Berlin patient, only one patient is known to have survived a similar therapy. Unfortunately in this patient a viral rebound occurred with a shift from a R5-tropic HIV before stem cell transplantation to a X4-tropic HIV after stem cell transplantation (SCT) [64]. Nevertheless, the case of the Berlin patient indicates that strategies targeting the CCR5 gene may eliminate or as in the case of the Boston patient, reduce the pool of latent reservoirs [65]. Recently, a European project was set up to monitor HIV-1 patients benefiting SCT due to life-threatening conditions (<http://www.icistem.org>). Preliminary results indicated that allogeneic SCT is systemically associated with a reduction of the HIV-1 reservoir size. However, stem cell transplantation cannot be practiced systematically due to the high mortality of this intervention (20–30% mortality) and therefore it is limited to HIV+ patients who have other associated malignancies [66]. Yet, the Berlin patient and observations from other studies suggest that it is important to eliminate the latent reservoirs. With the recent advent of new technologies in gene editing, these studies might pave the way to the development of new strategies leading to a cure [67].

Altogether, evidence is accumulating from patients who received allogeneic transplantation, from Elite Controllers and from PTC, that it is important to achieve a low reservoir size to defeat HIV. Theoretical considerations and modelling latently-infected cell activation help clinicians to predict the outcomes of treatments applied [68–72].

3. Cell and animal models to study HIV reservoirs

Many *in vitro* latency models use cell lines or primary cells but these models do not reflect the *in vivo* properties of the reservoirs found *in vivo* [73,74]. One of the major problems is that latency-reversal agents (LRA) do not produce the same effect in all models [75]. However, in one case, the drug phytohemagglutinin (PHA) although efficiently reactivates latent viruses from reservoirs in all cell models, it has serious side effects which prevents therapeutic use. Latency is a heterogeneous process involving different mechanisms in different models [75]. For example, memory CD4 + T cells are heterogeneous; they do not form a unique class of cells. The experimental procedure used to create the model system contributes also to differences in the reactivity of the latently infected cells. The cell models differ whether dividing or resting cells are infected (Table 1). The cell model depends on specific properties of the HIV-1 strain used for infection, as well. It is important that all T cell derived models can only reproduce the activities of the circulating fraction of latently-infected cells and not those, which are associated with tissues including gut associated lymphoid tissue (GALT), the lymph nodes or the central nervous system [26]. The integration sites of HIV-1 have also been shown to affect the response to LRAs [76]. Even if the models are imperfect, they help to better understand the specific aspects of the molecular mechanisms underlying the establishment and persistence of HIV latency. Moreover, *in vitro* models are very important to facilitate efficient drug screening and/or testing new drug designs. However, some LRAs do not reactivate all latently infected cells *ex vivo* despite positive results obtained in *in vitro* tests. The lack of complete reactivation might reflect the stochastic nature of the regulation of HIV transcription, which could be responsible for the inefficient provirus reactivation during a first round T-cell activation [77,78]. Current *in vitro* cell models do not reproduce all the processes involved in the establishment and the persistence of latency. Therefore new relevant *in vitro* latency models are needed that include the stochastic nature of HIV transcription [79]. Chen et al. have developed a new tool i.e. the barcoded viruses

Table 1
Cell and animal models to identify and characterize LRAs.

