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REVIEW

The Long and Winding Road Towards an HIV Cure

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SUMMARY

In the summer of 1981, a new deadly disease suddenly emerged targeting young men having sex with men (MSM); three years later, a new virus, an exogenous human retrovirus, later named human immunodeficiency virus (HIV), was demonstrated to be the causative agent of the new disease, the Acquired Immuno-Deficiency Syndrome (AIDS), affecting, in addition to MSM, also intravenous drug users, hemophiliacs, heterosexual individuals and children born to infected mothers. AIDS remained a dead sentence for >95% infected individuals until 1996 when the first combination antiretroviral therapy (cART) was shown to be effective saving the lives of countless people. Since then, cART has become extremely powerful and simpler to adhere (now down to one or two pills a day). However, virus eradication ("Cure") has been achieved thus far only in two individuals as a result of stem cell transplantation by an immunologically compatible donor homozygote for the CCR5Δ32 mutation; CCR5 is indeed the major entry coreceptor for the virus together with the primary receptor CD4. This represents the exception to the rule that none of the many experimental attempts of eliminating or silencing the virus reservoir unaffected by cART has achieved a significant proof of concept. In this article we will describe the essential aspects of the viral reservoirs and the current strategies to tackle it.

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INTRODUCTION

40 years of HIV/AIDS (Table 1)

In 1981 a bulletin from the CDC described the unusual observation of multiple casualties among young homosexual men living in the Los Angeles area due to an interstitial pneumonia caused by an "innocent" agent (*pneumocystis (p.) carinii*, later renamed as *p. jirovecii)* that rarely causes health problems in immunocompetent individuals (Centers for Disease 1981). Other opportunistic infections or cancer, including a surprisingly frequent detection of Kaposi'sarcoma of the skin, characterized these casualties in association with an unprecedented, extreme contraction of the CD4+ subset of circulating T lymphocytes (Masur *et al*., 1981).

It became rapidly clear that the new disease was infectious in nature and was transmitted by the sexual route (by and to both homo- and hetero-sexual persons) or by contaminated blood and blood products (such as factor VIII concentrates to hemophiliacs). In 1983, a research team from the Pasteur Institute in Paris, France, firstly identified a type C retrovirus in a cell culture established from a lymph node of an infected individual (Barre-Sinoussi *et al*., 1983), a study that was later judged worthy of the Nobel prize in 2008. In 1984, several papers published by Robert C. Gallo of the National Cancer Institute in Bethesda, MD, USA provided the definitive evidence of a causative link between this newly identified virus [later defined as the human immunodeficiency virus (HIV)] and the deadly disease, the acquired immunodeficiency syndrome (AIDS) causing death of >95 infected individuals in ca. 8-10 years (Gallo *et al*., 1984). In the same year, CD4 was demonstrated to be the entry receptor for HIV (Dalgleish *et al*., 1984; Klatzmann *et al*., 1984) thus explaining the selective and progressive disappearance of the CD4+ subset of circulating T lymphocytes known to play a role as "orchestra director" of the immune system.

The first antiretroviral agent, Zidovudine, an inhibitor of reverse transcriptase, the very enzyme peculiar of retroviruses that converts the virion RNA genome into a DNA version competent to integrate into the host cell chromosomes, was the first (Mitsuya *et al*., 1985) of many agents that, finally, in 1996, when used in combination proved to be very effective in blocking virus replication and disease progression (Palella *et al*., 1998).

