Establishment of latent HIV-1 reservoirs: what do we really know?

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Abstract

Despite our ability to suppress HIV-1 replication indefinitely in people on optimal combined antiretroviral therapy (cART), HIV-1 persists as a stably integrated and replication-competent provirus in a heterogeneous collection of long-lived cells (often referred to as 'latent reservoirs') in all individuals on treatment. Reactivation of these latent proviruses is believed to be responsible for the rebound viraemia that can be seen in nearly all people following treatment cessation. Hence, the persistence of HIV-1 in latent reservoirs remains one of the greatest challenges in current HIV cure research. Latent HIV-1 reservoirs are established early during the acute phase of the infection, possibly before the virus appears in the systemic circulation. As well as the issue of timing, we review the proposed hypotheses on the mechanisms by which this latent state is believed to be established early in the course of the infection and the effect of early initiation of cART on the size and stability of these reservoirs.

We conclude that prevention of the establishment of latent HIV-1 reservoirs by even extremely early initiation of cART proves to be practically impossible. However, early treatment initiation remains one of the crucial interventions needed to achieve the ultimate goal of a functional cure for HIV-1 infection because of its ability to reduce the overall size of HIV-1 reservoirs. Together with other interventions, early cART initiation may thus eventually lead to a state of better control over the residual amount of virus in the body, allowing people to stay off treatment for prolonged periods of time.

Introduction

The implementation of combination antiretroviral therapy (cART) has led to a dramatic improvement in outcomes for people living with HIV, turning a previously life-threatening illness into a chronic condition [1].

Despite its great success, it remains difficult to provide this revolutionary therapy to all people living with HIV, especially to those in resource-limited settings. This is particularly problematic given the need for strict therapeutic adherence. Taken together with the possibility of drug toxicity, drug–drug interactions, persistent immune dysfunction and ongoing low-grade inflammation in people on therapy, there is a growing interest in finding a cure for HIV rather than control of HIV replication [2]. The report of the Berlin patient, who is considered to be cured, and some cases of long-lasting remission have strongly fuelled the ongoing research in this field [3–6].

Indeed, cART is very effective in suppressing plasma virus levels to below the limit of detection of clinical commercial assays (20–50 copies HIV-1 RNA/mL), yet fails to be curative as viral loads rebound quickly upon cART cessation, typically within 2 weeks. This makes lifelong treatment an absolute necessity and the current standard of treatment for every individual living with HIV [7,8]. The main reason for viraemic rebound after cART cessation is believed to be the presence of stable reservoirs of HIV-1 provirus in resting CD4 T cells, even in patients on long-term cART [9–11].

The main focus of recent research has been towards eliminating viral reservoirs via a strategy called the 'shock and kill' [12]. Briefly, latent proviruses are reactivated with latency-reversing agents, restarting viral replication in the infected cell. This should lead to the elimination of these cells through viral cytopathic effects or by immune-mediated killing as they now express viral proteins. So far, attempts to purge the reservoir in this fashion

have been largely unsuccessful, since both parts of the strategy, shock and kill, still need optimising [13]. At the same time, other creative strategies (e.g. 'block and lock', which aims to create a permanent nonproductive state of infection) are also being investigated [14].

In order to optimise these approaches and to create new strategies to target viral reservoirs of HIV-1, a more thorough understanding of their biology, composition, distribution and dynamics will be of the utmost importance. We review the evidence on the establishment of latent HIV-1 reservoirs during the acute phase of the infection.

Background

HIV reservoirs

The term 'latency' in virology usually refers to a nonproductive state of infection of a pool of cells that are transcriptionally silent, but retain the capacity to produce infectious virus particles upon stimulation [15]. This is different from 'clinical latency', a term formerly used to describe the clinically asymptomatic phase following the acute infection with HIV-1 and before the development of AIDS.