Type of cellular model	Cell population	Cell subsets	Homogeneous cell population	Activation/proliferation status	Derived from	HIV strain	Readout	Advantages	Disadvantages
Cell lines	T lymphoid Monocytes/macrophages Microglial cells Astrocytes	No	Yes No Yes Yes	Based on activating cells Immortalized	HIV-1 negative donor	HIV subtype B Depending on the cell line: Full-length or deleted or mutated virus	Depending on the cell lines: Luciferase, Reverse transcriptase, HIV mRNA, intracellular or extracellular p24, fluorescence.	Easy to use, one or two HIV-1 copies per cell	Transformed, each cell line has a single integration site, some have defective virus.
Primary cell models	Resting CD4 ⁺ T cell	Yes	Depending on the model	Depending on the model: Based on dividing or resting cells Supplemental activation during the establishment processus Proliferation between 3 weeks and 2 months	HIV-1 negative donor	HIV subtype B Depending on the model: Full-length or deleted NL4-3 virus	Depending on the model: Luciferase, Reverse transcriptase, Tat mRNA, intracellular or extracellular p24, GFP.	Several integration sites, non-transformed	Some models have defective virus, difficult to set up
<i>Ex vivo</i> patient cell cultures	CD8 ⁺ -depleted PBMCs CD4 ⁺ T cells MDM	No Yes No	No Depending on the selection Yes	Activated and/or resting cells Proliferation at least for 1–2 weeks	HIV-1 positive patient	Endogenous	Intracellular or extracellular RNA, p24, DNA	Easy to prepare; study on several integration sites; depending on selected patients, study on different HIV-1 subtypes; physiological culture environment with CD8 ⁺ -depleted PBMCs	Sometimes aberrant activation
Murine Models	PBMC-transplanted Mouse HSC-transplanted humanized mouse BLT (HSC, Liver, Thymus) humanized mouse	No Yes Yes	No	Fully activated by xenoreaction Physiological Physiological	Mouse transplanted with tissues from HIV-1 negative donor	HIV	Plasma viremia, reservoirs size, histology	Relatively easy to construct A set of CD4 ⁺ T cell subsets A set of CD4 ⁺ T cell subsets, acquired immunity	Cost, Aberrant activation short-term Cost, Difficult to construct, no acquired immunity Cost, Difficult to construct, shortage of human fetal tissues
Non-Human Primates	All cell populations that can be infected by SIV	Yes	No	Physiological	SIV negative animal	SIVmac	Plasma viremia, reservoirs size, histology	Study on SIV reservoir distribution, study in presence of cART, obtention of anatomical reservoir tissues (such as CNS)	Cost, study not directly on HIV

to monitor a polyclonal cellular model at a single cell level. They have notably found a variation in the reactivation capacity of drugs which depends on the integration site [80] (comments in [81]).

Preclinical studies allow clinicians to test the efficiency of drugs and to characterize their pharmacokinetic and pharmacodynamic properties. Since none of the models mimics fully HIV-1 latency *in vivo*, LRAs must be tested in several models before use in clinical studies. Non-human primates (NHP) and humanized mice are the best-suited animal models to study HIV-1 persistence and reactivation by LRAs [82] (Table 1). Rhesus macaques and pig tailed macaques under cART are also excellent *in vivo* models to study HIV persistence and to test the efficiency of LRAs used alone or in combination. HIV-1 infected pig tailed macaques under cART are used to study neurocognitive impairments (NCI). The central nervous system (CNS) of the macaques under cART harbors latent simian immunodeficiency virus (SIV) genomes. One out of three animals under cART when tested with a combination of LRAs (Ingenol B and Vorinostat) presented a detectable viral load in plasma and SIV RNA in the cerebral spinal fluid (CSF) indicating that the brain is a potential reservoir for the virus [83]. However, serious side effects of the treatment appeared; three SIV-infected macaques had brain inflammation [83]. Reviews [26,84] discuss in detail how these side effects can be controlled. Moreover, the high cost of animals and animal resources and the very long time needed for the experiments (up to two years) limit seriously the use of NHP. Overall, new original strategies should be set up to reactivate SIV and HIV-1 from reservoirs with the goal to eradicate or, more reasonably, to decrease the pool of reservoirs, including those in sanctuaries like the brain and the lymph nodes. Another interesting model is the humanized mouse model, which mirrors HIV-1 infection [85]. It was used successfully to study LRAs and to map the *in vivo* distribution of reservoirs [86]. A humanized mouse model for HIV-1 infection of the CNS has been recently developed [87]. However, this model has also its limitations. For example, the main HIV-1 reservoirs in the brain are microglial cells, which are not infected in the humanized mice. This is a serious limitation since microglial cells are thought to be the major source of latent HIV-1 in the brain. Animal sampling is another important limitation of the use of humanized mice. At the same time, animal models have allowed the evaluation of newly characterized LRAs and new strategies such as the use of LRAs in combination with immune enhancing drugs to reactivate and eliminate latently-infected cells. Indeed, animal models allow clinicians to better define the route of administration and a better timing of drug delivery, which is crucial for the elimination of latent reservoirs from sanctuaries with poor drug penetration such as lymph nodes and the brain.