Also in 1996, the second entry receptors for the virus, two chemokine receptors, i.e. CCR5 (Alkhatib *et al*.,

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Table 1 - *Milestones in HIV research.*

Year	*Milestone*	*Refs*	*Note*
1981	Description of first AIDS cases	(Centers for Disease 1981)	
1983	Identification of a retrovirus as potential cause of AIDS	(Barre-Sinoussi *et al*., 1983)	Causative link between the virus and AIDS was established by R.C. Gallo (Gallo *et al.,* 1984)
1984	Discovery of CD4 as primary entry receptor for HIV	(Dalgleish *et al.,* 1984; Klatzmann *et al*., 1984)	
1985	Description of anti-HIV properties of AZT	(Mitsuya *et al*., 1985)	AZT monotherapy was soon abandoned, but it successfully prevented mother to child HIV transmission (Centers for Disease and Prevention 1994)
1996	Effectiveness of cART	(Palella *et al.*, 1998)	cART was earlier defined as "highly active antiretroviral therapy (HAART)"
1996	Discovery of CCR5 and CXCR4 as entry co-receptors for HIV	(Alkhatib *et al*., 1996; Feng *et al.,* 1996)	In December 1995, the HIV inhibitory capacity of chemokines later discovered to bind to CCR5 was reported (Cocchi *et al.,* 1995)
1996	Discovery of the protective effect of *ccr5Δ32* genetic deletion on HIV acquisition and disease progression	(Liu *et al*., 1996)	*ccr5Δ32*/*ccr5Δ32* homozygosis protects from infection, *ccr5Δ32*/*ccr5* heterozygosis is linked to a long-term natural control of HIV disease progression (Tamalet *et al.,* 2021)
1997-1999	Description of "resting memory" CD4+ T cells as cART-resistant HIV reservoir	(Finzi *et al*., 1999)	
2009	First and only partially successful preventative vaccination	(Rerks-Ngarm *et al*., 2009)	The RV144 trial in Thailand reported ca. 30% protection from HIV acquisition during the first 3 years post-vaccination
2009	Successful "HIV cure" by transplantation of stem cells from a *ccr5Δ32* homozygous donor	(Hutter *et al*., 2009)	The patient was *ccr5Δ32*/*ccr5* heterozygote, received total body irradiation and two rounds of stem cell transplantation
2019	Second case of "HIV cure" by transplantation of stem cells from a *ccr5Δ32* homozygous donor	(Gupta *et al*., 2019)	The patient was *ccr5*/*ccr5* homozygote, did not receive total body irradiation and had a single round of stem cell transplantation

1996) and CXCR4 (Feng *et al*., 1996), were discovered and provided a clear picture of how the virus evolved within a single individual and was transmitted to others. Most infections were caused by viruses with using CCR5 together with CD4 for cell entry whereas usage of CXCR4 were inefficiently transmitted to others while they emerge in ca. 50% individuals infected by subtype B HIV (dominant in Europe, North America and Australia) in late-stage disease. A homozygotic deletion mutation in the *ccr5* gene (*ccr5Δ32*) explained why certain individuals were surprisingly resistant to HIV infection (Liu *et al*., 1996) in spite of multiple sexual exposures whereas *ccr5*/*ccr5Δ32* heterozygosis was associated with a "long-term non-progressor (LTNP)" phenotype, i.e. people infected who showed an unusually long spontaneous control of disease progression, sometimes extended for decades after infection (Tamalet *et al*., 2021). These observations will be a clue for the few success stories of virus eradication, as later discussed.

The success of cART, that have become more and more potent and easy to adhere over the years, is contrasted by the two major failures in fighting the HIV pandemics: the lack of a preventative vaccine and the need to stay on cART in order to maintain control of virus replication. Concerning this second problem, it has become clear that the major obstacle is represented by a relatively small pool of infected cells carrying integrated, replication-competent proviruses that are capable of rekindling virus spreading soon after cART is suspended (International *et al*., 2012; Deeks *et al*., 2021). This obstacle has been named "the (pro)viral reservoir" and it will be the subject of this article.

One or more HIV reservoir(s)?

In the same years of cART breakthrough, the first evidence that a tiny pool of infected CD4+ T cells were persisting in individuals receiving the antiviral agents was published by Robert Siliciano and colleagues from the Johns Hopkins University in Baltimore, MD, USA (Chun *et al*., 1997; Finzi *et al*., 1997). At first, this observation was overlooked for the excitement of the discovery of cART that was firstly predicted to lead to virus eradication (by the combined effect of immune restoration and spontaneous death of residual infected cells). Unfortunately, this optimistic view was soon replaced by the evidence

that cART suspension, regardless of its composition and duration, lead to almost universal rapid rebound of virus replication to pre-therapy levels along with disease progression (Davey *et al.*, 1999). This hard lesson shifted the research community back to focusing on the HIV reservoir, its cellular composition and molecular features in perspective to identify ways to eliminate, curtail or control it.

