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A cure for AIDS: a matter of timing?

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Abstract

Despite the huge clinical success of antiretroviral therapy, several factors such as side effects, requirement of life-long adherence, high cost, incomplete access to therapies and development of drug resistance make the quest for an ultimate cure of HIV/AIDS a worldwide priority of biomedical research. In this respect, several sterilizing or functional cures have been reported in the last years in both non-human primates and humans. This review provides a summary of the main results achieved so far, outlining their strengths as well as their limitations. A synthetic interpretation of these results could be pivotal in order to develop an effective and widely available cure.

Keywords: Eradication, Functional cure, Reservoirs, Acute HIV infection, Stem cell transplantation, Vorinostat, Therapeutic vaccine, Auranofin, BSO

Review

Introduction

The quest of a cure for AIDS has been defined a "herculean task" [1], given the enormous complexities behind it and the numerous setbacks that have curbed early enthusiasms along the years. The ultimate goal of research for a cure is the complete eradication of the virus from the organism (*i.e.* a "sterilizing cure"), but a more feasible goal may be the achievement of spontaneous drug-free control of the infection without disease progression (*i.e.* a functional cure) [2]. The enormous difficulties that have been encountered in the quest of a cure for AIDS reside in the complex virus/host interplay that is a hallmark of this disease. Infection with HIV is initially characterized by a primary (acute) phase in which the virus is partially controlled by a robust immune response of the host [3]. Unfortunately, this immune response is not sufficient to eradicate the virus from the body, opening the way to the asymptomatic (chronic) phase. The chronic phase is characterized by an initial "steady state" between the virus and the immune system that is then slowly tilted in favor of the former, eventually leading to AIDS in the majority of the patients [4]. Treatment with antiretroviral drugs (ART) can reproducibly decrease viremia to levels below the limit of detection of the routine clinical assays and delays immune deterioration, but is not sufficient to tackle the viral reservoirs or to induce a strong immune response against the virus [5-7]. The viral reservoirs are formed early during acute infection [8] and are exceptionally stable sources of viral persistence [6,9], harboring latent copies of integrated virus that are "invisible" to the immune system and unharmed by ART (5,6,9, for a review on the latency mechanisms, see: [10]). Viral reservoirs can be of both myeloid and lymphoid lineage, allowing a widespread distribution to different compartments such as the central nervous system, the gut-associated lymphoid tissue and the reproductive tract [11]. At a cellular level, central and transitional memory T-cells (T_{CM} and T_{TM}) were recently identified as a crucial source of viral persistence during therapy [12]. Additionally, macrophages are regarded as important contributors to this persistence, as well [13].

This review provides an outline of the therapeutic successes in the pathway towards a cure for AIDS. Our description is focused on the results that have so far been obtained in humans or SIV/SHIV infected macaques, which are, among the allowed animal models, those phylogenetically nearest to humans and most closely recapitulating the pathogenesis of human AIDS [14,15]. Recent reports have provided substantial data supporting the view that the path to a cure is a viable research avenue. These new data allow attempting a re-evaluation of the paradigms that have oriented cure-related research and addressing some of the questions that have so far been left unanswered.