4. The “Shock and Kill strategy”

The strategy known as “shock and kill” aims to purge or at least reduce the size of cellular reservoirs to achieve a cure or a remission. In this strategy, first HIV transcription is reactivated by small molecules (shock) and then to eliminate the virus (kill) an intensive cART and/or other interventions are applied to enhance the immune system [88]. However, the implementation of the strategy is difficult, mainly because of the poor understanding of the molecular mechanisms underlying the establishment and persistence of HIV-1 latency in reservoirs such as in resting CD4⁺ T-cells or in microglial cells of the brain [17]. Virus production in latently-infected cells is blocked essentially on the level of HIV transcription. It is known that HIV transcription is under epigenetic control of the HIV-1 promoter. Reactivation of the transcription is prevented by specific inhibitory mechanisms and/or by the sequestration of positive transcription factors. However, post transcriptional

events such as mRNA export, splicing and translation might also be important in latency and deserve more attention [28,61].

4.1. The shock

4.1.1. Molecular mechanisms of HIV-1 latency

DNA methylation and post-translational histone modifications are the main forms of the epigenetic control exerted on the HIV-1 promoter. DNA methylation has been involved in DNA silencing and latency [89]. The importance of DNA CpG methylation in maintaining HIV-1 latency is well described *in vitro* [90,91]. Two CpG islands surround the HIV-1 transcription start site and were reported to be hypermethylated in latently-infected model T-cell lines [90,91]. However, numerous studies *in vivo* using patient's cells have shown that the methylation profile of the HIV-1 5'LTR during latency is more heterogeneous [77,90,92–94] and depends on the clinical characteristics of the infection such as the duration of the infection or of the antiretroviral treatment [94].

The local status of chromatin influences greatly the level of transcription. Indeed, a heterochromatin environment, which is more compact and structured than euchromatin, is repressive for transcription. This compaction of chromatin and its capacity for transcription depends on post translational modifications of histones such as acetylation, methylation, sumoylation, phosphorylation and ubiquitination [95]. DNA transcription also depends on the recruitment of chromatin-modifying enzymes onto the HIV-1 promoter. CTIP2/Bcl11b is a key factor in inducing a heterochromatin environment in CD4⁺ T cells and microglial cells [96]. It was suggested that in CD4⁺ T cells' repression of HIV-1 transcription involves posttranslational modifications by CTIP2 that represses HIV-1 promoter activity by recruiting the NuRD complex [97]. In contrast, another study shows that Protein Kinase C-Mediated phosphorylation of CTIP2 at Serine 2 negatively regulates its interaction with the NuRD complex during CD4⁺ T-Cell activation [98]. Moreover, CTIP2 was associated with histone acetyltransferase (HAT) such as P300. In microglial cells, CTIP2 recruits a chromatin modifying complex on Sp1 sites of the proximal promoter [99] which binds the histone deacetylases HDAC1, HDAC2 and the histone methyltransferase SUV39H1. This complex allows the deposit of the H3K9me3 epigenetic mark. This histone modification promotes heterochromatin protein 1 (HP1) binding, heterochromatin formation and thus HIV silencing [99–101]. In addition, CTIP2 interacts physically and functionally with the lysine specific demethylase (LSD1) and inhibits HIV-1 transcription and viral expression in a synergistic manner [102]. LSD1 in turn allows the enrolment of hSet1 and WDR5, belonging to the hCOM-PASS complex, on the HIV-1 promoter [102]. Interestingly epigenetic regulation of HIV-1 by HDACs and SUV39H1 was also described in astrocytes [103] and CD4⁺ T cells [104].