The best characterized reservoir of replication-competent HIV are CD4+ T lymphocytes surviving virus-induced cytopathicity. As the seminal work of Robert Siliciano and others, including Tae-Wook Chun (who was initially part of Siliciano's team and then moved to Anthony S. Fauci's laboratory), demonstrated these cells bear a "resting memory" phenotype implying a return to a homeostatic phase after their activation, likely caused by HIV infection itself (Finzi *et al.*, 1999). Within these cells, further studies have further dissected out subsets that were quantitatively more represented than others; among others, T helper follicular cells (Tfh), that participate to the formation of germinal centers of lymph nodes and other secondary lymphoid organs in close contact with B lymphocytes, are characterized by a superior resistance to virus-induced cytopathic effects and possess the highest levels of infection (Perreau *et al.*, 2013). The persistence of a resting memory T cell reservoir is supported by at least to independent mechanism: homeostatic proliferation in response to self-produced cytokines such as interleukin-7 (IL-7) and cell stimulation by low levels of antigens (Chomont *et al.*, 2009).

More recently, another mechanism of T cell reservoir maintenance and potential expansion was uncovered. As a consequence of the integration site of HIV proviruses in "DNA hot spots" frequently associated with protooncogenes the infected T cells can undergo clonal expansion thereby accounting for a substantial fraction of the viral reservoir and with the peculiarity that all cells of the individual clone bear the same integration site and general molecular features (Maldarelli *et al.*, 2014; Wagner *et al.*, 2014). Furthermore, a study from our institute has indicated the possibility that these integrated proviruses could generate chimeric transcripts and proteins capable of influencing the functional status of the infected cells, for example by favoring their differentiation towards a T regulatory (i.e. immunosuppressive) phenotype (Cesana *et al.*, 2017).

Siliciano's observations were published after several years of *in vitro* research on surrogate models of HIV latency and persistency in different cell types following infection. The earliest models were cell lines of both T cell and myeloid origin that survived the cytopathic effects of acute *in vitro* infection. As cell lines rarely express CCR5, most these models were generated with laboratory-adapted viral strains using CXCR4 as entry co-receptor. Research conducted in the late '80s in Anthony Fauci's laboratory at the National Institute of Allergy and Infectious Diseases (also directed by Fauci) by Thomas Folks, myself and others lead to the development of ACH-2 and U1 cell lines as models of reversible latency of T lymphocytic (Rodari *et al.*, 2022) and myeloid cells (Poli 2022), respectively. They were very useful and convenient tools, on the one hand, to delineate the earliest molecular features associated with latent and transcriptionally active proviruses and, on the other hand, to establish functional links between pharmacologic and immunologic agents, such as pro- and anti-inflammatory cytokines, and modulation of the state of latent vs. productive infection. Several molecules discovered with these and similar cell lines were also validated in primary cell models of *in vitro* infection, as reviewed in (Cassol *et al.*, 2006).

The existence of one or more "non-T cell" reservoir could be inferred by the simple observation that AIDS patients in the pre-cART era frequently had undetectable circulating CD4+ T cells reflecting *bona fide* also their profound depletion in tissues. Yet, they usually showed high levels of viremia and clear evidence of the consequence of virus replication in terms of disease progression. Where did the virus come from? Which was the "non T cell" reservoir of newly as well as chronically infected cells? In this regard, it is worthy to underscore that lentiviruses, the genus of retroviruses to which HIV belongs to, are characterized by a general tropism for myeloid cells (Sattentau and Stevenson 2016). The evolutionary "choice" of CD4 as primary entry receptor has caused an expansion of the tropism of HIV to a subset of T lymphocytes causing the profound immunodeficiency observed in individuals not treated with cART. There is evidence in animal models that the experimental depletion of CD4+ T cells leads to a significant "viral jump" into tissue macrophages that become the major source of virus production, likely mimicking the status of AIDS patients depleted of CD4+ T lymphocytes (Micci *et al.*, 2014).

