A Novel *in vitro* Assay to Measure Stochastic Reactivation of HIV-1 from Resting CD4+ T Cells: Implications for Measuring the Size of the Latent Reservoir in Infected Patients

by
Nina Narayan Hosmane

A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, Maryland
March 2016

Abstract

Human Immunodeficiency Virus-1 (HIV-1) is an enveloped retrovirus that preferentially infects activated CD4+ T helper lymphocytes. After completion of reverse transcription, HIV-1 cDNA integrates into the host genome. Latency arises when HIV-1 infects an activated CD4+ T cell as the cell is returning back to a resting memory state. Critical host cell transcription factors, NFκB and NFAT, are also required for HIV-1 transcription, but are inactive in resting T cells. This results in a stably integrated but transcriptionally silent form of the viral genome in a long-lived memory CD4+ T cell. Activation of a latently infected resting memory CD4+ T cell can give rise to replication-competent virus. Longitudinal analyses of latently infected cells purified from infected patients suppressed on ART (combination antiretroviral therapy) demonstrated that this infected cell population has a long half-life of 44 months and requires life-long ART to eliminate all infected cells. Therefore, the stability of the latent reservoir is the major barrier to curing HIV-1 infection. While there have been major efforts in the field to discover therapeutic strategies to reverse latency in order to reduce or eliminate the reservoir, there is a lack of an assay that can accurately quantify the true size of the latent reservoir. Without a reliable measure, eradication clinical trials will not be able to determine whether or not there has been a true reduction in the frequency of latently infected cells harboring replication-competent virus.

Here, I describe the advantages and disadvantages of current assays used in the field to measure the size of the latent reservoir. In addition, my thesis research involved developing a novel *in vitro* assay that provides a more accurate measure of the size of the latent reservoir of replication-competent virus. This work has revealed that latently infected cells, including clonally expanded cells, release replication-competent virus in a stochastic manner after multiple rounds of maximal T cell activation. My work highlights the need to develop additional assays that accurately measure the size of the latent reservoir and suggests the need to address reservoir

elimination strategies that include targeting clonally expanded latently infected cells harboring replication-competent viruses.

Advisor:

Dr. Robert F. Siliciano (Reader)

Thesis Committee:

Dr. Joel N. Blankson (Chair)
Dr. Janet D. Siliciano (Reader)
Dr. Ashwin Balagopal

Acknowledgements

I would first like to thank my mentor Dr. Robert F. Siliciano for always seeing my greatest potential and giving me so many growth-promoting opportunities throughout graduate school. From day one, he has always been incredibly positive, caring, supportive, and has always reminded me to look at the big picture of what I'm doing--an important trait to have as a successful scientist. Under his training, I truly feel like I have become an independent scientist. In addition, I am very grateful for Dr. Janet D. Siliciano and for all of her loving support, caring, optimism, and dedication to my wellbeing during my graduate career. I would like to thank Dr. Joel N. Blankson, who provided me with much needed comic relief but, more importantly, was an incredibly strong source of support during my graduate work. I would like to thank Bonita Grant and Jun Lai for being supportive and ensuring I had everything I needed to make my graduate school experience an enjoyable one. I would like to thank Kyungyoon J. Kwon because of her tremendous hard work, dedication, and perseverance in assisting me with experiments that contributed greatly to my thesis work.

Lastly, I would like to thank my parents, my older brother, and my sister-in-law for being extremely supportive, loving, and understanding of the sacrifices I had to make in graduate school. Without their positive influence, unconditional love, patience, and support, I would not be the person I am today. I would also like to thank the Cellular and Molecular Program for making my graduate experience such an enjoyable one. Colleen Graham and Leslie Lichter not only gave me their unconditional support, but they were two important motherly figures to me. They knew about my goals in graduate school and did everything to ensure that I would meet my goals and, for this, I am very grateful. I would like to thank all my CMM classmates and the new friends I made in Baltimore because these meaningful connections I had with them are what gave me the strength and drive to pursue what I am passionate about.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Introduction

There are currently 35 million people worldwide infected with Human Immunodeficiency Virus Type 1 (HIV-1). Without life-long antiretroviral therapy, most of these individuals will die of Acquired Immunodeficiency Syndrome (AIDS). However, to date, only 15 million infected people are on combination antiretroviral therapy (http://www.cdc.gov/hiv/statistics/basics/). Although combination antiretroviral therapy (ART) is effective in suppressing HIV-1 replication by blocking various steps in the viral life cycle, it is not curative due to the existence of a latent viral reservoir (LR) in resting memory $CD4^+$ T cells[1-5]. The LR has an extremely long half-life (44 months), necessitating lifelong treatment[6,7]. The LR is established when HIV-1 infects activated $CD4^+$ T cells as they are returning to a resting memory state as part of the normal physiological process after encountering cognate antigen[8,9]. Thus, latent proviruses are found predominantly in different subsets of resting memory cells. The virus does not replicate in resting CD4+ T cells due to blocks at numerous steps of the viral life cycle[1,10-12]. Latently infected resting memory $CD4^+$ T cells are nonpermissive for viral gene expression, and the viral genome persists as integrated proviral DNA that is transcriptionally silent[13]. For this reason, latently infected cells are not targeted by the immune system or ART. The LR in resting memory $CD4^+$ T cells is widely recognized as a major barrier to HIV-1 eradication.

The goal of HIV-1 cure strategies is to significantly reduce or eliminate the LR such that patients can successfully come off ART without subsequent viral rebound or have a significant delay in rebound once stopping ART. With no interventions to reduce the LR, most patients typically rebound within a few weeks after stopping ART. To date, only one person has been cured of an established HIV-1 infection. This individual (the "Berlin Patient") was HIV-1-infected and on ART when he developed acute myeloid leukemia. After an extensive conditioning chemotherapy regimen, he received a hematopoietic stem cell transplant (HSCT) from an HLA-matched donor whose cells were homozygous for a deletion in CCR5, a co-receptor used for HIV-1 entry into target cells[14]. This patient received a new immune system that

was resistant to HIV-1 infection and has had no viral rebound since stopping ART after his transplant nearly 8 years ago. Although a HSCT resulted in the only documented cure thus far, this procedure is not feasible for HIV-infected patients who do not have cancer. Due to the stability of the LR, the field has instead turned to identifying pharmacological agents that can force the virus out of latency and eliminate the LR, defined as the 'shock and kill' strategy[15].

In conjunction with identifying therapeutic strategies to eliminate the LR, there is a need to have an assay that accurately measures the size of the LR. Current PCR- and culture-based assays overestimate and underestimate the size, respectively, and don't correlate well with one another[16,17]. As cure trials move forward, there is a critical need to develop an assay that accurately measures the effectiveness of an eradication strategy[17].

For my thesis work, I hypothesized that repeated maximum stimulation of latently infected, resting CD4+ T cells would induce additional outgrowth of replication-competent virus and provide a more accurate measurement of the LR. To address this hypothesis, I designed a novel *in vitro* assay that involved isolating and culturing resting CD4+ T cells isolated from peripheral blood from 12 patients on suppressive ART. Resting CD4+ T cells were subjected to four subsequent rounds of maximal T cell activation at weekly intervals. In addition, I analyzed outgrowth viruses to determine whether certain inducible provirus would require only one round of activation vs multiple rounds for outgrowth. I sequenced two short regions within the viral genome in both the LTR and *env* genes and found that, in 9 out of 12 patients, different rounds of stimulation caused outgrowth of identical viral isolates by sequence. This result is consistent with previous work showing *in vivo* clonal expansion of infected cells carrying replication-competent virus[18-21]. Taken together, my work strongly supports the novel concept of stochastic reactivation of intact proviruses from clonally expanded resting CD4+ T cells in the latent reservoir.

Chapter 2

Towards an HIV-1 cure: measuring the latent reservoir

Bruner, K.B.*, **Hosmane, N.N.*,** and Siliciano, R.F. Towards an HIV-1 cure: measuring the latent reservoir. *Trends Microbiol*. 2015 Apr;4(23):192–203. ***contributed equally**.

Abstract

The latent reservoir of HIV-1 in resting memory CD4+ T cells serves as a major barrier to curing HIV-1 infection. While many PCR- and culture-based assays have been used to measure the size of the latent reservoir, correlation between results of different assays is poor and recent studies indicate that no available assay provides an accurate measurement of reservoir size. The discrepancies between assays are a hurdle to clinical trials that aim to measure the efficacy of HIV-1 eradication strategies. Here we describe the advantages and disadvantages of various approaches to measure the latent reservoir.

Measurement and composition of the latent reservoir

The latent reservoir (LR) was first measured and characterized in the mid-1990s using a viral outgrowth assay (VOA), which is still considered the gold standard for measuring the frequency of resting $CD4^+$ T cells that carry replication-competent proviruses in a latent state[3,22]. The VOA makes use of phytohemagglutinin (PHA), a strong T cell mitogen, to globally activate resting $CD4^+$ T cells in a manner that mimics antigen-driven activation. This renders the cells permissive for viral gene expression and induces release of HIV-1 from latently infected cells. Since the development of the VOA, other assays such as PCR-based ones that measure total HIV-1 proviral DNA, have been advanced as simpler measures of the LR[23-25]. Although both culture- and PCR-based methods are commonly used, there is little correlation between the two types of assays. Based on the VOA, approximately 1 per 10^6 resting $CD4^+$ T cells contain replication-competent virus, but PCR-based assays show that on average 300 per 10^6 resting $CD4^+$ T cells contain HIV-1 proviral DNA[16].

These discrepancies are attributed to the heterogeneous nature of the proviruses in resting $CD4^+$ T cells. These proviruses can be divided into two groups: those that are induced to release replication-competent virus after one round of T cell activation (induced proviruses) and those that are not (non-induced proviruses), as is shown in **Figure 2.1**. The VOA measures only the former population, whereas PCR-based approaches detect both groups of proviruses. This partially explains the lack of correlation between the two types of assays. Interestingly, most noninduced proviruses are defective[26]. The majority contain large internal deletions that arise by copy choice recombination between homologous regions in genomic viral RNA during reverse transcription[26-28]. Other defective proviruses contain inactivating G to A nucleotide substitutions introduced by APOBEC3G, a cytidine deaminase that is part of the host innate immune defense system[29-31]. A small percentage of proviruses contain other inactivating defects such as packaging signal deletions, small nucleotide insertions or deletions (INDELS), and missense mutations.

Defective proviruses will not produce infectious virus in the VOA but can be detected by PCR assays, depending on the assay specifics. In addition, a small percentage of noninduced proviruses have intact genomes and are potentially replication-competent[26]. These proviruses are termed intact, noninduced proviruses (INPs). In order to achieve a sterilizing HIV-1 cure, all of the proviruses capable of reinitiating infection must be purged from the LR.

Accurately measuring changes in the size of latent reservoir

Currently, the most widely discussed approach to eliminate the LR is to reverse latency pharmacologically so that the infected cells can be eliminated. This approach is termed 'shock and kill'[15,32,33]. The rationale is to first force HIV-1 out of latency by inducing expression of HIV-1 genes since high levels of certain HIV-1 proteins can cause death of the infected T cells through cytopathic effects. In addition, presentation of peptides derived from viral proteins can lead to lysis of infected cells by virus-specific cytolytic T lymphocytes (CTL). Cell surface expression of the HIV-1 envelope glycoprotein (Env) can, in principle, target infected cells for destruction by antibody-dependent cell-mediated cytotoxicity (ADCC). In addition, downregulation of MHC class I molecules by the HIV-1 accessory factor Nef can render productively infected cells susceptible to lysis by natural killer (NK) cells[34].

Clinical trials involving the 'shock and kill' strategy are in progress. Histone deacetylase inhibitors (HDACis) reverse HIV-1 latency in model systems and are being tested in infected individuals on ART[35-41]. However, recent studies have shown a discrepancy between the effects of latency reversing agents (LRAs) in *in vitro* HIV-1 latency models and in *ex vivo* assays with cells from HIV-1 infected individuals[42-44]. In order to determine if these LRAs are effective, it is important to develop techniques that accurately measure changes in the LR size *in vivo*.

Significant progress has been made in developing culture- and PCR-based assays since the initial discovery of the LR in the 1990s. However, a detailed comparison of several current

assays has shown no precise correlation[16]. Available assays are discussed below, and their advantages and disadvantages are summarized in **Table 2.1**. A high-throughput, reliable, and sensitive assay that can accurately measure the true size of the LR is urgently needed. Such an assay is essential for determining the efficacy of pharmacological agents used in current or future clinical trials and for reliably concluding that a sterilizing cure has been achieved in HIV-1-infected patients undergoing eradication treatments.

Culture-based assays to measure the latent reservoir

The LR was initially defined and measured using the VOA[3]. This assay is still considered the 'gold standard' for measuring the LR. In this assay, resting $CD4^+$ T cells are isolated from patients on ART and plated in fivefold serial dilutions. Phytohemagglutinin (PHA), a lectin, is added along with irradiated allogeneic peripheral blood mononuclear cells (PBMCs) to induce global T cell activation. The PBMCs ensure successful activation since they contain macrophages and dendritic cells, which act as antigen-presenting cells and enhance the PHA-driven activation of T lymphocytes[22]. These conditions give uniform activation of resting $CD4^+$ T cells as assessed by CFSE dilution[45]. Initially, $CD4^+$ lymphoblasts from HIV-1-negative donors were used to expand virus released from the patient cells to detectable levels. This process can be simplified by using the continuously proliferating cell line, MOLT-4/CCR5, which expresses high levels of CD4 and CXCR4 and has been stably transfected with CCR5, a co-receptor for HIV-1 viral entry[46]. The patient cells are co-cultured with lymphoblasts or MOLT-4/CCR5 cells for two to three weeks and then culture supernatants are analyzed for free virus using an ELISA assay for HIV-1 p24 antigen. Alternatively, a highly sensitive RT-PCR assay for virus in the supernatant can detect viral outgrowth as early as seven days after activation[46].

