RESIDUAL VIREMIA, CURE STRATEGIES, AND TISSUE RESERVOIRS IN HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 INFECTION

by
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ABSTRACT

Human immunodeficiency virus-1 (HIV-1) is a retrovirus that primarily infects immune cells bearing CD4 and either CCR5 or CXCR4. It is transmitted horizontally through contact with infected bodily fluids via intravenous drug use, sexual contact, or blood transfusion and is transmitted vertically via mother-to-child transmission during birth or breastfeeding. Untreated infection in all but very rare populations of patients leads to depletion of CD4+ T cells and a resulting lack of immune function that results in acquired immunodeficiency syndrome (AIDS), in which the infected individual is increasingly susceptible to opportunistic infections that lead to death.

Despite the insidious nature of the disease, effective treatment with antiretroviral therapy (ART) can substantially prolong the life of an individual living with HIV by blocking various stages in the HIV-1 replication cycle. Effective ART reduces viral loads to below the limit of detection of standard clinical assays by halting viral replication. Patients on an effective treatment regimen can therefore continue to live relatively normal lives thanks to ART.

However, ART is not a cure. Using very sensitive laboratory assays, HIV can be detected in patients on ART even below the limit of detection of clinical assays; and using a quantitative viral outgrowth assay (QVOA) developed in our lab, replication-competent HIV can be measured from patients with clinically undetectable viral loads on ART. Because ART is not a cure, infected individuals must continue to take ART for the remainder of their lives; otherwise, their viral load will quickly rebound to the levels that occurred before treatment with ART, and the disease will progress to AIDS. The need to take ART for a lifetime results in an extremely high cost of care; additionally, many of the
components of ART have undesirable side effects. Therefore, despite the effectiveness of ART at halting the disease’s progression to AIDS, a cure for HIV is still needed.

Finding a cure for HIV will require both an understanding of the reservoirs that constitute the barrier to curing HIV and an understanding of treatments that will purge these reservoirs. In this work, we discuss a study investigating a small molecule, disulfiram, as a potential latency-reversing agent, the development of a streamlined assay for measuring the latent reservoir, and the investigation of two different tissue sites (hematopoietic progenitor cells and cells in the gut-associated lymphoid tissue) as anatomic sites that could contain cells with replication-competent HIV in patients on ART.

Chapter 1 provides an introduction to HIV latency and the concepts that differentiate a reservoir from a compartment. Additionally, the introduction provides a discussion of various cell types and anatomic sites that have been considered potential barriers to HIV cure.

Chapter 2 describes a study investigating the potential use of disulfiram as a latency-reversing agent. This clinical trial involved measuring the latent reservoir before and after treatment with disulfiram using the QVOA and measuring the amount of residual viremia before, during, and after treatment with disulfiram to detect changes in residual viremia that might indicate latency reversal. Although no significant change in the size of the latent reservoir was detected, some study participants exhibited transient but dramatic increases in residual viremia, suggesting that some latency reversal occurred but that this effect was not substantial enough to decrease the size of the latent reservoir.
In Chapter 3, we investigate hematopoietic progenitor cells as a potential reservoir for HIV. Bone marrow aspirates were obtained from individuals on suppressive ART. Although HIV DNA was detected in CD4+ T cells from the peripheral blood and in bone marrow cellular fractions containing CD4+ T cells, no HIV DNA was detected in purified CD34+ cells (hematopoietic progenitor cells), and no replication-competent HIV was detected using the QVOA.

In Chapter 4, we develop a streamlined QVOA for measuring the size of the latent reservoir more efficiently. Like the original assay, the streamlined QVOA uses a limiting-dilution coculture assay to measure the size of the latent reservoir. However, the streamlined assay uses the MOLT-4/CCR5 cell line in place of activated lymphoblasts from healthy donors, and it uses real-time PCR to detect full-length HIV RNA several days earlier than could be detected in the original assay using an ELISA for HIV p24.

In Chapter 5, we investigate the gut-associated lymphoid tissue (GALT) as a tissue site that could serve as a barrier to HIV cure. We use aspects of the streamlined QVOA described in Chapter 4 to measure the size of the latent reservoir in circulating resting CD4+ T cells and in cells from the GALT of patients on suppressive ART; additionally, we use phylogenetic analysis to investigate whether GALT cells could contribute to residual viremia in these same patients. Although we detected replication-competent HIV in the circulating resting CD4+ T cells from most study participants, we detected replication-competent HIV in the GALT in only one participant. Despite the infrequency of this observation, however, our phylogenetic data are consistent with the concept that HIV in the GALT could contribute to residual viremia and therefore constitute a barrier to the cure of HIV in addition to circulating resting CD4+ T cells.
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My husband, David Gurule, has shown me what it means to be thoroughly known and thoroughly loved. He is a marvelous example of strength and vulnerability, intelligence and humility, hilarity and depth. He has helped me take myself less seriously while also showing me how precious I am to him; he has torn down emotional walls I had constructed while also helping me become stronger. He has helped me understand God’s love through the way he treats me. He is home to me. I did not even know to ask for a life partner like him: even though neither of us is perfect, we are each other’s Perfect, and I know he will forgive me for stealing that line from him because that is just how much he loves me. He has helped me grow immeasurably during the time we have been together, and I am unspeakably grateful that we have each other to comfort and challenge for the rest of our lives.
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CHAPTER 1 – INTRODUCTION

This chapter was originally published as Eisele and Siliciano, 2012. It has been updated to reflect more recent findings in the field.

Background

In 1997, it became possible for the first time to reduce plasma HIV-1 levels to below the detection limit of clinical assays (50 copies of HIV-1 RNA/mL) with combinations of three antiretroviral drugs (1). This approach, known as combination antiretroviral therapy (ART), dramatically decreased deaths from HIV-1 infection (2). With millions of life-years saved (3), ART is a major achievement of modern medicine, converting a uniformly fatal illness into one in which adherent patients starting treatment early now have near normal life expectancy (4).

The effective suppression of viremia initially inspired hopes that the virus could be eradicated with two to three years of ART (1). However, in 1995, a latent form of HIV-1 infection was demonstrated in vivo (5). A small fraction of resting memory CD4+ T cells was shown to carry integrated viral genomes. These cells do not release infectious virus in the resting state, but can do so following cellular activation (5, 6). This latent reservoir in resting CD4+ T cells persists even in patients on ART who have no clinically detectable viremia (6-8). Longitudinal analysis demonstrated that in patients on ART, the time to eradication of the population of latently infected cells would be over 60 years (9-11). This latent reservoir is now recognized as the major barrier to HIV-1 eradication (12). Even patients who have been on ART for several years experience viral rebound within weeks of treatment interruption (13), typically from an archival variant (14). Life-long ART is therefore required.
The latent reservoir for HIV-1 in resting CD4+ T cells differs in important ways from classic forms of latency observed for viruses such as those of the *Herpesviridae* family. For herpes simplex viruses, latency allows immune evasion and viral persistence between episodes of active replication (15). In contrast, in untreated HIV-1 infection, there is continuous viral replication throughout the disease course (Figure 1.1) (16). Rapid viral evolution allows escape from neutralizing antibodies (17) and cytolytic T lymphocyte (CTL) responses (18) and provides the principal mechanism of immune evasion.

**Figure 1.1. Plasma virus levels in untreated and treated HIV-1 infection**

Legend, Figure 1.1. Virus production continues throughout the course of untreated HIV-1 infection (top panel). Levels of viremia vary widely between patients but average between 10,000 and 100,000 copies/mL. Stable reservoirs make a very small contribution to overall virus production. Their contribution, apparent
only when active replication is halted by ART, is around 1 copy/mL (19, 20). ART induces a rapid biphasic drop in the level of viremia (bottom panel), reflecting the rapid turnover of the cells that produce most of the plasma virus (1, 21-23). Eradication strategies will likely be implemented in patients who have had suppression of viremia to below the limit of detection for >1 year to allow decay of these labile infected cell populations.

Why then does late HIV-1 infection occur? HIV-1 latency is best viewed as an accidental consequence of viral tropism for activated CD4+ T cells. Generally, infection leads to the rapid death of activated CD4+ T cells (21, 23), but rarely, activated CD4+ T cells become infected as they are reverting back to a resting memory state. Resting CD4+ T cells are much less permissive for HIV-1 gene expression due to profound differences in the transcriptional environment. Thus, latency can be established. Latent HIV-1 is found in resting memory CD4+ T cells but not in naïve CD4+ T cells (6, 24-26), supporting the idea that the cells become infected during the transition from activated effectors to resting memory cells. The establishment of latent infection is a rare event; hence, the frequency of latently infected cells is extremely low, typically around 1/10^6 resting CD4+ T cells (6, 7). Encountering antigen or other activating stimuli can induce latently infected cells to produce virus, but the rate of virus production from this and other stable reservoirs is typically <0.01% of the rate of virus production due to ongoing replication in untreated patients (Figure 1.1). Thus, this small pool of latently infected cells likely plays only a minor role in the natural history of infection. Essentially, HIV-1 latency is an epiphenomenon, but one that is extremely important in the context of ART because it represents a barrier to cure. This view of latency has significant implications for therapeutic efforts to purge the latent reservoir cells and for the search for other HIV-1 reservoirs.

In addition to the latent reservoir in resting CD4+ T cells there is another important indication of viral persistence in patients on ART. Although ART suppresses
viremia to <50 copies/mL, more sensitive assays with limits of detection as low as 1 copy/mL reveal trace levels of viremia in many patients (19, 20, 27). Because the half-life of plasma virus is on the order of minutes (22, 28), this low-level viremia, referred to as “residual viremia,” indicates ongoing virus production in patients on ART. At least some of these viruses appear to be replication-competent (29). In principle, residual viremia could result from a low degree of ongoing viral replication, the release of virus from latently infected cells that have become activated, or the release of virus from other stable reservoirs.

Although the discovery of a stable latent reservoir for HIV-1 diminished hopes for eradication, the recent cure of a single patient using an ablative bone-marrow transplantation strategy has renewed interest in cure research (30). The patient had stable suppression of viremia on ART when he developed acute myelogenous leukemia (AML). As part of the treatment for AML, he received chemotherapy and radiation followed by a bone marrow transplant from an HLA-matched donor. The unique aspect of the case was the choice of a donor who was also homozygous for a 32-base pair deletion in the HIV-1 coreceptor CCR5. Thus, the patient’s hematopoietic compartment was reconstituted with HIV-1-resistant cells. At the time of initial transplantation, ART was stopped, and there has been no rebound in viremia for over five years. This patient is thus considered to be the first and, to date, the only patient cured of HIV-1 infection. Although this approach will be difficult to generalize, the successful cure of a single patient has renewed interest in the possibility of eradicating HIV-1 infection. Here we will precisely define the viral reservoirs that represent a barrier to eradication.

Definitions
Discussions of HIV-1 eradication have been hindered by a lack of precision and consensus regarding the meaning of key terms such as latency, viral replication, reservoir, sanctuary, compartment, and cure. Therefore, we propose here a set of definitions that can be used as a starting point for refining the terminology in cure research (Table 1.1).

**Table 1.1. Proposed definitions for key terms in eradication research**

<table>
<thead>
<tr>
<th>Term</th>
<th>Brief definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Latency</td>
<td>Reversibly non-productive state of infection of individual cells. Latently infected cells retain the capacity to produce infectious virus particles.</td>
</tr>
<tr>
<td>Viral replication</td>
<td>New cycles of infection in which previously uninfected cells become infected and produce virus that goes on to infect additional cells. Evidence for viral replication includes the presence of labile products of reverse transcription, viral evolution during prolonged ART with drugs at therapeutic concentrations, and a decrease in residual viremia levels with the addition of a fourth drug to an ART regimen.</td>
</tr>
<tr>
<td>Reservoir (previous virologic definition)</td>
<td>Cell type or anatomical site in association with which a replication-competent form of the virus persists with more stable kinetics than the main pool of actively replicating virus.</td>
</tr>
<tr>
<td>Reservoir (practical definition)</td>
<td>Infected cell population that allows persistence of replication-competent HIV-1 in patients on optimal ART regimens on the order of years.</td>
</tr>
<tr>
<td>Compartment</td>
<td>An anatomical site for which there is limited exchange of viral genetic information with other sites; observable using phylogenetic tools. May contain compartment-specific viral sequences.</td>
</tr>
<tr>
<td>Sanctuary</td>
<td>An anatomical site with suboptimal free drug levels.</td>
</tr>
<tr>
<td>Cure:</td>
<td></td>
</tr>
<tr>
<td>Sterilizing</td>
<td>Complete eradication of all replication-competent forms of the virus.</td>
</tr>
<tr>
<td>Functional</td>
<td>Permanent viral suppression in the absence of therapy to levels that prevent immunodeficiency and transmission.</td>
</tr>
</tbody>
</table>

*Latency*

The standard virologic definition of latency is a reversibly non-productive state of infection of individual cells. Latently infected cells do not produce infectious virus particles but retain the capacity to do so. Resting memory CD4+ T cells carrying transcriptionally silent HIV-1 genomes clearly represent a form of latency because a fraction of these cells produce infectious virus following cellular activation (5-8, 31). The term latency is sometimes used in clinical settings to refer to the prolonged asymptomatic phase between acute infection and the development of AIDS. This usage is incorrect.
because viral replication continues throughout the course of untreated HIV-1 infection (Figure 1.1) (16).

**Viral replication**

The first step in HIV-1 eradication is to stop the virus from replicating. Surprisingly, there is considerable confusion regarding the term “viral replication” and the measures that can be used to determine whether replication has been halted by ART. Some authors erroneously equate virus production with viral replication. The mere detection of viral RNA in cells from patients on ART does not demonstrate ongoing replication, nor does the presence of residual viremia. Both can be explained by the reactivation of latently infected cells (Figure 1.2). Virus production by these cells can occur without new rounds of infection and does not propagate the infection. We suggest that the term “replication” be used to mean new cycles of infection in which previously uninfected cells become infected and produce virus that goes on to infect additional cells.

**Figure 1.2. Distinguishing ongoing viral replication from virus production by latently infected cells that have been activated**
**Legend, Figure 1.2.** A variety of parameters have been evaluated to determine whether a low degree of viral replication continues in patients on ART. These are indicated in red and include expression of HIV-1 RNA, residual viremia, labile products of reverse transcription such as linear unintegrated HIV-1 DNA, LTR circles, and the accumulation of mutations (viral evolution). Depending on the ART regimen, some of these parameters can also reflect the reactivation of latently infected cells without further replication (right panel). For example, in patients on regimens in which an integrase inhibitor is the most active drug, all of these features except evolution would be present. Only the accumulation of new mutations is uniquely characteristic of ongoing replication.

**Reservoir**

Viral reservoirs have been defined as cell types or anatomical sites in association with which replication-competent forms of the virus persist with more stable kinetic properties than the main pool of actively replicating virus (32). There are two important elements to this definition. First, a reservoir allows persistence of some replication-competent forms of the virus capable of replenishing the pool of infected cells in the future. Many HIV-1 genomes are defective, and thus the detection of proviral DNA by PCR does not establish that a viral reservoir is present. Second, there is a kinetic element to the definition. Classic studies of viral dynamics in HIV-1 infection (1, 21-23, 33) established that most of the plasma virus is produced by activated CD4+ T cells, which turn over very quickly in the productively infected state ($t_{1/2} \approx 1$ day). The mechanisms by which the cells are cleared have not yet been elucidated and may not involve direct lysis by CTL (34, 35). The rapid death of these cells causes the precipitous drop in viremia seen when new infection of susceptible cells is blocked by ART (Figure 1.1).

By the definition given above, any infected cell with a half-life longer than 1 day could be considered a reservoir. Careful analysis of the rate of decay of viremia following the initiation of ART revealed a minor population of virus-producing cells with a half-life of approximately two weeks (1). The nature of these cells is controversial. Productively infected CD4+ T cells with a resting phenotype are observed in vivo in acute infection (33), and in vitro studies suggest that chemokines can render resting CD4+ T cells
susceptible to infection (36). Macrophages are also infected *in vivo*, but whether either of these cell types is responsible for the second phase of decay remains unclear.

Eradication efforts will likely be attempted only in patients on ART who have had sustained suppression of viral replication for years, and therefore the cells responsible for the second phase may not represent a barrier to eradication. Clinically significant reservoirs are more stable. This element of long-term stability is captured in an elegant genetic definition of a viral reservoir proposed by Nickle and colleagues (37). Because sequences arising at different time points in the course of infection can be deposited and persist in a reservoir, the viral sequences in a reservoir will lack temporal structure and show less mean divergence from the most recent common ancestor (MRCA) than the contemporaneous pool of actively replicating virus. The latent reservoir in resting CD4+ T cells meets these criteria and can store the original wild type sequences as well as drug-resistant sequences arising due to suboptimal therapy (38-40). Extensive sequence analysis is required to define reservoirs in this way. We suggest that a simple and practical definition for an HIV-1 reservoir is an infected cell population that allows persistence of replication-competent HIV-1 in patients on optimal ART regimens on a time scale of years. To date, the latent reservoir in resting CD4+ T cells is the only reservoir shown to fit this definition.

*Sanctuaries and compartments*

There is also confusion over these terms. Both refer to anatomic sites in which the virus may be present. “Sanctuary” is typically used to refer to a site in which limited penetration of antiretroviral drugs allows persistent replication. Whether there are sanctuaries of this kind in patients on ART is unclear. Most studies of pharmacologic
sanctuaries measure the total drug concentration, including both protein-bound and unbound forms of the drug. However, according to the free drug hypothesis (41), only unbound forms distribute across membranes and determine the biological effect. An important recent study has shown that while the total concentration of the reverse transcriptase inhibitor efavirenz (EFV) is much higher in the blood than in the seminal plasma, the free EFT concentration is actually the same (42). Thus, the male genital tract is actually not a drug sanctuary for EFV despite having a much lower total EFV concentration. Similar arguments may apply to other putative drug sanctuaries such as the central nervous system (CNS), and it is critical that free drug concentrations be measured in these sites. Despite concerns about drug sanctuaries, there is no anatomical site where continued HIV-1 replication has been documented in the majority of patients.

Some antiretroviral drugs such as reverse transcriptase (RT) and integrase inhibitors act intracellularly. Nucleoside analogue RT inhibitors (NRTIs) must be phosphorylated by cellular enzymes to an active triphosphate form. Intracellular concentrations of NRTIs correlate significantly with virologic efficacy, whereas plasma concentrations do not (43). Because of the technical barriers to sampling intracellular drug concentrations in putative drug sanctuaries, other methods must be used to determine whether viral replication is ongoing, as is discussed below.