A central role of tissue-resident as well as of monocyte-derived macrophages (MDM) in the pathogenesis and as reservoir of persistent HIV in individuals under cART is highlighted in particular by the microglia in the central nervous system (CNS). Virus replication in the brain of AIDS patients with HIV encephalitis, causing a clinical condition known as AIDS-associated dementia, was early and clearly visualized in the microglia with negligible contribution of CD4+ T cells (Gartner *et al.,* 1986; Koenig *et al.*, 1986). Even in a significant fraction of individuals with viremia fully controlled by cART there is imaging-based evidence of virus replication and/or virus-associated neuroinflammation in their CNS (Kugathasan *et al.*, 2017), an observation corroborated in non-human primates (NHP) (Gama *et al.*, 2017).

Apart from the CNS, clear-cut evidence that tissue macrophages are a relevant source of replication-competent virus in cART suppressed individuals was recently published by studying the penile tissue of individuals undergoing surgery for sexual reassortment purposes. Evidence of active virus production in spite of cART were observed in periurethral tissue macrophages whereas no evidence of a relevant contribution of T cells was collected (Ganor *et al*., 2019). Furthermore, the infected macrophages showed morphological evidence of virus accumulation in peculiar subcompartments defined as "virus-containing compartments (VCC)" (Tan and Sattentau 2013), a distinctive feature of HIV infection in macrophages that will be further discussed.

Other potentially relevant "non-T cell" reservoirs in cART-treated individuals include myeloid dendritic cells and CNS astrocytes, as discussed elsewhere (Li *et al*., 2016; Mitchell *et al*., 2019).

A Trojan horse hypothesis for HIV-infected macrophages

There are additional elements to consider macrophages as a "first class" HIV reservoir like CD4+ T cells. These include the observation of their natural resistance to virus-induced cell death, as observed *in vitro*, in relevant animal models [including NHP infected with SIV (Micci *et al*., 2014) and immunodeficient mice reconstituted with human progenitor cells - (Honeycutt *et al*., 2017)] and, ultimately, in AIDS patients with near complete depletion of their CD4+ T cells (Masur *et al*., 1981).

A second peculiar feature of HIV infection of macrophages is the development of VCC as major hidden source of new progeny virions, a feature that characterizes their infection both *in vitro* and *in vivo*, as in the cited case of periurethral macrophages (Ganor *et al*., 2019) or of microglia in the brain of AIDS patients (Tan and Sattentau 2013). VCC were originally interpreted as an expansion of the Golgi apparatus (Folks *et al*., 1988), but they were more recently defined on the basis of specific markers as invaginations of the plasma membrane, eventually detaching their connection with the outer environment (Tan and Sattentau 2013; Graziano *et al*., 2016).

We originally reported that VCC were functionally regulated and could be expanded by cell stimulation with the pro-inflammatory cytokine interferon-γ (IFN-γ) using the promonocytic U1 cell line stimulated by phorbol esters to differentiate into macrophage-like cells (Biswas *et al*., 1992). Subsequently, different stimuli could lead to an increase of VCC with the concomitant reduction of virions associated with the external plasma membrane, as reviewed in refs (Cassol *et al*., 2006). More recently, we have reported that short-term stimulation with extracellular adenosine triphosphate (eATP), a well-known pro-inflammatory molecule, lead to the rapid discharge of VCC-accumulated virions both in PMA-differentiated U1 cells and in primary MDM without causing significant cell death (Graziano *et al*., 2015). eATP acted via its main purinergic receptor P2X7, as shown by specific pharmacologic inhibitors, while the antidepressant agent imipramine, known to suppress the generation of extracellular vesicles from the plasma membrane, prevented the eATP-mediated release