Other factors enable the persistence of HIV-1 latency. For example, the poor level of expression and/or sequestration of positive transcription factors prevent HIV-1 reactivation. Indeed, several transcriptional activators such as NFAT and STAT5 are weakly expressed, others (e.g. NF-KB) are sequestered in the cytoplasm in latently-infected CD4⁺ T cells [105]. NF-KB and its co-activator PTEFb are sequestered in two compartments in resting cells thus preventing HIV-1 reactivation. In quiescent cells NF-KB forms a complex with the inhibitor IKB and is sequestered in the cytoplasm [106,107], while the elongation factor PTEFb is in an inactive multiprotein complex including 7SK snRNA, CTIP2, the cellular protein high mobility group AT-hook 1 (HMGA1) and HEXIM1, which is anchored to viral and cellular gene promoters in the nucleus [108]. Interestingly, CTIP2 significantly decreases CDK9 kinase activity in the inactive complex thus inhibiting PTEFb from functioning. This huge inactive complex is anchored on the HIV-1 and cellular target promoters by HMGA1 [109]. The results suggest

Fig. 1. HIV-1 LTR transcriptional locks. HIV-1 provirus transcriptional extinction and latency maintenance are under multi-layered host molecular regulatory pathways. Combinations of inhibitory processes at the promoter level and activator sequestrations have been described to orchestrate these events. Transcriptional inhibition is notably the results of chromatin remodelling through DNMTs-driven CpG island DNA methylation and epigenetic modification LTR-flanking nucleosome Nuc-0 and Nuc-1. Histone deacetylation at Nuc-0 and Nuc-1 occurs through HDAC1 recruitment by either NF-KB homodimer p50/p50 or CBF-1, while concomitant deacetylation and methylation of Nuc-1 lysine residues is the results of multi-enzymatic complexes such as the CTIP2/LSD1/HDAC1/2/SUV39h1 complex. Shortage in quantity and availability of powerful activators also prevent reactivation and participate in HIV-1 transcriptional repression. NF-KB heterodimer p50/p65 interacting with I κ B α and phosphorylated NFAT remain in the cytoplasm while the heterodimer Cdk9/Cyclin T1 (the elongation factor pTEFb) is trapped and enzymatically inactive in a small nuclear ribonucleoprotein complex, 7sk snRNA/CTIP2/Hexim1. These molecular locks can be lifted by antagonizing key enzymes responsible for post-translational modifications with HMT inhibitors, HDAC inhibitors or DNMT inhibitors or by triggering release of powerful activators, such as NF-KB, NFAT and/or pTEFb with respectively PKC agonists and inducers of P-TEFb release.

that protein complexes containing CTIP2 regulate viral and endogenous gene expression and favor HIV-1 persistence. Further investigations are needed to decipher the precise molecular mechanisms involved in these processes [26]. Overall, the studies point to the importance of CTIP2, which appears to be a scaffold protein anchoring several protein complexes of different functions. At least two different complexes containing CTIP2 are involved in the establishment and the persistence of HIV-1 latency (Fig. 1). Importantly, CTIP2 is also implicated in the control of cellular genes that regulate viral expression. Among these factors, the cellular cyclin-dependent kinase inhibitor CDKN1A/p21^{waf} favors HIV-1 gene transcription in cells of the monocyte-macrophage lineage [110]. The viral protein Vpr induces p21 expression, which in turn favors HIV-1 gene transcription. However, when present, CTIP2 displaces the HIV-1 Vpr from the promoter of p21 [111]. It was suggested that CTIP2 creates a cellular environment precluding viral reactivation and hence favoring HIV-1 latency. Other mechanisms not related to CTIP2 might also contribute to the transcriptional repression of HIV-1. A recent report found that HIC1 inhibits the late phase of Tat-dependent HIV1 transcription [112].

Understanding the in-depth mechanisms underlying HIV-1 latency in latently-infected cells is essential to develop new and innovative therapies for viral eradication. Several drugs targeting

some of these cellular factors involved in HIV-1 latency, called Latency Reversing Agents (LRAs), are currently under investigation.

4.1.2. The latency reversing agents

Early experiments used growth factors such as IL-2, IL-7 and IL-15 and T cell activators such as anti-CD3 antibodies to reactivate HIV-1 expression. However, such drugs alone did not affect the size of the HIV reservoir. The combination of drugs such as IL-2 and anti-CD3 antibodies which significantly reduced the HIV reservoir was too toxic [113,114]. In our laboratory, we tested drugs raised against molecular targets known to be implicated in HIV latency i.e. HDACs, Histone methyltransferase, NF-KB, the positive elongation complex pTEFb and the DNA methylation status. A wide range of drugs targeting these factors, approved for their use in other pathologies have also been tested. Two main classes of drugs are distinguished from these experiences.