The term “compartment” is sometimes used to refer to an anatomical site in which the virus is present. More commonly, it refers to a site that has limited exchange of viral genetic information with other sites (37). Compartments can be identified using phylogenetic criteria. Typically there are compartment-specific sequences – viral variants that evolve in a particular site and are not distributed to other sites. The extensive
literature on compartmentalization of HIV-1 has been reviewed in (44). Many early studies of HIV-1 compartmentalization were flawed by PCR resampling (45). Compartments can only be defined with adequate sampling of individual viral genomes in different sites. HIV-1 compartmentalization has been explored mainly in untreated patients, and in terms of eradication, the key issue is whether there are compartments that function as reservoirs.

Cure

Definitions for two types of cures have been proposed (46). In a sterilizing cure, there is complete eradication of the virus. In light of the high fraction of defective viral genomes, this definition could be further refined as follows: A sterilizing cure eliminates all replication-competent forms of the virus; no viral reservoirs remain. By this definition, a patient who retains some defective viral sequences would still be considered cured. In a functional cure, there is permanent control of viral replication without therapy. Elite suppressors (ES), patients who control viral replication without therapy, are often considered examples of a functional cure (47). However, replication-competent virus can be isolated from ES (48). In addition, in can be shown with sensitive assays (49) that these individuals have low-level viremia, analysis of which reveals evidence for viral evolution (50, 51). Nevertheless, ES generally maintain stable CD4+ T cell counts and plasma HIV-1 levels below the limit of detection of clinical assays and are unlikely to transmit the virus. In this light, a functional cure could be an intervention that renders patients with progressive disease able to permanently control viral replication to below 50 copies/mL without therapy, thereby preventing clinical immunodeficiency and transmission.
The debate over ongoing virus replication in patients on ART

A critical issue is whether ART completely stops viral replication. This issue has been difficult to resolve because some indicators of viral replication can also reflect virus production from stable reservoirs (Figure 1.2). Certain virologic measures could provide conclusive evidence that HIV-1 replication is ongoing in patients on ART. The first is the detection of labile products of reverse transcription (52). In patients on effective regimens composed of protease inhibitors, entry inhibitors, and/or RT inhibitors, labile products of reverse transcription should be absent. The final product of reverse transcription is a full-length, linear, double-stranded viral DNA, which is inserted into host DNA by HIV-1 integrase. Pioneering studies by Mario Stevenson showed that linear unintegrated HIV-1 DNA is a prevalent species in resting CD4+ T cells in untreated patients (53). However, this form can be targeted by exonucleases and is labile until it is integrated into cellular DNA (54, 55). Thus, the detection of linear unintegrated HIV-1 DNA would indicate recent infection. Linear unintegrated HIV-1 DNA has not been conclusively demonstrated in patients on ART. Circular forms of the viral genome, specifically one- and two-LTR circles, can arise particularly when integration is blocked (56-58). These forms represent dead ends with respect to replication but could serve as indicators of recent infection if they are labile. However, the stability of these forms is controversial (59-61). In addition, as shown in Figure 1.2, these forms could be generated through the activation of latently infected cells in patients on regimens that rely on an integrase inhibitor to provide most of the antiviral effect. In summary, the analysis of unintegrated forms of the viral genome has not yet provided unequivocal evidence for ongoing replication in most patients on ART.
Progressive evolutionary change is an inevitable characteristic of HIV-1 replication due to the high error rate of RT (62). In each infected individual, one or a small number of transmitted founder viruses evolve over time into a complex quasispecies (63, 64). Thus, ongoing evolution would also be a clear indication that ART does not completely stop replication. However, most studies fail to detect evolution in the majority of patients on ART (65-68). Analysis is complicated by variable adherence in patient populations. In any population, there will be patients with poor adherence in whom viral replication occurs as a result of low drug levels. Thus, evolution in a small subset of patients cannot be taken as evidence that the virus continues to replicate in patients on optimal ART regimens. There is no study documenting evolutionary change in patients known to have therapeutic levels of antiretroviral drugs. As discussed above, residual viremia in patients on ART reflects ongoing virus production and might be expected to capture evolutionary change if new cycles of replication were occurring. Interestingly, detailed studies of residual viremia have shown that it is drug sensitive, archival in character, and non-evolving (65, 68-70). This lack of evolution is also consistent with the general clinical experience that adherent patients can maintain suppression of viremia on ART indefinitely.

A final experimental test of the hypothesis that viral replication continues in patients on ART would be the demonstration that intensification of ART with additional antiretroviral drugs further reduces some measure of viral persistence. Numerous studies with several different intensification drugs have all shown that intensification has no effect on residual viremia (71-73). These results strongly suggest that residual viremia originates from stable reservoirs rather than ongoing replication. A single study suggests
that intensification transiently increases the levels of 2-LTR circles in a subset of patients (74), but in light of uncertainty about the half-life of these circles, the significance of this result remains unclear.

In summary, the weight of the current evidence suggests that ART effectively halts ongoing viral replication as defined above. This conclusion is supported by recent pharmacodynamics studies that quantitate the inhibitory potential of ART regimens (75). Clinical concentrations of some antiretroviral drugs, specifically certain protease inhibitors, can produce 10 logs of inhibition of a single round of infection, and three-drug ART regimens have an even higher combined inhibitory potential. Given that there are only $10^{12}$ lymphocytes in the entire body, only a small fraction of which are infected, this degree of inhibition should effectively block all new infection events in any drug-accessible region of the body. One interesting study suggests that in the vicinity of a virus-producing cell, the exposure of adjacent cells to multiple infection events reduces the efficacy of antiretroviral drugs on a probabilistic basis (76). However, cells with multiple proviruses are rare in vivo (77). Any replication that does continue in adherent patients on ART is sufficiently limited that resistance does not evolve. The success of ART at blocking replication has led to a shift in the HIV-1 treatment field away from the development of new antiretroviral drugs and towards the eradication of reservoirs (12).

**The latent reservoir in resting CD4+ T cells**

The mechanisms by which latent infection is established and maintained in resting CD4+ T cells have been previously reviewed (78-80) and will be only briefly summarized here. In resting cells, an interrelated set of changes in the transcriptional environment prevents viral gene expression. These include both the sequestration of
critical host transcription factors and epigenetic modifications that inhibit transcription. The transcription factors NFκB and NFAT, both of which are involved in HIV-1 gene expression, are excluded from the nucleus in resting CD4+ T cells (81, 82). In addition, in resting cells there is also sequestration of pTEFb, a complex of CDK9 and cyclin T1 that, in association with the HIV-1 protein Tat, promotes elongation of HIV-1 transcripts (83-86). Even though HIV-1 proviruses are typically integrated within genes that are actively transcribed in resting CD4+ T cells (87, 88), repressive chromatin modifications of nucleosomes and DNA methylation at the HIV-1 LTR can interfere with expression of viral genes (89-91). Many of these mechanisms have been elucidated using transformed cell lines, and the relative importance of each mechanism in maintaining latency in vivo is unclear. The reversal of latency in vivo with agents designed to target these mechanisms may provide insight into this critical issue.

Several approaches to curing HIV-1 infection have been proposed (92). One involves reactivation of latent virus while patients are on ART. This could cause death of infected cells from viral cytopathic effects or allow the immune system to act against these cells. Classes of small molecules capable of reactivating latent HIV-1 include histone deacetylase inhibitors such as vorinostat (suberoylanilide hydroxamic acid, SAHA) (93, 94) and phorbol esters such as bryostatin and prostratin that induce transcription of HIV-1 through activation of the cellular protein kinase C pathway (95-98). One clinical study demonstrated increased HIV-1 RNA expression in resting CD4+ T cells of patients on ART after a single oral dose of SAHA, suggesting that SAHA is capable of perturbing the latent reservoir in vivo (99). However, more recent evidence indicates that simply reactivating latent HIV-1 may not be sufficient to induce immune-
mediated purging of latent reservoirs (100). *Ex vivo* experiments have shown that priming of CD8+ T cells with HIV-1 *gag* peptides permits these cells to kill HIV-1-infected CD4+ T cells whose virus had been reactivated with SAHA, suggesting that a combination of immune priming and reactivation of latent HIV-1 may be necessary for a cure.

As approaches for targeting the latent reservoir are developed, it is important to understand how the reservoir is measured. The original *ex vivo* measurements of the latent reservoir in resting CD4+ T cells were made using a viral outgrowth assay (6, 7). In this assay, serial dilutions of purified resting CD4+ T cells from an HIV-1 infected individual are activated with the mitogen phytohemagglutinin (PHA) and irradiated allogeneic peripheral blood mononuclear cells. This causes uniform T cell activation and thereby switches all of the cells carrying latent HIV-1 genomes into a state that is permissive for virus gene expression. CD4+ T lymphoblasts from healthy donors are added to amplify any virus released from the infected cells, and after a period of 2-3 weeks, the numbers of culture wells at each dilution that are positive for viral growth is determined by ELISA assay for HIV-1 p24 antigen. Using Poisson statistics, the number of positive wells can be used to calculate the number of latently infected cells per million resting CD4+ T cells (infectious units per million, IUPM). The advantage of this assay is that it quantifies the minimum number of latently infected cells harboring replication-competent virus. Disadvantages include cost and complexity and the fact that it does not distinguish between cells carrying integrated proviruses and recently infected cells carrying linear unintegrated HIV-1 DNA. However, because linear forms are labile, they may not confound the measurement in patients on ART. The most serious problem is the
range, which is limited by the low frequency of latently infected cells and the large amount of blood required to detect them. Eradication approaches that produce only small (1 log) reductions in the size of the latent reservoir may render the reservoir undetectable with this assay.

Alternative assays use PCR to quantify cells containing viral genomes. Some PCR assays are specific for integrated proviruses (5, 31, 101). A problem with all PCR-based assays is that they do not differentiate between cells that can produce infectious virus and cells containing proviruses that have been either permanently silenced through epigenetic mechanisms or rendered non-infectious through APOBEC3G-mediated hypermutation or some other form of mutation (6, 102). In patients on ART, the frequency of resting CD4+ T cells carrying HIV-1 DNA is 100- to 300-fold greater than the frequency of latently infected cells detected in the culture assay (103). Thus, although PCR assays are simpler, it is not yet clear that they will provide an effective way to monitor eradication efforts.

**Other reservoirs**

Although the existence of a stable latent reservoir in resting CD4+ T cells is clearly established, additional reservoirs could further complicate eradication efforts. Evidence for additional reservoirs comes from a detailed analysis of residual viremia. Although many viral sequences in the plasma of patients on ART are identical to sequences found in the latent reservoir, the residual viremia in some patients is dominated by oligoclonal populations called predominant plasma clones (PPC) that are profoundly underrepresented among proviruses in circulating CD4+ T cells (65). Sophisticated genetic tests for population structure also suggest that some of the residual viremia originates from cells other than resting CD4+ T cells found in the circulation (65, 104). It
is also possible that these clonal sequences originate from resting CD4+ T cells present only at low frequency in the circulation (105). In any event, residual viremia provides an important window into the state of virologic suppression because it reveals ongoing virus production in patients on ART and thus reports on the existence and persistence of viral reservoirs.

Macrophages

Among other cell types that could serve as HIV-1 reservoirs, macrophages are frequently mentioned. Early studies demonstrated HIV-1 infection of macrophages in vivo and showed that these cells are more resistant to viral cytopathic effects than are activated CD4+ T cells (106, 107). Macrophage-tropic HIV-1 variants utilize the CCR5 coreceptor (108, 109) but also have sequence changes in the Env protein that allow entry into cells that express low amounts of CD4, including macrophages (110, 111). Infection of macrophages is particularly prominent late in the course of infection when most of the CD4+ T cells have been lost (112). Although infected macrophages play a role in HIV-1 pathogenesis, it is not yet clear that these cells fit the definition for a reservoir proposed above.

Infection of macrophages should be viewed in the context of the normal differentiation of these cells, reviewed in (113) and (114). After less than one day, monocytes leave the circulation and undergo further differentiation. This differentiation is tissue-specific in the sense that it results in the generation of cells with different morphologies and functions depending on the tissue. These tissue forms include ordinary tissue macrophages, dendritic cells, Langerhans cells in the epidermis, alveolar macrophages in the lung, Kupffer cells in the liver, perivascular macrophages and
microglial cells in the CNS, and osteoclasts in bone. The tissue half-lives of these cell types are not well studied. Importantly, there is evidence that Langerhans cells and microglial cells take up residence in their respective anatomic sites and persist with less need for continuous replacement by monocytes (115).

Macrophages could in principle be infected at three different stages: during differentiation in the marrow, in the blood as monocytes, or in the tissues as mature macrophages. Infection of hematopoietic progenitor cells (HPC) is discussed below. Little is known about infection of bone marrow progenitor cells committed to the monocyte-macrophage lineage, but if it does occur, it must be rare. The number of circulating monocytes carrying HIV-1 DNA is extremely low, so low in fact that contaminating CD4+ T cells cannot be excluded as a source of the signal (116). Monocytes appear relatively resistant to \textit{in vitro} infection with HIV-1, due to inefficient entry and delays in early steps in the life cycle (117). In addition, the host restriction factor SAMHD1 may protect these cells from infection (118). Neither monocytes nor any of the committed progenitors in the marrow can be considered reservoirs by the definition proposed in Table 1.1 because these are transient stages of differentiation lasting only a matter of days before further differentiation occurs. In contrast, macrophages could serve as a reservoir if they can persist in an infected state on a time scale of years in patients on ART. Most studies documenting HIV-1 infection of macrophages in various tissues were from the pre-ART era (107, 119-121). Proving that macrophages function as a reservoir will require definitive demonstration that these cells harbor replication-competent HIV-1 in patients who have had prolonged suppression of viremia on ART.
Hematopoietic progenitor cells (HPCs)

HPCs express low amounts of CD4 and the coreceptors required for HIV-1 entry, CCR5 and CXCR4. Most studies have concluded that these cells do not harbor HIV-1 in vivo. For a review, see (122). Carter et al. reported HIV-1 infection in CD34+ HPCs in 4 of 9 patients on ART at a frequency of 2.5-40 copies of HIV-1 DNA/10^4 cells (123). Interestingly, the infection remained latent until the cells were driven to differentiate. A follow-up study indicated that CD133+ cord-blood-derived cells were susceptible to in vitro infection, but only with X4-tropic virus (124). Other more recent studies in patients on ART have used more rigorous purification of CD34+ cells and have not detected HIV-1 DNA by PCR (92). It is therefore not clear that hematopoietic stem and progenitor cells constitute a reservoir.

Anatomic sites of HIV-1 persistence

Central nervous system (CNS)

The CNS is an anatomic site in which viral replication has unique pathophysiologic consequences. In the pre-ART era, HIV-1-associated dementia was a devastating consequence of the disease, as reviewed in (125) and (126). HIV-1 has been detected in several areas of the brain as early as 15 days after infection (127). Within the CNS, infection is detected principally in perivascular macrophages and microglial cells (121). There is strong evidence for compartmentalization of HIV-1 in the CNS and concern that the CNS is a pharmacologic sanctuary. CNS-specific viral variants can be demonstrated in untreated individuals and may be associated with dementia (110, 128). With respect to the sanctuary issue, drug concentrations in brain parenchyma are hard to assess, but for some drugs, concentrations in the cerebrospinal fluid (CSF) are
substantially lower than in the blood. A sophisticated index for the CNS penetration and effectiveness of antiretroviral drugs has been developed (129), but the best predictor of neurocognitive disorders is the pretreatment CD4+ T cell count nadir (130). Studies of drug concentrations in the CSF are subject to the same caveats mentioned above for the male genital tract. Antiretroviral drugs are substrates for a variety of transporters that could potentially affect drug levels, as reviewed in (131). Arguing against the sanctuary concept is the general finding that initiation of ART rapidly reduces virus concentrations in the CSF, generally to undetectable levels, and prevents or improves many of the neurocognitive problems seen in untreated HIV-1 infection (132). However, there are rare patients in whom replication appears to continue, as evidenced by detectable free virus in the CSF (132). Given the prevalence of asymptomatic neurocognitive impairment in patients on ART (25-50% in some studies) (130), this is an extremely important area for future research.

Compelling evidence for viral persistence in the CNS comes from an elegant model of HIV-1 associated neurologic disease in SIV-infected macaques (133). In this model, infection with a neurovirulent SIV inoculum consistently produces neurologic disease within months of infection in untreated animals. Infection of microglial cells is particularly prominent (134). Antiretroviral drugs rapidly reduce plasma and CSF levels of virus, and viral RNA in the brain parenchyma (135). Interestingly, levels of SIV DNA remain constant. Because of the evidence from murine studies that microglial cells turn over less rapidly than other monocyte-derived cells (115), there is concern that these cells may serve as an important HIV-1 reservoir. The SIV model affords the best opportunity
to establish whether infected microglial cells can persist on a time scale of years in the setting of ART.

*Gut-associated lymphoid tissue (GALT)*

A substantial fraction of the total lymphocytes in the body are in specialized sites along the gastrointestinal tract. The GALT includes the Peyer’s patches, organized lymphoid structures in the small intestine that function as inductive sites. In addition, there are activated T cells in the lamina propria layer of the intestinal mucosa, beneath the epithelium. There are also lymphocytes within the epithelium. Pioneering studies by Veazey and colleagues in the SIV model (136) indicated that a dramatic loss of lamina propria CD4+ T cells occurs early in infection, much earlier than the loss of CD4+ T cells in other sites. The same is true for patients with HIV-1 infection (137, 138). This appears to reflect the high frequency of lamina propria CD4+ T cells that express the HIV-1 coreceptor CCR5 and that are in an activated state that is optimal for HIV-1 infection. The loss of CD4+ T cells from the lamina propria is not rapidly reversed by ART (138-140). In patients on ART, the fraction of cells in the lamina propria that carry HIV-1 DNA is substantially higher than in other sites (139, 141). Chun and colleagues reported an average of approximately 5000 HIV-1 DNA copies/10^6 CD4+ T cells in CD8-depleted biopsy samples from the terminal ileum of patients on ART, roughly 5 times greater than the HIV-1 DNA levels in resting CD4+ T cells from the peripheral blood. These findings raise the issue of whether CD4+ T cells in the GALT provide a unique reservoir for HIV-1. However, there are unresolved issues. Because many CD4+ T cells in the gut are in an activated state, it is important to know whether the infected cells in this site are expressing viral RNA, and if so, whether ongoing viral replication in this site contributes
to the high level of proviruses observed. One report describes a striking lack of evolution in the proviral sequences from recto-sigmoid biopsies (66). These results are inconsistent with continuous viral replication in this site. It is also important to determine the half-life of these infected cells and whether they contribute to residual viremia and viral rebound following treatment interruption. The results of our study, described in Chapter 5, suggest that these cells may contribute to residual viremia and that in some patients on ART, replication-competent virus can be measured from cells obtained from GALT biopsies using a quantitative viral outgrowth assay (QVOA).