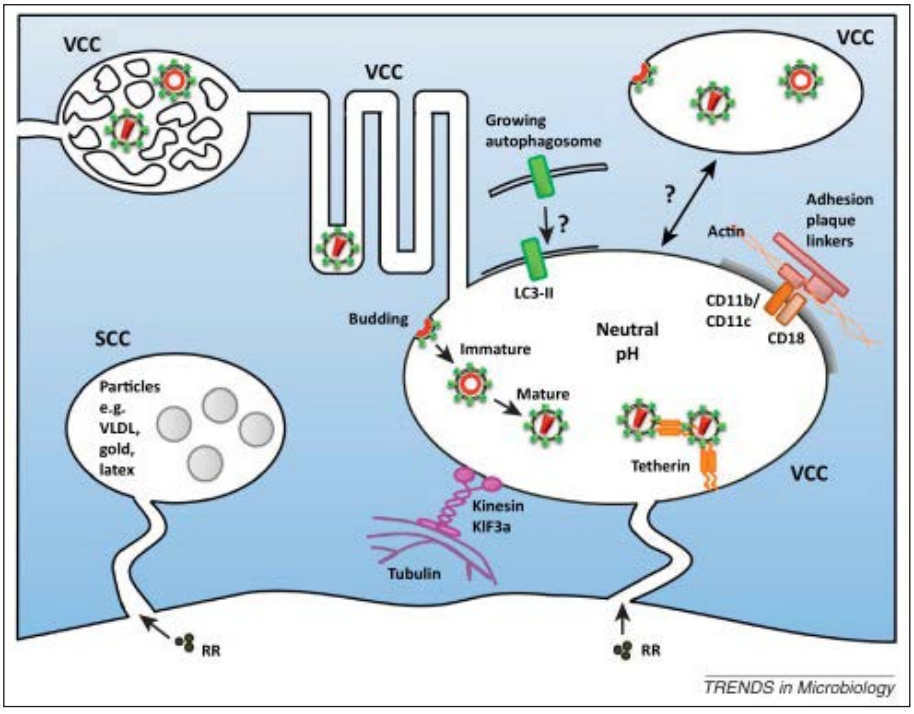

Figure 1 - Virus Containing Compartment in HIV-infected Macrophages.
The VCC is a structure generated by invaginations of the plasma membrane in macrophages where HIV-1 virions are assembled and stored; both surface-accessible and enclosed VCCs appear to be present. Modified from (Tan and Sattentau 2013).

of virions (Graziano *et al.*, 2015). Thus, the VCC of macrophages is a "druggable" target (Graziano *et al.*, 2016) that should be considered for clinical studies aiming at dissecting out the contribution of macrophages to the overall viral reservoir.

"Shock and kill" vs "block and lock" strategies to achieve a functional cure of HIV infection in cART-treated individuals

After the demonstration that cART was ineffective in even reducing the size of the viral reservoirs there has been a substantial international effort to tackle it by a strategy commonly referred to as "Shock (or "kick") and kill". The theoretical basis of this strategy is the stimulation *in vivo* of persistently infected CD4+ T cells with well-characterized pharmacological agents effective *in vitro* in reversing the proviral status from latent to productive ("shock phase"). In order to protect potential target cells from the infection by the newly released virions (at least some of which credited to be replication-competent) cART would be maintained. The "kill" phase was originally hypothesized to occur either simply as a consequence of virus production and/or by means of the restored immune response, particularly from CD8+ T cells endowed with cytotoxic capacity (CTL, cytotoxic T lymphocytes) (Cohn *et al.*, 2020). Unfortunately, this theoretical approach has not (yet) produced relevant results; the main reasons being:

1. Significant toxicity of the latency-reversing agents (LRA) not allowing a "proof of concept" of their efficacy *in vivo*.
2. No evidence of reduction of the latent pool of infected CD4+ T cells following distinct protocols involving different LRA.