A reservoir is defined as 'a cell type or anatomical site in which a replication-competent form of virus accumulates and persists' [16], in spite of long periods of cART-suppressed viraemia [15]. Since various types of cells in different anatomical locations can fulfill these criteria, it is more accurate to consider HIV reservoirs in a plural form. Of note, this definition does not include most integrated HIV-1 genomes, as many are defective [17]. Evidence seems to indicate that even on suppressive cART there may be some ongoing active viral replication, in selected patients and/ or in certain sanctuary sites [15]. These reservoirs may significantly contribute to the problem of viral persistence on therapy, but they do not qualify as 'latent' reservoirs in the stricter sense. As such, we will not discuss them in great detail.

It should also be noted that, since the focus of our review was on the timing of reservoir establishment, we did not perform a

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systematic review of the literature on cellular and anatomical reservoirs and sanctuary sites for HIV infection. When considering HIV infection, timing and location of reservoir formation cannot be separated, hence, we will provide a short overview of the current knowledge on cellular and anatomical HIV reservoirs (including possible sanctuary sites). For more extensive reviews we refer to [18–22].

Cellular reservoirs

HIV primarily infects cells of the immune system and in particular CD4 T cells. They also remain the best characterised reservoirs for HIV-1 in virally suppressed patients on cART [18–20]. T cells are classified according to their differentiation and memory status, or their effector functions, based on the expression of cell surface markers [18,19,23]. Naïve CD4 T cells are produced in the bone marrow and move to the thymus where they undergo antigenic selection. After stimulation, these cells proliferate and differentiate into active, specialised effector cells, including helper T cells (TH1, TH2, TH17 and TH9), follicular T helper cells (TFH) and regulatory T cells [18,23]. These activated CD4 T cells are the preferential targets for productive HIV-1 infection but they have short lifespans owing to cytopathic effects of the virus or cytotoxic T lymphocyte-mediated killing [24]. However, some effector cells can revert to a resting state and differentiate into long-lived memory CD4 T cells [25]. Different types of memory CD4 T cells exist : stem cell memory (TSCM), central memory (TCM), transitional memory (TTM), effector memory (TEM), migratory memory (TMM), tissue resident memory (TRM) and terminally differentiated (TTD) cells [18,19,23]. Studies have shown that the distinct CD4 memory T cell subsets are infected to a different extent by latent HIV-1 and may support viral persistence through various mechanisms [25,26]. Central memory CD4 T cells are still considered to be the most important reservoir of latent HIV [18,19]. Interestingly, a skewed distribution of HIV-1 reservoirs from the long-lived TCM compartment towards shorter-lived populations of TTM and TEM seems to be important for natural control of the virus, as can be seen in both 'elite-controllers' (EC) and in 'post-treatment controllers' (PTC) [6,27]. Early cART initiation seems to protect the frequency of infection of all memory T cell subsets [28–30], with one study showing a relative protection of the TCM subset [31], although another subsequent study failed to replicate these findings and instead found a higher contribution of memory stem cells and central memory T cells to the latent reservoir [30]. It has therefore not been consistently established whether the TCM subset would be more protected than others by early treatment. Even haematopoietic precursor cells may contribute to the latent reservoirs, although results are conflicting [32,33].

Other immune cells have been implicated as reservoirs as well, especially myeloid cells. Macrophages are a known target for HIV infection [34], but their role as a reservoir remains debated [18–22,35]. Macrophages are not killed by viral cytopathic effects *in vitro*, which would make them a likely reservoir [35,36]. However, studies on HIV infection and latency in macrophages have been complicated by the diversity of these cells. Monocyte-derived macrophages appear in tissues following a local injury and can be replenished from the blood compartment, whereas tissue resident macrophages are established during fetal development [37]. They are maintained independently and are highly specialised per organ (into Kupfer cells, alveolar macrophages, intestinal macrophages, microglia cells etc.) [37]. Each subset of TRMs has a different susceptibility to HIV infection. Macrophages in the gut-associated lymphoid tissue (GALT) appear to be quite resistant to infection, except in the rectum, whereas alveolar macrophages seem relatively permissive [38–41].