Conclusions

Recent developments have contributed to renewed optimism that HIV-1 infection can be cured, but the discussion of this issue has been hampered by lack of agreement regarding the meaning of important terms. Here, we suggest definitions to facilitate the discussion. The weight of evidence suggests that HIV-1 persistence in patients on ART is due to long-lived infected cells that function as a reservoir rather than viral replication that continues despite ART or in drug sanctuaries. Although previous studies have proposed the existence of multiple reservoirs for HIV-1, only the latent reservoir in resting CD4+ T cells meets the practical definition proposed here, a cell type in which replication-competent virus can persist on a time scale of years in patients on optimal ART regimens. Macrophages are clearly infected in vivo, but it is not yet clear that infected macrophages in tissue sites survive on a time-scale of years in patients on optimal ART regimens. It will be particularly important to understand viral persistence in compartments such as the CNS, where long-lived microglial cells may represent a reservoir, and in the GALT, which is a major site of HIV-1 replication in untreated
individuals and may be a source of residual viremia and replication-competent virus in patients on ART.
CHAPTER 2 – A PILOT STUDY ASSESSING THE SAFETY AND LATENCY-REVERSING ACTIVITY OF DISULFIRAM IN HIV-1-INFECTED ADULTS ON ANTIRETROVIRAL THERAPY

This chapter was originally published as Spivak, Andrade, Eisele, Hoh, Bacchetti, Bumpus, Emad, Buckheit, McCance-Katz, Lai, Kennedy, Chander, Siliciano, Siliciano, and Deeks, 2014. It has been updated to reflect more recent findings in the field.

Background

Combination antiretroviral therapy (ART) has dramatically altered the natural history of human immunodeficiency virus type 1 (HIV-1) infection for most infected individuals with access to treatment (142). ART reduces plasma HIV-1 RNA to below the limit of clinical detection. It was initially hoped that the virus could be eradicated with 2-3 years of effective ART treatment (1); however, a latent form of HIV-1 exists in vivo.

Stably integrated, transcriptionally silent viral genomes persist in long-lived resting memory CD4+ T cells (5-8, 31). The stability of this latent reservoir is the major barrier to eradication of HIV-1 (5, 7, 10), requiring patients to remain on ART indefinitely.

Given the concern for adverse effects of ART, as well as the financial burden of treatment and need for adherence, strategies to eliminate the latent reservoir have become an urgent research priority.

One eradication strategy that has attracted significant attention involves targeting the latent reservoir through the use of drugs that reverse latency without inducing global T-cell activation (143, 144). This strategy is based on the hypothesis that cells in which latency has been reversed will be targeted by cytolytic CD8+ T cells or will die by viral cytopathic effects (145). Previous attempts to target latently infected cells by inducing
global T-cell activation have proven too toxic for use in humans (146-148). Subsequent research has focused on identifying compounds that will induce HIV-1 gene expression in latently infected resting CD4+ T cells without activating the cell itself (149). To this end, several in vitro models have been described that appear to recapitulate the phenotype of HIV-1 latency in resting CD4+ T cells (150-152). We have described one such model that makes use of Bcl-2-transduced primary CD4+ T cells (152) and performed a high-throughput screen to identify compounds that induce viral gene expression without triggering cellular activation (153). One hit from this screen was disulfiram, a US Food and Drug Administration (FDA)-approved drug used to treat alcoholism (154).

Disulfiram (bis[diethylthiocarbamoyl]disulphide) inhibits aldehyde dehydrogenase, resulting in an increased concentration of acetaldehyde when alcohol is consumed (155). This leads to an unpleasant systemic reaction that serves as a deterrent to alcohol consumption (156, 157). Disulfiram has been in clinical use for several decades (158) and has a well-characterized safety profile (159, 160).

The molecular mechanism of in vitro disulfiram-induced HIV-1 latency reactivation is unclear. Disulfiram undergoes a complex metabolism (160) with the downstream metabolite N,N-diethylthiocarbamate sulfoxide (DETC-MeSO) primarily responsible for aldehyde dehydrogenase inhibition and resultant clinical effect (161). In contrast, reactivation of latent HIV-1 in vitro occurs only with the parent compound and first metabolite, diethyldithiocarbamic acid (DDTC) (153). Subsequent metabolites, including diethyldithiocarbamate methyl ester (DDTC-Me), induce no appreciable HIV-1 reactivation (153). One report found that intracellular depletion of the phosphate and tensin homolog (PTEN) protein by disulfiram led to upregulation of the Akt signaling
pathway, resulting in HIV-1 gene transcription in the U1 HIV-1-infected monocyte cell line (162). This potential molecular mechanism of disulfiram activity is under further investigation.

We describe a pilot trial in which 500 mg of disulfiram was administered daily for 14 days to HIV-1-infected patients who had suppression of viremia on ART to determine whether this compound could reactivate latent HIV-1 from resting memory CD4+ T cells. This FDA-approved dose was selected to achieve in vivo concentrations of disulfiram and its metabolites comparable to concentrations that resulted in latency reversal activity in vitro (153). The safety of higher doses is unknown. We hypothesized that disulfiram treatment would be safe and would result in a transient increase in residual viremia due to virus release from latently infected resting CD4+ T cells and a measurable decline in the size of the latent reservoir.

Methods

Participants

This was an open-label, single-arm, pilot clinical trial at Johns Hopkins Hospital (JHH) and the University of California, San Francisco (UCSF). Inclusion criteria included age >18 years, use of a Department of Health and Human Services-recommended ART regimen continuously for a minimum of 18 months, >90% adherence as determined by self-report, maintenance of undetectable plasma viral load using standard commercial assays (<50 copies RNA/mL) for the previous 12 months, and a CD4+ T cell count >200 cells/µL for 24 weeks prior to enrollment. Participants had to agree to abstain from alcohol during the 2-week period of disulfiram administration and the subsequent 2 weeks.
Exclusion criteria included the presence of an alcohol use disorder; use of any drug formulation containing alcohol or medications involved in clinically important drug interactions with disulfiram; serious illness requiring hospitalization in the 3 months prior to enrollment; severe myocardial or coronary artery disease; history of psychosis, peripheral neuropathy, seizure disorder or hypothyroidism; evidence of clinically active hepatitis with aspartate aminotransferase (AST) or alanine transaminase (ALT) serum concentrations >3 times the upper limit of normal; treatment with immunomodulatory drugs in the previous 16 weeks; pregnancy or breastfeeding; and allergy to rubber or thiuram derivatives. The protocol was approved by the institutional review boards of both institutions participating in the trial.

Study design

Potential participants underwent an initial screening visit followed by 2-3 pretreatment visits after enrollment, during which blood was obtained for baseline measurements of residual viremia using a highly sensitive quantitative real-time reverse transcriptase polymerase chain reaction assay, the single-copy assay (SCA), as previously described (19). Safety laboratory tests including chemistry and liver function profiles and complete blood counts were obtained weekly during disulfiram administration and at every visit before and after the disulfiram administration period. At day -14, a large peripheral blood sample (180 mL) was obtained to measure the frequency of latently infected resting memory CD4+ T cells using a previously described quantitative viral outgrowth assay (163).

Beginning on day 0, subjects received a directly observed oral dose of 500 mg of disulfiram. Disulfiram was administered daily for 14 days under direct observation on
weekdays and by participant administration on weekends. Participants were evaluated at every visit for potential adverse events using a standardized questionnaire and a detailed face-to-face interview with a study investigator. All antiretroviral medications were continued throughout the trial, with medical adherence determined by self-report. Beginning at day 0, residual viremia was measured every 2 days (Monday, Wednesday, and Friday) for 3 weeks using the SCA. SCA was performed at every subsequent study visit for an additional 9 weeks. Plasma samples were also used to quantify disulfiram concentrations. A second 180-mL blood sample was obtained at week 12 for a posttreatment measurement of replication-competent HIV-1 in resting memory CD4+ T cells (Figure 2.1).

**Figure 2.1. Timing of trial events**

![Figure 2.1 Timing of trial events](image)

**Legend, Figure 2.1.** After enrollment, participants underwent several baseline measurements of residual viremia using a highly sensitive quantitative real-time reverse transcriptase polymerase chain reaction assay (the single-copy assay). Five hundred milligrams of disulfiram was administered daily from day 0 to day 13. A 180-mL blood sample was obtained at day -14 and again on day 84 to estimate the frequency of latently infected cells in peripheral blood using a limiting-dilution coculture method. Safety laboratory tests including a complete blood count and metabolic panel were drawn weekly before, during, and 2 weeks after disulfiram administration, and monthly thereafter. Abbreviation: DSF, disulfiram.

**Disulfiram mass spectrometry**

An ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) assay was developed to measure disulfiram concentrations in plasma. The UPLC-MS system consisted of a Dionex Ultimate 3000 UPLC system coupled to a TSQ
Vantage Triple Stage Quadrupole mass spectrometer (Thermo Fisher Scientific). A deuterated analogue (d20-disulfiram; deuterated at 20 positions on the molecule) was used as the internal standard. The analytes were extracted via protein precipitation and were chromatographically separated on a Phenomenex Kinetex column (phase C18; diameter, 2.1 mm × 50 mm; particle size, 1.7 µm; pore size, 100 Å) using mobile phases consisting of (1) water, 0.1% formic acid and (2) acetonitrile, 0.1% formic acid delivered at a flow rate of 400 µL/minute. The resolved analytes were detected by mass spectrometry in selected reaction monitoring mode under negative electrospray ionization using the following transitions: disulfiram m/z 297.2/116.1 and d20-disulfiram m/z 317.2/126.1. The assay was linear from 15 ng/mL to 6400 ng/mL of disulfiram with an $r^2$ of 0.996 (SD 0.001). Interday and intraday precision ranged from 1.8% to 6.3% and 1.2% to 5.8%, respectively, whereas the accuracies were 95.4% to 104%, and 93.6% to 105%, respectively.

**Biostatistical analysis**

Maximum likelihood estimation of rates of latently infected resting CD4+ T cells was carried out using the NLMIXED procedure in SAS version 9.2 (SAS Institute, Cary, North Carolina), with Wald 95% confidence intervals (CIs) calculated for log IUPM (infectious units per million) and then back-transformed to the IUPM scale. For cases with no positive cocultures, the estimated IUPM was zero and an upper 95% confidence bound was calculated as $3/(\text{total number of cells tested})$ (164). We modeled the effect of the post-disulfiram time point (vs. predisulfiram) on log IUPM across all participants, using the primary data from each coculture of whether it was positive or negative and how many cells it contained. This model was fit by maximum likelihood in the
NLMIXED procedure in SAS, and included parameters for each participant’s baseline log IUPM to account for the matched pre-post nature of the data within individuals. Additional models allowed the postdisulfiram effect to differ for those who ever had a detectable disulfiram level compared to those who did not. The software provided Wald p values and 95% CIs. We back-transformed estimates and CIs to fold-effects on IUPM. We performed sensitivity analyses by excluding particular wells that had probability <0.001 given the estimated IUPM.

We modeled residual viremia measured by SCA using negative binomial regression, with a random intercept to account for within-person correlation, again using the SAS NLMIXED procedure. To prevent very large values from dominating the analyses, we set SCA values >56 to equal 56, which was the 97th percentile of all observed values. We initially fit a model with one parameter for how viremia during disulfiram administration differed from the baseline period and one parameter for how it differed postdisulfiram compared to baseline. We then fit models that examined a number of possible refinements: allowing viremia 2 hours after the first does (measured at JHH) to differ from viremia at other times during disulfiram administration; allowing viremia during and after administration to differ depending on whether disulfiram was ever detected in any of the patient’s blood specimens; and allowing viremia during administration to be influenced by the concurrently measured blood level of disulfiram. We chose the primary model for presentation as the simplest one for which all further refinements had a p value >0.05.

**Results**
Study participants and safety outcomes

We enrolled 16 participants (11 at JHH, 5 at UCSF; Table 2.1). The median CD4+ T cell count and percentage at the time of enrollment were 609 cells/µL (range, 224-1168 cells/µL; interquartile range [IQR], 366 cells/µL) and 30% (range, 12.6%-42.7%; IQR, 11%), respectively. The median time of viral suppression (<50 copies/mL) was 79 months (range, 16-162 months; IQR, 79 months). ART regimens for 8 participants combined 2 nucleoside reverse transcriptase inhibitors (NRTIs) with the nonnucleoside reverse transcriptase inhibitor efavirenz, and regimens for the other 6 patients combined 2 NRTIs with a ritonavir-boosted protease inhibitor. Two participants were taking regimens that included agents from >2 antiretroviral drug classes. One dropped out of the study after completing 12 days of disulfiram therapy.

Table 2.1 Participant baseline characteristics

<table>
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<tr>
<th>Participant no.</th>
<th>Age</th>
<th>Sex</th>
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<th>Duration of viral suppression</th>
<th>ART regimen</th>
<th>Screening CD4 count (%)</th>
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</tr>
<tr>
<td>2432</td>
<td>57</td>
<td>M</td>
<td>W</td>
<td>26</td>
<td>FTC/TDF/EFV</td>
<td>503 (34)</td>
</tr>
<tr>
<td>3037</td>
<td>52</td>
<td>M</td>
<td>AA</td>
<td>58</td>
<td>3TC/ABC/TDF/ZDV/ETV/DRV/r</td>
<td>504 (23)</td>
</tr>
</tbody>
</table>

Legend, Table 2.1. Abbreviations: 3TC, lamivudine; AA, African American; ABC, abacavir; ART, antiretroviral therapy; ATV/r, atazanavir boosted with ritonavir; DRV/r, darunavir boosted with ritonavir; EFV, efavirenz; ETV, etravirine; FPV/r, fosamprenavir boosted with ritonavir; FTC, emtricitabine; H, Hispanic; NVP, nevirapine; TDF, tenofovir; W, non-Hispanic white; ZDV, zidovudine. *Consecutive
months of documented viral load (plasma HIV-1 RNA) suppression below limit of clinical detection on ART. \(^b\)Absolute CD4+ T cell count measured in cells/µL.

Disulfiram was safe and well tolerated in all participants. Observed adverse events during the study were consistent with grades I and II toxicity. One participant had a single detectable viral load measured by a standard commercial assay (620 copies/mL) at a postdisulfiram time point that returned to an undetectable level (<50 copies/mL) at the next study visit and remained undetectable for the trial duration. All other participants maintained undetectable viral loads as measured by commercial viral load assays throughout the trial. No substantial changes in CD4+ T cell count or percentage were observed in any participant for the duration of the trial.

*Effect of disulfiram administration on the frequency of latently infected cells*

The size of the latent reservoir from each participant was measured by limiting dilution coculture assay (165) 2 weeks before and 10 weeks after disulfiram administration (Figure 2.2). There was little change in the geometric mean frequency of latently infected cells 10 weeks after disulfiram administration compared to baseline (postdisulfiram fold-effect = 1.16; 95% CI, 0.70 – 1.92; p = 0.56). A majority of participants had a latent reservoir size within the previously described dynamic range of this assay (10).
Figure 2.2. Effect of disulfiram (DSF) on latent reservoir size

Legend, Figure 2.2. No substantial change in the frequency of latently infected cells was observed 10 weeks after disulfiram administration compared to baseline as measured by limiting dilution coculture assay (post-DSF fold-effect = 1.16; 95% confidence interval, 0.70-1.92; p = 0.56). *Dashed lines/open circles represent 3 coculture assays in which no infected cells were identified (these cocultures had 12.5 million to 27.5 million cells assayed). Abbreviation: IUPM, infectious units per million.

Effect of disulfiram administration and plasma concentration on residual viremia

Residual viremia was measured by SCA in plasma samples obtained at enrollment, days -14, -7, 0, 2, 4, 7, 9, 11, 14, 16, and 18, and at weeks 3, 4, 8, and 12. Individual plasma virus and disulfiram concentrations through week 4 are shown in Figure 2.3. Our initial model estimated that residual viremia averaged 1.53-fold higher during disulfiram administration than during the predisulfiram baseline period (95% CI, 1.04- to 3.57-fold; p = 0.039).
Figure 2.3. Individual single-copy assay and disulfiram (DSF) plasma concentration results.
Legend, Figure 2.3. The gray bar (between days 0 and 13) represents the period of directly observed DSF administration. Half of Johns Hopkins Hospital participants (numbers 7151-7161) had an increase in viremia within several hours of the dose, but viremia during DSF administration after that time averaged only slightly higher than baseline and was not statistically significant. Six of 15 participants had detectable DSF plasma concentrations during the dosing interval. The lower limit of detection of DSF plasma concentrations using mass spectrometry was 15 ng/mL.

Participants at JHH (n = 10) received disulfiram 2 hours prior to plasma sampling at each visit. We observed an increase in residual viremia after the first dose of disulfiram (day 0) in several participants. Residual viremia on day 0 was estimated to average 3.81-fold higher than the predisulfiram baseline (95% CI, 1.43- to 10.18-fold; p = 0.01). Average residual viremia for the remainder of the disulfiram dosing interval did not differ significantly from baseline (1.30-fold increase; 95% CI, 0.74- to 2.27-fold; p = 0.33).

Table 2 shows our model, which takes into account these early increases in viremia.

Table 2.2. Single copy assay kinetics

<table>
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<tr>
<th>SCA time point</th>
<th>Fold change</th>
<th>% Change</th>
<th>95% CI</th>
<th>P value</th>
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<tbody>
<tr>
<td>DSF first dose (day 0)</td>
<td>3.81</td>
<td>281.30</td>
<td>42.8-917.7</td>
<td>0.01</td>
</tr>
<tr>
<td>DSF dosing interval (days 1-13)</td>
<td>1.30</td>
<td>29.90</td>
<td>-25.7-127.4</td>
<td>0.33</td>
</tr>
<tr>
<td>Post-DSF interval (days 14-84)</td>
<td>1.88</td>
<td>87.90</td>
<td>2.8-243.2</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Legend, Table 2.2. Abbreviations: CI, confidence interval; DSF, disulfiram; SCA, single copy assay. ^Fold-change and percentage changed compare average SCA values with the pre-DSF baseline SCA estimate of 2.2 copies/mL (95% CI, 0.9-5.4). ^Day 0 SCA comparison performed with Johns Hopkins Hospital (JHH) SCA values only (n = 10) because University of California, San Francisco subjects did not undergo plasma sampling immediately after day 0 DSF dose. ^JHH SCA values only (n = 10). ^Data for all subjects included (n = 15).

Subject-to-subject variability in disulfiram exposure

We measured plasma levels of disulfiram by mass spectrometry. There was substantial and unexplained variability in disulfiram concentrations that did not appear to be predicted by treatment regimen (Figure 2.2). Six of 15 subjects had detectable plasma disulfiram concentrations at some point during the 2-week dosing interval (subjects 7153, 7154, 7155, 7156, 2006, and 3037 shown in Figure 2.3; lower limit of detection 15 ng/mL). There was a nonsignificant trend suggesting higher average viremia in these 6
subjects compared to those in whom disulfiram concentrations were not detected (estimated difference, 0.47-fold; 95% CI, 0.20- to 1.10-fold, \( p = 0.077 \)). Comparing postdisulfiram average viremia to the predisulfiram baseline showed an estimated 2.96-fold increase over baseline among these 6 subjects (95% CI, 1.29- to 6.81-fold; \( p = 0.01 \)) compared to those without detectable disulfiram (1.39-fold; 95% CI, 0.69- to 2.79-fold; \( p = 0.33 \); difference detected vs. not detected, 2.13-fold; 95% CI, 0.85- to 5.4-fold; \( p = 0.10 \)).