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Hit fast, hit hard

Acute infection offers an ideal time window for effective therapeutic interventions [3]. A pioneering demonstration of the therapeutic potential of early treatment was the case report of spontaneous control of viral replication following treatment interruption in the first "Berlin Patient" [16] (not to be confused with Mr. Timothy Brown, the second "Berlin Patient", see next subchapter). This man was treated during acute infection with a non-standard ART regimen (containing hydroxyurea) and subsequently underwent two structured treatment interruptions (STI). Eventually, after the second STI, the man displayed a long-lasting (19 months, until he was lost to follow-up) spontaneous control of viral load below the assay detection limit (500 copies of viral RNA/mL). Moreover, viral load control was accompanied by immune restoration, with CD4 counts and CD4/CD8 ratio progressively increasing over time [16]. This striking result confirmed those of a previous study by Vila *et al.*, employing a similar drug regimen and achieving as well a long-lasting post-therapy viral load control in two human subjects [17]. However, both studies were uncontrolled, and the two clinical cases described by Vila *et al.* were associated with high CD4 counts and low viral loads before treatment initiation [17]. A fully controlled animal study employing a therapy containing hydroxyurea administered sequentially in the form of multiple ART/STI cycles strengthened these case reports and showed that post-therapy viral load control could be induced in macaques acutely infected with the HIV homolog SIVmac251 [18]. Of note, in all these studies, apart from the early treatment initiation, hydroxyurea may have played a role in the post-therapy viral load control obtained. Hydroxyurea exerts a cytostatic effect by inhibiting the activity of the ribonucleotide reductase enzyme, thus halting the cell cycle at the G1 phase [19]. This effect may hamper viral reservoir maintenance/expansion in T_{CM} and T_{TM} cells that mainly relies on antigen-driven and homeostatic proliferation respectively [12]. Despite these promising results, combinations of hydroxyurea and antiretroviral drugs displayed in some instances high pancreatic and hepatic toxicity [20,21] and consequently hydroxyurea is not recommended for routine treatment of HIV infection, although there is still ongoing research on this topic [22].

Another uncommon ART regimen administered during early infection yielded promising results in a recent study conducted in macaques infected with different SIV/SHIV strains [23]. In some of these animals, a prolonged (more than 8 years) tenofovir monotherapy proved able to induce a spontaneous control of the infection following the final treatment withdrawal [23]. Apart from the early treatment initiation, this result may be due to an effect of tenofovir in selecting suboptimal drug resistance mutations, and the result may also have been contributed by the additional interventions to which the macaques were subjected during follow-up (temporary depletion of $CD8^+$ cells and treatment at viral rebound).

Treatment during acute infection has provided some amount of clinical success also with more traditional ART regimens [24-30]. News such as the case report of the cure of an ostensibly HIV^+ baby treated in the very early phase of the disease [30] and, more importantly, the results of the ANRS VISCONTI study [29] have been hailed with widespread enthusiasm. Of particular note, up to ≈ 15% of the early treated individuals have been shown to display spontaneous control of viremia following STI [27]. However, the rate of post-therapy control following ART administration during the acute phase may be lower (≈5%) according to another report [28]. Moreover, no definite timing and drug composition has been proven to reproducibly induce post-therapy control even in a minority of patients, and several studies have failed to induce any significant reduction in the post-therapy viral set point following treatment during acute infection [31-33].

Despite these mixed results, the data available indicate that ART administration during acute infection can induce, in a minority of cases, a post-therapy control of the infection which is independent from known favorable genetic backgrounds [29] (Figure 1). Several hypotheses have been postulated to explain the enhanced efficacy of ART treatment during primary infection. The most frequently cited explanations are: 1) the preservation of an efficient immune response [34,35], 2) the induction of a "self-vaccination" after multiple STIs [16,18], and 3) the impairment of viral reservoir formation [27,29,36-38]. A reduced viral reservoir size does not *per se* guarantee successful ART withdrawal [39], but is, even in the most conservative scenario, a promising platform in the quest of a cure. The overwhelming majority of HIV^+ individuals, however, are diagnosed during the chronic phase of the infection, and a large body of evidence shows that STI protocols (even in the form of short "drug holidays") are not effective in improving the course of the disease once the chronic phase is established ([40-42], reviewed in: [43]).