Dendritic cells are a heterogenous group of antigen-presenting cells, some with lymphoid and some with myeloid origin, with different features depending on their anatomical location [42]. They are susceptible to HIV infection *in vitro*, but appear less so than CD4 T cells *in vivo*. Still, dendritic cells are thought to aid the dissemination of HIV by sequestering intact virions and presenting them to T cells, thus infecting them [43]. Follicular dendritic cells in B follicles actually derive from perivascular precursors and may contribute to the reservoir in a different way: they may carry a stable pool of HIV-1 virions on their surface without being infected [44,45]. Finally, other cell types such as epithelial cells, fibrocytes and astrocytes are sometimes implicated as reservoirs, although these claims still need to be further investigated [19–22].

Anatomical HIV reservoirs

After HIV enters the body and local replication occurs at the site of entry, HIV quickly disseminates to the lymph nodes (within days) and later on (within weeks) to the bloodstream [46]. At this point, the virus spreads throughout the entire body as individual virions, by the transport of infected immune cells and by cell-to-cell transmission [20,47]. Indeed HIV-infected cells have been found in the brain (and cerebrospinal fluid), the lungs, kidneys, liver, adipose tissue, in the gastrointestinal tract, the male and female genitourinary systems and bone marrow [20,22]. However, the lymphoid tissues (spleen, thymus, lymph nodes, GALT) are the most important sites of viral replication during active infection, and HIV DNA can still be detected in the lymph nodes after years of cART [22]. Within these organs, HIV is usually found within T cells and macrophages, but also in more organ-specific cell types (such as epithelial cells, microglia, astrocytes and podocytes) [19,20,22]. The contribution of all these anatomical compartments as latent reservoirs is still debated.

Overall, research on HIV reservoirs is still in its infancy. There is much data from *in vitro*, *in vivo* and animal models but as yet no gold standard to determine the size of the latent reservoir. Patient populations are highly heterogeneous (virus strain, route of transmission, ethnicity, lifestyle, timing of cART, duration and type of treatment, rate of suppression, etc.). All of these factors may influence HIV reservoirs and confound experimental studies.

Methods

We conducted a systematic literature search of all English-language articles published between January 1997 and November 2017 using MEDLINE (PubMed). Where available, medical subject headings (MeSH) were used as search terms and included: *HIV-1* and *virus latency*. Additional search terms consisted of *reservoir, latent, persistence, establishment, generation, formation* and *seeding*.

A total of 301 articles was found using the above search terms. Of these, 79 relevant articles related to HIV-1 latency establishment were collected and reviewed, with no limitation of study design. Additional studies (commonly referenced and/or older and more recent articles of significance) were selected from bibliographies and references listed in the primary resources.

Results

Mechanisms of latent HIV-1 reservoir establishment

In general, when exploring the literature, there are two main models that can be distinguished to explain latent memory cell infection: pre-activation and post-activation latency.

In the pre-activation model of latency, resting CD4 memory T cells are directly infected by HIV-1 before they are re-activated by stimuli from the environment. This process of direct resting cell infection is usually considered very inefficient due to blocks at various points in the viral life cycle [48,49]. It therefore resolves mostly in non-integrated linear and cytoplasmic forms of the viral genome with a half-life of approximately 1–6 days, due to instability of the pre-integration complex (also called 'pre-integration latency') [50–52]. However, it has been shown that certain cytokines and chemokines from the tissue microenvironment have the ability to make these resting CD4 T cells more permissive to HIV-1 infection, resulting in integrated proviruses without inducing cell proliferation or upregulation of T cell activation markers *in vitro* [53–55]. It cannot, therefore, be entirely excluded that such mechanisms could also occur *in vivo*, as it has been previously shown that mucosal CD4 resting T cells can at least be productively infected by HIV-1 *in vivo* [56,57].

Alternatively, the post-activation model for latency establishment is derived from studying normal T cell biology. As previously described, a small subset of antigen-stimulated active CD4 T cells will return to a resting state, awaiting activation upon re-encounter of the same antigen in the future, hence fulfilling their memory potential [58]. In theory, activated CD4 T cells can become infected with HIV-1 in the process of transitioning back to this resting state, allowing integration of the proviral genome in a host cell lacking favorable conditions for optimal viral gene expression, hence escaping rapid destruction of the infected T cell [10,52,59]. Evidence in support of this theory has come indirectly from *in vitro* models showing that HIV-1 is able to establish a latent infection in actively replicating T cells which, after being cultured with specific cytokines that induce a resting cell phenotype, are capable of producing active virus after stimulation with latency-reversing agents [54,60,61].