**Discussion**

We conducted a pilot clinical trial in which we administered the FDA-approved drug disulfiram for 14 days to HIV-1-infected individuals on ART to evaluate the safety and efficacy of this intervention as a means to perturb the HIV-1 latent reservoir. Disulfiram was well tolerated by all participants. The size of the latent reservoir, measured by a well-validated in vitro viral outgrowth assay (163), did not decrease after the intervention. We observed only a small and not statistically significant average change in residual viremia during disulfiram treatment compared to baseline. In a post hoc analysis limited to 10 subjects with frequent sampling, we observed an unexpected rapid and transient increase in plasma viremia. Disulfiram exposure varied substantially among subjects. There is much interest in understanding the kinetics of the later stages of viral replication, including proviral gene transcription, translation, viral budding, and release in resting CD4+ T cells. A recently published study in which a single dose of the histone deacetylase inhibitor (HDACi) vorinostat was administered to 8 HIV-1-infected patients with viral suppression estimated a mean 4.8-fold increase in cell-associated HIV-1 RNA within 4-7 hours of drug administration (99). Similar data have been reported by
Lewin and colleagues with vorinostat (166). Levin and Tolstrup reported rapid increases in plasma viremia after exposure to the HDACi panobinostat in an ongoing phase 1/2 clinical trial (NCT01680094) (167). In an _in vitro_ study assessing the impact of various antilatency drugs on the kinetics of HIV-1 RNA production, the impact of disulfiram was more rapid and transient than vorinostat (168), an observation consistent with the data presented here. Disulfiram appeared to have no effect on the size of the latent reservoir as measured by quantitative _in vitro_ viral outgrowth. The mechanism of action of disulfiram in inducing proviral transcription is not currently understood. Disulfiram may induce HIV-1 transcription by activating the Akt signaling pathway, as has been described in cell line models of HIV-1 latency (162). Alternatively, it is possible that even potent and sustained reversal of latency may not affect the reservoir in a durable manner. One important tenet of the “shock and kill” HIV-1 eradication strategy that makes use of latency-reversing agents targeting the reservoir is that virus-producing cells will be cleared by the immune system or will be eliminated by viral cytopathic effects following viral reactivation. However, it appears that reversing latency without T cell activation may not be sufficient to kill latently infected CD4+ T cells (100). The study presented here suggests that “shock and kill” strategies with drugs such as disulfiram will likely require another step to prime the immune system to clear virus-producing resting memory CD4+ T cells. Additionally, more recent studies have suggested that latency reversing agents individually are ineffective at inducing outgrowth from the latent reservoir (169) and that combinations of certain agents are necessary to induce this outgrowth _ex vivo_ (170).
The pharmacokinetics and pharmacodynamics of disulfiram appear to be highly variable among subjects. Up to 50% will not have a disulfiram-ethanol reaction with a 250-mg dose (171). For some individuals, doses of 500 mg are insufficient to instigate this reaction (171). A formal study of the elimination kinetics of disulfiram found marked intersubject variability in plasma levels of disulfiram and its metabolites (159). A separate study identified a 600-fold variation in disulfiram plasma concentrations among subjects (172). The mechanism for this variability remains unknown. Using highly sensitive mass spectrometry, we also found substantial subject-to-subject variability in drug exposure, and could detect plasma disulfiram concentrations in only 6 of 15 participants. These measurements take into account only the parent compound; mass spectrometry assays for downstream metabolites may illuminate intersubject disulfiram pharmacokinetics and are in development for future studies. These participants had a significant “post-drug” increase in low-level viremia that was sustained over the 2 months following the disulfiram dosing interval, and also demonstrated a nonsignificant trend toward decrease in the size of the latent reservoir, suggesting that higher exposures to the drug in vivo may have more pronounced and prolonged effects on HIV-1 production.

In summary, this trial attempted to safely translate in vitro discoveries affecting the latent reservoir into initial in vivo analysis. Disulfiram was safe and well tolerated, but did not appear to significantly perturb the latent reservoir. The apparent exposure-response effect observed in this study highlights significant intersubject variability in disulfiram pharmacokinetics and suggests that higher doses of disulfiram might be more effective. It is also possible that combining disulfiram with other latency-reversing agents
will have a more pronounced effect on the reservoir, and the favorable safety profile of disulfiram provides support for such combination approaches.
CHAPTER 3 – HIV-1 DNA IS DETECTED IN BONE MARROW POPULATIONS CONTAINING CD4+ T CELLS BUT IS NOT FOUND IN PURIFIED CD34+ HEMATOPOIETIC PROGENITOR CELLS IN MOST PATIENTS ON ANTIRETROVIRAL THERAPY

This chapter was originally published as Durand, Ghiaur, Siliciano, Rabi, Eisele, Salgado, Shan, Lai, Zhang, Margolick, Jones, Gallant, Ambinder, and Siliciano, 2012.

Background

Antiretroviral therapy (ART) stops replication of human immunodeficiency virus type 1 (HIV-1) and reduces plasma viremia to undetectable levels. Despite treatment, latent HIV-1 DNA is detected in resting CD4+ T cells; with therapy interruption, HIV-1 viremia rapidly rebounds to pretreatment levels, reviewed in (173). Longitudinal studies confirm that due to the long-lived nature of resting CD4+ T cells, the size of this reservoir does not appreciably decrease with time on ART (10).

In addition to latent HIV-1 DNA in resting CD4+ T cells, most patients on ART have residual HIV-1 RNA in the plasma that can be detected with sensitive real-time polymerase chain reaction (PCR) assays (19). HIV-1 sequence analysis and phylogenetic studies of residual viremia demonstrate that many sequences in the plasma are identical to sequences in resting CD4+ T cells. However, in some individuals, residual viremia contains predominant HIV-1 clones that are profoundly underrepresented in circulating CD4+ T cells (65). This suggests that other significant HIV-1 reservoirs may exist in cells capable of proliferating after infection.

Hematopoietic progenitor cells (HPCs) are a logical candidate for a second reservoir due to expression of coreceptors required for HIV-1 entry, that is, CCR5 and
CXCR4 (174). Most studies of HPCs over the past 2 decades suggest that these cells do not harbor HIV-1 \textit{in vivo}. This work has been reviewed in (122). However, other studies found HIV-1 DNA in HPCs in patients infected with non-subtype B virus (175, 176). A recent study was the first to address this in patients on ART with undetectable plasma HIV-1 (123). The authors reported HIV-1 infection in CD34+ HPCs in 4 of 9 patients at a frequency of 2.5-40 copies of HIV-1 DNA/10^4 cells. The reasons for these different findings are unclear. Determining whether CD34+ cells constitute a second HIV-1 reservoir in patients on ART is critical to ongoing HIV-1 eradication efforts, which currently focus on latently infected CD4+ T cells as the barrier to curing HIV-1 infection. We conducted a study in ART-treated patients with undetectable plasma HIV-1 using rigorous purification techniques and a real-time PCR assay to look for HIV-1 DNA in CD34+ HPCs. In a subset of patients, we also performed an HPC/lymphocyte coculture assay, which is less sensitive for total HIV-1 DNA but highly specific for replication-competent HIV-1.

\textbf{Methods}

\textit{Subjects}

We studied HIV-1-infected adults on ART with a plasma HIV-1 level <50 copies/mL for at least 2 years. Isolated HIV-1 plasma RNA measurements between 50 and 200 copies/mL were considered viral “blips” and did not exclude participation. All patients gave informed consent according to a Johns Hopkins School of Medicine institutional review board.
Cell purification

Study volunteers donated 40 mL of blood and 25-50 mL of bone marrow aspirated from the iliac crest. Bone marrow mononuclear cells (BMMCs) were obtained by Ficoll-Hypaque density gradient centrifugation. Cells were cultured in RPMI (Sigma-Aldrich) supplemented with 10% fetal bovine serum overnight. CD4+ T cells and CD34+ cells were purified within 24 hours by positive selection using a magnetic bead and column system (Miltenyi). The bead-enriched CD34+ population was labeled with phycoerythrin-conjugated anti-CD34 and fluorescein isothiocyanate-conjugated anti-CD3 monoclonal antibodies (BD Biosciences) and purified by fluorescence-activated cells sorting (FACS) on a MoFlo flow cytometer (Beckman Coulter). Viable CD34+ and CD3+ cells based on forward- and side-scatter properties were collected by FACS. Anti-CD3 was used to mark T cells because HPCs may express CD4 at low levels.

HIV-1 DNA quantification

DNA was extracted immediately following cell sorting using a QIAamp DNA Microkit (Qiagen). A single-step real-time PCR was used to quantify HIV-1 DNA in a 50-µL PCR reaction mix containing 25 µL of TaqMan Universal PCR Master Mix (Applied Biosystems), 20 µL of template DNA, and a primer-probe set designed to bind to a conserved region of gag: primers 6F (5’-CATGTTTTTCAGCATTATCAGAAGGA-3’) and 84R (5’-TGCTTGATGTCCCCCACC-3’) (600 nmol) and probe (5’-FAM-CCACCCACAAAGATTTAAAACACCATGCT) from a published single-copy assay to amplify HIV-1 RNA (19). Amplifications were performed with an Applied Biosystems 7300 real-time PCR system. Thermal cycling conditions were 95°C for 10 minutes, 50 cycles at 95°C for 15 seconds, and 60°C for 1 minute. HIV-1 copy number was
determined by extrapolation from a standard curve generated from serial dilutions of a cell line harboring 1 HIV-1 genome per cell (177), which were spiked into genomic DNA from HIV-1-uninfected peripheral blood mononuclear cells (PBMCs) in order to accurately represent the \textit{in-vivo} situation of low HIV-1 copy number in a high background. The limit of detection of this assay was determined to be 2 copies of HIV DNA in $10^5$ cell equivalents of DNA, defined as the minimum copy number that could be detected >90% of the time. To determine if CD34+ cellular DNA in particular inhibited amplification of HIV-1, we measured the ability of our assay to detect 100, 10, and 5 HIV-1-positive cells spiked into $10^5$ patient CD34+ cells. To quantify cell number, a separate reaction was performed to measure the human gene CCR5 using a published primer-probe set (178). All reactions, including negative controls with water and with uninfected PBMCs, were run in triplicate.

\textit{Culture assay for replication-competent HIV-1}

In patients from whom >$10^6$ CD34+ cells were obtained by FACS ($n = 7$), CD34+ cells were cultured to amplify replication-competent HIV-1 RNA. Two 4-mL wells with CD34+ cells at $10^5$ cells/mL were cultured in StemSpan (StemCell Technologies) with 100 ng/mL of stem cell factor, 100 ng/mL FMS-like tyrosine kinase-3 ligand, and 100 ng/mL thrombopoietin (R&D Systems) plus either (1) 100 ng of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and 2.5 ng/mL tumor necrosis factor α (TNF-α; Biolegend) or (2) 10 ng/mL of phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich) for 7 days as previously described (123). Then $5 \times 10^6$ BMMCs and CD34-depleted cells were cultured under the same cytokine conditions (concentration $10^6$ cells/mL). After 7 days, activated CD4+ lymphoblasts from uninfected
donors were added as described in a resting CD4+ T cell lymphocyte coculture assay (163). Lymphoblasts were added at days 7, 14, and 21. Culture supernatants were collected on days 7, 14, 21, and 28 and tested for p24 antigen (Perkin Elmer) and HIV-1 RNA (Roche Amplicor).

**Results**

*Patients*

Patient characteristics are shown in Table 3.1. Median duration since HIV-1 diagnosis was 21 years, and median duration of undetectable viremia on ART was 4.8 years. Patients had prior evidence of significant immunosuppression with a median CD4+ T cell nadir of 212 cells/µL. Four of 11 participants had anemia or thrombocytopenia at the time of sampling. The majority (10 of 11) of patients started ART during chronic infection, and 1 patient initiated ART within 2 months of acute HIV-1 infection.
Table 3.1. Patient characteristics

<table>
<thead>
<tr>
<th>Study ID (age in y, sex)</th>
<th>Current CD4 count, cells/mm³</th>
<th>CD4 nadir, cells/mm³</th>
<th>Years since diagnosis</th>
<th>Years on ART with HIV RNA &lt;50 copies/mL</th>
<th>White blood cell count, K/µL</th>
<th>Hematocrit, %</th>
<th>Platelet count, K/µL</th>
<th>No. of CD34+ cells assayed in PCR</th>
<th>Purity of CD34+ cells, %</th>
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</thead>
<tbody>
<tr>
<td>BM1 (65, M)</td>
<td>427</td>
<td>212</td>
<td>16</td>
<td>4.7</td>
<td>3.8</td>
<td>36.8</td>
<td>198</td>
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<td>642</td>
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<td>24</td>
<td>2.2</td>
<td>8</td>
<td>41.6</td>
<td>298</td>
<td>1.1 × 10⁶</td>
<td>98.7</td>
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<tr>
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<td>551</td>
<td>255</td>
<td>11</td>
<td>6</td>
<td>4.7</td>
<td>35.3</td>
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<td>1.6 × 10⁵</td>
<td>&gt;99.5</td>
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<tr>
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<td>21</td>
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<td>5.6</td>
<td>46.6</td>
<td>212</td>
<td>2.2 × 10⁵</td>
<td>99.3</td>
</tr>
</tbody>
</table>

Legend, Table 3.1. Abbreviations: HIV, human immunodeficiency virus; PCR, polymerase chain reaction. aIsolated measurements between 50 and 200 copies/mL were considered clinically insignificant viral “blips” and were not included. bNormal range, 2.4 – 10.8 K/µL. cNormal range for men, 40% – 54%. dNormal range, 150–450 K/µL. ePurity of CD34+ cells, expressed as the fraction of CD34+CD3- cells.
*Purification*

We isolated CD34+ HPCs using a 2-step process (Figure 3.1a). After bead enrichment, the average purity of CD34+ cells was 63% (range, 25%-87%). The average level of contaminating CD3+ T cells was 10% (range, 3%-17%). After FACS, the purity of the CD34+ cell population was >98.7% (Figure 3.1b), with contaminating CD3+ T cells <0.2% in all patients.
Figure 3.1. Analysis of human immunodeficiency virus type 1 (HIV-1) DNA in CD34+ hematopoietic progenitor cells

Legend, Figure 3.1. a) Purification methods used. CD34+ cells were first enriched from bone marrow mononuclear cells (BMMCs) using anti-CD34-conjugated magnetic beads. Highly pure populations were then obtained by flow-cytometric cell sorting using anti-CD34 and anti-CD3 antibodies. b) Representative flow cytometric analysis corresponding to CD34+-enriched vs. CD34+-sorted cell populations from (a); phycoerythrin (PE) and fluorescein isothiocyanate (FITC). c) HIV-1 DNA copies/100,000 cells in peripheral CD4+ T cells (CD4+), sorted CD34+ cells, bone marrow depleted of CD34+ cells (CD34-depleted), CD3+ cells sorted from enriched CD34+ cells (CD3+), unfractionated BMMCs, and peripheral blood mononuclear cells from uninfected donors (HIV-PBMCs). Each color square represents a different patient designated by BM (bone marrow) and study number. The corresponding color HIV-PBMCs are the negative controls for the specific patient experiment. For patient 1, only sorted CD34+ and CD34-depleted fractions were tested.
**HIV-1 DNA quantification**

To measure HIV-1 DNA, we used a real-time PCR assay with a limit of detection of 20 copies of HIV-1 DNA/10^5 cells. We compared HIV-1 copy numbers in sorted CD34+ cells to copy numbers in peripheral CD4+ T cells and other bone marrow fractions: CD34-depleted cells, sorted CD3+ cells from the bead-enriched CD34+ cells, and BMMCs. Table 3.1 lists the number of CD34+ cells assayed per patient (median, 5.5 × 10^5 cells; range, 1.1 × 10^5 to 1.1 × 10^6).

The copy number of HIV-1 per 10^5 cells is shown in Figure 3.1c. HIV-1 DNA was detected in CD4+ T cells in 11 of 11 patients, with a geometric mean of 64 copies/10^5 cells. In many patients, HIV-1 DNA was detected in the other bone marrow fractions. In 11 of 11 patients, no HIV-1 DNA was detected in sorted CD34+ cells. Lack of signal was not due to PCR inhibition because the CCR5 gene was amplified in all samples. In addition, an internal positive control using 100, 10, and 5 HIV-1-positive cells spiked into 10^5 sorted CD34+ patient cells confirmed that small amounts of HIV-1 could be detected in these cells (data not shown).

**CD34+ HPC/lymphocyte coculture**

To assess whether production of HIV-1 is induced with *ex vivo* differentiation of CD34+ HPCs, we cultured cells from 7 patients with cytokines and added activated CD4+ lymphoblasts as target cells for HIV-1 replication. In resting CD4+ T cells, this coculture assay detects replication-competent virus released from a single infected cell (10). In 7 of 7 patients, neither HIV-1 antigen nor HIV-1 RNA was detected in culture supernatants from CD34+ cells over 28 days. We cultured BMMCs and CD34-depleted cells under the same conditions and detected replication-competent HIV-1 in 3 of 7
patients (2 in BMMC in GM-CSF/TNF-α and PMA, respectively, and 1 in CD34-depleted cells in GM-CSF and TNF-α).

**Discussion**

We have found that in patients undergoing ART, HIV-1 DNA can be frequently detected in bone marrow fractions that contain mature T cells but not in highly pure populations of CD34+ HPCs. Our quantitative PCR assay detected HIV-1 in CD4+ T cells in 11 of 11 patients but found no evidence of HIV-1 in CD34+ cells with a sensitivity down to 2 copies of HIV-1 DNA/10^5 cells and a median of 5.5 × 10^5 CD34+ cells assayed per patient. Because it has been reported that latent infection of CD34+ cells can be reversed by cytokine-directed differentiation, we cultured bone marrow fractions from a subset of 7 patients with uninfected lymphocytes. After culture for 1 month, we detected replication-competent HIV-1 in CD34+ cells from 0 of 7 patients and in bone marrow fractions containing T cells in 3 of 7 patients. This assay only detects replication-competent HIV-1 and thus is less sensitive for total HIV-1 DNA.

This study was limited by the small sample size and sensitivity of our PCR assay. We had >90% power to detect infection in 20% of patients. We can conclude that in the majority of HIV-1 subtype-B-infected patients on ART, the frequency of HIV-infected CD34+ is <0.002%. These conclusions extend a large body of clinical and translational data reported over the past 2 decades indicating that CD34+ cells do not constitute a significant reservoir for HIV-1 *in vivo*. This work has been reviewed in (122).