Hit later, hit harder

The mainstream approach to purge the viral reservoirs during chronic infection is a multi-step "shock and kill" therapy [44]. During the "shock" phase, the latent virus harbored in the reservoirs is expected to be pharmacologically reactivated and prompted to resume productive infection. During the"kill" phase, the newly produced virions would be blocked by ART, while the HIV-infected cells are expected to be eliminated by viral cytopathogenicity, or recognized and killed by the immune system. A plethora of compounds have been put forward as candidates to induce the "shock" phase (recently reviewed in:

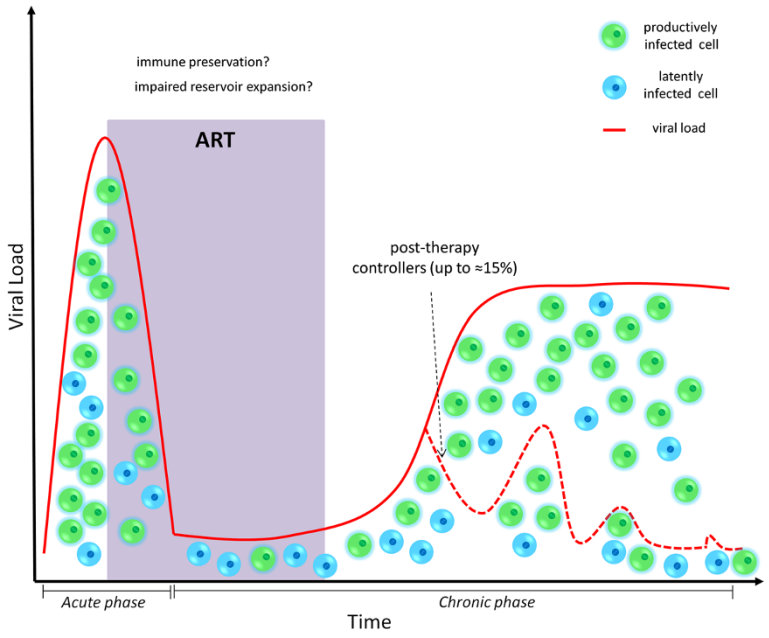

Figure 1 ART administration during acute infection results in spontaneous control of viral load in a minority of individuals following therapy suspension. The dotted line depicts representative viral load dynamics of patients controlling the infection following ART withdrawal, as compared to non controllers (solid line). The upper limit of the percentage of controllers is taken from [27]).

[45,46]). Among these, the most thoroughly investigated are histone deacetylase inhibitors (HDACI's). Several HDACI's (*e.g.* valproic acid, vorinostat, panobinostat) have been tested or are currently under investigation in both pre-clinical studies and clinical trials (reviewed in: [47]). Vorinostat [*i.e.* suberoylanilide hydroxamic acid (SAHA)] was recently reported to have a moderate latency disrupting effect in a group of patients previously selected for the responsiveness of their resting $CD4^+$ memory T-cells to treatment with this drug *in vitro* [48]. However, preliminary data do not show significant effects of vorinostat on viral reservoir size [49,50], while no data on post-therapy viral dynamics are available so far. Moreover, treatment with combined ART/vorinostat regimens on SIVmac-infected macaques led to mixed or disappointing results [51,52]. More data on the *in-vivo* effects of vorinostat will be available from the two ongoing clinical trials investigating the effects of this drug on individuals under ART (NCT01319383, NCT01365065). For the remaining HDACI's only data obtained from cell cultures are available at present [53-55], although panobinostat is currently under investigation in a Phase I/II clinical trial (NCT01680094).

Another approach aimed at HIV reactivation from latency involves the use of cytokines (reviewed in [56]). In particular, the use of IL-7 in combination with ART intensification is currently being investigated (NCT01019551). Unfortunately, in two recent clinical trials, the addition of IL-7 to standard ART protocols did not result in viral reactivation from latency [57], and increased the size of the viral reservoir [58], in line with the well-known effects of this cytokine, favoring homeostatic proliferation of T_{CM} and T_{TM} cells [12,58,59].