The VOA has a number of advantages, including the ability to detect individual latently infected cells. The clonal viruses that grow out in wells seeded at limiting dilution can be

characterized by sequencing and functional studies. The VOA has the additional advantage of not detecting defective proviruses, which vastly outnumber replication-competent proviruses and which are detected in standard DNA PCR assays[26]. However, some recent studies have shown that the VOA underestimates the true size of the LR because viral outgrowth is not observed in all wells that contain cells with intact proviruses. When wells negative for viral outgrowth were stimulated a second time with PHA, outgrowth of replication-competent virus was observed[26]. There are several potential explanations for the initial failure of these INPs to give rise to viral outgrowth following cellular activation. Viral induction could represent a stochastic process such that each intact provirus has only a finite probability of becoming induced following cellular activation. Most HIV-1 proviruses integrate into introns of actively expressed cellular genes[47], and transcriptional interference from the host gene could reduce the probability that a given intact provirus will be induced by a single round of T cell activation. However, the same provirus may be induced by subsequent rounds of T cell activation. Additionally, some of these INPs could have been successfully induced to produce viral RNA and release infectious virions. However, the released virus may have failed to establish a spreading infection that is required for detection by the VOA. For these reasons, the LR may actually be larger than the average value of one replication-competent provirus per 10^6 resting $CD4^+$ T cells, as measured by the VOA[26]. Thus, the VOA is best regarded as a definitive minimal estimate of the frequency of latently infected cells.

In addition to underestimating the size of the LR, the VOA can be problematic because it uses large volumes of blood (120-180 mL), is labor intensive and expensive, and requires 1-3 weeks of culture in a BSL3 laboratory. Hence there has been great interest in developing alternative assays.

T cell activation assays with viral RNA readout

Other culture-based assays used to measure the LR include ones in which patient resting $CD4^+$ T cells are plated and maximally activated at limiting dilution such that there is less than one virus-producing cell per well. Following maximal T cell activation, the frequency of cells producing viral RNA is directly measured by a quantitative PCR (qPCR) specific for either cell-associated unspliced mRNA[43], multiply spliced mRNA[45], or HIV-1 mRNA in the culture supernatant (presumably reflecting virion release)[43,46]. These assays measure different forms of RNA present throughout the HIV-1 life cycle. It is important to identify what form of HIV-1 RNA is being detected since these represent different stages in replication. Early in the replication cycle, multiply spliced mRNA transcripts are generated, but with time the transcripts are increasingly exported as unspliced and singly spliced mRNA species. Some mRNA transcripts are translated to make viral proteins necessary for virion assembly while other full-length transcripts are packaged into newly assembled virions for subsequent infection. When virions are released from the infected cell, certain assays can measure the two full-length mRNA copies that are present within a single virion. A recent study compared virion release with intracellular RNA measurements and found that, on average, 1.5% of proviruses could be reactivated to produce HIV-1 virions, whereas 6.8% and 8.2% of proviruses from two different patients could be reactivated to produce unspliced cell-associated HIV-1 RNA[43]. Since HIV-1 mRNA present in the culture supernatant reflects virus production, this measurement is likely a more accurate quantification of the LR. In summary, these RNA-based assays allow for detection of infected cells with no need for outgrowth of the virus before PCR measurement, shortening the length of required culture time. However, the same caveats that apply to the VOA also apply to these assays. Since they only involve a single round of T cell activation, they do not detect INPs within the LR. Additionally, some defective proviruses may be able to produce RNA and even release virions even though they are unable to produce infectious virions. These defective proviruses

could be detected by these assays. Thus, this class of assays may be confounded by both false negative and false positive results and do not accurately measure the size of the LR.

PCR-based assays to measure the latent reservoir

PCR-based assays are commonly used to measure persistent HIV-1 and provide a complementary approach to the VOA. These methods provide a quicker and easier way to study viral persistence and can be applied to a variety of immune cell types. The most common PCR method for measuring the LR is a qPCR for proviral DNA in either unfractionated PBMCs[23,48-52], $CD4^+$ T cells[53,54], or resting $CD4^+$ T cells[55]. All methods involve isolating the desired cell populations from the peripheral blood of infected individuals and subsequently extracting the DNA. A qPCR using primers located in conserved regions of the HIV-1 genome is carried out on the DNA extracts. The number of infected cells is calculated using a standard curve constructed with known copy numbers of proviral DNA, typically from a plasmid standard such as pNL4-3. A qPCR assay for a cellular gene present in two copies per diploid genome (frequently the ribonuclease RNase P), is used to determine the total number of cells in the sample. The proviral DNA copy number, combined with the total number of cells present, can give an estimate of the frequency of cells that harbor HIV-1 DNA. These methods have also been adapted to measure proviral DNA in the gut-associated lymphoid tissue (GALT)[56-58], a site which contains a high frequency of infected cells, as well as in $CD4^+$ T cells in the bone marrow[59]. Other PCR methods use the droplet digital PCR technique to measure HIV-1 DNA in $CD4^+$ T cells[16,25] as well as in PBMCs[16,60,61]. This method permits absolute quantification of HIV-1 DNA rather than relative quantification derived from a standard curve, as with qPCR.

In measuring the LR, it is important to detect only stably integrated proviruses and not extra-chromosomal HIV-1 DNA forms, which are unstable or replication-defective. Pioneering studies by Stevenson and colleagues showed that most of the HIV-1 DNA in resting $CD4^+$ T cells

from viremic patients is in a linear, unintegrated form which represents the end product of reverse transcription prior to integration[62]. Following cellular activation, this unintegrated viral DNA is integrated and transcribed, ultimately giving rise to infectious virus. Since the VOA involves cellular activation, it also detects cells with linear, unintegrated HIV-1 DNA if carried out on samples from viremic patients. Several studies suggest that in the absence of integration, the linear, unintegrated form of the viral genome is labile[63,64]. Therefore, cells with unintegrated HIV-1 DNA should not be considered as part of the stable LR. Following initiation of ART, labile, unintegrated viral genomes in recently infected cells decay, and after six months, the frequency of cells detected in the VOA falls to a stable plateau[65]. Importantly, because it can detect cells with linear unintegrated viral genomes, the VOA does not give a reliable estimate of the LR in untreated patients or in patients who have been on therapy for less than six months.

Due to the problem of linear, unintegrated HIV-1 DNA, there has been considerable interest in PCR assays that can distinguish integrated viral genomes from unintegrated genomes. The most common method used for this purpose is Alu-PCR, which has been applied to either purified $CD4^+$ T cells or PBMCs[24,66-69]. Alu-PCR selectively amplifies integrated HIV-1 genomes by using one primer targeting Alu elements, which are found in high copy numbers in the human genome, and a second primer located in the HIV-1 *gag* gene. A nested real-time PCR is then performed using a second set of primers in the HIV-1 LTR. Controls in which the Alu primer is excluded are important to demonstrate that the observed signal is in fact coming from integrated HIV-1 DNA. Proviruses integrated in close proximity to Alu elements give shorter first round PCR products and will amplify to a greater extent. A standard curve with a mixture of DNA from cells with different HIV-1 integration sites is used to account for differential amplification of proviruses integrated at different distances from an Alu element, and a correction factor is used to account for proviruses that are too far from an Alu sequence to be detected. When compared to droplet digital PCR for HIV-1 DNA in patients on suppressive ART, there was a strong

correlation, indicating that the majority of HIV-1 DNA in ART patients is integrated[16]. This conclusion is further supported by additional studies showing that unintegrated forms of HIV-1 DNA are typically found in low levels in patients on suppressive ART[48,51,68]. Alternative methods for measuring integrated proviruses have also been developed and include linker ligation PCR[70] and inverse PCR [1].

Recently, two different research groups[18,19] have improved methods to study the specific integration sites of proviruses. Their findings show that a substantial fraction of proviruses (40% in one study)[18] are integrated into the genomes of cells that have undergone clonal expansion after infection. In one patient, approximately half of the infected cells contained a single viral clone with the same integration site[18]. In addition, integration sites were shown to be favored in genes that are associated with cancer[18,19]. These studies suggest that clonal expansion may occur as a result of integration into growth promoting genes[18], which will cause an expansion in the number of CD4+ T cells carrying HIV-1 proviruses. However, it remains unknown whether or not the proviruses present in these expanded clones are replication-competent. PCR-based assays will detect clonally expanded proviruses regardless of whether or not they are replication-competent, while culture-based assays will only detect outgrowth of replication-competent virus.

Total HIV-1 DNA PCR measurements can also detect other forms of unintegrated proviruses that are not a part of the LR. Two-long terminal repeat circles (2-LTR circles) result when integration fails and non-homologous end-joining (NHEJ) occurs between the two LTRs of linear, unintegrated HIV-1 DNA[71]. 1-LTR circles also are present during the course of HIV-1 infection and arise when homologous recombination occurs between linear HIV-1 DNAs at the LTRs[72]. This results in a circularized form of HIV-1 containing only a single LTR. Neither of these forms can integrate nor produce infectious virus. Although these forms are not considered to be part of the LR, 2-LTR circles have been used as a measure of recent infection or ongoing replication, based on the assumption that 2-LTR circles are labile. However, the stability of 2-

LTR circles remains controversial[49,73-75]. Several assays have been developed to study 2-LTR circles, including a droplet digital PCR assay on resting $CD4^+$ T cells and PBMCs[16,61], as well as a qPCR assay on PBMCs[49,76] and $CD4^+$ T cells[73]. These assays use primers flanking the 2-LTR circle junction. 1-LTR circles are more difficult to quantify using PCR[1,77,78]. It remains unclear how measurements of these replication-defective circular forms will contribute to our understanding of HIV-1 reservoirs.

It is important to understand the relationship between PCR-based assays for proviral DNA and other assays of the LR. As discussed above, PCR assays for HIV-1 proviruses give infected cell frequencies that are at least two logs higher than those obtained by the VOA[16]. This is due to the fact that PCR methods detect many defective proviruses as well as replication-competent proviruses. A recent study by Ho and colleagues[26] characterized INPs and found that almost 90% of these proviruses are defective and contain large internal deletions, APOBEC3G-induced hypermutations, or other inactivating mutations (**Figure 2.1**). Detection of these defective proviruses helps explain the overestimation of the LR size by PCR-based assays.

An additional concern with PCR-based assays is that they may fail to detect changes in LR size since most of the PCR signal is from defective proviruses that may respond differently to LRAs. Many defective proviruses may be unable to produce viral proteins even if latency is successfully reversed. Elimination of infected cells is likely dependent upon viral protein production, and thus cells containing defective proviruses may not be eliminated even by successful strategies. Thus, large numbers of cells with defective viruses could potentially mask successful clearance of latently infected cells by eradication strategies. In summary, the VOA underestimates the true size of the LR since one round of activation does not induce outgrowth of all replication-competent proviruses. On the other hand, PCR assays for HIV-1 DNA dramatically overestimate the LR since they cannot discriminate between defective and replication-competent proviruses.

Measuring residual viremia

While ART is effective in suppressing viremia to below the clinical limit of detection (50 copies of HIV-1 RNA/mL of plasma), HIV-1 RNA can still be detected at low levels in the plasma, indicating persistent residual viremia[79]. To measure residual viremia, Palmer and colleagues developed a highly sensitive single-copy assay (SCA). This assay can detect HIV-1 RNA down to one copy per mL of plasma[80-82]. The SCA is an RT-PCR assay that uses primers located in a conserved region of *gag* in the HIV-1 genome. It measures ongoing viral production from $CD4^+$ T cells or from other stable reservoirs[83]. Another version of the SCA that uses a larger volume of plasma and primers located in a conserved region of integrase has recently been described[84].

Sequence analysis of residual viremia show that it is archival in character and sensitive to the current ART drugs[85,86]. These results suggested that residual viremia results from release of virus from stable reservoirs rather than ongoing viral replication. This hypothesis was substantiated in 2009 by the addition of a fourth drug to the three drug ART regimens of patients who had stable suppression of viremia to below 50 copies/mL This addition did not further reduce residual viremia[87] and further treatment intensification studies have confirmed this result[51,88-91]. Thus, residual viremia reflects virus production by a stable reservoir of cells infected prior to the initiation of therapy. In this sense, it can be used as a measure of viral reservoirs. However, the nature of the cells that produce the residual viremia is not yet clear. It is certainly possible that residual viremia results from the activation of a small fraction of the resting $CD4^+$ T cells that constitute the LR. However, residual viremia in patients on ART is frequently oligoclonal[83,92]. Despite the enormous sequence diversification that occurs during HIV-1 infection, the residual viremia in many patients is dominated by a single clone, termed a predominant plasma clone (PPC). Proviruses with the same sequence can be found in resting $CD4^+$ T cells in the blood, but there is not a 1:1 correspondence between plasma and cellular

sequences[83,93]. This may reflect the large fraction of defective proviruses[26] or possibly the presence of an additional reservoir. The relationship between the PPC and the expanded clones of infected cells described above remains unclear. Thus, the relationship between residual viremia and the LR is complex. While the SCA is a useful tool for measuring viral persistence in the plasma, it may not accurately quantify changes in the LR in patients in eradication trials.

