One Step Closer to HIV Eradication?

Brigitte Autran, Chiraz Hamimi, Christine Katlama

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Brigitte Autran[1,2,3], Chiraz Hamimi[2], Christine Katlama[4,5,6]

1 : UPMC Univ Paris 06, Laboratory of Immunity and Infection, Paris, F-75013, France
2 : INSERM, U1135, Laboratory of Immunity and Infection, Paris, F-75013, France
3 : AP-HP, Hôpital Pitié-Salpêtrière, Département d'Immunologie, Paris, F-75013, France
4 : Sorbonne Universités, UPMC Univ Paris 06, F-75013, Paris, France
5 : UMR_S 1136, Institut Pierre Louis d'Epidémiologie et de Santé Publique, F-75013, Paris, France
6 : AP-HP, Hôpital Pitié-Salpêtrière, Service des Maladies Infectieuses et Tropicales, Paris, F-75013, France

Introduction

The successes of anti-retroviral therapy (ART) are counter-balanced by their failure to eradicate HIV-1 infection [1], imposing a life-long treatment still associated with potential toxicities and cumulative costs. HIV cure, consisting at best in viral eradication or at least in a functional cure,[long term control of HIV in the absence of ART] has emerged as the ultimate goal [2-5] and has been validated with the report of an apparent cure in the “Berlin” patient [6] . Even if the concept of HIV eradication appears by far too elusive, it has been re-inforced more recently by the probable cure in the Mississippi baby [7]. In contrast, reaching a functional cure or remission may be more realistic and has been illustrated by the “Post-Treatment Controller” (PTC) patients [8-11]. PTCs durably control HIV-1 after stopping a treatment initiated at primary infection and share some characteristics with the rare Elite Controllers (EC) [12]. These successes have opened the way for research of drugs capable in vitro of re-activating the provirus to clinical trials testing novel therapeutic strategies purging the HIV-1 reservoirs [4, 5, 13-17] However, the modest results obtained so far illustrate the major difficulties in eradicating HIV and the need for more basic and translational research to further understand the mechanisms of HIV-1 persistence during ART, and to identify the characteristics in those rare cases of HIV “cure”. This review of the recent key articles will show that if we are not yet one step closer to HIV eradication some recent advances have paved the way towards a step closer to HIV remission.

Defining the HIV reservoirs

Understanding the sources of HIV-1 persistent reservoirs in individuals treated with combination ART (cART) is crucial in developing novel therapeutic strategies enabling elimination or durable control of the virus in the absence of treatment. The levels of HIV-1 reservoirs appear to be one of the major factors influencing the duration of virologic suppression after ART cessation. The HIV-1 reservoirs are made up of cells containing stably-integrated, transcriptionally-silent but replication-competent proviruses and most infected cells are memory CD4+ T cells. They can be estimated most easily by the amount of the total cell-associated HIV-1-DNA in peripheral blood mononuclear cells (PBMCs) that roughly parallel the plasma plasma viral load (pVL) during the course of the HIV-1 infection [18, 19] and strongly correlate with tissue viremia levels *[20, 21]*. In those on long term cART the total HIV-DNA levels associated with purified total CD4+T cells are less than 1-log lower in PBMCs compared to rectal CD4 T cells [22]. The HIV-1 reservoir decrease to a much

lesser extent with cART than pVL [1-5], except in some instances where cART is initiated early during acute infection [8, 19]. Other methods have been proposed to quantify HIV reservoirs. A recent comparison of 11 different approaches for quantification of persistent HIV-1 infection in 30 patients on cART, using the viral outgrowth assay for resting infected CD4 T cells as a reference [23]. This study showed that PCR-based assays quantifying cells containing HIV-1 DNA demonstrated infected cell frequencies about 2 logs higher than the viral outgrowth assay and correlated roughly with this latter assay, achieving significance for integrated HIV-1 DNA in PBMCs and HIV-1 RNA/DNA ratio in rectal CD4 T cells. However these various methods require extensive amounts of biological material and cannot be easily applied to large cohort studies or clinical trials, while quantification of cell-associated total HIV-DNA is feasible on limited amounts of cells and has been strongly and clinically validated for HIV diagnosis in newborns from infected mothers [20, 21]. In addition the variations observed between the ratios of infected cell frequencies determined by viral outgrowth and PCR-based assays among patients [23] reflect the low quantitative reproducibility inherent to any complex function-based assay, and the presence of a large and variable pool of cells infected with defective proviruses that are not sensitive to reactivating agents [24]. A recent molecular characterization identified HIV defective viruses in 88% of 213 non-induced proviral clones from treated patients [25]. In addition full-length intact proviral clones could be reconstructed and became replication-competent, suggesting that the size of the latent reservoir may be up to 60-fold greater than previously estimated based on the replication-competent viral outgrowth assay [25]. This latter finding might reconcile the discrepancies observed between PCR-based and virus outgrowth assays. Two other studies showed also the accumulation of rare defective proviruses bearing APOBEC-3G signatures in various compartments [26, 27] , i.e. PMBCs, cerebral spinal fluid and rectal or renal tissues from 30 long-term treated chronically-infected patients [27]. HIV hypermutated sequences were detected in 36% cases in at least one viral reservoir, and more often in viral sanctuaries than in peripheral blood. Altogether the accumulation of defective viruses might be falsely re-assuring if activation of replication-competent, non-induced viruses may occur in vivo.