The only prior study to examine this issue in ART-treated patients with undetectable plasma HIV-1 RNA levels found PCR evidence of HIV-1 DNA in CD34+ cells in 4 of 9 patients (123). There are several potential explanations for the conflicting
data. This could be the result of CD34+ cells being infected at a level below the limit of detection of our assay and/or be due to insufficient sampling of cells per patient. However, our reported sensitivity is comparable to the prior report, and the number of cells assayed per patient is larger. We hypothesize that contamination with HIV-1-infected mature T cells could produce false positive results in bead-enriched HPCs. The purity of the bead-enriched CD34+ cells in patients on ART studied by Carter et al. (123) ranged from 30% to 97% (median 85%) and was 30%, 57%, 83%, and 0% in the 4 patients with detectable HIV-1 DNA. This purity is typical of column enrichment methods, and we had a similar range of purity (25% – 87%) after CD34+ bead enrichment. We did not perform PCR on the CD34-enriched cells in order to maximize our yield from FACS. As an alternative control, we tested other bone marrow fractions known to contain CD4+ T cells and detected HIV-1 in a significant proportion of patients.

Lack of HIV-1 detection in our cohort could be explained by HIV-1 coreceptor tropism. CD34+ cells are heterogeneous and include rare hematopoietic stem cells as well as committed myeloid and lymphoid progenitors. These cells vary in their expression of CCR5 and CXCR4 used for HIV-1 entry. More primitive cells express CXCR4 but not CCR5 (174). An in vitro study by Carter et al. (124) showed that HIV-1 vectors pseudotyped with X4 envelope infect progenitor cells more efficiently than vectors pseudotyped with R5 envelope. However, whether this occurs in vivo is less clear. We do not know the tropism of viruses present in the majority of patients in our cohort. In vivo studies that focus on patients with different frequencies of X4 virus may clarify this issue.
CHAPTER 4 – RAPID QUANTIFICATION OF THE LATENT RESERVOIR FOR HIV-1 USING A VIRAL OUTGROWTH ASSAY

This chapter was originally published as Laird (co-1st author), Eisele (co-1st author), Rabi (co-1st author), Lai, Chioma, Blankson, Siliciano, and Siliciano, 2013. It has been updated to reflect more recent findings in the field.

Background

Combination antiretroviral therapy (ART) has significantly reduced the morbidity and mortality associated with HIV-1 infection. However, while ART can reduce plasma viral loads to below the clinical limit of detection (50 copies of HIV-1 RNA/mL) in adherent patients (1, 179, 180), this treatment is not curative. Even in individuals on prolonged suppressive ART, HIV-1 persists as a latent but replication-competent provirus integrated in the genomes of a small percentage of resting memory CD4+ T cells (1, 5-9). These latently infected cells are extremely long lived as a consequence of the biology of memory T cells, with an estimated half-life of 44 months (10, 181). The extreme stability of this HIV-1 reservoir precludes eradication with ART alone and suggests that without disruption of this reservoir, infected individuals must remain on ART for the remainder of their lives (10, 181).

Recent studies have identified small molecules capable of reactivating HIV-1 gene expression (93-95, 152, 153, 182, 183). While resting CD4+ T cells harboring a latent HIV-1 provirus are not susceptible to viral cytopathic effects of immune clearance, pharmacological reactivation of latent proviruses could lead to depletion of this latent reservoir. Recent in vitro work suggests that reactivation paired with a T cell vaccination strategy could be used to eradicate the latent reservoir of HIV-1 in resting CD4+ T cells.
Several clinical trials are investigating the ability of small molecule reactivators to perturb the latent state of the provirus and reduce the size of the latent reservoir (12, 99).

Assessing strategies to perturb or eliminate the latent reservoir requires assays that can accurately quantitate the size of the latent reservoir and that can be scaled for use in large clinical trials. The reservoir was originally identified using a viral outgrowth assay carried out on highly purified resting CD4+ T cells isolated from patient peripheral blood mononuclear cells (PBMC) (7, 9, 10, 163). These resting cells do not actively produce virus without stimulation (5). Limiting dilutions of the resting CD4+ T cells are activated with the mitogen phytohemagglutinin (PHA) in the presence of irradiated allogeneic PBMC. This activation reverses latency and reinitiates the production of infectious HIV-1 from the subset of resting CD4+ T cells harboring replication-competent proviruses. The viruses that are produced are expanded in PHA-stimulated CD4+ lymphoblasts from uninfected donors, which are added to the culture at two time points. After two weeks, viral outgrowth is assessed by an ELISA assay for HIV-1 p24 antigen in the culture supernatant. The frequency of latent infection, expressed as infectious units per million (IUPM) resting CD4+ T cells, is determined using Poisson statistics. Typically, patients on long term ART exhibit IUPM values between 0.1 and 1 (7, 9, 10).

While this viral outgrowth assay is widely recognized as the definitive assay for determining the minimum frequency of CD4+ T cells harboring replication-competent proviruses, it is time-consuming, labor-intensive, and expensive, requiring two weeks of cell culture and PBMC from at least 3 uninfected blood donors per assay. In its current form, this assay is not suitable for use in large clinical trials evaluating the efficacy of
eradication strategies, and the identification of simpler assays for the latent reservoir has been identified as an AIDS research priority (184). A recent study compared 11 different approaches for measuring persistent HIV-1 in patients on ART (185). Infected cell frequencies measured by PCR-based methods were generally at least two orders of magnitude higher than and poorly correlated with IUPM values. This likely reflects the presence of large numbers of defective proviruses that are detected by PCR-based assays. The results raise doubts about whether PCR-based assays can be used to assess the frequency of cells harboring replication-competent proviruses. Therefore, we developed a rapid and simple viral outgrowth assay that can nevertheless detect and provide at least a minimal estimate of the frequency of cells that must be eliminated to cure HIV-1 infection.

Methods

Patient cohort

Twenty HIV-1-infected patients were enrolled in this study; 19 were recruited from the Moore Clinic at The Johns Hopkins Hospital, and 1 patient was recruited from the SCOPE cohort at the University of California San Francisco. All study participants provided written informed consent for participation. This study was approved by the Johns Hopkins Institutional Review Board. Seventeen of the 20 patients were recruited on the basis of prolonged continuous suppression of plasma HIV-1 viremia on ART to below the limit of detection of standard clinical assays (<50 copies HIV-1 RNA/mL). Three of the 20 patients were recruited on the basis of detectable plasma HIV-1 viremia; two patients were reported non-adherent to their ART regimens, and one patient had not yet initiated therapy.
Isolation of resting CD4+ T lymphocytes

Peripheral blood mononuclear cells (PBMC) were isolated using density gradient centrifugation. CD4+ T lymphocytes were enriched by negative depletion (CD4+ T Cell Isolation Kit, Miltenyi Biotec). Resting CD4+ T lymphocytes were further enriched through negative depletion of cells expressing CD69, CD25, or HLA-DR (CD69 MicroBead Kit II, Miltenyi Biotec; CD25 MicroBeads, Miltenyi Biotec; Anti-HLA-DR MicroBeads, Miltenyi Biotec). Resting CD4+ T cells purified using the above described two-step bead depletion procedure were stained with CD4-PE and HLA-DR-APC (BD Biosciences). The purity of these cells was analyzed by flow cytometry using a FACS Canto II (BD Biosciences) and FlowJo software (Treestar).

Limiting dilution viral outgrowth assay

For each patient, both a standard viral outgrowth assay and a MOLT-4/CCR5 viral outgrowth assay were performed using freshly purified resting CD4+ T lymphocytes obtained from a single blood draw as described above. Briefly, five-fold serial dilutions of resting CD4+ T lymphocytes from HIV-1-infected patients were stimulated by coculture with a 10-fold excess of γ-irradiated allogeneic PBMC from uninfected donors and the mitogen PHA (Remel) in RPMI containing 10% fetal bovine serum, 100 U/mL IL-2 (Novartis), and 1% T-cell growth factor, which was produced in-house, as described previously (163). These conditions are sufficient to activate 100% of the resting CD4+ T lymphocytes, as previously demonstrated by CFSE dilution and expression of cell surface activation markers (7, 186). T cell activation reverses HIV-1 latency in at least a fraction of the latently infected cells. After one day of stimulation, the mitogen-containing media is removed and either MOLT-4/CCR5 cells or healthy donor CD4+ lymphoblasts are
added in fresh media to propagate replication-competent HIV-1 in the culture wells. The standard viral outgrowth assay utilizes two additions of CD4+ lymphoblasts from uninfected donors as target cells for HIV-1 outgrowth on days 2 and 9. The MOLT-4/CCR5 viral outgrowth assay utilizes a single addition of MOLT-4/CCR5 cells on day 2. The ratio of target cells added is the same for both assays (163), with $1 \times 10^7$ target cells added to wells containing $1 \times 10^6$ patient resting CD4+ T cells and $2.5 \times 10^6$ target cells added to all other wells. Five days after initial mitogen stimulation of input resting CD4+ T lymphocytes, the culture media was changed, and the cells in each well were split. Supernatants from each well were tested for HIV-1 RNA and/or HIV-1 p24 protein at various time points by RT-PCR and ELISA (Alliance HIV-1 p24 antigen ELISA Kit, Perkin Elmer), respectively. The frequency of latently infected cells among the input resting CD4+ T lymphocytes was calculated by a maximum likelihood method as described previously, and is expressed as infectious units per million cells (IUPM) (163). The 95% confidence intervals for individual IUPM determinations are ±0.7 log IUPM, or 5 fold (163). With a sample size of 25, this assay can detect with 80% power a 0.2-log reduction in the reservoir assuming a type 1 error of 0.05.

**HIV-1 RNA detection by RT-PCR**

After reversal of latency and subsequent release of HIV-1, virion-associated HIV-1 RNA was isolated from 60 µL of culture supernatant using the ZR-96 Viral RNA Kit (Zymo Research), a 96-well column-based RNA isolation kit. The cDNA was synthesized from the isolated HIV-1 RNA using the SuperScript III First-Strand Synthesis System (Invitrogen) with oligo-dT primers. Isolated cDNA was assayed for HIV-1 by RT-PCR as described previously (187) using an Applied Biosystems 7300 Real Time PCR System
RNA copy number was determined using a standard curve generated with the DNA plasmid pVQA, described previously (187). RNA copy numbers below 10 copies or above $10^6$ copies were extrapolated based on the standard curve generated.

**Statistics**

Infected cell frequencies in limiting dilution assays were calculated as described previously (188). Where appropriate, results were expressed as mean ± standard deviation. A Wilcoxon rank sum test (paired comparison) was used for statistical analysis. All statistical analyses were performed with MedCalc software, v.12.4.0.0. A p value of <0.05 was considered significant.

**Results**

**Patient characteristics**

A rapid and simple viral outgrowth assay was developed and evaluated using samples from 20 patients with HIV-1 infection. The baseline characteristics of the patient cohort enrolled in this study are summarized in Table 4.1. Seventeen patients were recruited on the basis of prolonged suppression of viremia to <50 copies of HIV-1 RNA/mL on ART, with a duration of suppression from 12 to 156 months. We intentionally enrolled three additional patients who were viremic at the time of blood draw with viral loads of 5,392, 452,059, and 31,238 copies/mL. Of the three viremic patients, one patient had not yet started ART, and two were known to be non-adherent. The average age of the cohort (± SD) was 50.8 ± 10.8 years, and the cohort was overwhelmingly composed of black males. The CD4 nadir of the cohort ranged from 6 to 755 cells/µL, and 8 patients had a history of an AIDS diagnosis.
Table 4.1 Characteristics of study patients

<table>
<thead>
<tr>
<th>Pt. ID</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Race</th>
<th>Duration of HIV-1 infection (months)</th>
<th>CD4 cell count (cells/µL)</th>
<th>Viral load at enrollment (copies/mL)</th>
<th>Time on ART (months)</th>
<th>ART regimen</th>
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<tr>
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<td>157 806</td>
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<td>144</td>
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<td>54</td>
<td>M</td>
<td>W</td>
<td>132</td>
<td>321 554</td>
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<td>120</td>
<td>TDF, FTC, EFV</td>
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<tr>
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<td>M</td>
<td>W</td>
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<td>&lt;50</td>
<td>24</td>
<td>DRV/r, RAL</td>
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<td>B</td>
<td>240</td>
<td>6 721</td>
<td>&lt;50</td>
<td>156</td>
<td>TDF, FTC, RAL/r</td>
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<td>B</td>
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<td>182 357</td>
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<td>48</td>
<td>TDF, 3TC, RAL</td>
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<td>&lt;50</td>
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<td>84</td>
<td>TDF, FTC, EFV, ATV/r</td>
</tr>
<tr>
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<td>W</td>
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<td>243 563</td>
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<td>15</td>
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<td>B</td>
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<td>422 513</td>
<td>&lt;50</td>
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<td>TDF, FTC, NVP</td>
</tr>
<tr>
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<td>M</td>
<td>H</td>
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<td>N/A 354</td>
<td>5,392</td>
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</tr>
<tr>
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<td>M</td>
<td>B</td>
<td>168</td>
<td>210 223</td>
<td>452,059</td>
<td>Viremic Non-adherent</td>
<td></td>
</tr>
<tr>
<td>V3</td>
<td>42</td>
<td>M</td>
<td>B</td>
<td>168</td>
<td>43 468</td>
<td>31,238</td>
<td>Viremic Non-adherent</td>
<td></td>
</tr>
</tbody>
</table>

Legend, Table 4.1. aRace abbreviations: W, white, non-Hispanic; H, Hispanic; B, black. bTime of documented continuous suppression of plasma viremia to <50 copies/mL on ART. cDrug abbreviations: DRV/r, darunavir boosted with ritonavir; FTC, emtricitabine; TDF, tenofovir disoproxil fumarate; EFV, efavirenz; RAL, raltegravir; RAL/r, raltegravir boosted with ritonavir; ATV/r, atazanavir boosted with ritonavir; 3TC, lamivudine; MVC, maraviroc; FPV/r, fosamprenavir boosted with ritonavir; NVP, nevirapine.
Highly purified resting CD4+ T cells can be obtained using a two-step bead depletion procedure.

The original viral outgrowth assay used to define the latent reservoir was performed on resting CD4+ T cells purified from PBMC in a multistep process that included fluorescence activated cell sorting (FACS) and required a BSL-3 sorting facility. Resting CD4+ T cells were differentiated from activated CD4+ T cells by the absence of cell surface markers CD69, CD25, and HLA-DR. To simplify the purification of resting CD4+ T cells, we devised a two-step bead depletion purification procedure. Initially, CD4+ T cells were purified from PBMC by negative selection as described in the Methods section. Subsequent bead depletion of cells expressing CD69, CD25, or HLA-DR yielded a highly purified, unmanipulated population of resting CD4+ T cells. The purity of these cells was routinely assessed by staining with antibodies to CD4 and HLA-DR. Representative examples are shown in Figure 4.1. Typical purities were 96-97% with less than 0.1% contamination with activated (HLA-DR+) CD4+ T cells. As is discussed below, IUPM values obtained with these purified resting CD4+ T cells were in the same range as observed with sorted cells.
Figure 4.1. Two-step bead depletion procedure yields highly purified resting CD4+ T cells from HIV-1-infected patients

Legend, Figure 4.1. A two-step negative selection strategy to purify resting CD4+ T cells from patient PBMC. (A) Representative FSC/SSC plot indicating live cell population after the two-step bead depletion procedure. (B) Representative dot plot indicating purity of resting CD4+ T cells. Purified cells were stained with antibodies to CD4 and HLA-DR.

The use of MOLT-4/CCR5 cells for virus expansion gives statistically comparable results at 14 days

The standard viral outgrowth assay requires a minimum of 3 separate blood samples from healthy donors in addition to a blood sample from the test patient (Figure 4.2). CD4+ T lymphoblasts from two of these samples are added to cultures at days 2 and 7 to expand virus released from patient cells in which latency has been reversed by T cell activation. These donor lymphoblasts are prepared by PHA stimulation of donor PBMC for 2 days followed by depletion of CD8+ T cells. Eliminating the need for donor lymphoblasts in virus expansion would significantly simplify the assay. We hypothesized that a single addition of a cell line expressing high levels of CD4 and the coreceptors CCR5 and CXCR4 would allow for efficient expansion of viruses released from latently infected cells. We chose the MOLT-4/CCR5 cell line (189). This cell line was derived
from MOLT-4 cells (190), which express high levels of CD4 and CXCR4, and has been engineered to stably express CCR5. It is thus capable of supporting replication of both X4-tropic and R5-tropic variants of HIV-1. To verify that the MOLT-4/CCR5 cells are an acceptable alternative for donor-derived CD4+ T lymphoblasts, we compared IUPM values obtained using the standard viral outgrowth assay with donor-derived lymphoblasts to IUPM values obtained using a simplified assay in which a single addition of MOLT-4/CCR5 cells was used in place of donor lymphoblasts. All 3 viremic patients (V1, V2, and V3) and 14 of the 17 patients on suppressive ART (S1-S14) were included in this comparison. Resting CD4+ T cells obtained from each patient using the bead depletion method described above were split and tested using the standard assay and the simplified MOLT-4/CCR5 assay as outlined in Figure 4.2. The p24 ELISA at day 14 was used as an assay endpoint. Replication-competent HIV-1 was isolated from purified resting CD4+ T lymphocytes in all 3 of the viremic patients and 10 of the 14 patients on ART by both assays (Figure 4.3a). In 3 of the 14 patients on ART, replication-competent HIV-1 was isolated in only one of the two viral outgrowth assays (Figure 4.3a, Patients S4, S7, and S14). No replication-competent HIV-1 was recovered from purified resting CD4+ T lymphocytes by either viral outgrowth assay in 2 of the 14 patients on ART (Figure 4.3a, Patients S8 and S13). These results are expected because splitting the sample reduces the input number of resting CD4+ T cells. The frequency of latently infected resting CD4+ T lymphocytes was markedly higher in the viremic patients compared to patients on ART (Figure 4.3a), consistent with our previous results (181).
The frequency of HIV-1 latent infection of resting CD4+ T cells can be measured using a viral outgrowth assay. PBMC are collected from HIV-1-infected individuals, and resting CD4+ T cells (CD25-, CD69-, HLA-DR-) are purified. Resting T cells are plated in 5-fold serial dilutions in duplicate, such that the input number of patient cells ranges from 1,000,000 to 320 cells per well. To reverse latency in the cells that harbor a replication-competent HIV-1 provirus, patient cells are activated with PHA and a 10-fold excess of irradiated PBMC from healthy donors. The next day, target cells for HIV-1 infection are added to allow outgrowth of replication-competent HIV-1 released from infected cells in which latency has been reversed. In the standard viral outgrowth assay, CD4+ lymphoblasts from healthy donors are added on days 2 and 7 of the assay. In the MOLT-4/CCR5 viral outgrowth assay, MOLT-4/CCR5 cells are added on day 2 only. For the standard assay, HIV-1 p24 antigen ELISA is used to identify wells positive for HIV-1 outgrowth at 14 days. For the MOLT-4/CCR5 assay, RT-PCR is used to identify wells positive for outgrowth at 7 days. The frequency of latently infected cells can be determined using limiting dilution statistics based on the input number of patient cells in the wells positive for outgrowth. This frequency is reported in infectious units per million (IUPM).
Figure 4.3. The standard and the MOLT-4/CCR5 viral outgrowth assays yield comparable frequencies of latent infection

Legend, Figure 4.3. (A) The frequency of latently infected resting CD4+ T cells was measured in 3 viremic patients and 14 ART-suppressed patients using both the standard and the MOLT-4/CCR5 viral
outgrowth assays, with HIV-1 p24 antigen ELISA used as the endpoint assay of viral outgrowth at day 14. (B) Statistical comparison of the IUPM values measured using the standard viral outgrowth assay and the MOLT-4/CCR5 viral outgrowth assay by Wilcoxon rank sum test. (C) The correlation of IUPM values measured using the standard viral outgrowth assay and the MOLT-4/CCR5 viral outgrowth assay (Pearson’s correlation coefficient, r).