Despite the enormous efforts that have been put in the study of HIV reactivating HDACI's and cytokines, the most promising results so far obtained in the quest of a cure for AIDS are not derived from these approaches. The most astonishing result in the field to date, and the first proof of concept for the feasibility of a sterilizing cure during chronic HIV infection, is the case report of the treatment of Mr. Timothy Brown, the aforementioned second "Berlin Patient" [60,61]. Apart from being chronically infected with HIV, this man was diagnosed with acute myeloid leukemia and consequently treated with an aggressive combination of ablative chemotherapy/radiotherapy, immune suppression through drugs and allogeneic stem cell transplantation. Importantly, the donor selected for the transplantation was homozygous for the Δ32 deletion of the *CCR5* gene [60]. This gene encodes for the main coreceptor employed by HIV for entry into cells, and individuals homozygous for the Δ32 deletion (about 1% of the caucasian population) are protected from HIV infection [62]. Following stem cell transplantation, Mr. Brown stopped taking antiretroviral drugs, and has remained off-ART since then, with no signs of disease progression [60,61]. Of note, in spite of an extensive sampling throughout the years, most of the analyses have failed to detect HIV RNA or DNA in blood and tissues, and the HIV-specific antibody titers have steadily decreased over time,

thus hinting that a complete eradication may have been achieved [61,63]. Despite the enormous excitement generated by the news of this cure, the scarcity of HLA-DR-compatible *CCR5* Δ32 donors makes it very difficult to replicate the whole experiment. Consequently, several attempts have been made to isolate the contribution of each of the different therapy components. Allogeneic bone marrow transplantation had been employed for treatment of HIV since the first years of the epidemics (reviewed in [64]) and had been even advocated as a possible curing strategy [65]. The most visible difference between these early attempts and the treatment of Timothy Brown is the favorable genetic background of the cells received by the latter, bearing the homozygous *CCR5* Δ32 deletion. Thus, it is not surprising that many investigators have used this observation as a starting point for further studies. In this regard, a gene therapy approach aimed at disrupting the *CCR5* gene (virtually recreating the Δ32 deletion) is currently under investigation in clinical trials (NCT01252641, NCT00842634). In these studies, the disruption of *CCR5* is performed employing zinc finger nucleases in previously isolated autologous cells that are afterwards re-transplanted in the host. The preliminary results released so far do not allow drawing a definite conclusion on post-therapy viral load dynamics, which seem to be quite variable among study subjects, although post-therapy viral load containment may have been achieved in a small subset of individuals that were heterozygous for *CCR5* Δ32 at baseline [66]. Anyway, the zinc finger treated $CD4^+$ T-cells have been shown to be able to persist in the organism at least one year after the transplant and have had an enhancing effect on CD4 counts in immunologic non-responders [67].

On the other hand, recent data indicate that allogeneic stem cell transplantation may possibly lead to a cure also in the absence of the *CCR5* Δ32 mutation. This is suggested by the outcome of the treatment of two HIV^+ individuals (the "Boston patients") that had received an allogeneic transplantation of stem cells from *CCR5* wild-type donors. After transplantation, while still receiving ART, these individuals displayed a reduction of viral DNA in peripheral blood to undetectable levels [68]. Further investigation proved that viral DNA could not be detected with large scale analysis in PBMCs and in rectal tissue, and thus STI was attempted in both patients [69]. Following ART interruption, no viral rebound was observed, and, despite the relatively short follow-up, the data available suggest that a cure may have been achieved [69]. An important element of the strategy employed for treating the "Boston patients" may have been the long-term ART maintenance following transplantation which may have blocked viral reseeding before the establishment of a full donor chimerism. This aspect differentiates the "Boston patients" from the second "Berlin patient" in which ART was discontinued from the day of transplantation and viral reseeding was likely hampered by the *CCR5* Δ32 mutation of the transplanted cells. On the other hand, a common feature between these case reports is that the "Berlin patient" and the two "Boston patients" were all heterozygous for *CCR5* Δ32 before transplantation [60,68] (see Figure 2). Although their blood cells were fully replaced afterwards (by homozygous *CCR5* Δ32 cells in the "Berlin patient" and by wild type *CCR5* cells in the "Boston patients"), it cannot so far be excluded that their original *CCR5* Δ32 heterozygous status may have played a role in the clinical outcome. It is known that heterozygosity for *CCR5* Δ32 is associated with slower disease progression [62,70] and the results of a recent study conducted in non-human primates suggest that *CCR5* expression levels may be associated with the size of the viral reservoir [71]. Despite the necessity of conducting further studies on this topic, the high treatment-related mortality of allogeneic transplant [72] hampers the use of this technique as an HIV curing strategy, unless salvage therapies are required due to life-threatening comorbidities. On the whole, the studies hereto reviewed support the hypothesis that decreasing the viral reservoir size through drugs, coupled to immune system renovation, may be a key to the achievement of a cure.