The first class includes LRAs targeting cellular factors involved in the epigenetic control of HIV-1 (Fig. 2). This class comprises HDAC inhibitors (HDACi) such as Valproic acid, Vorinostat, panobinostat and romidepsin; Histone methyl transferases inhibitors (HMTi) such as chaetocin and BIX 01294; and DNA methylation inhibitors such as 5-AzaC. HDACi and HMTi have been shown to reactivate to some extent HIV-1 expression both *in vitro* [115–

Fig. 2. Classes of latency reversal agents. Compounds, which previously displayed a positive impact on HIV-1 latent proviruses reactivation, have been designated “latency reversing agents” (LRAs). LRAs are targeting different molecular locks implicated in HIV-1 latency establishment and maintenance: Histone deacetylase (A) and methyltransferase (B) inhibitors relieve epigenetic blocks at HIV-1 promoter, DNA methylation inhibitors (C) prevents provirus extinction, PKC activators (D) and inducers of P-TEFb release (E) increase the recruitment of transcriptional activators, such as NF-KB and P-TEFb, at the viral promoter.

118] and *ex vivo* **[119]** (Fig. 1). Several teams have also shown that 5-AzadC induces HIV-1 gene expression *in vitro* **[90,91]**. Interestingly, only 5-AzadC, but not its oxidized analog 5-AzaC, is able to reactivate HIV-1 **[118]**.

The second class of LRAs impacts positive cellular factors such as NF- κ B and pTEFb, which is the coactivator of NF- κ B [106] (Fig. 2). These drugs induce the expression of positive cellular factors and/or their release from the inactive complex [120,121]. Pros-

tratin, bryostatin and ingenol B by activating the PKC pathway release both NF- κ B and pTEFb from inactive complexes and increase pTEFb expression [122–124] which ultimately leads to HIV-1 reactivation [125]. Bromodomain inhibitors such as JQ1 and others are able to release pTEFb from the BRD4-pTEFb complex [125–127]. However, these drugs used alone are inefficient to fully reactivate HIV-1 expression *ex vivo* [128,129]. This might reflect the multi factorial mechanisms involved in promoting latency and the stochastic nature of latency. It was suggested that combination of drugs has synergistic effect improving the efficiency of reactivation and reducing toxicity because of the lower doses used.

In this context, we investigated the reactivation potential of compounds releasing active pTEFb in combination with PKC agonists (Fig. 1). Treatments of a combination of HMBA/BETi and PKC agonists lead to strong synergistic activation of HIV-1 expression in several *in vitro* post-integration latency cell line models [125]. Remarkably, in *ex vivo* cultures of resting CD4+ T cells isolated from HIV-1+ cART-treated aviremic patients, the combinations of bryostatin-1+JQ1 and ingenol B+JQ1 efficiently activated latent HIV-1 [125]. These results constitute the first demonstration of anti-latency drug combinations exhibiting such a potent effect. It was also shown that a sequential treatment with a demethylating agent (5-AzaC) and a clinically tolerable HDACi was more effective both *in vitro* and *ex vivo* to induce HIV gene expression than the corresponding simultaneous treatment [118]. These data demonstrate the importance of the treatment time schedule in the reactivation of HIV by combinations LRAs. Interestingly, the reactivation capacity of LRAs *ex vivo* correlates with the size of the HIV-1 reservoir [130]. However, some patients had very low or extremely high reactivation capacities relative to their reservoir size [130]. Timely administration of LRAs in reactivation assays and a better understanding of the variability of reactivation in patients is another important issue. A defective Cas9 (dCas9) protein fused to activators (such as herpes simplex VP16 activator domain) or to the synergistic activator mediator could be an interesting new tool to reactivate latently-infected cells. A potential application of CRISPR/dCas9 in the reactivation of latent HIV was suggested by *in vitro* experiments [131]. This strategy showed reactivation of HIV expression in CD4+ T cells and in microglial cell lines [132]. Similarly, CRISPR/dCas9 when used in combination with HDAC inhibitors and PKC activators reactivated HIV in a synergistic manner [133].