Moreover, quantification of total HIV-1 DNA is considered to be integrated as a provirus in silently-infected CD4+T cells in patients with undetectable pVL [18, 19, 28]. Those cells have been estimated to contain only 1-2 HIV-1 provirus copies per cell [18], thus allowing to estimate frequencies of stably infected reservoir cells. Those frequencies are generally low, though with broad ranges from 1/100,000 to 1/100 or less depending on the stage of infection [19], and on the cell compartment measured. It is assumed that the CD4+T cells harbor the vast majority of those reservoirs, although monocytes and macrophages can also be infected [2-5, 19, 28, 29]. A typical hierarchy has been shown among CD4+T cells with

the lowest levels observed in naïve T cells (TN) compared to the heterogeneous memory CD4+T cell compartment, composed of the central- (TCM), transitional- (TTM) and effector- (TEM) memory CD4+T cells [10, 28-31]. Levels of infection also differ whether they are measured only in quiescent resting CD4+T cells or in mixtures of activated and quiescent T cells. Heterogeneous levels of HIV-1 persistence among CD4+T cell subsets appear to reflect a composite of low-level virus production and persistence of latent integrated proviruses, two mechanisms that are strongly influenced by the heterogeneity in CD4+T cell subset homeostasis, activation and turn-over, while reservoir clearance depends on cell death or recognition by the immune system [5, 6, 10, 28, 29, 32]. These mechanisms might also be influenced by host-related factors and patient's "history" of HIV-1 infection, such as duration of HIV-1 infection prior to ART initiation and CD4 nadir , suboptimal intracellular penetration of the potent modern ART and duration of viral suppression, as well as residual immune activation among sufficiently treated patients [33], though such hypothesis has not been proven yet. Besides anatomical sanctuaries, HIV-1 reservoirs are thought to be preserved through HIV-1 DNA persistence in quiescent cells and cycles of residual viral replication arising from viral reactivation in latently-infected cells exposed to antigenic and homeostatic stimuli in an uncontrolled pro-inflammatory environment.

Barriers to an HIV Cure

Persistence of true (i.e. non-defective) HIV-1 latency represents certainly the biggest challenge in finding a cure for HIV [2-5] and depends mainly upon mechanisms inducing post-integration latency. Multiple viral and cellular factors have been described so far, but it remains unclear which are the most efficient at preventing effective proviral transcription in patients' host cell genome and allow some HIV-1-infected CD4+ T cells to survive and revert to a resting memory state. In addition, most of those mechanisms have been defined in cell lines and cellular models of latency but remain poorly defined in primary cells from treated patients.

HIV-1 itself manipulates its target cells to convert them into effective virus producers rather than carriers of transcriptionally silent proviruses. The early and late regulatory proteins (Nef and Vpu) affect control of HIV-1 transcription by modulating the key NF-κB host transcription factor [34, 35]. Control and repression of HIV-1 transcription is certainly crucial to the establishment and maintenance of post-integration latency [3, 4]. HIV transcription is known to be controlled at various levels such as the: i) site of integration and mechanisms of transcriptional interference, ii) inducible host transcription factors and transcriptional repressors, iii) nucleosomal organization of the HIV-1 promoter, iv) epigenetic level including