In the latter view, latency is considered as being merely an epiphenomenon of the activation status of the cell. Recent studies, however, show that T cell activation status and HIV-1 latency might not be as tightly coupled as previously thought. In this respect, Calvanez *et al.* have repeatedly shown that HIV-1 latency can be established directly in both activated and resting CD4 T cells, but with a different ratio of productive versus latent infection in both cell types [54,62]. This view gives rise to yet another possible model, whereby HIV-1 latency established in activated CD4 T cells shortly after entry confers a greater potential to survive and possibly return to a resting memory state, contributing to the long-lived latent reservoirs [54]. HIV-1 integration-site selection may play a role in the mechanisms of establishment of HIV-1 latency in these activated CD4 T cells. Indeed HIV-1 integration is not random but targeted towards active genes [63–67]. Lens epithelium-derived growth factor (LEDGF/p75) is the main cellular co-factor of HIV-1 integration (reviewed in [68]). As a chromatin reader and transcriptional co-activator, LEDGF/p75 recognises nucleosomes associated with actively transcribed genes thereby tethering integrase to active chromatin [69–71]. Our lab developed small-molecule inhibitors of the LEDGF/p75-integrase interaction, called LEDGINs [72,73]. We also showed that these LEDGINs inhibit viral replication and integration [72,74–80] and also retarget residual integration sites away from active genes, making the residually integrated provirus more resistant to reactivation (and thus more latent) [80]. Another possible explanation for latency can be found in the theory of stochastic gene expression. According to this theory, it is believed that stochastic (or random) mutations in the critical HIV-1 transcriptional activator molecule (Tat) can hinder active HIV-1 transcription, influencing the latency decision independent of the target-cell activation state [81,82].

This could offer a plausible explanation for the fact that there remains a small but significant pool of replication-competent HIV-1 provirus in resting CD4 T cells that cannot be induced by the known 'deterministic' host cell-associated factors that influence its transcription process [83]. In addition, a relatively recent *in vitro* study has shown that removing activation stimuli from the environment of primary CD4 T cells infected with HIV-1 predictably lowers the cellular activation state, but surprisingly leaves the viral transcription activity unaffected [84]. In the same study, the investigators showed, via the development of a synthetic circuit, that the Tat positive feedback is sufficient to control viral transcription without the need for cellular activation. These data are in support of previous findings that the establishment of latency can both be prevented and reversed by sufficient levels of intracellular HIV-1 Tat [85].

These different proposed models of HIV-1 latency establishment may not necessarily be mutually exclusive, but could also be complementary. Most studies on this topic are *in vitro*, leaving many questions about the extent to which, or if, these different mechanisms contribute to this complex picture *in vivo*.

Time of seeding of latent HIV-1 reservoirs

The discovery of long-lived stable cellular reservoirs for HIV-1 has led to a pessimistic outlook on strategies aimed at eradicating residual latent HIV-1 with cART alone [11,86]. However, initiating cART before the establishment of latent HIV-1 reservoirs can prevent lifelong acquisition of HIV, as this is the principle behind post-exposure prophylaxis (PEP). This brings us to the vital question of how early reservoirs are seeded.

The easy answer to this question would be 'very early in the course of the infection', at least before the symptomatic primary infection phase occurs and the available commercial diagnostic assays can confirm the diagnosis [50,87]. The initiation of cART as early as 10 days after the presentation of symptoms of a primary HIV-1 infection could not prevent the generation of latently infected resting CD4 T cells carrying replication-competent provirus [87]. Even more recently, a study investigating the effect of treatment interruption on eight participants who acquired HIV-1 and started cART in Fiebig stage I (corresponding to the first 2 weeks of infection, Table 1) found that there was rapid viral rebound in all individuals who had a median of 2.8 years of treatment [88]. Moreover, it was previously shown in rhesus monkeys that very early initiation of cART on day 3 after intrarectal SIV infection and before systemic viraemia occurred, could not prevent viral rebound after 24 weeks of treatment [89]. Although not in humans, this study reflects the strikingly early seeding of viral reservoirs during the 'eclipse phase', when the infection is still limited to the mucosa and local lymphoid tissue.