Overall, it is crucial to consider the nature and the distribution of latently-infected cells in the choice of a strategy to reduce the size of the HIV reservoir. Indeed, resting T cells tested *ex vivo* are circulating cells but are also located in poor drug access locations such as lymph nodes. Moreover, we believe that reservoirs other than CD4+ T cells, e.g. macrophages, exist as well and these are found in sanctuaries such as the brain [19]. The importance to reduce the pool of all latently-infected reservoirs has been discussed recently [29]. The main challenges one expects to encounter when targeting reservoirs located in a sanctuary such as the brain are [26,134]:

- i. In the CNS sanctuary barriers (blood brain barrier and choroid plexus) lower the access of some of the presently used drugs [135].
- ii. The key cellular targets in the brain are astrocytes and microglial cells. However, few drugs are able to target the monocyte-macrophage lineage [136].
- iii. Reactivation of the virus is often associated with the activation of macrophage/microglial cells responsible for CNS inflammation [84].

Of note, only few LRAs currently tested can attain lymph nodes and cross the blood brain barrier. Among them, bryostatin-1 is

promising since it is able to activate Protein Kinase C in microglial cells and astrocytes, which are the main HIV reservoirs of the brain [125,137].

4.2. The kill

Although LRAs are not toxic in clinical trials they are also unable to reduce significantly the size of the latently-infected reservoir [126,138–140]. Many reasons were stated [141]: (i) induction of HIV expression does not lead to the death of the cells, (ii) the impaired cytotoxic activity of CD8+ T cells is not restored by cART, (iii) drugs and CTLs are unable to reach reservoirs in sanctuaries. Furthermore, following the “shock” therapy the immune system needs to be boosted to “kill” HIV-1 infected cells. There is now mounting evidence that clearing of latently-infected reservoirs in HIV infected patients involves humoral and cell mediated immune responses. It was observed that B-cell depletion by the CD20-specific antibody rituximab is associated with the failure of the HIV control [142]. Interestingly, the failure of HIV-1 control has been associated with a decrease in titers of neutralizing antibodies targeting the CD4 binding site. This observation supports the crucial role of the humoral mediated immune response in the control of HIV.

Experiences on cART-treated macaques and elite macaques also suggested that an efficient cell-mediated immune response is capable to eliminate the reservoirs. Indeed, CD8+ T cell depletion increased while recovering CD8+ T cells decreased viremia in these animal models [143–145]. Major causes of the failure of cell-mediated immune response are high mutation rate of the virus changing epitopes targeted by CTLs leading to viral escape, and poor penetration of immune cells in sanctuaries such as the brain and the lymph nodes. Several recent works emphasized that HIV specific CD8+ T cells play a crucial role in eradicating HIV reservoirs [146].

Overall, the kill strategy should enhance both humoral-mediated and cell-mediated immune responses and consequently purge HIV reservoir.

4.2.1. Humoral-mediated immune response

Humoral immune response plays an important role in controlling HIV infection [147]. It has been a field of intense research in the development of a vaccine since the discovery of HIV-1. To date all vaccine trials preventing HIV-1 infection have failed. Over the past years, a new class of antibodies raised against conserved regions of the protein Env was identified and isolated from infected patients [148,149]. These antibodies, called broadly neutralizing antibodies, neutralize a wide range of HIV strains. An intense field of research is the identification and characterization of new broadly neutralizing antibodies against HIV. Several methods, including single B cell culture coupled to high throughput neutralization screening, are currently used to identify new broadly neutralizing antibodies from large cohort of HIV infected patients [150]. Several new anti- HIV-1 neutralizing monoclonal antibodies have been isolated and shown to block HIV-1 and SHIV infection in animal models [151]. Indeed, they efficiently cured SHIVs infected macaque monkeys and HIV-1 infected humanized mice. Also they were shown to suppress HIV-1 from latently infected CD4+ T cells in patients and in a humanized mouse model [152–154]. Some improvement for their use are still awaited since in all clinical trials a resistance to the antibodies developed [155]. Moreover, the mechanisms of action of these broadly neutralizing antibodies are mainly unknown which are essential for their use in a kill strategy [155]. Besides their utilization in strategies aiming to target latently-infected reservoirs, these broadly neutralizing antibodies raised the hope to develop vaccine against HIV-1 [156–158]. Their efficiency is improved when applying in combination [159] and

with the development of multi-specific engineered antibodies like bi and tri-specific antibodies [160,161]. Non-neutralizing antibodies deserve also attention since they can also be used to target the reservoirs. Indeed they can direct antibody-dependent cellular cytotoxic response which controls virus replication in Elite Controllers [162].