histone posttranslational modifications and DNA methylation, or v) positive-transcription-elongation-factor-b (P-TEFb) [36-42]. The involvement of these elements in post-integration latency, particularly the epigenetic modifications, certainly depends on the status of activation and differentiation of the heterogeneous CD4+ T cell subsets hosting the HIV-1 reservoirs, but these aspects have not been defined in patients on cART. In vitro, cellular activation has resulted in the expression of cell restriction factors capable of suppressing HIV replication with an increase interferon-stimulated genes such as ISG15, but also the well known HIV restriction factors TRIM5, tetherin, some APOBEC3 family members[43]. Other Type-I interferon stimulated genes (ISG) also seem to play a key role in the innate immune response against HIV such as Schlafen-11 (SLFN11), an ISG and restriction factor acting by down-modulating HIV-1 mRNA translation [44] and found to be elevated in CD4+T cells from Elite Controllers (EC) compared to viremic and treated patients. These findings suggest that SLFN11 may play a role in the suppression of HIV-1 *in vivo* [45]. Another protein, 90K, has recently been described as an antiviral factor associated with the release of poorly infectious virion and induces defective maturation of HIV-1 Env during the novo virus production and reduced incorporation of Env into progeny virions [46]. Finally the Myxovirus-resistance-2 molecule (MX2) has recently been demonstrated to be an IFN-induced inhibitor of HIV-1 infection by probably inhibiting capsid-dependent nuclear import of subviral complexes [47]. In addition, the RNA-associated early-stage anti-viral factor (REAF) has been reported to block HIV and SIV at an early post-entry stage during or following initiation of reverse transcription [48]. The cyclin-dependent kinase inhibitor $p21^{Waf1/cip-1}$ also impairs HIV/SIV reverse transcription in macrophages [49, 50]. These factors acting at early steps of viral replication might reduce HIV-1 provirus integration, thus further limiting the size of HIV reservoirs.

Overall, these numerous pathways illustrate the multiple challenges in HIV eradication and requires a better understanding of how these mechanisms operate in optimally-treated patients. The pleiotropic mechanisms involved also show that designing future therapeutic strategies to eliminate latent HIV-1 infection or to restrict the latent pool down to a size the host immune system can control might require a combination of approaches.

One step closer into Therapeutic strategies towards a cure ?

Despite these multiple barriers to an HIV cure and the complex pathogenetic mechanisms involved, two broad clinical strategies are currently being proposed to purge HIV reservoirs,

either by limiting further the residual replication or by activating virus production from the latent compartment. Although still far from an ideal goal, strategies based upon very early initiation of cART have recently shown some very promising, though still debated results.

Therapeutic strategies to limit residual HIV production

Early ART initiation and HIV cure of the Mississippi baby: This second highly publicized case of an apparent cure of HIV was announced last year at CROI. It is as appealing as the "Berlin patient", though much simpler and more practical with the use of conventional ART regimen. The innovation seems to lie in the very early initiation of cART, 31 hours after the newborn's delivery from an untreated mother who was diagnosed with HIV at delivery with a moderate pVL below 2,500 copies/mL, and normal CD4 counts [7]. The infant's pVL rapidly became undetectable and remained so during the 18 months of cART as well as during the 18 months after ART interruption with only traces of HIV DNA in PBMC at month 26 confirmed that the baby had truly been infected. The extended follow-up of this child will tell us whether this is the 2nd known eradication of HIV or simply a functional cure.

What can we learn from this case? First, that a very early cART intervention within the first day after delivery might interfere with the establishment of a persistent reservoir. Second, that this case may perhaps not be that exceptional. Since its disclosure, all paediatricians following HIV-infected children treated at birth have initiated studies evaluating the size of HIV reservoirs in those children and some ART interruption studies in highly selected children are likely to be performed.

The Post-Treatment Controllers (PTC) from the Visconti study: A few HIV patients who were diagnosed early in primary infection and started cART almost immediately and for prolonged period, were able to durably control viremia after treatment interruption. This key population has been compared to standard non controllers patients and to the rare Elite Controllers. PTCs display a symptomatic primary infection with pVL and CD4+T cell counts comparable to non controller patients and represent 8-15% of early-treated patients [9, 10]. Genetic analysis indicates an enrichment of some HLA-class I molecules in PTCs such as HLA B*07 and B*35 associated with accelerated progression to AIDS, but no increase in the protective HLA-B*27 or *57 like in Elite Controllers [10]. Accordingly the PTCs anti-HIV CD8+T cells frequency and suppressive capacity are lower than in ECs, with less activated total CD8+T cells, suggesting altogether that CD8+T cell responses might not be implicated in viral control in those PTCs. The early treatment could favor the spontaneous control of the infection by

limiting the establishment of viral reservoirs [51] and the diversity of the virus [52, 53], as well as the immune alterations [54, 55]; three conditions that could facilitate later control by undamaged immune responses. Along these lines, PTCs display a very small viral reservoir comparable to ECs, particularly in the long-lived naïve and central-memory CD4+T cells [9]. Interestingly, during the follow-up, the viral reservoirs were shown to shrink overtime in some PTCs. This encouraging PTC phenomenon suggest that limiting the number of infected cells early-on after infection is crucial for the maintenance of viral control in absence of therapy. Nevertheless more investigations are needed to understand the mechanisms of viral control in these peculiar patients.