No significant difference was observed between the frequency of latently infected resting CD4+ T lymphocytes measured in the standard viral outgrowth assay vs. the MOLT-4/CCR5 viral outgrowth assay (Figure 4.3b, Wilcoxon Rank sum test, p = 0.9032). Furthermore, the frequency of latently infected cells as measured by the MOLT-4/CCR5 viral outgrowth assay correlates highly with that of the standard viral outgrowth assay (Figure 4.3c, Pearson’s correlation coefficient, r = 0.9381, p<0.0001). When only patients on suppressive ART were considered, the correlation remained highly significant (r = 0.7602, p = 0.0016).

**HIV-1-specific RT-PCR assay detects HIV-1 outgrowth from the latent reservoir earlier than p24 antigen ELISA**

To shorten the time required to measure latently infected cells by the viral outgrowth assay, we explored the use of RT-PCR as an alternative to the p24 ELISA to detect virus production. Twenty-nine replicate wells were set up with 200,000 patient resting CD4+ T cells/well from a patient on suppressive ART (S15). The cells were activated with PHA and irradiated feeders and then cultured with MOLT-4/CCR5 cells over 14 days using the protocol developed for the viral outgrowth assay. Culture supernatants were assayed for released virus at multiple time points during the 14-day culture using both the p24 antigen ELISA and a novel RT-PCR assay. This assay detects polyadenylated HIV RNAs without interference from proviral or plasmid DNA, and when applied to virion-containing supernatants, detects mainly genomic viral RNA, allowing accurate quantitation of virus release (187). For both assays, positive wells
showed an exponential increase in the amount of virus in the supernatant (Figures 4.4a and 4.4b). Among the wells positive for outgrowth, there was complete concordance between HIV-1 p24 antigen ELISA and HIV-1-specific RT-PCR at 14 days. All of the wells that were positive for outgrowth by RT-PCR eventually tested positive by p24 ELISA (Figure 4.4c). Under conditions in which a majority of the positive wells are predicted to contain a single latently infected cell, HIV-1-specific RT-PCR detected viral outgrowth significantly earlier than HIV-1 p24 antigen ELISA (Figure 4.4d, Wilcoxon rank sum test, p = 0.0020), with the average days (± S.D.) of detection being 6.1±2.1 and 9.6±2.7 days, respectively. These results indicate that HIV-1-specific RT-PCR accurately detects viral outgrowth in a shorter time frame.
Figure 4.4. Kinetics of HIV-1 outgrowth from latently infected CD4+ T cells measured by HIV-1 p24 antigen ELISA and HIV-1-specific RT-PCR

Legend, Figure 4.4. Resting CD4+ T cells were isolated from ART patient S15, whose latent reservoir was previously measured to be 3.25 IUPM. Twenty-nine replicate wells were plated in which 200,000 resting cells were activated with PHA and irradiated PBMC from a healthy donor and subsequently cultured with MOLT-4/CCR5 cells. Outgrowth of reactivated HIV-1 was measured in positive wells over 14 days using both (A) HIV-1 p24 antigen ELISA and (B) HIV-1 specific RT-PCR. (C) The difference between the day on which a particular well becomes positive by RT-PCR vs. p24 ELISA. (D) The day of detection of HIV-1 outgrowth from the latent reservoir is shown for HIV-1 p24 antigen ELISA and HIV-1-specific RT-PCR.

Accurate measurement of the frequency of latently infected cells can be obtained in 7 days using RT-PCR to detect outgrowth in the MOLT-4/CCR5 viral outgrowth assay.

Given that HIV-1 specific RT-PCR accurately detected HIV-1 outgrowth from the latent reservoir significantly earlier than the HIV-1 p24 antigen ELISA, we sought to
determine whether HIV-1-specific RT-PCR could be used to detect positive wells more rapidly in the MOLT-4/CCR5 viral outgrowth assay. As shown in Figure 4.4b, nearly all wells that eventually became positive by p24 ELISA were positive by RT-PCR on day 7. Therefore, supernatants from viral outgrowth cultures from patients S8-S14 and V1-V3 were tested at day 7 using the HIV-1 specific RT-PCR assay. For comparison, an HIV-1 p24 antigen ELISA was also performed on culture supernatants from these viral outgrowth cultures on day 7. The frequencies of latently infected cells obtained using both assays on day 7 of the viral outgrowth assay were compared to the frequencies determined on day 14 using the HIV-1 p24 antigen ELISA (Figure 4.5a). The frequencies determined with HIV-1 p24 antigen ELISA on day 7 were significantly lower than the frequencies determined with the same assay on day 14 (Figure 4.5a, Wilcoxon rank sum test, p = 0.0010). However, the frequencies determined with HIV-1-specific RT-PCR on day 7 and with HIV-1 p24 antigen ELISA on day 14 were not significantly different (Figure 4.5a, Wilcoxon rank sum test, p = 0.9219). These results suggest that the use of a sensitive assay for free virus on day 7 of the culture may effectively substitute for an ELISA assay on day 14.
Figure 4.5. Accurate measurement of IUPM at day 7 using HIV-1-specific RT-PCR

A

B

C

D
Legend, Figure 4.5. (A) Using the rapid MOLT-4/CCR5 viral outgrowth assay, the frequency of latently infected cells was measured for ART patients S1-S14 and viremic patients V1-V3 at day 7 with the HIV-1-specific RT-PCR assay and at both days 7 and 14 with HIV-1 p24 antigen ELISA. Statistical significance of the differences in IUPM values was assessed by Wilcoxon rank sum test. (B) Correlation of the IUPM measured at day 7 using HIV-1 p24 antigen ELISA with the IUPM measured at day 14 using HIV-1 p24 antigen ELISA (Pearson’s correlation coefficient, r). (C) Correlation of the IUPM measured at day 7 using HIV-1-specific RT-PCR with the IUPM measured at day 14 using HIV-1 p24 antigen ELISA (Pearson’s correlation coefficient, r). (D) Correlation of the IUPM measured at day 7 using the rapid MOLT-4/CCR5 outgrowth assay with the IUPM measured at day 14 using the standard outgrowth assay (Pearson’s correlation coefficient, r).

We next sought to evaluate the agreement between the day 7 and day 14 endpoint assays. A total of 74 wells from patients S8-S14 and V1-V3 were positive for HIV-1 outgrowth by either HIV-1 specific RT-PCR or HIV-1 p24 antigen ELISA. Outgrowth was detectable on day 7 using HIV-1 p24 antigen ELISA in only 60% of the positive wells. However, outgrowth was detectable in 81% of the positive wells on day 7 using HIV-1-specific RT-PCR. The correlation of the frequency of latently infected resting CD4+ T cells calculated at day 7 and day 14 was evaluated using Pearson’s correlation coefficient. The correlation between the frequency of latent infection calculated at day 14 using HIV-1 p24 antigen ELISA and the frequency determined on day 7 was markedly higher when the HIV-1 specific RT-PCR assay was used rather than the HIV-1 p24 antigen ELISA (Figure 4.5c vs. Figure 4.5b, r = 0.9698 vs. r = 0.9133). When only patients on suppressive ART were considered, the frequency determined at day 7 using the RT-PCR assay was positive correlated with the frequency determined at day 14 by HIV-1 p24 antigen ELISA (r = 0.8516, p = 0.0001), while the frequency determined at day 7 using the HIV-1 p24 antigen ELISA no longer correlated with the frequency determined at day 14 by ELISA (r = 0.4448, p = 0.1110). Two additional patients on suppressive ART (S16 and S17) were included in a final comparison of the rapid MOLT-4/CCR5 viral outgrowth assay and the standard viral outgrowth assay. A statistically significant positive correlation was seen when the frequency of latently infected resting
CD4+ T cells determined using the rapid MOLT-4/CCR5 viral outgrowth assay was compared to the frequency determined using the standard viral outgrowth assay (Figure 4.5d, r = 0.9591, p<0.0001). When only patients on suppressive ART were considered, a statistically significant positive correlation was still observed (r = 0.7522, p = 0.0194).

**Discussion**

Latent HIV-1 infection of resting CD4+ T cells remains the major barrier to HIV-1 eradication. A number of small molecules have been identified that are capable of reactivating transcription of otherwise silent HIV-1 proviruses (93-95, 153, 182, 183). Some of these compounds have already entered clinical trials (99), and drug discovery efforts to find additional compounds that can perturb or eliminate latent HIV-1 continue. Concurrently, immunological approaches are being investigated and have shown promise (100). However, a key hurdle facing HIV-1 eradication efforts has, until recently, been largely ignored: the development of a reliable and simple assay to measure the size of the HIV-1 latent reservoir. Such an assay is absolutely required for evaluating the effectiveness of an eradication strategy. PCR-based assays are being used to quantify proviruses in T cell subsets and the level of residual viremia in HIV-1 infected patients (19, 20, 27, 99, 101, 191). A recent study has compared results of various PCR-based assays with those obtained with the viral outgrowth assay using a set of samples from two well-characterized cohorts of patients on ART (185). Because current PCR assays detect both replication-competent and defective proviruses, the correlation between infected cell frequencies measured by PCR and viral outgrowth was not strong, with the exception of an assay measuring integrated HIV-1 DNA in PBMC (185). The measurement of integrated HIV-1 DNA by *Alu* PCR (191) is of particular interest because the stable
reservoir for HIV-1 consists of resting CD4+ T cells harboring integrated HIV-1 DNA (5, 6). It is likely that this and other PCR-based assays will play an important complementary role to viral outgrowth assays. Prior to the present study, the standard viral outgrowth assay was the only assay available to directly quantify the frequency of resting CD4+ T cells harboring latent but replication-competent viral genomes.

The development of a rapid isolation procedure to obtain unperturbed resting CD4+ T cells was an essential first step toward creating a viral outgrowth assay suitable for widespread use in eradication studies and clinical trials. The latent reservoir was originally defined using viral outgrowth assays performed on highly purified populations of resting CD4+ T cells obtained through a combination of magnetic bead depletion and cell sorting. Thus, the assay required a BSL-3 cell sorting facility. While feasible for small-scale studies, this approach cannot be utilized for large-scale studies in which numerous measurements of the size of the latent reservoir must be taken across many patients. As we have demonstrated here, our isolation procedure yields a highly purified resting CD4+ T cell population. Moreover, the frequencies of latently infected cells measured in these populations are very similar to those obtained with the sorting method (5-7).

The standard viral outgrowth assay relies on a minimum of 3 separate blood samples from healthy donors. PBMC from two of the subsequent donations are added to the cultures to propagate the HIV-1 released following the reversal of latency. These healthy donor cells may also provide subsequent allogeneic stimulation to the patient cells in culture. We sought to replace these healthy donor cells with a cell line that supports infection by both X4-tropic and R5-tropic HIV-1. Furthermore, we believed that
a cell line would provide greater uniformity to the viral outgrowth assay, since every viral isolate growing out of a patient’s latent reservoir would propagate in identical culture conditions. As we have clearly demonstrated, the MOLT-4/CCR5 cell line performs robustly in place of mitogen-stimulated CD8-depleted healthy donor PBMC in the viral outgrowth assay. This modification significantly simplifies the assay and allows the assay to be more easily scaled for large studies or clinical trials. Other cell lines that support replication of both X4 and R5 isolates could potentially be used as target cells in this assay. Of note, our rapid assay in its current form still requires a single blood donation from a healthy donor for the generation of irradiated PBMC used in the initial mitogen stimulation. It is possible that the replacement of mitogen stimulation with costimulation via anti-CD3 and anti-CD28 monoclonal antibodies could alleviate the need for any healthy blood donors.

Interestingly, the success of MOLT-4/CCR5 cells in propagating reactivated HIV-1 suggests that the allogeneic stimulation provided by the healthy donor CD4+ T lymphoblasts was not required. As MOLT-4/CCR5 cells do not express MHC class II (192, 193), no allogeneic stimulation of patient CD4+ T cells should occur. The lack of allogeneic stimulation was noted in the early characterization of the MOLT-4 cell line (194-196). Thus, we can infer from our study that the initial mitogen stimulation alone is generally sufficient for reactivation of latent HIV-1 and viral outgrowth. However, we cannot conclude that this initial mitogen stimulation is sufficient for reactivation of all latent proviruses. More recent studies have shown that some replication-competent proviruses remain non-induced after a single round of mitogen stimulation (197). Therefore, any measurement of the size of the latent reservoir that relies upon
reactivation of latent proviruses through a single round of T cell stimulation may in fact be underestimating the size of the reservoir. The strategic use of both culture- and PCR-based assays may allow us to bracket the true size of the latent reservoir.

The standard viral outgrowth assay requires 14 days to complete. This 14-day period includes two additions of CD4+ T lymphoblasts from healthy donor PBMC as well as multiple media changes. The length of time required for the standard viral outgrowth assay is a function of the endpoint assay used to measure viral outgrowth: the HIV-1 p24 antigen ELISA. With the goal of reducing the length of time required for detecting viral outgrowth, we adapted a recently developed HIV-1-specific RT-PCR assay for use as an endpoint assay. On average, viral outgrowth from the latent reservoir under conditions resembling the MOLT-4/CCR5 viral outgrowth assay was detectable by the HIV-1 specific RT-PCR assay after 6.1±2.1 days (± S.D.) vs. 9.6±2.7 days (± S.D.) for HIV-1 p24 antigen ELISA. This result indicated that utilizing a more sensitive endpoint assay for viral outgrowth could indeed reduce the length of time required to complete the MOLT-4/CCR5 viral outgrowth assay. The data presented here demonstrate that no significant difference exists between the frequency of latent infection of resting CD4+ T cells as measured at day 7 using HIV-1-specific RT-PCR and at day 14 as measured by HIV-1 p24 antigen ELISA. The utilization of an RT-PCR-based measurement for HIV-1 outgrowth not only allows more sensitive detection of viral replication, but also allows more high-throughput measurement of HIV-1 replication. Of note, extremely high sequence conservation has been observed at the primer and probe binding sites, especially amongst subtype B isolates (187). The degree of conservation is actually higher than is observed in the regions of gag that are amplified in many other
PCR assays (187). It remains possible that in rare patients, sequence variation in these conserved regions could interfere with PCR detection. In these rare cases, negative results in the PCR assay may be due to primer mismatch rather than low frequency of latent infection.

Given the greater sensitivity of the HIV-1-specific RT-PCR assay, it is possible that small amounts of replication-defective virus released after mitogen stimulation of resting CD4+ T cells might be detected, resulting in a false positive readout of viral outgrowth. Our data suggest that the release of replication-defective virus is not widely detected by the RT-PCR assay at day 7. In rare cases (patient S13), we observed weak positive signals by RT-PCR at day 7 in wells that remained negative by p24 ELISA at day 14. These data could represent viruses that have low fitness and a slower replication rate than needed to expand to beyond the limit of detection for HIV-1 p24 antigen ELISA on day 14. It is possible that such viruses could be detected by HIV-1 p24 antigen ELISA with a longer culture period. As shown in Figure 4.4c, only 11 out of 29 wells containing an input of 200,000 resting CD4+ T cells from patient S15 were positive for viral outgrowth. The frequency of latent HIV-1 infection measured in the resting CD4+ T cells of patient S15 (3.25 IUPM) suggests that each of the 29 replicate wells likely contained dozens of integrated, defective proviruses. However, only 11 wells were positive for viral outgrowth when tested by RT-PCR. Furthermore, of the wells that were positive for viral outgrowth, detection by HIV-1-specific RT-PCR was not possible until 6.1±2.1 days (± S.D.), and these wells remained positive for the duration of the outgrowth time course, with an exponentially increasing amount of virus. These data suggest that our HIV-1-
specific RT-PCR requires a level of viral replication achieved after nearly one week and is not sensitive enough to detect replication-defective viruses that do not expand further.

The MOLT-4/CCR5 viral outgrowth assay does require a single large blood sample of 140-200 mL. This is due to the low frequency of replication-competent proviruses harbored within resting CD4+ T cells. Given this fundamental aspect of the biology of HIV-1 latency, it is unlikely that any version of a viral outgrowth assay can be performed without a large input of resting CD4+ T cells. However, because no alternative assay exists that specifically measures latent, replication-competent proviruses in resting CD4+ T cells, viral outgrowth assays will likely continue to play an important role in evaluating HIV-1 eradication strategies.

The MOLT-4/CCR5-utilizing viral outgrowth assay presented here is the most rapid and scalable assay available for measuring the size of the HIV-1 latent reservoir. As such, we believe that this assay will be an indispensable tool in evaluating the success of strategies to perturb or eradicate the HIV-1 latent reservoir.
CHAPTER 5 - SEQUENCE SIMILARITY BETWEEN HUMAN IMMUNODEFICIENCY VIRUS FROM DIFFERENT ANATOMIC SOURCES DESPITE INFREQUENT DETECTION OF REPLICATION-COMPETENT VIRUS IN TISSUE CELLS

Background

Despite the effectiveness of antiretroviral therapy (ART), which can reduce plasma HIV-1 RNA levels in infected patients to below the limit of detection of standard clinical assays, the virus persists in a small population of resting memory CD4+ T cells that harbor latent viral genomes. The long-lived nature of these memory cells contributes to the long half-life of this latent reservoir. As a result of this long half-life, ART alone is insufficient to cure HIV-1 infection (10). The persistence of infection can be observed through viral outgrowth from resting CD4+ T cells from patients on suppressive ART using a quantitative viral outgrowth assay (QVOA) (6, 9); through viral “blips,” which occur when plasma HIV-1 RNA levels briefly become detectable in patients on ART (198); and through the presence of residual viremia – trace levels of free virus in the plasma of patients on suppressive ART that fall below the limit of detection of clinical assays but are detectable by more sensitive laboratory methods (20).