Turning back time

From the aforementioned studies, it is evident that the therapies that have resulted in a cure during the chronic phase of the infection bear a much higher degree of risk as compared to the strategies adopted in the acute phase. Thus, the possibility of inducing an acute infection-like scenario in an advanced stage of the disease may represent a unique option to open a new window of opportunity for the therapeutic interventions adopted during the acute phase. A good candidate for this strategy is the gold-based compound auranofin [52] which has been employed for many years in treatment of rheumatoid arthritis [73]. Our group has recently shown the potential of auranofin to act as an anti-reservoir compound *in vivo* when administered to chronically-SIVmac251 infected macaques [52]. Of note, auranofin is able to preferentially induce differentiation/cell death of the memory T-cell compartment including the T_{CM} and T_{TM} $CD4^+$ cells which encompass the main viral reservoir [52] (for a schematic representation of the mechanism of action of auranofin, see Figure 3). Beside its anti-reservoir effect, the addition of auranofin to ART was able to prompt a drastic modification of the post-therapy viral load dynamics in chronically SIVmac251-infected macaques [52,74]. Following treatment interruption, the macaques that had received the ART/auranofin combination displayed a sharp viral load rebound reminiscent of an acute infection peak which was in turn accompanied by an increase in specific immune responses in accordance with the typical acute infection scenario [3,74]. From a therapeutic perspective, treatment with ART/