In most documented cases of children with perinatally acquired HIV-1 where cART is initiated within 30 hours of delivery, rebound viraemia occurs at some point after cART discontinuation. This seems to suggest indirectly that such an early seeding of viral reservoirs as seen in rhesus monkeys could also occur in humans [90–92]. However, the precise moment of infection could not be defined in these children and could have occurred *in utero* sometime before delivery.

It is particularly challenging to study the precise time at which reservoirs are established in humans because we cannot reliably detect the virus this early in the infection because our methods of detection mainly focus on the systemic viraemia phase. However, the possibility of the viral reservoirs being seeded during the mucosal eclipse phase of the infection calls for a better understanding of the early events after transmission of the virus. Much

Table 1. The Fiebig stage of primary HIV infection based on the emergence of a specific set of viral markers in the plasma of individuals who have acquired HIV. Classifications are based on a parametric Markov model

Fiebig stage	Cumulative duration		HIV RNA	P24 antigen	Antibody (EIA)		Western blot
	Days	95% confidence interval			NS	S	
I	5.0	3.1–8.1	+				
II	10.3	7.1–13.5	+	+			
III	13.5	10.0–17.0	+	+		+	
IV	19.1	15.3–22.9	+	+		+	Indeterminate
V	88.6	47.4–129.8	+	+	+	+	+ (p31 band negative)
VI	Open ended		+	+	+	+	+ (p31 band positive)

EIA: enzyme immunoassay: NS: not sensitive (refers to second generation not IgM-sensitive immunoassay); S: sensitive (refers to second generation IgM-sensitive immunoassay).

of our current knowledge on this topic, comes from studies with a rhesus macaque model of SIV vaginal transmission by Haase *et al.* [57].

In these studies, it is shown that there is an eclipse phase of 1–3 days post-infection in which SIV DNA is detectable in the tissues at the portal site of entry before systemic viraemia occurs, with only few cells producing viral RNA representing progeny virus [93]. During these early stages of the infection, it appears that mostly resting CD4 T cells are productively infected by SIV at the portal site of entry. The same findings were observed in lymph node and tonsillar biopsies performed during the acute stage of HIV-1 infection in humans [56].

It is reasoned that because resting CD4 T cells greatly outnumber other potential target cells at the portal site of entry (here, the vaginal mucosa tissue) there is a greater possibility of virus encountering and infecting a resting cell until immune activation causes an influx of activated CD4 target cells to which the infection is later preferentially disseminated [94].

A more thorough characterisation of the activation state of these T cells productively infected by both SIV and HIV-1 in the early stage of the infection was performed by testing them for the activation markers CD25, CD71, CD30, CD38, CD134 and Ki67. Results showed that although cells with and without these activation markers were present, most cells were negative for each marker and could be categorised as resting [56].

Only a small fraction of the resting CD4 T cell population is infected during the first week after initial acquisition and a nearly 20-fold decrease in SIV RNA+ Ki67- cells occurs between week 1 and 4 [94]. These findings suggest that an as yet undefined subpopulation of resting cells – possibly those in a slightly higher state of activation – can support productive infection. These cells are then either eliminated by viral cytopathic or host immune-effector mechanisms or can be activated. Alternatively, this decrease may reflect a conversion of the pool of initially productively infected cells to a state of latent infection [94]. Although it is very hard to provide direct evidence for these theories in humans, these studies show that the initial environment during the mucosal eclipse phase of the infection after sexual transmission of HIV-1, could potentially support the generation of a pool of latently infected resting CD4 T cells as early as a few days after initial acquisition. Whether these potentially latently infected cells have the ability to persist during the lifetime of the individual has not been clearly established.

The effect of timing of antiretroviral therapy initiation on size and stability of HIV-1 reservoirs

In spite of the previously described evidence for very early seeding of viral reservoirs during HIV infection, numerous studies have focused on the effect of early cART initiation on the size of the reservoirs and the possibility of achieving HIV remission when treatment is interrupted (post-treatment control).