4.2.2. Cell-mediated immune responses

Several strategies were proposed to enhance cell-mediated immune responses, e.g. CD8+ T cell or natural killer cell activities [163,164]. The adoptive transfer of virus specific CD8+ T cells prevents viral rebound of the cytomegalovirus and Epstein Barr virus in cancer therapy [165,166]. Other procedures aim to redirect HIV-specific cell mediated immune responses [167]. In one strategy, T cells are redirected and expanded *ex vivo* to recognize various HIV antigens such as the proteins Gag, Env and Pol. This approach is not toxic and generates poly-specific CD8+ T cells targeting several HIV antigens. In other assays artificial T cell receptors (TCRs) or chimeric antigen receptors (CARs) are expressed from genetically modified lentivirus [167]. Another possibility is to increase the specificity and the affinity of the epitopes of the receptors to achieve broader HIV epitope recognition [167]. CD8+ T cells that express artificial receptors significantly reduce HIV infection in a humanized mouse model [168]. The use of CARs is also promising [169]. Currently, clinical trials test the efficiency of these molecules on patients affected by CD19+ hematologic malignancies. Recent studies suggest that adoptive transfer of SIV specific CD8+ T cells most likely reduce the level of infection [170]. However, the expression of TCRs and CARs cause severe off target effects which today limit their use [171,172]. A recent work using an adoptive T-cell therapy in combination with LRAs is also promising. It notably showed that *ex vivo* expanded CD8+ T cells derived from treated patients target more efficiently the HIV-1 reservoir than bulk CD8+ T cells [173]. The feasibility to boost HIV-specific CD8+ T cell responses with heterocyclic peptides is currently tested. It is expected that these peptides, which are a subset of sequence variants, stimulate stronger the cell-mediated immune responses than native epitopes [174]. As in cancer therapy, one expects to improve the HIV-specific cell-mediated immune response by increasing the avidity of the peptides for HIV epitopes. The purge of escape variants accomplished by increasing drug avidity for pathogen epitopes contributed to the decrease of viremia in a murine hepatitis virus infection [175].

Another approach exploited the importance of co-stimulatory and co-inhibitory molecules involved in the regulation of T-cell responses [176]. Among co-inhibitory molecules, the role of the checkpoint inhibitor programmed death 1 (PD-1) in HIV-1 infection is of interest. Indeed, PD-1+ CD4+ T cells constitutes the major cell reservoir of HIV-1 in viremic and aviremic cART-treated patients [24,177]. Treatment by specific antibodies targeting PD-1 is thought to decrease the size of the latently-infected reservoir and help to recover CD8+ T cell function from exhaustion. In patients with sepsis blocking PD-1 restored the function of innate immune cells [178]. In chronic infection inhibition of PD-1 was shown to boost CD8+ T cell activity against HIV-1 [179]. Such an approach is currently tested in a clinical trial in cART-suppressed patients with the aim to counter-act cell-mediated immune response failure [164]. Interestingly, the significant decrease of a fraction of PD1+ CD8+ T cells following stimulation with heterocyclic peptides helps to restore potent HIV-specific CD8+ T cell responses [180]. The use of therapeutic vaccines, which aim to enhance virus-specific CTL activities is also a promising approach [181]. Therapeutic vaccines could help CTLs to target cells infected by HIV-1 derived from latent reservoirs [182]. The CD32a low affinity receptor for immunoglobulin G Fc fragment is a recently discovered specific biomarker for CD4+ T cell HIV-reservoir, which might

be a future target [183]. However, the marker might not stain all HIV reservoirs since specific for CD4+ T cells, excluding other important cell reservoirs such as macrophages. The discovery of specific biomarkers for microglial cells, the CNS resident macrophages, may lead to the development of original strategies targeting specifically brain HIV reservoir. Indeed, the brain has long been considered as an immunologically privileged site. However, strong immune activation of cytotoxic T lymphocytes to eradicate potential reservoirs may be challenging or even detrimental in the brain [184].