ART intensification: The concept of treatment intensification has been supported by cART pharmacodynamic limitations. It is crucial to have constant optimal concentrations of antiretroviral drugs in compartments such as in lymph nodes or central nervous system where concentrations of some widely used drugs are far from optimal. Most cART intensification strategies utilized the integrase inhibitor raltegravir either alone or in combination, and have been shown in some reports to increase production of 2-LTR circles, suggesting an impact on the residual HIV production [14, 56]. More recently, a small study reported that a combination of an early integrase inhibitor-based treatment with an entry inhibitor such as maraviroc resulted not only in a faster reduction of 2-LTRs in newly infected cells and a faster CD4+T-cell recovery, but also in a modest reduction in total reservoir size after 48 weeks of treatment [57]. However, a larger randomized clinical trial did not confirm the benefits of a similar treatment intensification initiated within a month after infection compared to a standard triple Cart [58]. Similar disappointing results of a dual intensification strategy have been reported after a 48 week intensification in chronically-infected patients [17].

Strategies repressing HIV transcription and production:

Even in optimally-treated HIV-infection, chronic immune activation can persist with pro-inflammatory cytokines, IFN-α production and increased frequencies of activated $CD4^+$ T cells and may contribute to a continuous low level, though “undetectable” virus production. Some strategies targeting inflammation have been proposed such as drugs blocking type I Interferons, or the PD-1-PDL interactions that, enhance innate and specific immunity [4, 5]. Another option might be to use agents interfering with some tyrosine-kinase (TK) activation pathways, such as inhibitors (TKIs) of intracellular tyrosine-kinases known to be well

tolerated when chronically administered. This is the logic for *Dasatinib*, *Imatinib*, ABL tyrosine-kinase inhibitors (TKIs) used in treatment of chronic myelogenous leukemia and considered for use in inflammatory diseases. While HIV-1-cell fusion process depends also on the TK Abl, TKIs can block *in vitro* Env-mediated cell-cell fusion and virus-cell fusion in cell lines, our group recently showed, that *Dasatinib* could block in vitro HIV production in primary CD4+T cells from HIV-infected patients by inhibiting cell activation and proliferation, without inducing cell death, while the most commonly used *Imatimib* drug was too toxic for T cells [59]. The good safety profile and ability of this molecule to hinder persistent immune-activation and residual viral production deserves to be further investigated in vivo in strategies for HIV cure.

Finally, while administration of strong immunosuppressive drugs in HIV-infected patients is feasible and necessary as in transplanted HIV-infected patients, increasing immunosuppression in patients at high risk of immunodeficiency in case of cART interruption is an issue. Testing first whether these immunosuppressive agents help decrease viral reservoirs in non-human primate animal models might be a valid option before translation into clinical trials.

Purge strategies targeting HIV latency with or IL-7

Finally ‘‘flushing out’’ procedures were proposed over the last few years as an option to try purging the HIV reservoirs by de-repressing HIV-1 gene expression and reverting the transcriptional silence lying at the core of HIV-1 proviral persistence in latently-infected cells [2, 36, 37, 60]

Histone de-acetylase (HDAC) inhibitors and other classes of epigenetic modifiers: The most commonly tested agents are the HDAC inhibitors, particularly *vorinostat* which promote histone acetylation and is used for treatment of cutaneous T cell lymphoma. Culture of latently infected cells with this drug *in vitro* induces a significant but modest increase in HIV-1 transcription and/or production, though depending on the cellular model used, and usually far below the level induced by classical activation pathways [61-63]. Two pilot clinical trials tested *vorinostat*, one using a single dose [15] and the other one a repeated dose for 14 days (NCT01365065) [16]. Though still controversial, both showed some enhancement in HIV-1 transcription *in vivo,* particularly after repeated doses of *vorinostat* , but none succeeded at reducing the levels of the HIV-1 reservoirs. Another, more potent HDACi, *parabinostat*, is currently being tested in a clinical trial (NCT01680094) [64] while more selective class I

HDAC inhibitors such as *entinostat* might be even more attractive but has not been tested in vivo yet [65].