To date, the best-characterized barrier to the cure of HIV-1 infection is the latently infected pool of circulating resting CD4+ T cells (9). As a result, many cure studies test potential latency-reversing agents on these cells or on in vitro systems designed as models of these cells (103, 152, 199). However, there are other anatomic sites and cell types that can harbor replication-competent HIV-1. Most of the CD4+ T cells in the body at a given time are not in the peripheral blood. Previous studies of viral sequences in the
plasma and proviral sequences in circulating resting CD4+ T cells from patients on suppressive ART have shown that viral sequences in some patients exhibit a population structure that suggests that these viruses originate from a source in addition to circulating resting CD4+ T cells (200). Additionally, the residual viremia in some patients exhibits a predominant plasma clone (PPC), which is a viral sequence that represents the majority of the plasma sequences from a patient but is absent or underrepresented in the proviral sequences from circulating resting CD4+ T cells (65). The existence of the PPC also suggests that residual viremia could originate from cells that have undergone clonal expansion after infection (201, 202) and that are localized to tissue sites.

In addition to CD4+ T cells, macrophages are also susceptible to HIV-1 infection but are more resistant to viral cytopathic effects as compared to CD4+ T cells (106, 107). Although macrophages are infectable and can survive infection for relatively long periods of time, it has not yet been demonstrated that infected macrophages can serve as a reservoir in the same way that resting CD4+ T cells can: it is not clear that infection becomes latent in the same way that it does in CD4+ T cells, nor is it clear that macrophages harbor replication-competent HIV-1 in individuals on suppressive ART on a time scale of years. The issue is complicated by the fact that detection of HIV-1 DNA in these cells may represent phagocytosis of dying T cells (203). However, it is still possible that very long-lived infected tissue-resident macrophages could represent a barrier to a cure.

Previous studies have shown that in HIV-1 infection, CD4+ T cells in the GALT are depleted earlier than in other anatomic sites, including the peripheral blood, axillary and mesenteric lymph nodes, and the spleen (33, 137, 138, 204-206). Additionally, in
HIV+ individuals on suppressive ART, there are high levels of HIV-1 DNA in CD4+ T cells in the GALT, approximately 5 times higher than the HIV-1 DNA levels in circulating resting CD4+ T cells (139). The existence of HIV-1 DNA in cells is not sufficient to demonstrate the presence of replication-competent virus, however. Due to G-to-A hypermutation induced by APOBEC3G and large internal deletions that occur during reverse transcription, many proviruses are not replication-competent (197, 207-210). Therefore, measurements of HIV-1 DNA in cells typically overestimate the amount of replication-competent virus that serves as a barrier to cure (185). Replication-competent HIV-1 has been previously obtained from GALT tissue explants from viremic patients, but not from patients on ART (211). Here, we explore the relationship between plasma virus and proviruses in CD4+ T cells from the blood and GALT in patients on ART to better assess the contributions of the GALT to HIV-1 persistence.

**Methods**

Experiments were carried out in accordance with the Johns Hopkins Institutional Review Board regulations, protocol numbers NA_00049923 and NA_00077826.

*Verification of predominant plasma clone existence*

To verify the existence of a source of plasma viremia other than circulating, resting CD4+ T cells, plasma and purified resting CD4+ T cells were isolated from a volunteer whose viral load had been lower than 50 RNA copies/mL on ART for over 6 months.

*Isolation of plasma and resting CD4+ T cells*

Plasma and PBMC were isolated from 180 mL of whole blood using Ficoll-Hypaque density centrifugation. The plasma was subsequently centrifuged at 1200 rpm.
for 10 minutes at 4°C to remove any residual cells. The plasma was then stored at -80°C in 10-mL aliquots for later use. The buffy coat containing the PBMC was removed and washed twice with 30 mL of Wash Media (phosphate-buffered saline, pH 7.4, 2% heat-inactivated newborn calf serum, 0.1% glucose, 100 U/mL penicillin, 100 µg/mL streptomycin, 12 mM HEPES, pH 7.4). Then resting, CD4+ T cells were purified using the Miltenyi CD4+ T cell Isolation Kit, CD69 MicroBead Kit II, Anti-HLA-DR MicroBeads, and Anti-CD25 MicroBeads, according to the manufacturer’s protocols.

**Isolation of viral RNA from plasma virus**

Virus was isolated from the plasma via ultracentrifugation at 170,000 rcf for 30 minutes at 4°C. The virus pellet was resuspended in 400 µL of phosphate-buffered saline (PBS), and HIV RNA was isolated using the QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer’s protocol. The RNA was eluted with 60 µL of water, and the resulting RNA was DNase treated using Amplification-Grade DNase I (Life Technologies).

**Isolation of proviral DNA from circulating resting CD4+ T cells**

DNA was isolated from the peripheral resting CD4+ T cells using the Gentra Puregene Cell Kit (Qiagen), according to the manufacturer’s protocol.

**Limiting-dilution amplification of the C2-V4 region of HIV-1 Env from viral RNA**

To ensure that each amplicon represented amplification from a single molecule of HIV-1 RNA, we carried out limiting-dilution PCR on viral RNA. The RNA was diluted to limiting dilution and subsequently subjected to one-step reverse transcription and amplification using the SuperScript III One-Step RT-PCR System with Platinum Taq High-Fidelity Polymerase (Life Technologies), followed by nested PCR using Platinum
Taq High-Fidelity Polymerase (Life Technologies). The outer forward primer was 5’-CTGTTAAATGCGAGCTAGC-3’; the outer reverse primer was 5’-CACTTCTCGAATTGTCCCTCA-3’; the inner forward primer was 5’-ACAATGCAGGAAAAACCATAATAGT-3’; and the inner reverse primer was 5’-CATACGGGCTTTTCTCTACT-3’, as previously described (200). Thermal cycling was also carried out as previously described (200).

Limiting-dilution amplification of full-length HIV-1 Env from proviral DNA

Proviral DNA from circulating, resting CD4+ T cells was diluted to limiting dilution and subjected to nested PCR for full-length HIV-1 Env using Accuprime Pfx Polymerase (Life Technologies). The outer forward primer was 5’-ATGGCAGGAAAGCAGCGAGACAG-3’; the outer reverse primer was 5’-GCTCAACTGGACTAGCTTAGCACC-3’; the inner forward primer was 5’-GATAGACGCCTAGAAGGAGACAGTGGAATG-3’; and the inner reverse primer was 5’-CCTTGTACCGGCTCTTTAAAGGTTACTGAGGTCTGACTGG-3’.

Thermal cycling was carried out as previously described (200).

Sequence analysis

The products of the inner reactions were separated on 1% agarose gels, and the bands of the appropriate size were excised. DNA was extracted from the gel slices using the QIAquick Gel Extraction Kit (Qiagen). Because the reactions were performed at limiting dilution, with a probability of clonality greater than 90%, the products could be directly sequenced without a cloning step. After sequencing, the resulting chromatograms were inspected to ensure clonality. Any chromatograms containing doublet peaks were discarded, and those sequences were excluded from the analysis.
The remaining sequences were then checked for contamination with lab strains of HIV using BLAST (http://blast.ncbi.nlm.nih.gov/). Any contaminating sequences were removed from the analysis. The sequences were then aligned using Gene Cutter, preserving codon positions and using three clade C virus sequences as an outgroup. (http://www.hiv.lanl.gov/content/sequence/GENE_CUTTER/cutter.html). Any hypermutated sequences were identified using Hypermut (http://www.hiv.lanl.gov/content/sequence/HYPERMUT/hypermut.html) and were removed from the analysis. Prior to phylogenetic reconstruction, gaps were removed from the alignment using Gap Strip/Squeeze (http://www.hiv.lanl.gov/content/sequence/GAPSTREEZE/gap.html), with the gap tolerance set to zero. Phylogenetic reconstruction was carried out in MEGA using the maximum likelihood method based on the Tamura-Nei model (212, 213).

Investigation of the GALT as an additional source of residual viremia

Participant recruitment

Participants were recruited from patients of providers affiliated with The Johns Hopkins Hospital. Interested participants provided written informed consent and were then screened via verbal interview and blood tests for conditions that would contraindicate blood donation and colon biopsy donation. Also at the screening visit, participants’ viral load was measured to ensure that their viral loads were well controlled (<50 RNA copies/mL). Eligible participants were scheduled for three separate study visits at which they donated both peripheral blood and colon biopsies. Of the 16 volunteers screened, 10 participants were eligible and were enrolled in the study.

Biological sample acquisition
At each study visit, participants donated 120 mL of peripheral blood in 60-mL syringes prepared with 8 mL of acid citrate dextrose (ACD) per syringe as an anticoagulant. Participants self-administered a normal saline enema to minimize stool contamination of tissue biopsies. Colon biopsies were collected into 30 mL of Collection Media (RPMI containing 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin B, and 0.5 mg/mL piperacillin/tazobactam) using an Olympus adult sigmoidoscope and Boston Scientific Radial Jaw 3 Maximum Capacity Single-Use Biopsy Forceps. Biological samples were then processed in the lab.

Isolation of tissue cells from colon biopsies

After removing the collection medium from the biopsies, the biopsies were washed 3 times with 10 mL of Collection Media. Any remaining fecal contamination was then removed from the biopsies using pipet tips in a petri dish. The biopsies were then subjected to 30 minutes of enzyme digestion at 37°C while shaking at 225 rpm. The enzymes employed for the digestion consisted of 0.5 mg/mL collagenase (Sigma), 0.07 U/mL elastase (Worthington Biochemicals), and 0.083 U/mL DNase I (New England Biolabs). The tissue cells released by the enzyme digestion were collected by passing the biopsy suspension through a 70-µm mesh cell trainer (BD Biosciences). The biopsy pieces that were retained on the strainer were manually disrupted on the strainer using pipet tips. The collected tissue cells were pelleted by centrifugation at 1200 rpm for 10 minutes at 4°C, then resuspended in 3 mL of Collection Media and stored at 4°C for later use. The remaining biopsy pieces were then subjected to two more rounds of enzyme digestion, filtration through a cell strainer, and manual disruption.
All of the released tissue cells were then pooled and pelleted at 1200 rpm for 10 minutes at 4°C. They were then washed by resuspension in 10 mL of Collection Media and subsequently pelleted as described. The cell pellets were resuspended in 9 mL of room-temperature Collection Media, and red blood cells were removed from this cell suspension using Ficoll-Hypaque density gradient centrifugation. The buffy coat was collected, and the cells were washed twice with 30 mL of Wash Media. To prevent the antiviral effects of CD8+ T cells, CD8+ cells were then removed by incubation with CD8 Dynabeads (Life Technologies), per the manufacturer’s protocol. The remaining cells were then allowed to rest overnight on a 0.4-µm PTFE filter (Millicell) in Collection Media. Tissue cells were then removed from the PTFE filter using a cell scraper followed by trypsinization.

Isolation of plasma and PBMC from peripheral blood

Red blood cells were removed from whole blood using Ficoll-Hypaque density gradient centrifugation. The buffy coat containing PBMC was collected and washed twice with 30 mL of wash media. The cells were then resuspended in 30 mL of Collection Media and allowed to rest overnight in a T75 flask at 37°C. The plasma was collected and centrifuged at 1200 rpm for 10 minutes at 4°C to remove any remaining cells. The plasma was then stored at -80°C for subsequent virus isolation.

Quantitative viral outgrowth assay

Resting CD4+ T cells were isolated from study participant PBMC using the Miltenyi CD4+ T Cell Isolation Kit, CD69 MicroBead Kit II, Anti-HLA-DR MicroBeads, and Anti-CD25 MicroBeads, according to the manufacturer’s protocols. The outgrowth assay was carried out as previously described, using MOLT-4/CCR5 cells as target cells.
Briefly, GALT cells and circulating resting CD4+ T cells were plated at limiting dilution in activating conditions, which included 0.5 µg/mL phytohemagglutinin (PHA) and irradiated PBMC from a healthy donor. Assay Media (RPMI with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL of streptomycin, 0.25 µg/mL of amphotericin B, 2% T-cell growth factor, and 100 U/mL recombinant human IL-2) included amphotericin B due to the risk of fungal contamination from GALT cells. The media was changed on day 2 to prevent toxicity due to the PHA, and the target cells (MOLT-4/CCR5 cells) were added to amplify the viral signal. The media was changed on days 5 and 9 to promote cell viability. HIV-1 p24 was detected in the culture supernatant using the Alliance HIV-1 p24 Antigen Elisa Kit (Perkin-Elmer) according to the manufacturer’s protocol. The frequency of cells containing replication competent virus (infectious units per million, IUPM) was calculated using an IUPM calculator (215).

**Cell population identification**

An aliquot of PBMC was stained with anti-HLA-A2-FITC (BD Pharmingen). GALT cells were stained with anti-CD3-BV421 (BD Pharmingen), anti-CD4-PE (BD Pharmingen), and anti-CD68-APC (BioLegend). The corresponding isotype controls were used as negative controls (BD anti-G2bk-FITC, BD anti-G1k-BV421, BD anti-G1k-PE, and BioLegend anti-G2bk-APC, respectively).

**Viability staining during the quantitative viral outgrowth assay**

On days 0, 3, 6, and 13 of the QVOA, an aliquot of cells from a well containing the 1 million study participant cells was taken and stained using Annexin V-PE and 7-AAD (BD Pharmingen) according to the manufacturer’s protocol. The study participant cells were distinguished from the MOLT-4/CCR5 cells by size, and they were
distinguished from the feeders by HLA-A2 positivity (see “Cell population identification” section above). Study participant cells that were negative for both Annexin V and 7-AAD were considered live cells.

**Activation experiment**

PBMC from a healthy donor and PBMC and GALT cells from each study participant were stained with carboxyfluorescein succinimidyl ester (CFSE) (Life Technologies) according to the manufacturer’s protocol. The cells were then plated at 0.5 million/mL in Assay Media in a six-well plate. Experimental wells received PHA at a final concentration of 0.5 µg/mL; control wells received no PHA. The cells were then incubated at 37°C, and CFSE levels were read on days 0, 3, 6, and 13.

**Sequence analysis of the C2-V4 region of HIV-1 Env from plasma, GALT QVOA outgrowth, and provirus from GALT and resting CD4+ T cells**

RNA was isolated from virus produced by GALT cells in the QVOA using the Qiagen Viral RNA Mini Kit, according to the manufacturer’s protocol. The resulting undiluted RNA was subjected to one-step reverse transcription and amplification using the SuperScript III One-Step RT-PCR System with Platinum Taq High-Fidelity Polymerase followed by nested PCR using Platinum Taq High-Fidelity Polymerase (Life Technologies). The outer forward primer was 5’-CTGTTAAATGCGCAGTCTAGC-3’; the outer reverse primer was 5’-CACTTCTCCAATTGCTCCCTCA-3’; the inner forward primer was 5’-ACAATGCTAAAACCATAATAGT-3’; and the inner reverse primer was 5’-CATACATTGCTTTTCCTACT-3’, as previously described (200). Thermal cycling was also carried out as previously described (200). Negative controls (lacking reverse transcriptase and template RNA) were used to ensure that any amplification was due to
reverse transcription of viral RNA rather than proviral DNA or other contaminants. The resulting amplicons were separated on a 1% agarose gel. Bands of the appropriate size were excised from the gel, and the DNA was isolated using the QIAquick Gel Extraction Kit (Qiagen). Because the patient cells in the QVOA had been plated at limiting dilution, the virus from the positive supernatants was clonal, and the amplicons were sequenced directly. Clonality was confirmed via inspection of the sequencing chromatograms.

Virus was isolated from the plasma of study participants as described above. Undiluted RNA was subjected to one-step reverse transcription and amplification using the SuperScript III One-Step RT-PCR System with Platinum Taq High-Fidelity Polymerase (Life Technologies) using the outer forward primer 5’-CTGTTAAATGGCAGTCTAGC-3’ and the outer reverse primer 5’-CACTTCTCCAATTGTCCCTCA-3’, as previously described (200). Thermal cycling was also carried out as previously described (200).

Resting CD4+ T cells were isolated from study participant PBMC as described above. DNA was also isolated from the peripheral resting CD4+ T cells as described above. The outer PCR reaction was carried out using Accuprime Pfx DNA Polymerase (Life Technologies) and using the same outer and inner primers as were used to amplify the C2-V4 region from viral RNA. Each reaction contained 1.5 µL each of the 10 µM forward and reverse primer, 5 µL of 10X Accuprime reaction mix, 0.5 µL of Accuprime Pfx DNA Polymerase, 5 µL of undiluted DNA, and water to 50 µL. Thermal cycling was carried out as follows: denaturation at 94°C for 3 minutes, followed by 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 1 minute.
Products of the outer reactions from plasma RNA and cellular DNA were then subjected to inner PCR with barcoding to allow for differentiation of pooled samples during deep sequencing. The barcodes used were 10 of the 14 Roche 454Standard MID set and are listed in Table 5.1. Each inner reaction for the product derived from viral RNA was carried out with the following contents: 5 µL each of the 10 µM forward and reverse primers, 5 µL of the 10X Hi-Fi buffer, 1 µL of 10 mM dNTP mix, 2 µL of 50 mM MgSO₄, 0.5 µL of Platinum Taq Hi-Fi, 2.5 µL of the outer PCR product, and water to 50 µL. The cycling conditions were the same as previously described (200). Each inner reaction for the product derived from proviral DNA was carried out with the following contents: 1.5 µL each of the 10 µM forward and reverse primers, 5 µL of the 10X Accuprime reaction mix, 0.5 µL of the Accuprime Pfx DNA Polymerase, 2.5 µL of the outer PCR product, and water to 50 µL. These reactions were carried out under the same cycling conditions as the outer reactions. The inner forward primer was 5’-barcode-ACAATGCTAAAACCATAATAGT-3’, and the reverse primer was 5’-CATACATTGCTTTTCTACT-3’.