It has been repeatedly shown that patients who start cART early during the acute HIV-1 infection phase (up to 6 months after acquisition) exhibit a lower overall reservoir size compared to those who start treatment later, for example during chronic infection [29,30,95–103]. Although these studies differ in patient characteristics, timing of cART initiation, total treatment duration and assays for reservoir size measurement, they all show a significant decrease in total HIV burden when cART is started early. It does appear that the earlier treatment is initiated during acute HIV-1 infection (AHI), the smaller the size of viral reservoirs after viral suppression, suggesting an escalation of HIV-1 reservoir seeding over time [96,100]. Moreover, Ananworanich *et al.* recently showed how during the acute stage of the infection in participants who received no treatment, total HIV-1 DNA in PBMCs peaked 2 weeks after enrollment, reaching a set point 2 weeks later with little change thereafter, while integrated HIV-1 DNA seemed to increase during untreated infection [101]. Very early cART could reduce the frequency of HIV-1 DNA-positive cells significantly, but this effect is probably less pronounced if treatment is initiated in later stages of the infection [100].

Even when cART is started during the acute infection phase of the disease, no further decay of the frequency of cells harbouring replication-competent HIV-1 to less than 0.5 cells per million could be achieved after long-term treatment, suggesting a subset of very early latently infected cells may persist indefinitely [97].

Starting treatment early may, however, be clinically relevant since high HIV-1 DNA levels are an independent predictor of disease progression [104,105] and pre-treatment total HIV-1 DNA and cell-associated HIV-1 RNA levels predicted time to viral rebound upon cART interruption in several studies [105,106]. Additionally, the inability to detect HIV-1 DNA does not seem to exclude the possibility of rebound plasma viraemia occurring at some point following treatment interruption [4,90,107].

Since lower overall reservoir size has been associated with increased time to viral rebound and early cART reduces the size of viral reservoirs, it has been hypothesised that early cART initiation

during AHI would delay viral rebound following treatment interruption. Yet cART initiated as early as Fiebig stage I (HIV-1 RNA+, p24-, HIV-1 IgM-; Table 1) did not result in a significantly longer time to viral rebound after treatment interruption when compared to cohorts with chronic HIV-1 infection. This suggests that early cART alone is not sufficient to induce HIV-1 remission and additional strategies to eliminate or control latently infected cells are required [88]. In contrast, long-term virological remission after interruption of cART has been achieved in three children with perinatally acquired HIV-1 who started treatment early [5,108,109], and several adults who started treatment during primary infection [6]. These cases do suggest that as well as the size of the reservoirs containing replication-competent proviruses, other factors such as the absence of ongoing replication and HIV-1 specific immunity are also of great importance in achieving durable HIV-1 remission [110].

Discussion

The focus of HIV research has shifted from 'care' to 'cure' with the aim of finding alternatives for the current standard of treatment: chronic suppressive antiretroviral therapy for the lifetime of the individual living with HIV [2].

Two distinct interpretations of a possible cure have been introduced. In the first, the ultimate goal remains the absolute and complete eradication of the virus from the body of individuals leading to permanent virological remission off treatment, an approach often referred to as a 'sterilising cure' [111]. It is believed that the Berlin patient was successfully cured using this approach. This case shows the great challenges that go with this strategy and raises questions concerning the safety, cost, feasibility and scalability of this approach [3]. This, combined with the inability to replicate this success story, has led to the second interpretation for cure, the aspiration for achieving a state of 'sustained virologic remission' or 'functional cure' in which individuals living with HIV can remain without treatment despite the objective persistence of replication-competent proviruses without rebound of plasma viraemia [8]. As discussed above, early initiation of cART is likely to be one of the crucial steps in achieving long-lasting remission without treatment and the containment of the establishment of the viral reservoirs during the early stages of the infection appears to be a major contributing factor. Yet, some important questions remain unanswered.