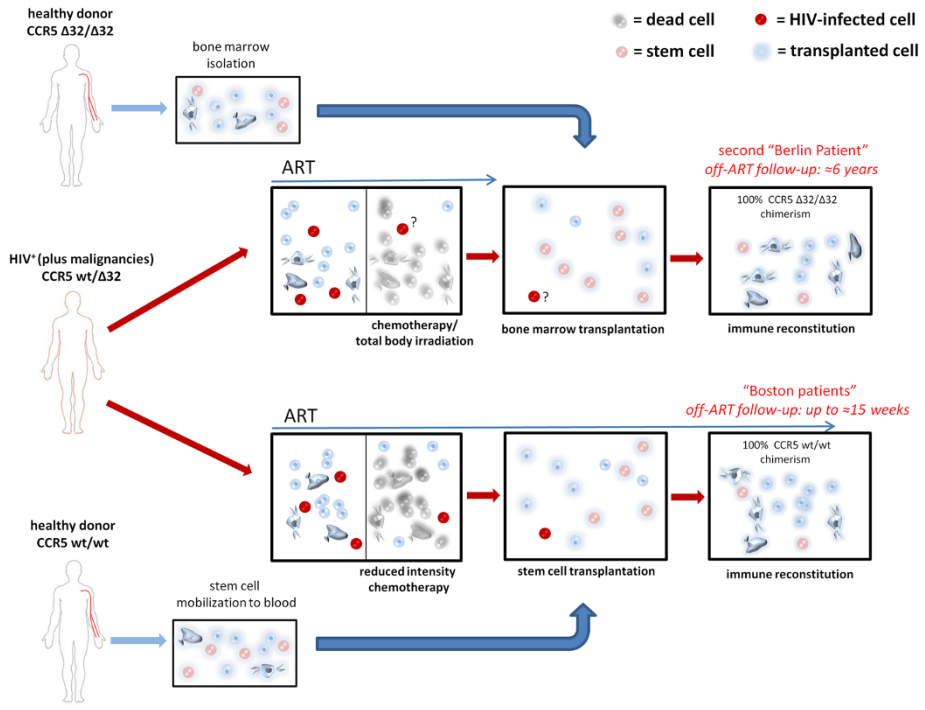

Figure 2 Schematic representation of the therapeutic interventions received by the second "Berlin patient" and the "Boston patients". Although the second "Berlin patient" had received two stem cell transplants, only one is shown for clarity purposes. Note that the length of the arrows indicating the period under ART is meant to provide a qualitative comparison between the ART and transplantation schedules and is not in scale.

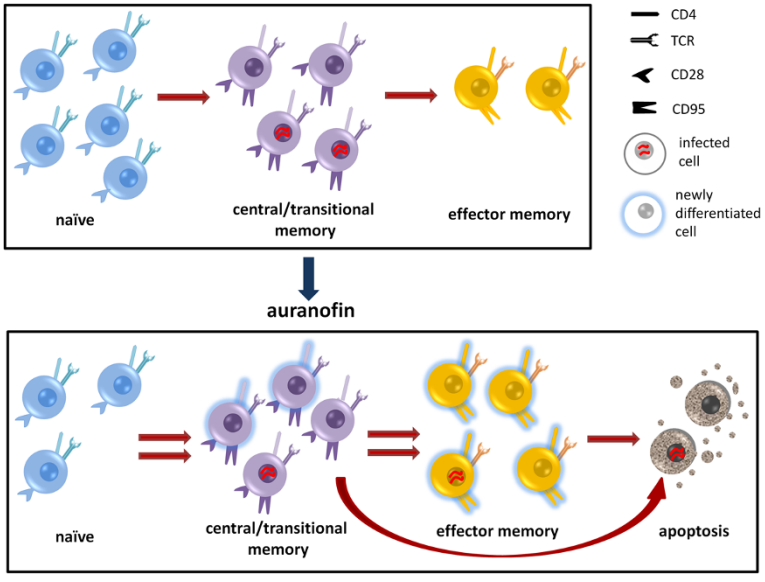

Figure 3 Treatment with auranofin increases the turnover of CD4$^+$ T-cell subsets and induces a partially selective apoptosis of the memory compartment. The cell subsets are identified by the expression of the surface markers CD28 and CD95 (naïve: CD28$^+$ CD95$^-$; central and transitional memory: CD28$^+$ CD95$^+$; effector memory: CD28$^-$ CD95$^+$).

auranofin induced a reduction in post-therapy viral load set point (≈1 Log_{10} viral RNA copies/mL of plasma) [52] and, importantly, treatment during the acute infection-like viral rebound showed the potential to mimic the aforementioned effects of early ART administration [74]. Indeed a short cycle of ART during the acute infection-like peak induced a further reduction in post-therapy viral load set-point [74] and, despite being attempted in a low number of macaques (n = 2), would prove effective in the long-term follow-up [74]. The viral load control induced with this therapeutic protocol may have been contributed to by the previous reservoir reduction prompted by auranofin and by the use of an ART regimen containing maraviroc, which, by blocking CCR5, may inhibit antigen-driven proliferation of the viral reservoir [74]. Although the mechanism behind the drastic modification of the viral rebound pattern induced by auranofin remains partially unclear, its effects on the macaque AIDS model suggest that this drug may offer an attractive possibility of successfully applying, to the chronic phase of the infection, strategies that would have been effective only in the early stages.

Immune enhancement: rejuvenating the immune system?