**Table 5.1. Barcodes used for identification of patient samples and time points during deep sequencing analysis**

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<th>ID</th>
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</tr>
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</tr>
<tr>
<td>MID2</td>
<td>ACGCTCGACA</td>
</tr>
<tr>
<td>MID3</td>
<td>AGACGCACCTC</td>
</tr>
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<td>ATCAGACACG</td>
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</tr>
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<td>CGTGCTCTCTA</td>
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</tbody>
</table>
The products of the barcoding/inner PCR were pooled into 5 batches to generate a SMRTbell sequencing library following the Pacific Biosciences template preparation. The sequence reads were demultiplexed using Fastx-Toolkit (216). The sequence reads for each patient were analyzed using a custom script in R so that only those sequences that occurred with a frequency greater than 0.5% were carried on to the subsequent analysis (217). The script has been uploaded to GitHub (https://github.com/Eitan177/Evelyn.Gurule.Publication.Supplement/blob/master/generic CleanupTemplate.R). Identical reads from each time point and anatomic site/cell type were collapsed to avoid erroneously reporting multiple reads due to PCR resampling. Sequences were subsequently processed using BioEdit version 7.2.5 (Tom Hall, Ibis Biosciences, Carlsbad, CA). Barcodes were removed, and any sequences that differed from the expected amplicon length by greater than 10% were removed from the analysis. BLAST was then used to ensure that no contamination with laboratory strains of virus had occurred (http://blast.ncbi.nlm.nih.gov/). The sequences were then subjected to multiple sequence alignment using Gene Cutter (http://www.hiv.lanl.gov/content/sequence/GENE_CUTTER/cutter.html), using three Clade C sequences as an outgroup. The translated alignments were inspected, and sequences containing non-codon-length gaps were deleted, as these are most likely the result of PCR error during the sequencing reaction. Prior to estimating phylogenies, gaps were removed using Gap Strip/Squeeze v.2.1.0 (http://www.hiv.lanl.gov/content/sequence/GAPSTREEZE/gap.html). Phylogenies were estimated using a maximum likelihood approach in MEGA (212). Nodal support was determined via bootstrap analysis using 500 replicates, and trees were visualized in
MEGA. A phylogenetic reconstruction was performed using all of the sequences from all of the study participants to ensure that no contamination between study participants had occurred.

Results

Verification of predominant plasma clone existence

We confirmed the existence of the predominant plasma clone (PPC) through extensive sequence analysis of an HIV+ individual whose viral load was well controlled on ART during the year that she volunteered to take part in the study (Figure 5.1). Although there are two cellular sequences that match the 74 plasma sequences, neither of these cellular sequences is from the earlier two time points, whereas the plasma sequences were observed at those time points. The existence of the PPC suggests that residual viremia could originate from cells other than circulating resting CD4+ T cells. Tissue cells in the GALT are an example of one such potential source for residual viremia.
Figure 5.1. Phylogenetic reconstruction of sequences obtained using limiting-dilution PCR from an HIV+ volunteer whose viral load was well controlled on ART.

<table>
<thead>
<tr>
<th>Days after entering study</th>
<th>0</th>
<th>89</th>
<th>152</th>
<th>251</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequences from plasma</td>
<td>▲</td>
<td>▲</td>
<td>▲</td>
<td></td>
</tr>
<tr>
<td>Sequences from resting CD4+</td>
<td>⬜</td>
<td>⬜</td>
<td>⬜</td>
<td></td>
</tr>
</tbody>
</table>

The phylogenetic reconstruction exhibits a predominant plasma clone (PPC), a sequence that represents the majority of the viruses in the plasma and persists over time but is unrepresented or underrepresented by corresponding sequences from circulating resting CD4+ T cells. Only two proviral sequences match the PPC sequence, neither of which is from time points 1 or 2, even though many of the viral sequences are from these time points. The existence of the PPC confirms that residual viremia could originate from a source other than circulating resting CD4+ T cells.
Legend, Figure 5.1. This phylogenetic reconstruction exhibits a predominant plasma clone (PPC), a sequence that represents the majority of the viruses in the plasma and persists over time but is unrepresented or underrepresented by corresponding sequences from circulating resting CD4+ T cells. Only two proviral sequences match the PPC sequence, neither of which is from time points 1 or 2, even though many of the viral sequences are from these time points. The existence of the PPC confirms that residual viremia could originate from a source other than circulating resting CD4+ T cells.

Quantitative viral outgrowth assay (QVOA)

The QVOA was performed on GALT cells and circulating resting CD4+ T cells. In order to retain as many GALT cells as possible, we did not purify any population of cells from the tissue cells. Previously performed experiments indicated that enriching for CD4+ T cells reduced cell yields to such an extent that carrying out the quantitative viral outgrowth assay was no longer feasible (Table 5.2). Therefore, we carried out the QVOA on bulk GALT cells. We chose to use amphotericin B in the Assay Media because of the risk of fungal contamination from the GALT cells. To ensure that the addition of amphotericin B did not negatively affect the viral outgrowth in the QVOA, the assay was carried out in parallel using circulating resting CD4+ T cells from each study participant. We detected replication-competent virus in the GALT cells of one of the 10 study participants and in the circulating resting CD4+ T cells of 6 of the 10 study participants (Figure 5.2).

Table 5.2. Quantitative viral outgrowth assay results from a prior feasibility study in which CD4+ T cells were isolated from GALT cells

<table>
<thead>
<tr>
<th>Study participant</th>
<th>Peripheral resting CD4+ IUPM</th>
<th>GALT CD4+ IUPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>G01</td>
<td>14.4</td>
<td>&lt;0.41</td>
</tr>
<tr>
<td>G02</td>
<td>2.93</td>
<td>ND: insufficient cells</td>
</tr>
<tr>
<td>G03</td>
<td>0.29</td>
<td>ND: insufficient cells</td>
</tr>
<tr>
<td>G04</td>
<td>2.78</td>
<td>ND: contamination</td>
</tr>
<tr>
<td>G05</td>
<td>14.4</td>
<td>&lt;0.32</td>
</tr>
</tbody>
</table>
**Figure 5.2. Frequency of cells containing replication-competent HIV-1, measured in infectious units per million assay cells (IUPM)**

<table>
<thead>
<tr>
<th>Participant Number</th>
<th>Log (IUPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>352</td>
<td>-1</td>
</tr>
<tr>
<td>355</td>
<td>-2</td>
</tr>
<tr>
<td>362</td>
<td>-1</td>
</tr>
<tr>
<td>365</td>
<td>-2</td>
</tr>
<tr>
<td>370</td>
<td>0</td>
</tr>
<tr>
<td>374</td>
<td>1</td>
</tr>
<tr>
<td>375</td>
<td>2</td>
</tr>
<tr>
<td>381</td>
<td>1</td>
</tr>
<tr>
<td>383</td>
<td>0</td>
</tr>
<tr>
<td>389</td>
<td>-1</td>
</tr>
</tbody>
</table>

**Legend, Figure 5.2.** Error bars represent the error inherent in the assay. For experiments that produced detectable virus, error bars represent the 95% confidence interval. For experiments that did not produce detectable virus, error bars represent the upper 95% confidence limit for the IUPM value. In other words, there is 95% confidence that the true IUPM value is below the upper bound.

**Cell population identification**

To determine whether the scarcity of replication-competent HIV-1 in the GALT is due to a dearth of infectable cells isolated from the biopsies, GALT cells were stained for markers of CD4+ T cells (CD4 and CD3) and macrophages (CD68 and CD4) (218, 219). Levels of CD4+ T cells and macrophages were all below 5% (Figure 5.3). These low levels were somewhat expected because other than depleting CD8+ cells, we did not enrich for any cell population during the cell isolation in order to retain as many cells as possible. Additionally, using bulk GALT cells allowed us to include macrophages in the experiment, which have been shown to be capable of surviving infection over long periods of time despite active virus production (220).
**Figure 5.3.** Frequency of CD4+ T cells and macrophages observed in GALT cells, measured by FACS analysis

*Legend, Figure 5.3.* Values are the percentage of total cells that were CD3/CD4 double positive (for CD4+ T cells) or CD4/CD68 double positive (for macrophages), normalized to the values exhibited by the isotype controls.

*Activation experiment*

To determine whether the difficulty in detecting replication-competent HIV-1 in GALT cells was due to low activation of these cells, healthy donor PBMC, study participant PBMC, and study participant GALT cells were stained with CFSE and activated with 0.5 µg/mL PHA. Control wells for each sample received no PHA. CFSE levels were read at days 0, 3, 6, and 13. The percent activatability of each sample was calculated as the percentage of cells that had divided (lost CFSE) as compared to the cells that had stained positively for CFSE on day 0 (Figure 5.4). Using a paired t-test to determine significance, the mean activatability of the GALT cells was significantly lower than that of PBMC at days 3, 6, and 13 (p = 0.002, p = 0.0002, and p = 0.02, respectively) (Figure 5.5).
Figure 5.4. Sample CFSE plot for activated, unactivated, and unstained PBMC

Legend, Figure 5.4. Decreased amounts of CFSE indicate cell division and therefore cellular activation. Percent activation was calculated as the percent of cells that had lost CFSE as compared to the stained cells at Day 0.
Figure 5.5. Mean activatability for GALT cells and PBMC from study participants

Legend, Figure 5.5. Values are measured as the percentage of total cells that have divided above the baseline of CFSE-stained cells. Error bars for average values represent ± 1 standard deviation. Using a paired t-test to calculate significance, the activatability of GALT cells was significantly lower than the activatability of PBMC on days 3, 6, and 13 (p = 0.002**, p = 0.0002***, and p = 0.02*, respectively).

Viability experiment

From the quantitative viral outgrowth assay, study participant cells in culture with feeders and MOLT-4/CCR5 target cells were identified using differential HLA-A2 staining and forward/side scatter on FACS plots. After identifying study participant cells, viability was determined based on Annexin V/7-AAD staining (Figure 5.6). Statistical significance between the two groups was calculated using a paired t-test. Although the
mean viability of the GALT cells from the resting CD4+ T cells differed on day 0 (p = 0.02), there was no significant difference between the viability of the GALT cells and the viability of the resting CD4+ T cells on days 3, 6, and 13 of the quantitative viral outgrowth assay (p = 0.8, p = 0.4, and p = 0.1, respectively).

**Figure 5.6. Viability of study participant culture cells**

![Viability graph](image)

**Legend, Figure 5.6.** Viability was measured using Annexin V and 7-AAD staining. The mean viability only differed significantly between resting CD4+ T cells and GALT cells on day 0 of the quantitative viral outgrowth assay (computed using a paired t-test, p = 0.02*). There was no significant difference between the mean viability of resting CD4+ T cells and GALT cells on days 3, 6, and 13 of the assay (p = 0.8, p = 0.4, and p = 0.1, respectively.

**Phylogenetic reconstruction**

Sequences from positive QVOA wells, plasma, GALT cells, and resting CD4+ T cells were analyzed using phylogenetic reconstruction using a maximum likelihood approach in MEGA (212) (Figures 5.7-5.9). To ensure that no contamination between study participants occurred, the sequences from all of the study participants were visualized on a single phylogenetic tree and inspected for sequences that comingled with
sequences from other study participants (Figure 5.10). In all of the trees, sequences from GALT and CD4+ T cells appear to comingle (Figures 5.7-5.9). In participants 370 and 381, however, some sequences from the GALT and the CD4+ T cells appear to occupy different populations (Figures 5.7c and 5.9c). Although sequences from the plasma often comingle with cellular sequences (Figures 5.7 and 5.9), some study participants also exhibit sequence groupings that suggest that sequences in the plasma are distinct from cellular sequences (Figures 5.8 and 5.9). There appear to be only two study participants in whom certain plasma sequences comingle exclusively with sequences from the GALT (Figures 5.8a and 5.9c); however, in several instances, certain plasma sequences seem to comingle with sequences from both GALT and resting CD4+ T cells (Figures 5.7a, 5.7b, 5.9a, 5.9c, and 5.9d). Although there is some comingling between CD4+ T cell sequences and GALT sequences in all participants, there is one case in which some of the GALT sequences appear to cluster separately from the CD4+ T cell sequences (Figure 5.9c). Notably, of the two sequences obtained from p24-positive supernatants using GALT cells in the QVOA, one was identical to a sequence observed in the plasma, in the GALT, and in resting CD4+ T cells; the other clustered with other sequences from GALT and CD4+ T cells (Figure 5.9d).
Figure 5.7. Phylogenetic reconstruction of sequences from study participants whose plasma virus primarily comingled with proviral sequences.

Legend, Figure 5.7. The evolutionary histories were inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The trees with the highest log likelihood are shown. Nodal support was determined via bootstrap analysis using 500 replicates, and the percentages of trees in which the associated taxa clustered together are shown next to the branches. Initial trees for the heuristic searches were obtained automatically by applying Neighbor-Join and BioNJ algorithms to matrices of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site.
Figure 5.8. Phylogenetic reconstruction of sequences from study participants whose plasma virus primarily grouped separately from proviral sequences

Legend, Figure 5.8. The evolutionary histories were inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The trees with the highest log likelihood are shown. Nodal support was determined via bootstrap analysis using 500 replicates, and the percentages of trees in which the associated taxa clustered together are shown next to the branches. Initial trees for the heuristic searches were obtained automatically by applying Neighbor-Join and BioNJ algorithms to matrices of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site.
Figure 5.9. Phylogenetic reconstruction of sequences from study participants whose plasma contained some viral sequences that comingle with proviral sequences and some that grouped separately from proviral sequences.

Legend, Figure 5.9. The evolutionary histories were inferred by using the Maximum Likelihood method based on the Tamura-Nei model. The trees with the highest log likelihood are shown. Nodal support was determined via bootstrap analysis using 500 replicates, and the percentages of trees in which the associated taxa clustered together are shown next to the branches. Initial trees for the heuristic searches were obtained automatically by applying Neighbor-Join and BioNJ algorithms to matrices of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site.
log likelihood value. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site.

Figure 5.10. Phylogenetic reconstruction of sequences from all study participants

Legend, Figure 5.10. To ensure that no contamination between study participants occurred, the sequences from all of the study participants were visualized on a phylogenetic tree constructed using the Maximum Likelihood method based on the Tamura-Nei model. Nodal support was determined via bootstrap analysis using 500 replicates, and the percentages of trees in which the associated taxa clustered together are shown next to the branches.

Discussion

To our knowledge, this study represents the first documentation of HIV outgrowth from tissue biopsies of an HIV+ individual on suppressive antiretroviral therapy. In this
individual, the one of the viruses that was obtained in the outgrowth experiment appears identical to provirus in the circulating resting CD4+ T cell population, in addition to virus that is free in the plasma. This observation suggests that provirus in GALT cells can contribute to residual viremia in HIV+ individuals on suppressive antiretroviral therapy and that these tissue cells could comprise part of the anatomic barrier to curing HIV, in addition to latently infected circulating resting CD4+ T cells. As a result, cure studies may need to take into account the unique cellular milieu of infected tissue cells, as well as the ability of any potential cure agents to penetrate tissues.

We observed viral outgrowth from tissue cells in only one of the ten participants studied (Figure 5.2). Some potential reasons for this infrequent observation include a lack of infectable cells (CD4+ T cells and macrophages) included in the QVOA, a lack of viability in the GALT cells used for the QVOA, and a lack of activatability in the conditions used to activate the cells in the QVOA.

In the standard QVOA, circulating resting CD4+ T cells are purified from peripheral blood before being activated and placed into culture; however, when we performed the QVOA on GALT cells, we did not purify any particular population of cells to minimize loss of cells. As a result, the fraction of infected cells is likely much lower when the QVOA is performed on unpurified GALT cells than when it is performed on purified circulating, resting CD4+ T cells. In fact, in all five of the study participants whose GALT cells we stained for CD4 and CD68, less than 5% of the GALT cells stained positive for these markers (Figure 5.3). This decreased frequency of infectable cells may mean that more cells from the GALT need to be isolated to obtain a more readily detectable level of replication-competent virus from this anatomic location. In the interest
of safety, we were unable to obtain more biopsies from the study participants; therefore, animal studies involving necropsy of GALT from animals on suppressive antiretroviral therapy may be helpful.

In our hands, the GALT cells experienced significantly less activation under the conditions of the QVOA than did PBMC on days 3, 6, and 13 of the experiment (Figure 5.5). If infected cells are truly experiencing a lower level of activation, then it is possible that GALT cells containing replication-competent provirus may not be induced to produce virus in the QVOA. However, it is also likely that the majority of the GALT cells are not cells that divide upon exposure to PHA and therefore are not likely infected with HIV-1. If this is the case, then the measured lack of activatability likely has little effect on the amount of replication-competent virus detected using the QVOA.

The only difference in viability that we observed between the GALT cells and the circulating resting CD4+ T cells occurred on day 0 of the QVOA (Figure 5.6). Therefore, it is unlikely that the infrequent viral outgrowth from GALT cells is due to a lack of viability in the GALT cells. Despite the infrequent detection of replication-competent virus from the GALT cells, we observed comingling of proviral sequences from GALT cells and circulating resting CD4+ T cells, in addition to comingling of proviral sequences from GALT cells and viral sequences from the plasma in many of the study participants (Figures 5.7-5.9). This observation is consistent with the idea that GALT cells contain replication-competent virus, even though this was only directly observed in one of the ten study participants. Additionally, in the study participant whose GALT cells did produce replication-competent virus in the QVOA, one of the two viral sequences observed is identical to a viral sequence from the plasma, a proviral sequence from
peripheral resting CD4+ T cells, and a proviral sequence from the GALT (Figure 5.9d).
This observation is also consistent with the idea that infected cells in the GALT can
contribute to residual viremia in HIV+ individuals on suppressive antiretroviral therapy
and therefore constitute a barrier to the cure of HIV-1 in addition to circulating resting
CD4+ T cells. Taken together, the results of this study suggest that potential cure
strategies will need to be effective not only in peripheral blood, but also in tissue cells.
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EDUCATION & TRAINING

2009 – 2015  **Ph.D. in Pharmacology and Molecular Sciences**  
The Johns Hopkins University School of Medicine, Baltimore, MD  
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Advisor: Rebecca Alexander, Ph.D.

RESEARCH EXPERIENCE

2011 – 2015  **Laboratory of Robert Siliciano,** The Johns Hopkins University School of Medicine

2008  **Laboratory of Benjamin Harrison,** Wake Forest Institute for Regenerative Medicine

2007 – 2008  **Laboratory of Rebecca Alexander,** Wake Forest University

PUBLICATIONS


SELECTED HONORS AND AWARDS

2009  Summa Cum Laude, Wake Forest University

2009  Phi Beta Kappa Member

2008  Summer Research Scholar, Wake Forest Institute for Regenerative Medicine

2007  Summer Research Scholar, Wake Forest University

LEADERSHIP

2012 – 2015  Thread, Baltimore, MD
High school drop-out prevention program: led and trained mentors and mentor leaders for working with underprivileged high school students

SERVICE

2011 – 2015  Thread, Baltimore, MD
Worked with underprivileged students at Paul Laurence Dunbar High School to assist with homework tutoring and non-classroom challenges

2013 – 2014  Baltimore Rescue Mission Clinic, Baltimore, MD
Provided basic primary care to residents of the Baltimore Rescue Mission and the affiliated Karis Home under the supervision of John Dalton, M.D.

2013 & 2014  Short-term mission trips, Managua, Nicaragua
Led discussions on hygiene and women’s health issues in severely underprivileged communities

2013  LINK Summer Program, Baltimore, MD
Planned curriculum and taught in a summer science program for underprivileged middle school students

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