How early does treatment need to be started? The findings of Buzon *et al.* suggest that initiating treatment as early as 6 months might not suffice [30]. Most evidence suggests that the earlier treatment is started, the better [100,101]. Using the Fiebig classification for AHI to define the point of treatment initiation [112], treatment during the earliest stages of the infection (e.g. Fiebig I and II) logically offers the most likely chance for an eventual stable HIV remission (Table 1). However, the exact window of opportunity for treatment initiation to achieve this goal remains to be elucidated. Most of the post-treatment controllers of the VISCONTI cohort [6] were treated in the later stages of AHI (Fiebig IV and V), highlighting the importance of additional parameters, such as immune control, that should be considered when plans for treatment interruption are being made. Thus, an accurate AHI staging system based upon a thorough characterisation of patients at time of treatment initiation (including factors such as HIV reservoir size, HIV specific immunity markers, HLA profile) to identify those more likely to benefit from HIV cure efforts is still lacking in the field.

Currently, as most widely available diagnostic assays are antibody-dependent and based on showing seroconversion, with most tests returning positive results 4–6 weeks after infection, there is also the practical problem of identifying individuals as early as a few days after infection. Very early detection of HIV-1 infection is possible using nucleic acid testing (NAT), directly identifying viral genetic material in the systemic circulation [113]. The use of these assays is limited owing to their cost, availability and the stigma or lack of awareness of risk factors that prevents individuals from seeking early testing. Additionally, symptoms of the acute retroviral syndrome do not occur in every individual and since symptoms are very non-specific, individuals do not always recognise them as signs of possible HIV acquisition [114].

Finally, the length of treatment required before interruption is defensible is unknown and we do not yet have the tools to determine the appropriate time for safe treatment interruption. For example, Chun *et al.* published a case report showing viral rebound 50 days after cART discontinuation in a person who initiated therapy during acute infection and remained on treatment for 10.5 years, despite undetectable levels of HIV-1 DNA in peripheral blood CD4 T cells or in sigmoid colon biopsies at the moment of treatment interruption [107]. Similarly, in the cases of the Mississippi child [5] and the Boston patients [4], investigators also failed to detect HIV-1 DNA, yet rebound viraemia eventually occurred in all of the individuals.

A small HIV-1 reservoir does seem to be important for achieving post-treatment viral control as a reduced reservoir size is a common finding in those PTC and EC identified to date. A critical shortcoming that remains, however, is a gold-standard method to measure intact replication-competent proviruses [115]. The current gold standard for measuring this form of the viral reservoir, the viral outgrowth assay (VOA), appears to underestimate the amount of replication-competent proviruses by 60-fold compared to direct detection methods of intact proviruses by sequence analysis [83]. There is a growing interest in finding more accurate detection methods [116]. Recent innovations include the Tat/rev induced limiting dilution assay (TILDA) [117] and the use of a reporter cell-based assay based upon the TZM-bl cell line [118], both of which have increased sensitivity, require less blood volume, are faster, less labour intensive and less expensive compared to the VOA. A noteworthy improvement to the PCR-based strategies is the development of the intact proviral DNA assay (IPDA) [119,120]. This digital droplet PCR assay detects intact, cell-associated, full-length genomic HIV DNA with increased sensitivity. Further validation, however, is needed to confirm the applicability of these assays in cART interruption studies.

A recent study by Henrich *et al.* reports the case of two extremely early HIV-1 diagnoses and subsequent treatment initiations, namely at the end of the eclipse phase and at the beginning of the start of Fiebig I, preceding the acute infection phase [121]. Despite the complete loss of detectable HIV-1 in blood, colorectal and ileal tissue, an excised inguinal lymph node, PBMCs, cerebrospinal fluid and a bone marrow biopsy in one individual following 32 weeks of continuous cART, rebound viraemia occurred 225 days following treatment interruption. This study highlights the extremely early seeding of HIV-1 reservoirs in humans, making future efforts aimed at the prevention of the establishment of viral reservoirs very likely to fail. On the other hand, it also provides an affirmation that increased time to viral rebound can be seen in those starting treatment very early. Future studies aimed at achieving a prolonged HIV remission state are therefore more likely to be successful if they focus on a combination strategy including early cART initiation, prolonged treatment duration, eliminating or silencing infected CD4 T cells and increasing the anti-HIV immune response [111,122].

Acknowledgements

Declaration of interests

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