In sum, combination of approaches should help to improve the kill strategy. The use of a wide range of broadly neutralizing antibodies combining with therapeutic vaccine treatment, which enhance CTL responses has shown to improve HIV therapy. Other combination approaches combine shock and kill strategies. The first evidence of the feasibility of a combined shock and kill strategy came from a therapeutic HIV immunization associated with romidepsin treatment [185]. Such a combination which reduced by approximately 40% of the size of the reservoir is encouraging and deserves further investigations. Starting very early cART treatment also improves immune responses [186]. Indeed, the number of CTL escape mutants is also drastically reduced due to a more efficient cell-mediated immune response [182].

5. Conclusion

An extensive number of clinical trials in the field of HIV reactivation and immunomodulatory molecules have been initiated from which beneficial effects are eagerly awaited over the next years [167,187,188]. Beside the shock and kill strategy, cell and gene therapy are promising approaches which will need however more investigations. Indeed, the advent of new technologies such as gene editing based on CRISPR/cas9 could be crucial to achieve cure. However, often the proof of concept is more difficult than initially hoped. Following the initial study establishing the proof of concept of the use of gene editing to target specifically HIV-1 [189], it soon appeared that the virus is able to subvert the DNA repair machinery to evolve rapidly into CRISPR/Cas9 resistant strains [31,190–192].

Over the past years much efforts have been made to improve the reactivation (the shock therapy) and the removal of the virus (the kill strategy) but further improvement of both strategies are still needed (Table 2). Regarding the shock therapy, it appears that to achieve complete HIV reactivation a combination of several LRAs acting at different levels is needed. Moreover, the delivery of these molecules will need to follow a precise time schedule to increase efficiency. Another important issue is to improve drug delivery into sanctuaries such as the lymph nodes and the brain. Several original strategies, e.g. the use of nanotechnology or ultrasound to enter drugs into the brain, are currently tested [193,194]. New strategies that enhance humoral and cell mediated immune reactions in response to the shock therapy and reinforce the effect of cART are still needed (Table 2).

A better understanding of the molecular mechanisms underlying latency in various reservoirs will certainly help the identification and characterization of new potential targets of dormant HIV-1. Translational approaches, for instance mathematical modelling predicting the outcome of treatments will help researchers and clinicians to design new and original strategies [71,72,195]. Mathematical models highlighted the importance of the HIV-1 Tat positive-feedback circuitry in the persistence of HIV-1 latency [78]. The models suggested that establishment and persistence of HIV-1 latency enhance viral transmission thus conferring an evolutionary role to latency [196]. The therapeutic implications of these studies indicate that suppression of reactivation during the first

Table 2

Perspectives to improve the shock and kill strategy.

Identification and characterization of new cellular factors involved in HIV-1 latency
Screening for molecular inhibiting factors involved in HIV-1 latency (LRAs)
Designing derivatives of inhibitors more efficient and less toxic
Development of new and original cell and animal models to test LRAs
Development of strategies to target all viral reservoirs including myeloid cells
Development of strategies to target sanctuaries
Identification and characterization of new broadly neutralizing antibodies against HIV: boosting humoral immune response
Improving the adoptive transfer of virus specific CD8+ T cells
Exploiting the importance of co-stimulatory and co-inhibitory molecules involved in the regulation of T-cell responses
Development of therapeutic vaccines which aim to enhance virus-specific CTL activities
Searching for specific biomarkers for all reservoirs including myeloid cells

week of infection followed by a shock and kill therapy could drastically reduce the size of the HIV-1 reservoir. Surprisingly, the evolutionary theory of latency could explain why it is so difficult to reactivate all the reservoirs. Theoretically it might be easier to favor latency rather than reactivation. Inducing a state of deep latency by drugs which inhibit Tat are currently under investigations [197–200].

Competing interests

None.

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