Treatment interruption and time to rebound as a measurement of latent reservoir size

Initially, following the introduction of ART, it was thought that only a few years of treatment would be sufficient to cure HIV-1 infection[94]. However, the discovery of the LR and its long-term stability indicates that patients must remain on ART for their entire lives to prevent a rebound in viremia[6,7], which typically occurs within a few weeks of stopping treatment[95]. As viral rebound reflects release of virus from stable reservoirs, there is a growing trend to evaluate reservoir reductions in HIV-1 eradication studies by stopping ART and observing the time it takes for viral rebound to occur[14,60,96,97]. This was done in the case of the two HIV-1 positive patients who received allogeneic stem cell transplants from CCR5-wild type donors while on ART. The transplants were done to treat Hodgkin's lymphoma in one case and diffuse large B-cell lymphoma and subsequent Hodgkin's lymphoma in the other case[97]. Although the patients (known as the 'Boston patients') had undetectable levels of HIV-1 DNA, both experienced a rebound of viremia several months after stopping ART[98]. In another example, a baby born to an HIV-positive mother (the 'Mississippi baby'), was given ART 30 hours after birth and, subsequently, viremia fell to undetectable levels and remained below the limit of detection. ART was discontinued against medical advice 18 months after initiation of treatment[99]. The baby showed no signs of virus in her blood following treatment cessation for over two years, but had a sudden rebound in viremia after 27 months off ART[100]. Interestingly, in all three cases, HIV-1-specific immune responses were absent. The long-term persistence of replication-competent HIV-1 in the absence of HIV-1-specific immune responses provides dramatic proof that the virus can

persist *in vivo* in a latent form. These examples demonstrate that with a very small LR resulting from transplantation or early treatment, there is a substantial delay in the time to rebound.

Although treatment interruption is ultimately the only way to determine if a patient is cured, there are precautions and ethical issues to consider when using 'time to rebound' as a measurement of LR size. Drug resistance can occur if the interruption or subsequent reinitiation of ART is done in a way that allows suboptimal drug concentrations to be present for a significant period of time[101,102]. One of the Boston patients developed a new resistance mutation while restarting therapy[98]. The Strategies for Management of Antiretroviral Therapy (SMART) study, conducted in 2006, compared the effects of episodic treatment interruption to continuous ART and found that the patients who underwent treatment interruption experienced higher levels of morbidity, opportunistic diseases, and malignancies compared to those on continuous ART[103-106]. Additional difficulties with this approach include the enormous variability in time to rebound. If the LR is reduced significantly in size but not entirely eliminated, rebound could be governed by stochastic processes and may occur unpredictably months or years after treatment interruption[107]. Thus, treatment interruption is problematic in evaluating reservoir reductions.

Current measurements in eradication clinical trials

As LRAs are meant to induce transcription of the latent provirus, methods to detect changes in HIV-1 RNA levels are used to evaluate their efficacy in eradication studies. Methods using qPCR have been developed for measuring steady state HIV-1 RNA levels in PBMCs[50,57], resting $CD4^+$ T cells[35], and in the GALT[16,57]. Droplet digital PCR has also been used to quantify HIV-1 RNA [108], and a recently described method detects polyadenylated HIV-1 mRNAs in resting $CD4^+$ T cells[42]. Most studies also incorporate careful measurements of HIV-1 RNA in the plasma to detect transient increases in viral production. Recently, small increases in HIV-1 RNA in the detectable range have been documented in patients receiving the HDACi panobinostat[41]. In

addition to determining whether or not a LRA has been effective in 'shocking' latently infected cells to produce viral RNA, it is also important to determine whether or not the infected cells have been 'killed' and eliminated from the reservoir[109]. In order to do this, an accurate measure of the LR before and after treatment(s) is necessary. Currently, the VOA provides the most definitive way to do this. To date, no significant reduction in the LR has been achieved by any 'shock and kill' strategy. Current and future eradication trials involve HDACis[38,40,41], gene therapies[60], and treatment intensifications[110]. Most current and future trials will measure LRA efficacy by decreases in the frequency of latently infected cells as measured by the VOA, increases in transient LRA-induced cell-associated HIV-1 RNA, or the appearance of plasma HIV-1 RNA. Some studies also measure total and integrated HIV-1 DNA or utilize a combination of multiple measures (**Table 2.2)**. In interpreting the outcomes of these trials, it will be important to keep in mind the differences in what is actually measured by different assays of the LR.

Discussion

Following the discovery of the LR in resting memory $CD4^+$ T cells and its long-term stability, identifying ways to eliminate the LR has become a research priority. It is equally important to develop better ways to measure the LR[111], since current and future eradication trials lack a universal measure to assess whether or not a decrease in the LR has been achieved. Although many assays have been developed to quantify the LR, there is little correlation between the methods[16] and none measure the true LR size as is shown in **Figure 2.2**. Additionally, there are also disadvantages to both PCR- and culture-based assays that need to be considered when measuring the LR or evaluating eradication therapies. The VOA is still considered the gold standard for measuring the LR since it provides a definitive minimal estimate of reservoir size. However it does not detect all latently infected cells that contain replication-competent virus[26]. Underestimating the size of the LR may give the false impression that a patient is 'cured' when the patient may still have latently infected cells. If taken off ART, a patient may experience a rebound in viremia, as was the case with the Boston patients[98] and the Mississippi baby[100]. PCR-based assays are also commonly used to quantify proviral DNA, but they cannot distinguish between replication-competent and defective proviruses. A failure to distinguish between the two would make it impossible to know when the LR has been successfully cleared and when or if patients can safely interrupt therapy. Treatment interruption is also used to measure a reduction in reservoir size; however, there are many concerns with this approach including increased risks for drug resistance and disease progression. Thus, there remains a need for assays that can measure the effects of therapeutic interventions on reservoir size without the need for treatment interruption. When applied to the 'shock and kill' strategy, neither culture- or PCR-based assays can adequately measure both the 'shock' and the 'kill' aspects. However, it may not be necessary to use the same assay to measure both the 'shock' and the 'kill'. Certain assays may be better suited to measure each aspect. For instance, RNA-based culture assays may provide a rapid

measure for the 'shock', but assays such as the VOA may be best suited for measuring the 'kill'. In the absence of a universal assay, clinical trials are using a combination of culture- and PCR-based methods to identify and measure changes in the LR (**Table 2.2)**. However, the conclusions drawn from these trials will vary depending on the measurements used. Without a high throughput, sensitive, and well-validated assay, it will remain difficult for researchers to identify novel LRAs, move forward with clinical trials, and develop strategies to eradicate HIV-1.

.

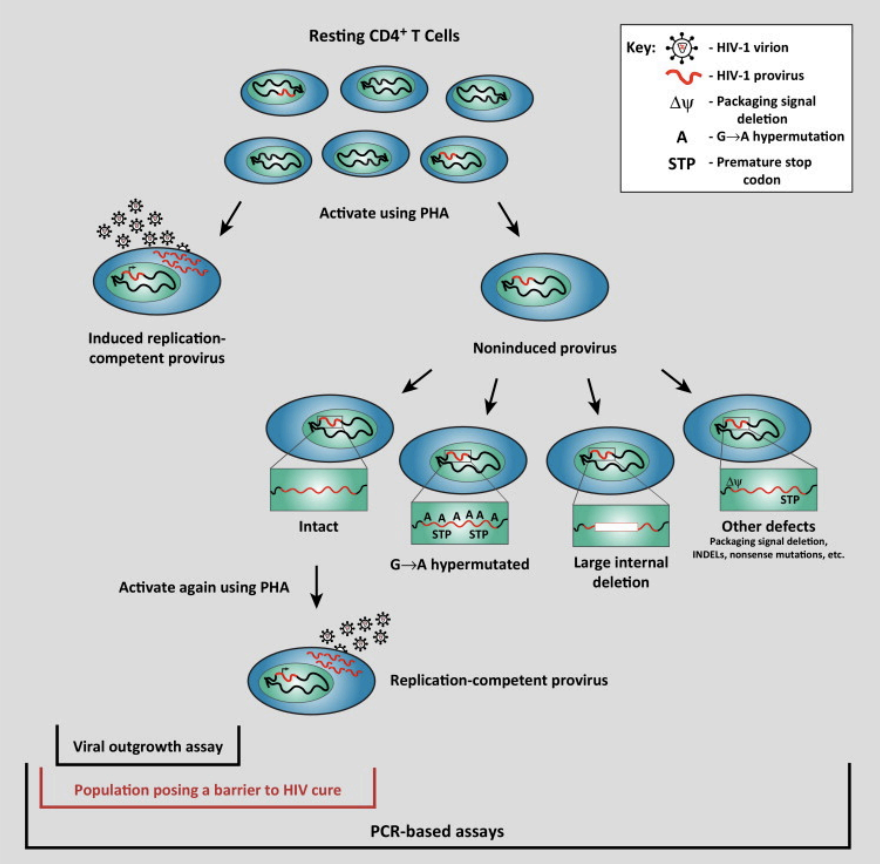


Figure 2.1. Profile of different types of HIV-1 proviruses in resting CD4$^+$ T cells.

The major types of proviruses are shown, including those that pose a barrier to an HIV-1 cure and those that do not. Following a single round of T cell activation, some proviruses are induced to produce virions, which can infect other cells (induced, replication-competent proviruses). Proviruses that are not induced to produce replication-competent virions following a single round of T cell activation are termed noninduced proviruses[26]. Many of these noninduced proviruses are defective and contain large internal deletions, G→A hypermutations, or other inactivating defects. However, some noninduced proviruses have fully intact genomes and, on subsequent rounds of cellular activation, can produce virions. These proviruses are termed intact, noninduced proviruses (INPs). Culture-based assays detect induced replication-competent proviruses only, while PCR-based assays detect all types of proviruses. Only induced replication-competent proviruses and INPs pose a barrier to an HIV-1 cure.

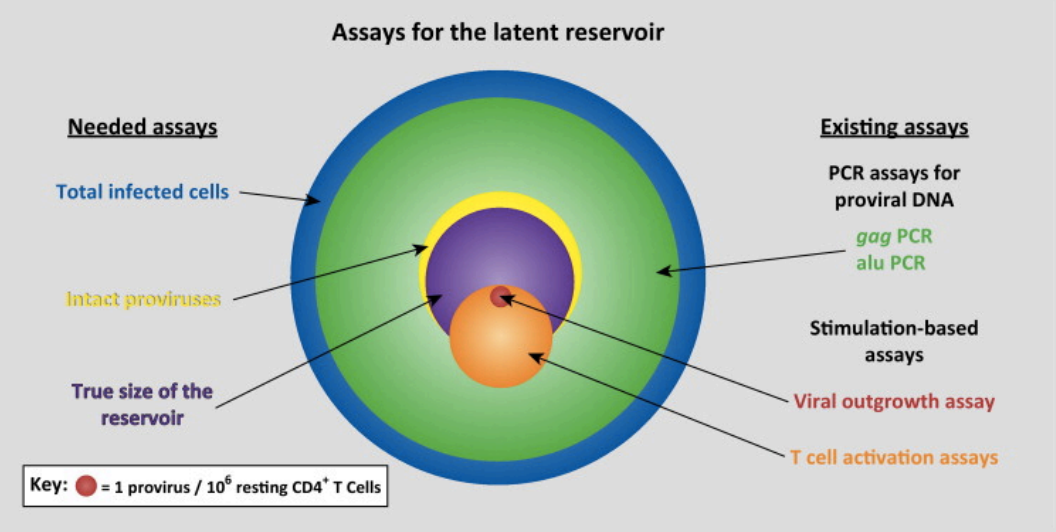


Figure 2.2. Venn diagram comparison of proviral populations measured by different methods of assessing the latent reservoir (LR).

Typical or estimated results from various PCR- and culture-based assays are shown relative to a prediction of the true LR size. The frequency of infected cells detected by the different assays is represented by the area of each circle. PCR-based assays overestimate the LR since most of the templates amplified represent defective viruses[26]. Additionally, some proviruses are likely to be deleted in the primer binding sites for the PCR, so PCR-based assays are likely to underestimate the total number of infected cells. The viral outgrowth assay (VOA) underestimates the LR size because not every replication-competent virus is induced by a single round of T cell activation[26]. Assays involving T cell activation with a viral RNA readout give intermediate values but suffer from both of the problems mentioned above: some of the viral RNA detected is derived from defective proviruses and not all of the replication-competent proviruses are induced by a single round of activation. There is a need for assays that measure only those proviruses that pose a threat to an HIV-1 cure.