Apart from these discouraging results, some corollary interesting evidence was nonetheless accumulated. The observation that latently infected cells would not die just as a consequence of virus expression was earlier demonstrated by our group in the

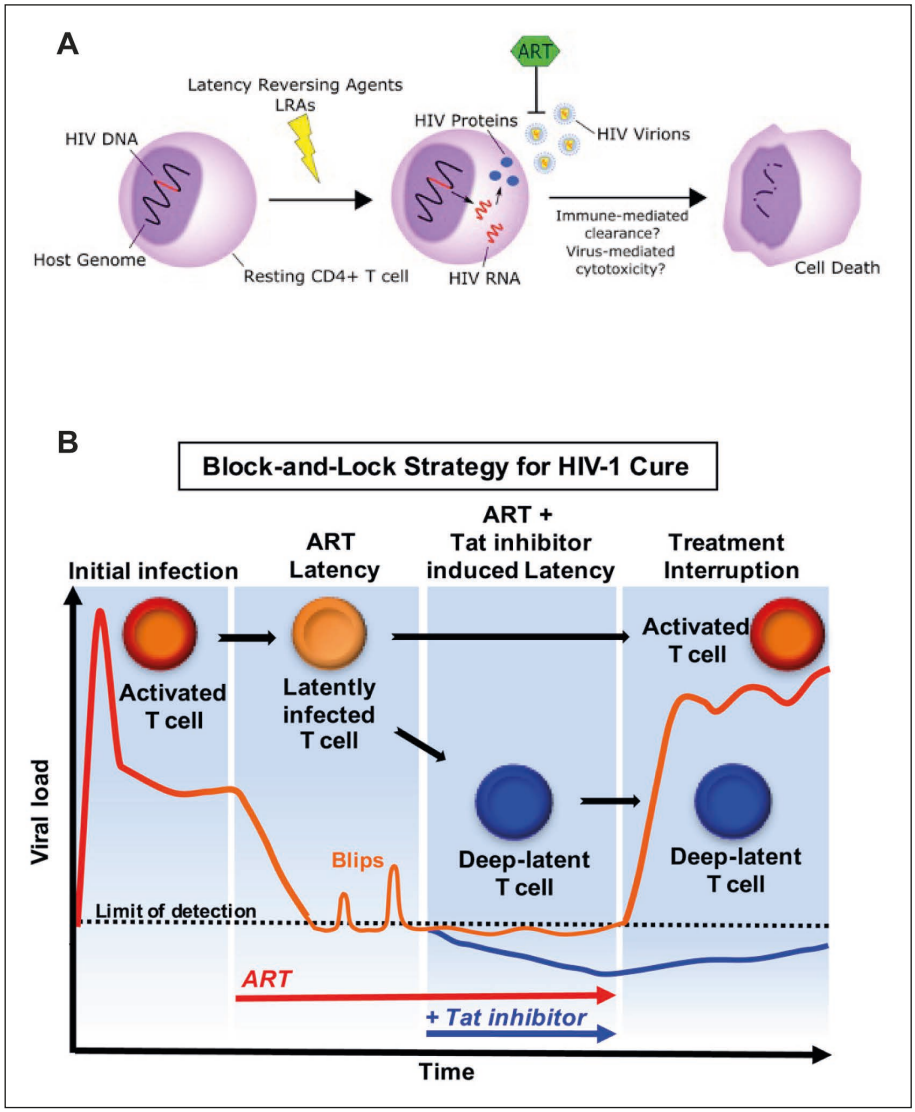

Figure 2 - Opposite Strategies to Eliminate HIV Latently Infected Cells.
A. *The "shock and kill" strategy uses LRAs to increase HIV transcription, protein expression, and virion production. The "kill" phase is conceived to be carried out by CTL or other cytotoxic cells. LRAs, latency reversing agents; ART, antiretroviral therapy. Modified from (Kim et al., 2018).*
B. *Block and Lock strategy for HIV-1 Cure, after: (Kessing et al., 2017).*

case of persistently infected cell lines (Biswas *et al*., 1994) and confirmed in more relevant primary T cell infected either *in vitro* or purified from infected individuals (Huang *et al*., 2018). Elegant publications demonstrated that elimination *ex vivo* of "shocked" cells required the presence of CD8+ CTL. However, most of the eliminated cells were harboring defective proviruses, whereas those with replication-competent proviruses were resistant (for unclear reasons) (Huang *et al*., 2018; Board *et al*., 2021).