Enormous efforts have been put in the development of strategies able to boost antibody and/or cell-mediated immune responses against HIV (reviewed in [75]). The curative potential of broad and robust cell-mediated immune responses, in particular by $CD8^+$ T-cells, is suggested by the association of such responses with better disease progression resulting in spontaneous drug-free control of viral load in a minority of individuals [76-79]. Thus, drugs able to bolster immunity against HIV-infected cells could represent an ideal tool for prompting, or supporting, a spontaneous control of the infection [75]. A promising compound for enhancing cell-mediated immune responses against HIV may be buthionine sulfoximine (BSO), a glutathione-depleting agent previously tested for cancer treatment in phase I clinical trials [80]. We recently showed that the addition of BSO to the aforementioned ART/auranofin combination is able to promote a significant and long-lasting enhancement of specific immune responses directed against SIVmac Gag [81]. Boosting immunity against Gag is an attractive achievement because several studies have shown that strong anti-Gag immune responses are associated with low viral loads and high CD4 counts both in macaques and humans [82-86]. Moreover, the results of a recent study suggest that $CD8^+$ T-cells may reduce the viral reservoir by recognizing Gag antigens produced by latently infected resting $CD4^+$ T-cells [87]. In accordance with these studies, enhancement of the immune responses against Gag following suspension of treatment with ART/auranofin/BSO was associated with the obtainment of a functional cure-like condition in a study conducted on a small number of chronically SIVmac251-infected macaques [81].

Partially similar results were obtained using a therapeutic vaccine based on dendritic cells pulsed with whole inactivated virus [88-91]. This vaccine proved able to achieve drug-free control of viral load in a subset of chronically SIVmac251-infected macaques [88] and to induce a reduction of viral load, although moderate, in ART-naïve HIV^+ subjects [89,90]. Moreover, coupling the vaccine administration to ART induced a reduction in post-therapy viral load set point in some individuals [91]. Of note, the highest viral load reductions observed in ART-naïve subjects were associated with high numbers of Gag-specific $CD8^+$ T-cells [89].

A proof-of-concept that strong $CD8^+$ (in particular T_{EM})-mediated immune responses can even lead to viral eradication was recently furnished by a preventive vaccine study conducted on macaques challenged with SIVmac239 [92]. Despite all vaccinated macaques becoming infected following multiple challenges with the virus [93], about half of them proved able to spontaneously control the infection and, strikingly, to get rid of the virus completely in the long run [92]. Interestingly, an involvement of T_{EM} cells was also shown, by multiple correlation analysis, in the effects of the auranofin-based therapeutic approach [52].

On the other hand, antibody-mediated immune responses have also proven the ability of inducing post-therapy viral load control [94,95]. In particular, in the recent study of Barouch *et al.*, a cohort of SHIV(env)-infected macaques was treated with wide spectrum neutralizing antibodies [95]. This treatment produced a functional cure in those macaques starting from viral loads of less than 3.5 Log_{10} viral RNA copies/mL of plasma [95]. Of note, this experiment provides an artificial substitution of a "non-functional" immune system with a surrogate functional immunity, *i.e.* the passive antibody transfer. The capability of adoptive antibody transfer to induce a functional cure only in those macaques displaying low baseline viral set points, supports the view that a limited viral reservoir should accompany the immune system renovation.

Finally, also the effects of transplantation strategies on viral load control may be associated with enhancement of immune responses. A study by Villinger *et al.* conducted in chronically SIVmac239-infected macaques showed that adoptive transfer of activated autologous $CD4^+$ T-cells may result in spontaneous post-therapy control of the infection [96]. This approach can hardly be employed in humans since it requires cells isolated before the infection, but it suggests that renovation of the immune system is important for obtaining effective immune responses [96]. Of note, autologous stem cell transplantation did not result in a cure of HIV^+ individuals [97], suggesting that cells isolated following infection may not be apt to prompt immune enhancement. Instead, the likely cures observed