Table 2.1. Comparison of assays for measuring the HIV-1 latent reservoir

Assay	What it measures	What it excludes	Detection Method	Advantages	Disadvantages
Viral Outgrowth Assay	Replication-competent virus	Defective virus Noninduced replication-competent virus Unintegrated DNA including 2-LTR circles	p24 ELISA or RT-PCR	Allows for quantification of induced replication-competent provirus without detecting defective proviruses.	Expensive, requires a large sample volume (120-180mL), and is time consuming requiring 2-3 weeks in a BSL3 laboratory. Does not detect all proviruses that potentially pose a barrier to a cure.
T cell activation assays with viral RNA readout	Fraction of proviruses that can be induced to make either cell associated unspliced or multiply spliced HIV-1 RNA or full length HIV-1 mRNA in the culture supernatant	Some defective virus Proviruses not induced to make HIV-1 RNA after a single round of activation	RT-qPCR	No need for amplification of virus before measurement. Less culture time is required than the VOA.	Requires 2-7 days of culture in a BSL3 laboratory. Does not detect all proviruses that potentially pose a barrier to a cure. May detect some defective proviruses.
qPCR for HIV-1 DNA	Total proviral DNA including replication-competent, defective provirus, and unintegrated DNA	None	qPCR	Easy, quick, and does not require any extended culture time in a BSL3 laboratory.	Detects many proviruses that do not pose a barrier to an HIV-1 cure. Provides a quantitation relative to a standard curve, but not an absolute value. Different qPCR assays use different standards.
Alu PCR for Integrated HIV-1 DNA	Integrated proviral DNA	Unintegrated proviral DNA including 2-LTR circles and linear unintegrated HIV-1 DNA	qPCR	Is useful in measuring integrated proviruses in untreated patients, when large numbers of unintegrated DNA can confound total proviral DNA measurements.	Does not detect integrated proviruses that are too far from an Alu sequence to be detected, but uses a standard curve to correct for these. Also detects defective proviruses.
Digital droplet PCR for HIV-1 DNA	Total proviral DNA including replication-competent, defective provirus, and unintegrated DNA	None	ddPCR	Highly precise. Provides an absolute quantitation rather than a relative one.	Detects many proviruses that do not pose a barrier to an HIV-1 cure.
qPCR for HIV-1 DNA rectal biopsies	Total proviral DNA in the GALT including replication-competent, defective provirus, and unintegrated DNA	Proviruses located in sites other than the GALT	qPCR	Allows for the measurement of viral persistence in the GALT of patients.	Assay input requires cells isolated from patient rectal biopsies.
qPCR for 2-LTR circles	2-LTR circles	Integrated proviral DNA	qPCR	Is useful in measuring ongoing replication in an untreated patient. Has been used as a measure of time since infection.	Doesn't detect integrated provirus, which is the only form that can contribute to latency.
Digital droplet PCR for 2-LTR circles	2-LTR circles	Integrated proviral DNA	ddPCR	Highly precise. Provides an absolute quantitation rather than a relative one. Measures ongoing replication in an untreated patient.	Doesn't detect integrated provirus, which is the only form that can contribute to latency.
qPCR for HIV-1 DNA in the bone marrow	Total proviral DNA in CD4+ T cells located in the bone marrow including replication-competent and defective provirus	Proviruses located in sites other than the bone marrow	qPCR	Allows for the measurement of viral DNA in sites other than CD4+ T cells in the peripheral blood.	Assay input requires a large sample volume of bone marrow aspirate isolated from patients.
Single Copy Assay	Residual viremia in plasma	Proviral DNA within cells or compartments	RT-PCR	Highly precise and requires a very small sample volume (8mL of plasma).	Time consuming and doesn't provide information about proviruses within the LR.

Table 2.2. Measurements used in past, current, and future HIV-1 eradication clinical trials

Name	Clinical Trial ID	Sponsor	Category	Measures	Est. Completion
BMS-936559	NCT02018510	NIAID	anti-PD1 antibody	Plasma RNA by SCA, total DNA, 2LTR circle DNA, cell-associated RNA, RNA/DNA ratios in total CD4s	April 2016
Lisinopril	NCT01535235	UCSF	ACE inhibitor	Change in RNA in total CD4s and in GALT, and DNA in total CD4s AND GALT	August 2014
Vacc-4x + Romidepsin	NCT02092116	Bionor Immuno AS/Celgene	Vaccine + HDAC inhibitor, stopping HAART treatment	VOA, total and integrated DNA in CD4s, cell-associated unspliced RNA in CD4s, and plasma RNA	December 2015
CD4-ZETA +/- IL-2	NCT01013415	University of Pennsylvania	Modified T cells with or without IL-2	Plasma RNA by SCA, tissue RNA, frequency of latent replication-competent HIV-1 in PBMCs	December 2015
Vorinostat	NCT01365065	Bayside/Merck	HDAC inhibitor	Plasma RNA by SCA and unspliced RNA in CD4s	March 2016
Disulfiram	NCT01944371	UCSF/Monash University/amfAR	acetaldehyde dehydrogenase inhibitor	Cell-associated RNA in total and resting CD4s, plasma RNA by SCA, proviral DNA, mRNA expression	August 2014
Poly-ICLC	NCT02071095	Nina Bhardwaj, MD/Campbell Foundation/Oncovir, Inc.	TLR-3 agonist	Cell-associated RNA, plasma RNA by SCA, and proviral DNA	June 2017
Romidepsin	NCT01933594	AIDS Clinical Trials Group/NIAID/Gilead	HDAC inhibitor	Cell-associated RNA in total and resting CD4s, plasma RNA by SCA, total and 2-LTR DNA circles in resting and total CD4s	January 2015
GTU-multiHIV + LIPO-5	NCT01492985	French National Institute for Health and Medical Research-French National Agency for Research on AIDS and Viral Hepatitis (Inserm-ANRS)	DNA + lipopeptide vaccines	Plasma RNA by SCA during and after stopping treatment, and proviral DNA	January 2015
AGS-004	NCT02042248	University of North Carolina at Chapel Hill/Argos Therapeutics/U.S. National Institutes of Health (NIH)	Autologous dendritic cells and HIV strain	Plasma RNA by SCA and VOA	January 2016
New Era Study	NCT00908544	MUC Research GmbH	2 NRTI + 1 PI/r + Maraviroc + Raltegravir	Cell-associated proviral DNA in PBMCs and total CD4s, plasma RNA by SCA	November 2019
Peg-Interferon-a2b	NCT01935089	University of Pennsylvania	Cytokine	Alu-HIV gag PCR to detect change in HIV-1 DNA in total CD4s	July 2016
ULTRASTOP	NCT01876862	Objectif Recherche VACcin Sida (ORVACS)/Fondation Bettencourt Schueller	HAART treatment interruption	DNA in sorted CD4 subsets, plasma RNA by SCA, HIV-1 DNA in PBMCs and total CD4s, defective DNA sequences and presence of stop codons	December 2015

Chapter 3

Repetitive stimulation reveals stochastic reactivation of latent HIV-1 from infected CD4+ T cells including expanded cellular clones carrying replication-competent virus

Abstract

Human Immunodeficiency Virus-1 (HIV-1) establishes latency in resting memory CD4+ T cells. These latently infected cells serve as a major barrier to eradication. The standard measure of the size of this latent reservoir is the viral outgrowth assay (VOA), in which maximum activation of resting CD4+ T cells is used to reverse latency and induce viral outgrowth in samples from patients on suppressive antiretroviral therapy (ART). Previous studies have shown that the VOA underestimates the size of the latent reservoir because some cells carrying intact proviruses do not release replication-competent virus after a single round of maximal T cell activation. We show here in studies of 12 patients on suppressive ART that additional viral outgrowth can be observed after each of three additional sequential rounds of T cell activation. Repetitive stimulation yielded large numbers of independent isolates of replication-competent virus from individual patients, thereby providing a better indication of the true size of the latent reservoir. Sequencing revealed that many isolates from a given patient were identical, consistent with dramatic *in vivo* clonal expansion of infected cells carrying replication-competent virus. Identical replication-competent viruses were often obtained from different rounds of maximal T cell activation, strongly supporting the concept of stochastic reactivation of intact proviruses from clonally expanded resting CD4+ T cells in the latent reservoir.

Introduction

More than 35 million people in the world are living with Human Immunodeficiency Virus-1 (HIV-1) infection, and approximately 47,352 new infections occur every year in the United States alone (http://www.cdc.gov/hiv/statistics/basics/). The development of combination antiretroviral therapy (ART) in the mid 1990's significantly reduced mortality for those infected. However, ART alone is not curative due to the ability of HIV-1 to persist in a latent form within immune cells, specifically resting memory CD4+ T cells[1-5]. HIV-1 replicates preferentially in activated CD4+ T cells[112,113]. Resting CD4+ T cells are less permissive for HIV-1 replication due to low expression of the CCR5 coreceptor[114], low dNTP levels maintained by the restriction factor SAMHD1[115], a rigid actin cytoskeleton[116], and lack of functional forms of key activation-dependent transcriptional activators including NFκB, NFAT, and pTEFB[117-121]. HIV-1 may establish latent infection when activated CD4+ T cell are infected as it as they reverting back to a resting memory state that is non-permissive for viral gene expression[4,8,122]. Direct infection of resting cells may also occur, especially with viruses that can utilize the CXCR4 coreceptor for entry[123,124]. In resting memory CD4+ T cells, the integrated viral genome (provirus) is not transcribed, making it difficult to detect or quantify latently infected cells[125]. Because of its stability[6,7], the latent reservoir (LR) in resting memory CD4+ T cells is the major barrier to eradication of HIV-1.

The standard assay used to measure the size of the LR is the viral outgrowth assay (VOA) [3,6,22]. This assay requires the isolation and limiting dilution culture of resting memory CD4+ T cells from patients on suppressive ART in the presence of PHA (phytohemagglutinin) and irradiated healthy donor peripheral mononuclear blood cells (PBMCs). This causes maximum activation of the patient CD4+ T cells[45,126], which reverses latency and allows release of infectious virus. Healthy donor CD4+ T lymphoblasts or a transformed CD4+ T cell line (MOLT-4) [46] are added to the culture to amplify virus that is released from infected patient cells following

reversal of latency. The standard VOA is carried out for 2 weeks, and the readout for viral outgrowth is a p24 ELISA assay that detects the capsid protein present in the virion. RT-PCR can also be used to detect exponentially increasing levels of virus in the supernatant[46]. Viral outgrowth is quantified in terms of infectious units per million (IUPM) resting CD4+ T cells. The resulting IUPM value represents the frequency of latently infected cells containing readily inducible, replication-competent virus.

In multiple laboratories, the frequency of latently infected cells measured by the VOA falls in a two log range, 0.1-10 IUPM[6,7]. These measurements are based on the number of wells in which viral outgrowth is observed after one round of maximum T cell activation with PHA. Interestingly, the frequency of cells with HIV-1 DNA, as measured by PCR-based methods, is approximately 300-fold higher than the frequency of latently infected cells measured by the VOA[16]. Ho et al. demonstrated that this discrepancy is due to the presence of large numbers of proviruses that are not induced to produce replication-competent virus in the VOA[26]. The majority of these noninduced proviruses have lethal mutations, such as large internal deletions or APOBEC3G-induced hypermutation[26]. However, full-length sequencing of non-induced proviruses revealed that a small fraction of these (12%) are genetically intact with no obvious defects. When reconstructed by gene synthesis, these intact, non-induced proviruses replicate well *in vitro*. To determine whether any of these intact, non-induced proviruses could be induced, cells in wells negative for viral outgrowth were subjected to a second round of maximum T cell activation and outgrowth of additional replication-competent viruses was observed. This study revealed that one round of T cell activation does not induce all of the replication-competent proviruses in the LR. Thus, the size of the LR is larger than previously estimated. This work highlights the need to understand the extent to which these intact, noninduced proviruses pose a risk of viral rebound in patients who stop ART[107,127].

Recent work has shown that expanded clones of infected cells are present in some patients on ART[18,19,21]. These studies suggested that integration of HIV-1 into growth-promoting and cancer-associated genes might drive clonal expansion of infected cells. Although the majority of clonally expanded cells are likely to contain defective proviruses, the possibility that cells carrying replication-competent proviruses can undergo extensive proliferation could further complicate eradication efforts. In a recent study of a patient with HIV-1 infection and squamous cell carcinoma, infectious virus has been recovered from a clone of CD4+ T cells that may have proliferated in response to the cancer[20].

In this study, we have addressed the issue of the true size of LR by isolating and characterizing inducible, replication-competent viruses that are missed by the standard VOA. We developed the multiple stimulation VOA (MS-VOA) that subjects resting memory CD4+ T cells from patients on suppressive ART to four rounds of maximum T cell activation. In addition, we performed an extensive phylogenetic analysis of all replication-competent viruses that grow out in this assay. Because this MS-VOA allows the isolation of large numbers of viral clones from each patient, we were able to demonstrate in most patients the presence of independently derived viral clones with identical sequences in the highly variable *env* gene. These results are consistent with the presence of expanded cellular clones carrying inducible, replication-competent viruses in patients. Taken together, our work supports a mechanism of stochastic reactivation of intact proviruses, including clonally expanded proviruses, and provides a new approach for estimating the minimum size of the LR.

Materials and Methods

Study subjects

Peripheral blood was obtained from healthy volunteers and infected donors who had suppression of viremia to <20 copies HIV-1 RNA/ml for >6 months on ART. This study was approved by the Johns Hopkins Institutional Review Board. Written informed consent was obtained from all participants.