Other epigenetic strategies might be proposed by targeting the hypermethylation of the HIV-1 promoter shown in some long-term cART-treated aviremic individuals [61], though this is still debated [25]. Accordingly some Histone-Methyl-Transferase (HMT) inhibitors yield *in vitro* results roughly comparable to the HDACi [61, 62], while classical HMT inhibitors, such as 5-Azacytidine or decitabine, two well-tolerated drugs used for decades in other indications, have been proposed for *in vivo* trials [4, 5, 16, 66]. However a two weeks administration of decitabine failed at reducing the size of the HIV reservoirs in fully suppressed cART treated patients [66].

Interleukin-7: Another purging candidate was interleukin-7 (IL-7), a human cytokine targeting the Stat-5 and Akt pathways and involved in intra-thymic T cell differentiation and homeostatic proliferation of mature T cells. This cytokine was shown to increase in vitro transcription and production of HIV provirus by many groups including ours [2, 10, 29, 66-69], although others failed at demonstrating generation of virus diversity [28]. This cytokine had been used *in vivo* for its proliferative homeostatic activity to augment CD4+T cell counts and displayed a good clinical safety profile despite some transient “blips” of plasma viremia in patients on conventional ART [69]. Our group therefore conducted a clinical trial, the ERAMUNE-01 study, to investigate in chronically-infected patients with suppressed HIV viremia, the capacity of interleukin-7 plus a dual intensification strategy with raltegravir/ maraviroc or intensification alone to decrease the HIV reservoirs [17]. Like HDACi or *decitabine*, results were disappointing and demonstrated this cytokine was inefficient at disrupting HIV latency and at purging the HIV reservoirs.

Thus, none of those “purging” strategies succeeded at reducing the HIV reservoirs so far, suggesting the mechanisms targeted were not the only ones responsible for HIV persistence *in vivo*. In addition, a recent comparison of thirteen stimuli reactivating HIV by defined mechanisms of action, including HDAC Inhibitors and IL-7, showed very discordant results in several culture models including latent primary T cell models, latent T cell lines and infected patients primary T cells [63]. These results as others [10, 29] indicate that the activation and differentiation status of the reservoir cells determine the mechanisms involved in HIV latency and the various cell sensitivity to these stimuli. Thus barriers to HIV eradication are more diverse than initially thought.

So far, only the early treatment initiation has brought us one step closer to HIV eradication, with at the best the cure of the Mississipi baby, and at the least the functional cure of the PTC cohort. Whether these successes are exceptional and early cART initiation the only explanation for inducing PTCs, or whether this goal is achievable in patients treated during the chronic phase remains a key question currently under investigation in our group. Altogether these results are encouraging enough to continue exploring the concept by defining novel, alternative or complementary strategies targeting the mechanisms of HIV-1 repression listed above. While no strategy aimed at purging the reservoir has been successful, this should not discourage continuing research to further understand the main obstacles towards HIV eradication. Ultimately, the strategy chosen should be dictated by the predominant mechanisms in CD4+T cells from the patients eligible for these innovative therapeutic strategies. Meanwhile, one of the major lessons in recent years is that early initiation of antiretroviral therapy is the best way to preserve immune cells and limit viral reservoirs and prevent deleterious immune activation and inflammation from uncontrolled HIV viremia.

The massive research efforts in the 90s lead to major progress in therapy. Significant advances in our knowledge of the mechanisms of HIV persistence will undoubtedly come from basic , translational and clinical research. The most frequent wish from patients remains " Doctor , When will we be able to stop therapy ??

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**7 . This report of the “Mississipi baby” suggests sterilization cure is at hand after extremely early HIV treatment of newborns from HIV-infected mothers.

*9 and **10 This study demonstrated that “The Hope” of functional cure could be achieved with early and durable treatment of acute infection in in up to 12-15% early-treated HIV patients .

*15 The first human trial suggesting on a very limited number of patients that a first generation of HDAC inhibitor might be able in some patients to favor transcription of HIV in vivo, a result that needs to be confirmed on a much larger scale and is not associated with purge of the HIV reservoirs.

**25 and *27. These two studies showed the accumulation of HIV hypermutated sequences were detected in 36% cases in at least one viral reservoir[27][5] and of rare defective proviruses in viral sanctuaries[27], an information that might be falsely re-assuring if activation of non-induced viruses occurs in vivo.

**32 The first human study demonstrating the anti-HIV CD8 T cell responses are associated with a limited infection of the key subset of central-memory CD4 T cells in HLA-B27/B57+ LTNPs.

** 47 The demonstration of a novel restriction factor of HIV induced by type-I IFN.

*66. The first study demonstrating that a novel property of an old drug to inhibit histone methylation might be able to favor transcription of HIV in vivo, without purging effects of the HIV reservoirs, yet.