An alternative approach was also developed that aimed at maintaining latently infected CD4+ T cells in their molecular state indefinitely, making them insensitive to potential LRA. The assumption at its basis was that harboring a few infected cells incapable of spreading their virus would equal to a state of "functional cure", i.e. the impossibility of HIV to spread in the infected person and, consequently, to others without requiring cART. At present, this hypothesis is mainly supported by a pharmacologic agent, Didehydro-Cortistatin A, that blocks the transcriptional activity of the regulatory viral protein Tat (Mousseau *et al*., 2015; Mediouni *et al*., 2019). However, other compounds are in the line to further substantiate this approach, as reviewed (Mori and Valente 2022).

In summary, we are still far away for a response to the question of whether viral eradication ("cure"), or at least a "functional cure", will become a complementary modality to treat infected individuals rendering them free from the chronic need to take cART.

From the "Berlin patient" to the "London patient". Proof of concept of a functional cure

A breakthrough observation was published in 2009 [the same year of the publication of the results of the only partially successful preventative vaccination trial, the RV144 study - (Rerks-Ngarm *et al*., 2009)] *(Table 1)*. A US citizen, Timothy Ray Brown, living in Germany, received two rounds of stem cell transplantation to cure a leukemia that was resistant to conventional therapy; the treatment was preceded by total body irradiation. The novelty was represented by the fact that the donor of stem cells was not only HLA-compatible, but was also naturally lacking CCR5 expression being homozygous for the CCR5Δ32 deletion mutation whereas the patient was heterozygous for this genetic condition. cART was interrupted before the intervention and was never resumed *et al*., 2009). Deep investigation in Tim Brown's (that self-defined himself as "the Berlin patient") tissue and organs (including a brain biopsy) failed to reveal the presence of infected cells. He lived "HIV-free" without need of cART until 2020 when he died because of a relapse of his original malignancy.

For several years, the key question in the scientific community was whether Tim Brown was just a lucky case, essentially irreproducible. The hypothesis that a central reason of this individual success was actually linked to the heavy total body irradiation was formally proven untrue. Finally, a second case, "the London patient", affected by a Hodgkin lymphoma requiring stem cell transplantation reproduced what observed with the Berlin patient (Gupta *et al*., 2019). There are important differences between the two patients, as highlighted in the *Table 1*. The fact that total body irradiation was not necessary and that the "London patient" was homozygote for *ccr5* excludes that these two variables are crucial to achieve a success.

Other candidate patients are being observed to understand whether this approach may be indeed extended to others, although highly selected based on their clinical condition.

CONCLUSIONS

Lessons learned and perspectives

It is difficult to predict whether the search for a functional cure of HIV infection that would unleash the requirement for chronic cART will ever achieve its goal (Dybul *et al*., 2021). The extreme potency coupled with the easiness of cART (down to 1-2 pills a day, like statins for cholesterol control or low-dose aspirin for prevention of cardio-vascular events) and the availability of long-term acting antiretrovirals may relegate the search for a cure in a niche of basic and translational research.

In this article, we did not discuss some extreme phenotypes related to spontaneous viral control after suspension of cART, globally referred to as "post-treatment controllers (PTC)", as reviewed elsewhere (Schwarzer *et al*., 2020), and individual cases where the viral reservoirs seem to have been spontaneously cleared (Jiang *et al*., 2020; Turk *et al*., 2022). Furthermore, the few examples of "cure" provided by the two transplanted patients highlights the centrality of CCR5 in this disease and may lead to a therapeutic approach that could mimic the results of stem cell transplantation without actually requiring its execution (Dybul *et al*., 2021).

Note

At the time of writing this article, a third person has been reported to be "cured" by transplantation of umbilical cord blood derived stem cells, although no scientific articles have been published yet.

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