Multiple Stimulation Viral Outgrowth Assay (MS-VOA)

The MS-VOA was performed as described previously[22], except that 200,000 patient cells were plated in 12-well transwell plates (Corning). A pure population of resting memory CD4+ T cells was isolated using a negative selection magnetic bead depletion (Miltenyi Biotec). PHA was added at a final concentration of .5 ug/mL and half the media from each transwell was removed 2x the following day after PHA addition and replaced with fresh media. MOLT-4 cells were added to the bottom chamber of the transwell on day 1[46]. Half the volume in the transwell was split into a new transwell before receiving an additional round of PHA and was done in a similar manner for all four rounds of PHA. The supernatant from each transwell was examined for p24 capsid by ELISA (PerkinElmer) after 21 days.

CFSE staining

Staining was performed as described previously[128,129]. A 5mM CFSE stock was made by adding 18 uL of DMSO into an aliquot of CFSE. A working stock of 10uM CFSE was made by adding 2 uL of the stock into 1000 uL of PBS. The final concentration added to cells was 5 uM CFSE per 1 million cells. The dilution of CFSE was analyzed by flow cytometry using a 488 nm laser on a BD FACSCANTO II cytometer (BD Biosciences) with an emission of 492⁄517 nM.

The results were read using the BD FACSDiva™ software (BD Biosciences) and analyzed using FlowJo software.

Activation and exhaustion marker staining

A week after each round of PHA activation, cells were stained with anti-CD4 (FITC), anti-CD25 (APC), anti-CD69 (APC), and anti-HLA-DR (APC) antibodies (BioLegend, San Diego, CA) and incubated at 4 C for 15-30 minutes. The stained cells were read by a 488 nM laser on a BD FACSCANTO II cytometer (BD Biosciences). Similarly to activation marker staining, cells were stained a week after each round of PHA activation with the exhaustion markers anti-PD1 (FITC), anti-CTLA-4 (PE), and anti-TIM-3 (P3/Cy7) antibodies (BioLegend, San Diego, CA) and were incubated at 4 C for 15-30 minutes. The results were read using the BD FACSDiva™ software (BD Biosciences) and analyzed using FlowJo software.

Cell viability analysis

The Zombie Red™ Fixable Viability Kit (BioLegend, San Diego, CA) was added to cells a week after each round of PHA activation. Cells were washed in PBS and 1 uL of the Zombie Red™ dye was added to 1 million cells. The stained cells were incubated at room temperature for 15-30 minutes and then washed with PBS before being read by a 561 nM laser on a BD FACSCANTO II cytometer (BD Biosciences) with an emission of 624 nM. The results were read using the BD FACSDiva™ software (BD Biosciences) and analyzed using FlowJo software.

RNA isolation and cDNA synthesis

Viral RNA was isolated by adding 200 uL of the supernatant from each transwell in the MS-VOA and collected using a a ZR-96 Viral RNA Kit™ (Zymo Research Corporation, Irvine, CA). 4 uL of RNA from p24-positive wells were DNase-treated (Life Technologies, Carlsbad, CA) and reverse transcribed using the qScript cDNA synthesis kit (Quanta Biosciences, Gaithersburg, MD).

Amplification of e*nv* and LTR from outgrown replication-competent viruses

A nested PCR for the V3/V4 region of env was performed on the cDNA from p24-positive wells. Approximately 600ng of cDNA was used for the outer PCR reaction. The outer PCR products were diluted 1:50 and 5 uL of this dilution put into an inner PCR reaction. For *env* amplification, outer primers ES7 (CTGTTAAATGGCAGTCTAGC) and ES8 (CACTTCTCCAATTGTCCCTCA) and inner primers Nesty8 (CATACATTGCTTTTCCTACT) and DLoop (GTCTAGCAGAAGAAGAGG) were obtained from Integrated DNA Technologies (Coralville, IA). Thermocycler settings were as follows for both outer and inner *Env* PCR reactions: 94C 3 min, 94C 30 sec, 55C 30 sec, 68C (1 min), Repeat cycles 2-4 39X following by 68C for 5 min. PCR products were run on a 1% agarose gel and extracted using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Extracted DNA was then Sanger sequenced at Genewiz, Inc. (Frederick, MD). Everything was done the same for LTR, except that for LTR amplification, outer primers 5LTROut (CACACAAGGCTAYTTCCCTGA) and mod_VQA_R (TTTTTTGAGGCTTAAGCAGTGGGTTCCCTA) and inner primers 5LTRIn (ACTGTCTAGATGGATGGTGCTWCAAGYTAGT) and mod_VQA_R (same as used in outer) were obtained from Integrated DNA Technologies (Coralville, IA). Thermocycler settings were as follows for both outer and inner LTR PCR reactions: 94C 3 min, 94C 30 sec, 55C 30 sec, 68C (1 min), Repeat cycles 2-4 39X following by 68C for 5 min.

Amplification of e*nv* from proviral DNA in resting memory CD4+ T cells

The limiting dilution PCR was performed as previously described[26], except the outer PCR primers are the exact same ES7/ES8 primers used on the outgrown replication-competent virus in this study. 2 uL of the outer PCR product was added to the inner PCR reaction, which also uses the same Nesty8/DLoop primers used on the outgrown replication-competent virus. Thermocycler settings were as follows for both outer and inner *Env* PCR reactions: 94C 3 min,

94C 30 sec, 55C 30 sec, 68C (1 min), Repeat cycles 2-4 39X following by 68C for 5 min. PCR products were run on a 1% agarose gel and extracted using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Extracted DNA was then Sanger sequenced at Genewiz, Inc. (Frederick, MD).

Phylogenetic tree generation and sequence alignments

Forward and reverse sequences for each sample were aligned into one consensus contig per sample using default assembly parameters on CodonCode Aligner software (CodonCode Corporation). Each sample consensus sequence was aligned with reference sequences of catalogued viruses from the Los Alamos National Laboratory online HIV sequence database (http://hiv.lanl.gov) using default assembly parameters that were adjusted to accommodate all sample and reference sequences. For phylogenetic tree generation, sequences were trimmed to the same length. Gaps were replaced with degenerate bases for both *env* and LTR sequences and aligned using BioEdit software. Maximum likelihood trees were created using 20 reference sequences from clades B and C viral sequences along with sequences from four stimulations done in all twelve patients. Trees were generated with a maximum composite likelihood algorithm and default parameters using MEGA5 software (Molecular Evolutionary Genetics Analysis Program)[130,131] and a bootstrap analysis was performed with 1000 bootstrap samples.

Clonal Prediction Score (CPS)

The CPS is performed as described (Laskey S.B. et al. Manuscript submitted).

Results

The viral outgrowth assay can be modified to include multiple rounds of maximal T cell activation (MS-VOA)

Reservoir quantitation by the VOA is based on the assumption that a single round of maximal T cell activation is sufficient to induce all of the replication-competent proviruses present in a given cell sample. Although the conditions used in the VOA activate 100% of patient resting $CD4^+$ T cells, a recent study described the presence of large numbers of intact proviruses that are not induced in this assay, but are capable of robust *in vitro* replication when reconstructed by gene synthesis. Some of these intact, noninduced proviruses can be induced following a second round of T cell activation[26]. Based on these results, we modified the VOA to allow for four sequential rounds of T cell activation with PHA. In this multiple stimulation VOA (MS-VOA), resting $CD4^+$ T cells from patients on ART are seeded in 12-well transwell plates at 200,000 cells per well, which represents a limiting dilution for viral outgrowth for most patients. The cells are activated with PHA and irradiated allogeneic PBMCs (**Figure 3.1**). Transwell plates are used to separate patient cells from the MOLT4–CCR5 cells, which are added to expand virus released from patient cells. Previous studies have shown that viral outgrowth is not affected by separating the cell populations by a semi-permeable membrane[26]. One week following the first round of activation, half of the volume in both the top and bottom chambers of each well of the initial set of transwell plates (designated Group A) is transferred into new transwell plates (labeled Group B) that receive a second round of PHA activation. The original wells are cultured without additional activation for a total of 21 days to allow for viral outgrowth. PHA induces T cells to undergo several rounds of division, and thus daughter cells in a given clonal population should be present in both the original and newly split wells[26]. A week following the second round of PHA activation, the Group B wells are split further to produce Group C wells which receive a third round of PHA activation, and the same is done a week later to generate Group D wells

which receive the fourth, and final, round of T cell activation. The readout for viral outgrowth from each round of activation is the same: a p24 ELISA performed three weeks following each respective round of PHA activation.

Multiple rounds of maximal T cell activation induce outgrowth of additional replication-competent viruses

The MS-VOA was performed on purified resting $CD4^+$ T cells from 12 patients on long term suppressive ART who had viral loads of below 20 copies per mL at the time of sampling. An equal number of male and female patients were recruited for this study. Patient characteristics are given in **Supplemental Table 3.1**. Strikingly, in 12 of 12 patients we demonstrated additional outgrowth of replication-competent viruses with additional rounds of maximal T cell activation **(Figure 3.2).** In 11 of 12 patients, two rounds of maximal T cell activation were insufficient to induce all of the proviruses that were ultimately induced with the third and fourth rounds of T cell activation (**Figure 3.2A**). As is discussed below, the PHA stimulation protocol was chosen to ensure activation of 100% of the patient $CD4^+$ T cells present. Thus, these results indicate that replication-competent proviruses can remain latent despite multiple rounds of maximal T cell activation and then subsequently reactivate. On average across 12 patients, one round of PHA resulted in reactivation of 19.31% of wells initially seeded with 200,000 resting $CD4^+$ T cells, while the cumulative effects of four rounds of PHA stimulation resulted in viral outgrowth in cultures derived from of 30.81% of the initially seeded wells (**Figure 3.2B**). These results were used to calculate the frequency of latently infected cells detected after one round of PHA stimulation and after a total of four rounds of PHA stimulation (**Figure 3.2C**). The initial IUPM measured after one round of PHA represents the value that would be determined in the standard VOA[132] while the total IUPM represents the best estimate of the total frequency of latently infected cells. On average in 12 patients, the total IUPM was two-fold larger than the initial IUPM value. These results show that while the standard VOA gives a minimal estimate of the

size of the LR, the MS-VOA gives a larger estimate that is close to the true frequency of latently infected cells.

Analysis of cell division, activation marker expression, and exhaustion marker expression by cells in the MS-VOA

The results presented above suggest that each round of stimulation with PHA induces additional latent proviruses to produce replication-competent virus. We next examined the effects of this repetitive stimulation on the patient $CD4^+$ T cell populations harboring these proviruses (**Figure 3.3**). To determine whether the patient $CD4^+$ T cells undergo normal cell division after each round of mitogen stimulation, we stained the cells with a fluorescent dye, carboxyfluorescein succinimidyl ester (CFSE), which is diluted with every round of cell division[128,129]. We have previously used CFSE to show that 99.8% of patient $CD4^+$ T cells have undergone division one week after the addition of PHA[26]. In this study, we found that a week following the first addition of PHA, >99% of the cells had undergone division (**Figure 3.3A**). The number of cells increased an average of 8-fold during this period (**Supplementary Figure 3.1A**). After the first round of PHA stimulation, cells continued to proliferate, perhaps due to the presence of IL-2 in the media, and CFSE dilution was observed both in wells that received additional PHA stimulation and in wells that did not. In later rounds of stimulation, this proliferation was balanced by cell death as the total number of viable patient $CD4^+$ T cells in the cultures showed smaller increases after the 2^{nd}, 3^{rd}, and 4^{th} rounds of PHA stimulation (3.5-, 2.5-, and 2-fold respectively; **Supplemental Figure 3.1**).

Although cells continued to proliferate throughout the course of the experiment, each additional round of PHA stimulation caused activation of the patient $CD4^+$ T cells as evidenced by increased cell surface expression of CD25, CD69, and HLA-DR, which are expressed on activated T cells[26]. There was minimal expression of these markers on purified resting $CD4^+$ T

cells prior to the PHA stimulation (**Figure 3.3B**). One week following the first stimulation, virtually all of the cells were positive for one or more markers. The activated cell populations showed a loss of activation markers if not given additional PHA stimulation (**Figure 3.3B**). Each additional round of stimulation caused an increase in activation marker expression relative to cells that did not receive that round of stimulation (**Figure 3.3B**). Thus, additional mitogen stimulation alters host gene expression, consistent with the induction of additional latent proviruses demonstrated above. The above result seen with CFSE and activation marker staining was seen in additional patients examined (**Supplemental Figure 3.2**).

We also explored whether repetitive PHA stimulation caused T cell exhaustion[133,134], possibly accounting for the lower number of latent proviruses induced by later rounds of stimulation. To determine if the cells in the MS-VOA developed an exhaustion phenotype, we stained patient cells with antibodies to PD-1, TIM-3, and CTLA-4, which are expressed on the surface of exhausted CD4+ T cells[135-139]. We found no increased expression of PD-1 and CTLA-4, but a small increase in expression of TIM-3 after three rounds of PHA stimulation, with a further increase in TIM-3 after the fourth round of activation (**Figure 3.3C**). This result is consistent with previous work showing an increase in TIM-3 expression on CD4+ T cells that have undergone a strong stimulation via the T cell receptor[140]. In addition, cell viability decreased with each subsequent round of PHA stimulation (**Supplemental Figure 3.1**). Thus, the additional viral outgrowth obtained with multiple stimulations, while greater than that seen in the standard VOA, may represent only a minimum estimate of the true reservoir size due to the effects of increasing cell exhaustion and decreasing viability in long term *in vitro* culture.