Table 1 Summary of the main characteristics of the therapeutic strategies described in this review

	Notable Results	Stage	Safety	Scalability
ART during acute infection	Long-term post-therapy viral load control in a minority of individuals [16,17,23-30].	Clinical/pre-clinical	High	Low (few patients are detected HIV^+ at acute infection)
Viral reactivation with HDACI's	Possible disruption of latency [48,49,53-55]. No viral reservoir reduction [49].	Clinical/pre-clinical	Medium	High
Viral reactivation with cytokines	IL-7 might disrupt latency but replenishes the viral reservoir [57-59].	Clinical	Medium	Medium/high
Gene therapy for disruption of *CCR5*	Mixed impact on viral load (depending on the genetic background) [66]. Possible immunologic improvement [67].	Clinical	Medium (long-term effects unknown)	Very low
Allogeneic stem cell transplant	Likely sterilizing cures in the second "Berlin Patient" [60,61,63] and in the "Boston Patients" [68,69].	Clinical	Very low	Very low
Addition of auranofin and BSO to ART	Long-term post-therapy control in chronically SIVmac251 infected macaques [74,81].	Late pre-clinical	Medium/high (good safety profile for individual drugs in humans)	High
Therapeutic vaccine with whole virus-pulsed dendritic cells	Post-therapy viral load control in a subset of macaques [88]. Viral load and viral load set-point reduction in a subset of ART-naïve [89,90] and ART-treated patients [91], respectively	Clinical	High	Medium
Administration of broadly neutralizing antibody/ies	Long-term post-therapy control in chronically SHIV(env) infected macaques starting from low viral loads [95]	Late pre-clinical	High	High

In square brackets are the references describing the main results for each strategy.

following allogeneic transplantation in the "Boston patients" [69] may have been induced or facilitated by the strong immune responses resulting from graft versus host disease, exacerbated by a partial HLA donor/receiver mismatch in one of the two patients, which may have played a critical role for the elimination of the viral reservoirs [68].

Conclusions

The studies reviewed herein indicate that curing, and even eradicating primate lentiviruses, including HIV-1, could be possible, at least in certain cases (see Table 1). However, it is important to point out that the majority of the "functional" cures that have so far been reported have been obtained during the acute phase or a short time thereafter, *i.e.* at a time in which the viral reservoir and the immune damage are still limited. The design of future therapeutic strategies should address the chronic phase of the disease, affecting the large majority of the HIV^+ individuals. The most successful approaches tested so far, though still preliminary and/or based on a small number of cases, strongly suggest that the path to a cure involves two key players: the viral reservoir and the immune system. In the typical scenario of chronic infection with an average/large viral reservoir and an impaired immune system, some of the approaches that have been successful showed the ability to target both the viral reservoir and the immune system through gradual (auranofin) or abrupt (chemotherapy/allogeneic transplant) immune system renovation, followed by enhanced immune responses either against conserved viral antigens (auranofin + BSO) or the host's infected cells (graft versus host disease). In this regard, further studies, fully controlled and with larger number of subjects, will be required to assess the curative potential of the aforementioned strategies. The ultimate goal will be to obtain with scalable drug combinations, the cure that has been induced with more aggressive approaches.

Abbreviations

ART: Antiretroviral therapy; STI: Structured treatment interruption; HDACI: Histone deacetylase inhibitor; SIV: Simian immunodeficiency virus; SHIV: Simian/human immunodeficiency virus; BSO: Buthionine sulfoximine; T_{CM}: T central memory; T_{TM}: T transitional memory; T_{EM}: T effector memory.

Competing interests

The Istituto Superiore di Sanità has requested patent rights on the use of the auranofin/BSO combination for treatment of HIV/AIDS.

Authors' contributions

AS and ILS developed the overall theory disclosed in this review, analyzed the literature and drafted the manuscript. ILS drew the original figures. All authors read and approved the final manuscript.

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