The MS-VOA supports stochastic activation of latent proviruses

The MS-VOA allowed us to obtain large numbers of clones of replication-competent virus from most patients. To analyze the relationship between viral clones that grew out after

different numbers of PHA stimulations, we first asked whether there were any genetic differences in the U3 region of the LTR region (**Figure 3.4**). This promoter region is critical for transcription of the provirus since it contains binding sites for essential transcription factors[141,142], such as NF-kB[117] and SP1[143]. We hypothesized that mutations in the transcription factor binding sites might explain why some viruses require additional rounds of T cell activation for outgrowth. Therefore, we sequenced the U3 region of the LTR from each of the viral isolates from five patients. In phylogenetic analysis, sequences from each patient clustered separately from each other and from reference Clade B sequences and more distantly related Clade C viruses (**Figure 3.4A**). We found no significant changes in the transcription factor binding sites amongst the viruses that had grown out with different number of stimulations in each patient (**Figure 3.4B**). These results suggest that the U3 region of the LTR does not account for why some viruses are only induced after additional rounds of T cell activation.

We next hypothesized that viruses arising earlier in the course of infection may have resided in a latent state for a longer period of time, making them harder to reactivate due to progressively deeper levels of epigenetic silencing[144] To address this issue, we sequenced a 500 bp region of the *env* gene (V3/V4) from each of the outgrowth viruses from all 12 patients (**Figure 3.5A**). Sequences from each patient clustered separately from each other and from reference Clade B sequences and more distantly related Clade C viruses. Within individual patients, there was substantial diversity within the *env* sequences, consistent with the fact that most patients initiated ART during chronic infection (**Figure 3.5F and Supplemental Table 3.1**). Sequence variants arising earlier in the course of infection are expected to show a lower degree of evolutionary divergence from the ancestral sequence[145]. We examined the relationship between the distance from the most recent common ancestor (MRCA) and the number of stimulations required to induce viral outgrowth (**Figure 3.5G**). We found that there was no

relationship between divergence and the number of stimulations, further supporting the mechanism of stochastic reactivation of HIV-1 from the LR.

Expanded clones of CD4+ T cells harboring replication-competent virus are readily detected with the MS-VOA in most patients on suppressive ART

Because the MS-VOA allowed us to obtain large numbers of independent isolates of replication-competent virus from each patient, we were able to examine the issue of whether clonal expansion of infected cells contributed to the LR. Studies of HIV-1 integration sites have provided evidence for clonal expansion[18,19], but the majority of expanded cellular clones may contain defective proviruses[20,21,26]. Surprisingly, we found that despite the high level of sequence variability in the V3/V4 region of *env*, 9 of 12 patients had one or more sets of replication-competent viral isolates with identical sequences in this region (**Figure 3.5A**). For representative patients with multiple identical isolates, we also carried on limiting dilution amplification and sequencing of the V3/V4 regions of proviruses present in resting $CD4^+$ T cells isolated directly from the same patients without culture (**Figure 3.5B-D**). For patient 2, we found two sets of identical sequences among the replication-competent isolates from the MS-VOA (designated here Set 2.1 and Set 2.2) (**Figure 3.5E**). Aside from patient 2, we found sets of identical viral sequences from 8/12 other patients (**Figure 3.5F**). Together, these results suggest that expanded cellular clones with identical viral sequences are present in many patients and that some of these clones harbor replication competent virus.

A caveat to this conclusion is that viruses with identical *env* sequences may differ elsewhere in the genome and thus may not originate from an expanded cellular clone. For patients 1 through 5, we showed that isolates with identical *env* sequences also had identical LTR sequences (**Figure 3.4A**), further supporting the conclusion that they originated from an expanded cellular clone. In addition, for these isolates we determined the Clonal Prediction Score

(CPS), an estimate of how much false-positive clonality should be expected in a phylogenetic tree generated using a given primer set (**Figure 3.6A**). It is based on an analysis of available sets of full genome sequences from individual patients (Laskey S.B. et al. Manuscript submitted). In **Figure 3.6A-C**, the CPS values for the primer sets used to generate the sequences are plotted as black lines representing the slope of a plot of the number unique sequences in a phylogenetic tree vs. the number of total sequences in the tree. The dashed red lines indicate one standard deviation above or below the CPS. The phylogenetic tree for each patient is plotted as a point. Points falling far below the black line in the green-shaded region (*e.g.* the dots representing Patient 2) are more likely to represent true clonal expansion. These results show that the identical viral isolates obtained from approximately half of the patients that were sequenced for both *env* and LTR are likely to represent true clones. The proportion of clonal sequences did not increase with increasing time on ART (**Figure 3.6B**) or with the final IUPM as measured by the MS-VOA (**Figure 3.6C**). Interestingly, identical isolates, including those with a high probability of representing true clones, were often obtained after different numbers of stimulations (**Figure 3.5A-F)**, consistent with the stochastic activation of otherwise identical proviruses in the LR. Taken together, our results show that in patients on suppressive ART, there are populations of latently infected cells, including clonally expanded cells, that release replication-competent virus in a stochastic manner after maximal T cell activation and that multiple rounds of maximal activation may be required to fully purge the LR.

Discussion

Multiple Stimulation Viral Outgrowth Assay (MS-VOA) enables up to four rounds of maximal T cell activation

The latent reservoir (LR) remains the major barrier to eradication of HIV-1[146-148]. Since the LR is stable and would require suppressive ART for the lifetime of a patient in order to eliminate all latently infected cells[6,7,149], there is not only an urgent need to find therapeutic strategies to eliminate the LR, but a need for assays that can accurately measure the size of the LR[148]. While there are many PCR- and culture-based assays that are used to measure the size of the LR, none correlate with one another[16,17] and poses a problem when accurately interpreting results of eradication clinical trials. A true cure cannot be confidently assessed if a decrease in the size of the LR has not been accurately observed. In our study, we developed the MS-VOA, which is a modified viral outgrowth assay that includes up to four consecutive rounds of maximal T cell activation given at weekly intervals. We performed the MS-VOA on 6 male and 6 female patients that initiated ART during the chronic phase of infection. We observed additional outgrowth of replication-competent virus past a single round of T cell activation in all 12 patients and had 10/12 patients that required more than two rounds of activation. In addition, the IUPM after four rounds of T cell activation was shown to be twice as large versus just one round of T cell activation in the standard VOA, giving a better estimate of the frequency of latently infected cells within patients on suppressive ART. These results are consistent with previous studies showing that the size of the LR is larger than originally estimated[26] and provides a more accurate IUPM measurement.

The MS-VOA is comprised of four consecutive viral outgrowth assays with cells showing continuous division and expression of activation markers with the administration of each round of T activation. In addition, we found that expression of an exhaustion marker, TIM-3,

increases after the third round of T cell activation and increases even more in the fourth round. This increase was associated with some degree of resistance to reactivation and may provide an explanation for the reduced viral outgrowth observed with later rounds of PHA stimulation. Even with limitations inherently present with the VOA[22,132,150], the MS-VOA provides both a more accurate IUPM measurement and strongly supports a stochastic mechanism of HIV-1 reactivation from the LR.

Replication-competent viral clones are released from different rounds of maximal T cell activation

We constructed a phylogenetic tree by sequencing both the U3 region of LR as well as the V3/V4 region of the *env* gene in all outgrown replication-competent virus in the MS-VOA. We wanted to see if there was a correlation between regions of the viral genome and the round of T cell activation the virus grew out in, but found no correlation. To our surprise, we found distinct sets of matching viral sequences that share sequence similarity of the V3/V4 region of *env* in 10/12 patients. In one particular patient, all the replication-competent outgrown virus divided into two distinct sets of clones that were 100% similar by nucleotide composition. This observation supports previous work on clonal expansion of HIV-infected cells[18-21]. With our extensive phylogenetic analysis showing a set of replication-competent viral clones present in 10/12 patients, we wanted to determine how likely these identical *env* and LTR sequences represent true viral clones. We performed a Clonal Prediction Score (CPS) on these sequences and confirmed that a population of clonally expanded cells present in some patients on suppressive ART. Taken together, our results not only strongly support the stochastic reactivation of virus, but shows that stochastic reactivation can occur from clonally expanded resting CD4+ T cells in the LR.

Clonal expansion of HIV-infected resting CD4+ T cells complicates eradication efforts

Further studies will need to examine the role of clonal expansion as an even larger barrier to the cure of HIV-1[151]. Recent studies have shown HIV-1 integrates into growth-promoting genes in the human genome and suggests that this could contribute to clonal expansion of infected cells[18,19,21]. Although one of the studies suggests the presence of defective HIV-1 in clonally expanded cells, a recent longitudinal study has shown matching plasma viral sequences to integrated DNA seen in resting T cells, suggesting the contribution of replication-competent virus released from clonally expanded cells to residual viremia[152]. Furthermore, Simonetti and colleagues showed the presence of replication-competent virus from a population of clonally expanded tumor infiltrating T cells in a patient that had squamous cell carcinoma[20]. Our work not only supports the presence of clonally expanded cells containing replication-competent virus, but also further shows a stochastic mechanism of reactivation from these clonally expanded cells. Further studies will need to be done in order to identify the integration sites that the outgrown viruses in the MS-VOA. The integration site the replication-competent viruses come from would provide key insight into why some replication-competent viruses come out in one round of maximal T cell activation versus multiple rounds. If viruses that replicate from different rounds of activation arise from the same integration site in clonally expanded cells, this would even further strongly support stochastic reactivation of viruses from the LR as observed in previous studies[26] and would strongly urge the field to focus efforts on employing different strategies to eliminate the LR. In addition, it is important to elucidate whether the latency reversing agents currently studied in the field may contribute to the clonal expansion of infected cells, which would increase the size of the LR and further complicate eradication efforts. Thus, the mechanism of stochastic reactivation of replication-competent virus from clonally expanded cells in the LR needs to be further studied in order to better modify strategies to eradicate the LR.

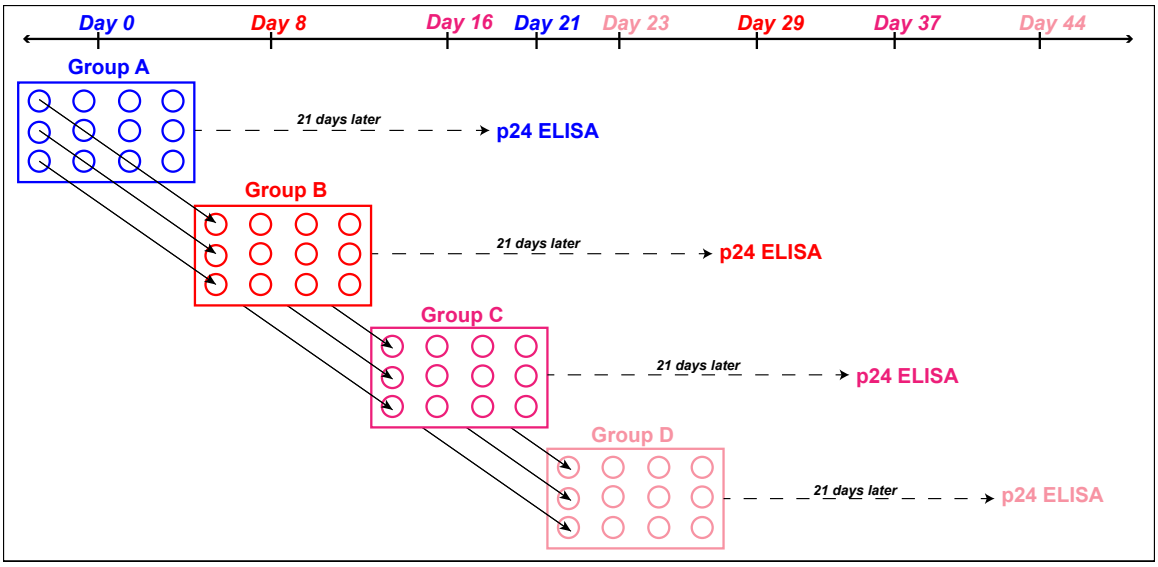


Figure 3.1. Multiple Stimulation Viral Outgrowth Assay (MS-VOA).

Purified resting CD4+ T cells from patients on suppressive ART are plated at 2 x 105 cells/well in the top chambers of 12-well transwell plates in media supplemented with growth factors and IL-2. PHA and irradiated allogeneic healthy donor PBMCs are added to each well. PHA is removed after 24 hours and 106 MOLT4-CCR5 cells are added to the bottom chamber of each transwell. The initial set of plates (Group A) are cultured for 8 days following the first round of activation, and then half of the volume from both the top and bottom chambers of each transwell is transferred to a fresh set of transwell plates (Group B) for a second round of PHA activation. The Group A plates are cultured without further activation for a total of 21 days. The group B plates are cultured for 8 days following the second round of PHA activation and then split as above to generate Group C plates, which receive a third round of activation. Similarly, these plates are split 8 days after activation to generate the Group D plates, which receive a fourth round of activation. A p24 ELISA for the capsid protein of the virion is performed 3 weeks after each respective round of PHA activation to quantify viral outgrowth.

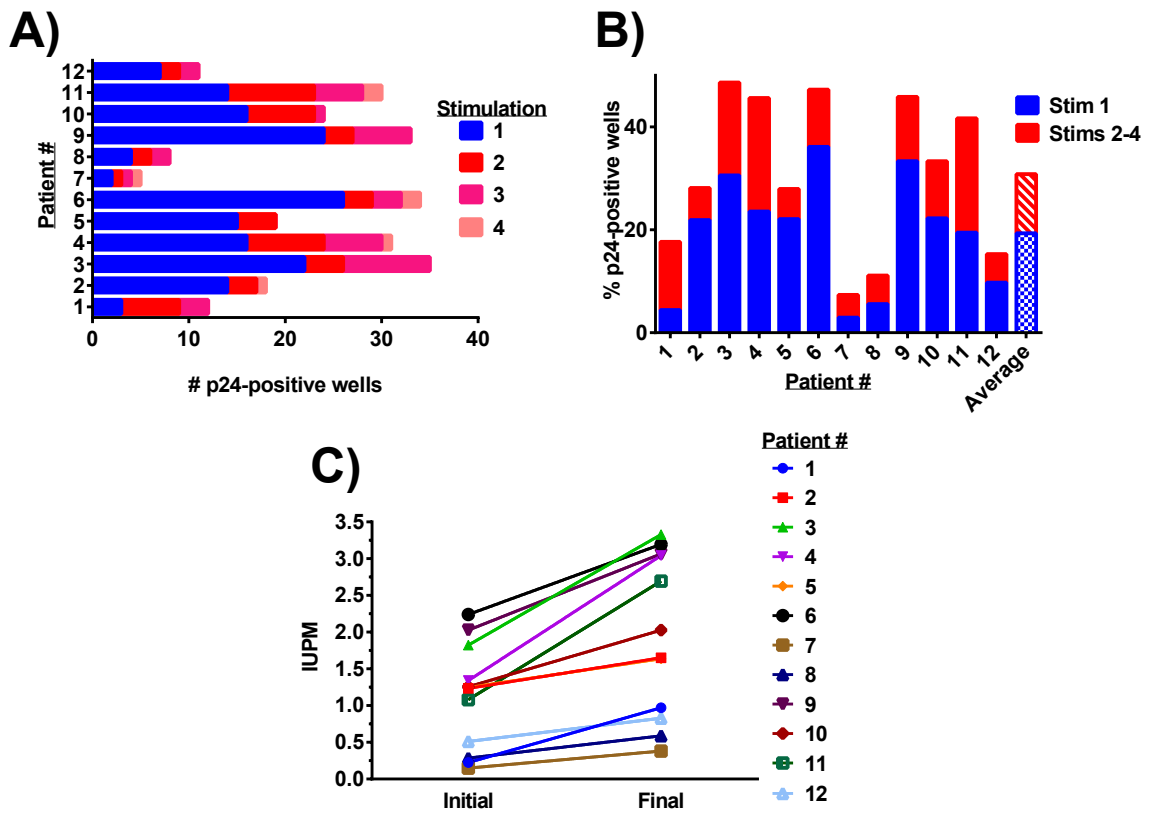


Figure 3.2. Further rounds of T cell activation in the VOA induce additional proviruses to produce replication-competent virus.

(A) The number of p24-positive wells resulting from of each round of T cell activation. For each patient, a total of 64-72 wells were initially seeded with $2x10^5$ cells/well. Cells from each well received 4 rounds of T cell activation (except for cells from Patient 1 which only received three total rounds of PHA activation). **(B)** The percent of p24-positive wells detected after the first round of T cell activation or after subsequent rounds. The denominator is the total number of wells initially seeded with 200,000 resting $CD4^+$ T cells. **(C)** The initial and final IUPM values calculated from the number of p24-positive wells after one round of activation and after a total of 4 rounds of PHA stimulation.

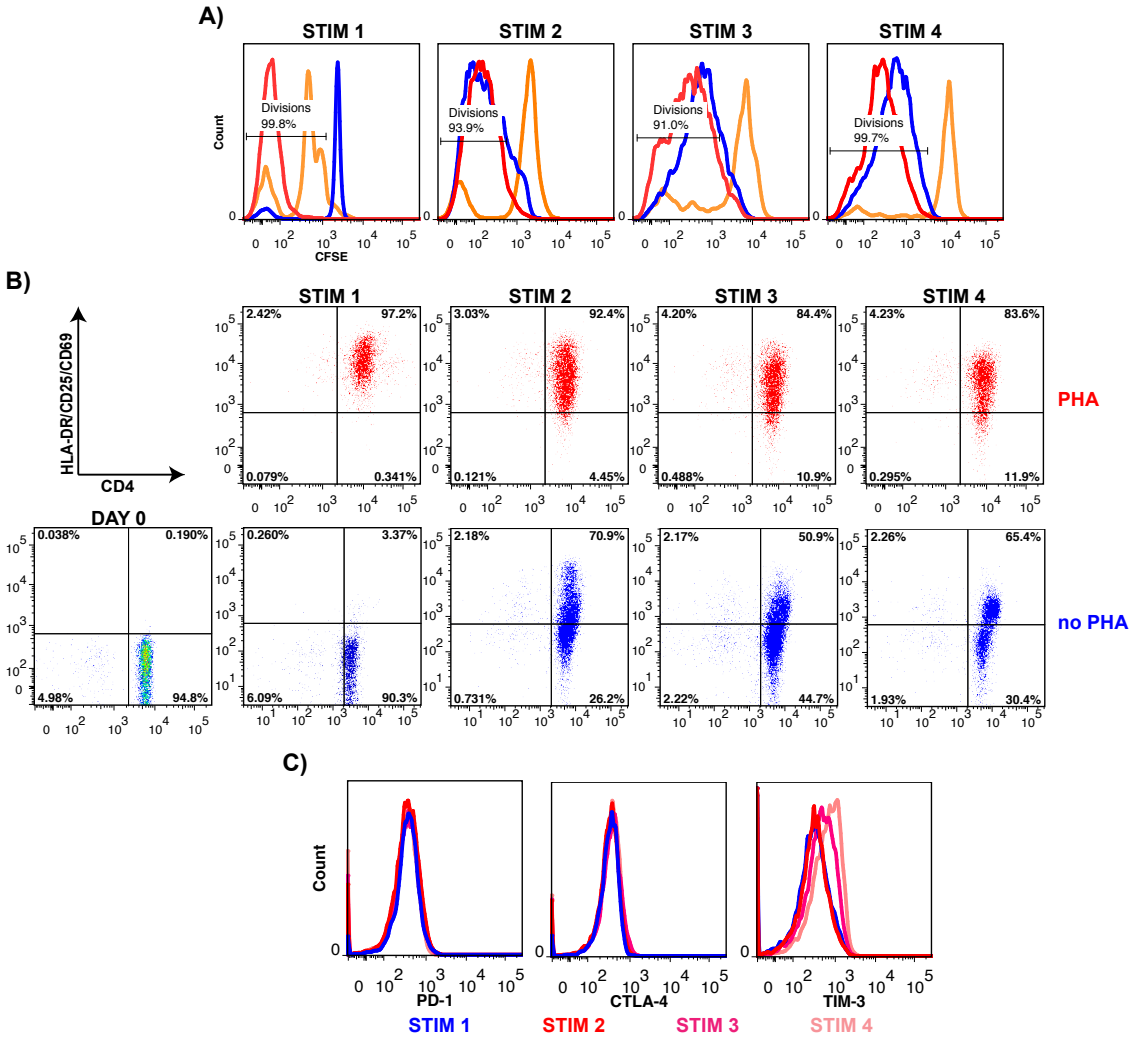


Figure 3.3. Cells divide and express activation markers after each round of activation and express TIM-3 after the third round of activation.

(A) Effect of each round of PHA stimulation on proliferation of patient CD4+ T cells. Immediately prior to PHA stimulation, patient cells were stained with CFSE. An aliquot of cells were analyzed 1 day after stimulation (orange histograms). Cells were analyzed by flow cytometry one day after addition of PHA as an internal gating control for the decrease in CFSE (orange histograms). Another aliquot of cells was analyzed 7 days activation to determine the percentage of cells that had undergone division (red histograms). Cells that did not receive the additional round of PHA stimulation served as controls (blue histograms). Results are shown for

Patient 8 and are representative of 2 other patients analyzed. **(B)** Expression of activation markers following each round of PHA stimulation. Cells were stained with antibodies to CD4 (x-axis), and the activation markers CD25, CD69, and HLA-DR (y-axis) one week after each round of PHA stimulation (red dot plots). Cells that did not receive the relevant round of PHA stimulation were analyzed in parallel (blue dot plots). Results are shown for patient 10 and are representative of 2 other patients analyzed. **(C)** Expression of exhaustion markers after each round of PHA stimulation. Cells were stained with antibodies to the exhaustion markers PD-1, CTLA-4, and TIM-3 one week after each round of T cell activation. Results are shown for patient 10 and are representative of 2 patients studied.

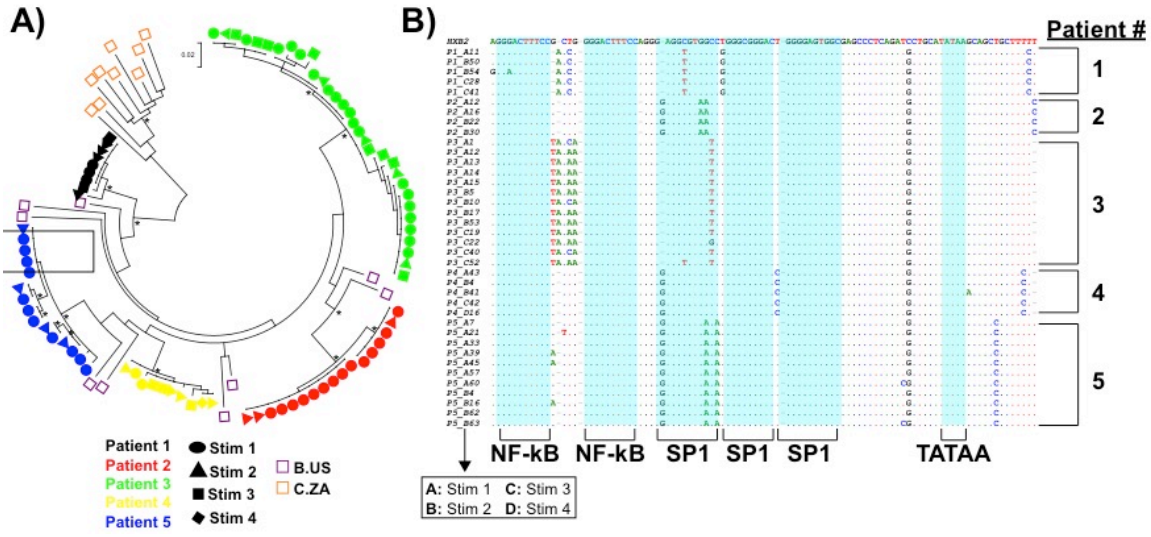


Figure 3.4. The U3 region of LTR does not account for viruses growing out in different number of stimulations.

(A) The U3 region of LTR containing the transcription factor binding sites was sequenced for all outgrown replication-competent viruses in patients 1 through 5 in the MS-VOA. The sequences were aligned in CodonCode software and a neighbor-joining phylogenetic tree of all outgrown sequences in all 12 patients was generated using the MEGA software. The orange open boxes represent consensus sequences from a Clade C virus and the purple open boxes represent consensus sequences from a Clade B virus; all consensus sequences were retrieved from the Los Alamos National Laboratory HIV website (http://hiv.lanl.gov). An asterisk (*) indicates bootstrap support ≥75%. **(B)** A representative set of viral sequences from different stimulations in each patient shown in (A) was aligned using BioEdit software. The different letters indicate which stimulation the virus grew out from: A is Stim 1, B is Stim 2, C is Stim 3, and D is Stim 4. The sites for NF-kB, SP1, and TATA box are marked in the alignment.

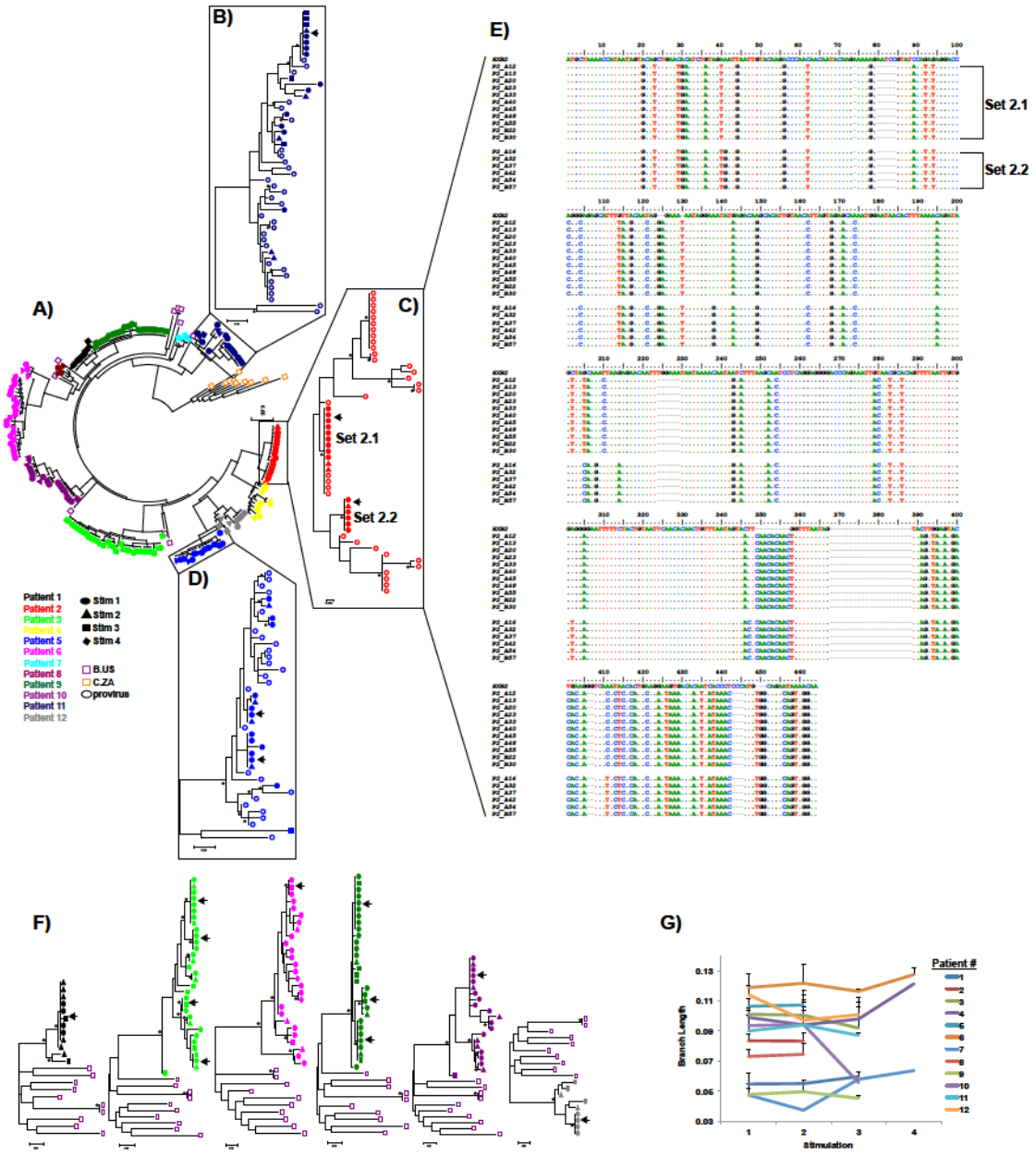


Figure 3.5. Patients on suppressive ART contain a distinct set of expanded replication-competent viral clones.

(A) The V3/V4 region of *env* was sequenced for all outgrown replication-competent viruses in all 12 patients in the MS-VOA. The sequences were aligned in CodonCode software and a neighbor-joining phylogenetic tree of all outgrown sequences in all 12 patients was generated using the MEGA 5 software. The orange open boxes represent consensus sequences from a Clade C virus

and the purple open boxes represent consensus sequences from a Clade B virus; all consensus sequences were retrieved from the Los Alamos National Laboratory HIV website (http://hiv.lanl.gov). Circles represent Stim 1 viruses, triangles represent Stim 2, squares represent Stim 3, and diamonds represent Stim 4 viruses. Arrows indicate a set of >2 outgrown virus that contain the same V3/V4 region of *env*. An asterisk (*) indicates bootstrap support ≥75%. **(B)** The V3/V4 region was sequenced on proviral DNA from resting memory CD4+ T cells isolated from Patient 11 at the time the MS-VOA was done. A limiting dilution method was done to ensure that the proviral sequences are clonal as described in [26]. The proviral sequences are depicted as open circles, while the MS-VOA outgrown replication-competent viruses are represented in shaded symbols. An asterisk (*) indicates bootstrap support ≥75%. The same limiting dilution proviral sequencing for V3/V4 was done for **(C)** Patient 2 and **(D)** Patient 5. **(E)** V3/V4 sequences from the two sets of distinct replication-competent clones from Patient 2 in (C) were aligned using BioEdit software. The different letters indicate which stimulation the virus grew out from: A is Stim 1 and B is Stim 2. **(F)** Phylogenetic trees of the V3/V4 region of *env* from left to right: Patient 1, Patient 3, Patient 6, Patient 9, Patient 10, and Patient 12. Arrows indicate a set of >2 outgrown virus that contain the same V3/V4 region of *env*. An asterisk (*) indicates bootstrap support ≥75%. **(G)** The average branch length from the most recent common ancestor (MRCA) in the phylogenetic trees for each patient shown in (A) was plotted against the stimulation that resulted in outgrowth of replication-competent virus in each culture well.

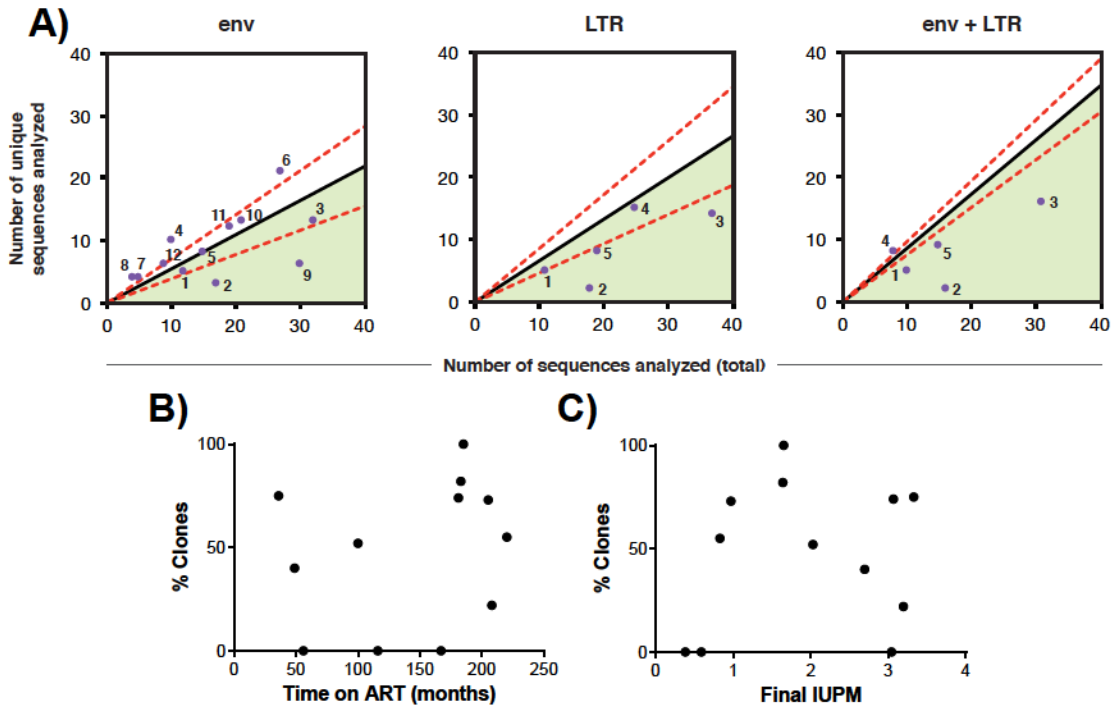
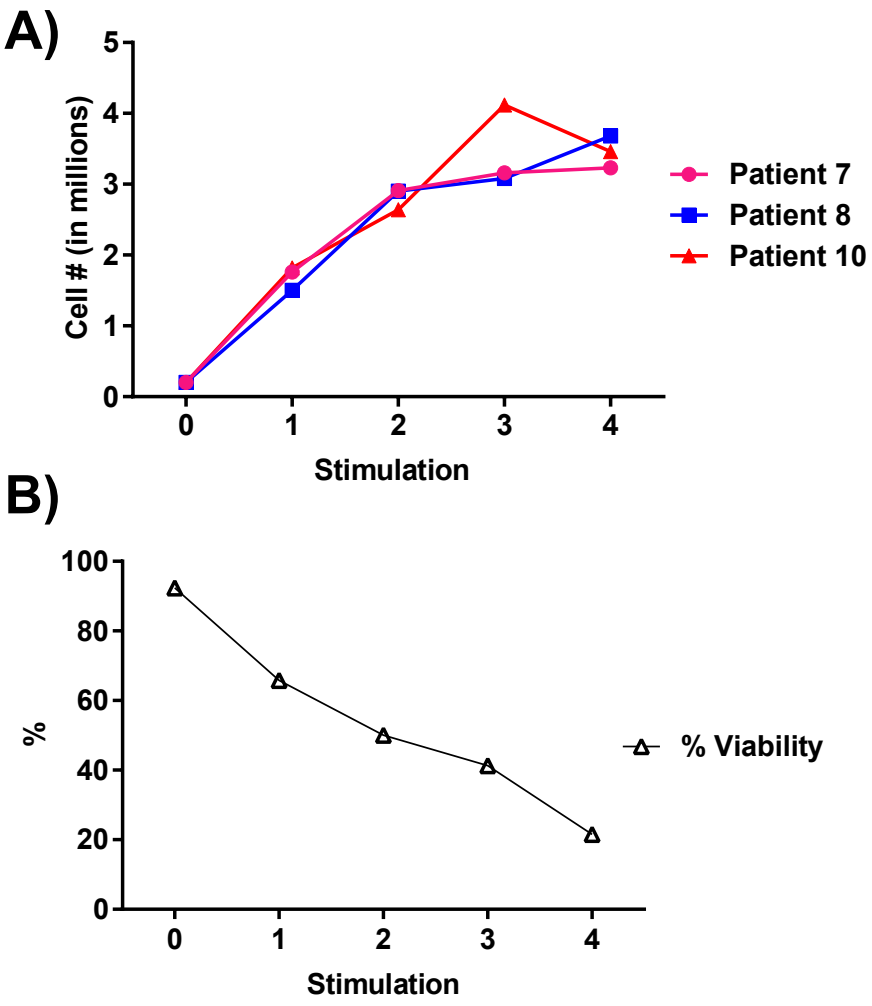


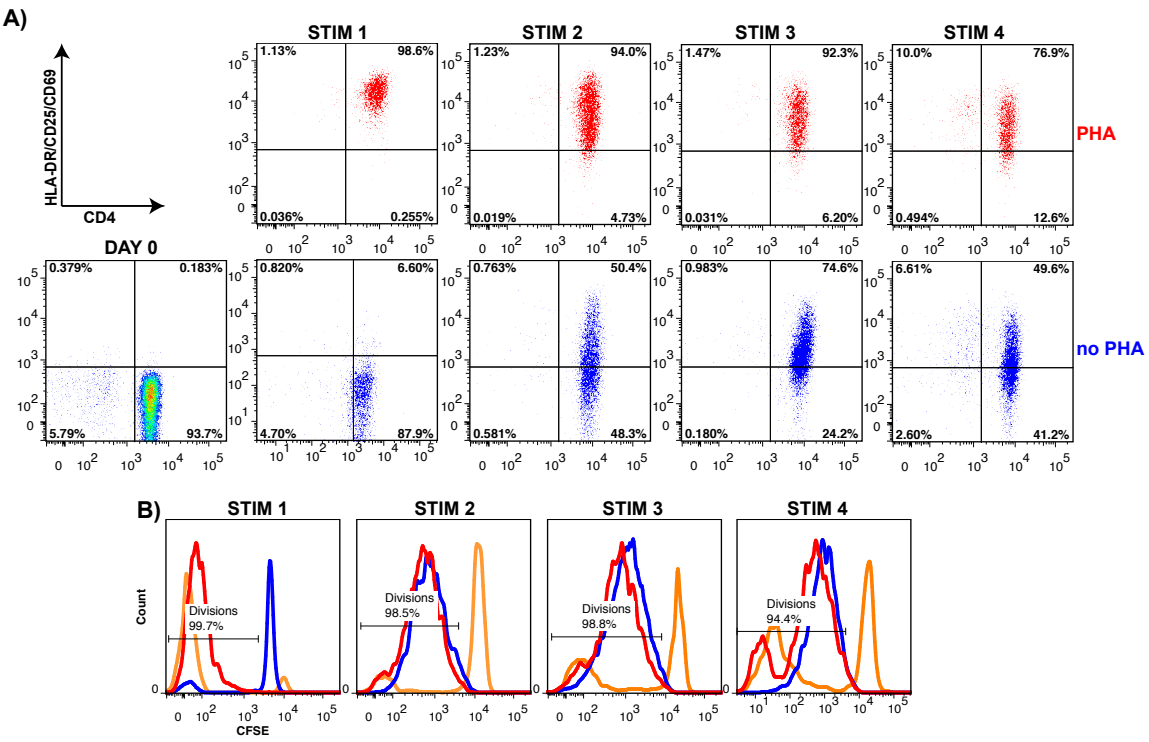
Figure 3.6. Quantitative analysis of clonally expanded replication-competent viral clones.

(A) A quantitative analysis of clonal expansion was performed using the Clonal Prediction Score (Laskey S.B. et al. Manuscript submitted). The alignments used to generate the *env* phylogenetic trees in Figure 3.5A, LTR phylogenetic tree in Figure 3.4A, and a combination of the two are plotted as dots, indicating the relationship between the total number of sequences and the number of unique sequences in each alignment. The black line shows the expected false-positive clonality rate for the amplicons used to generate these alignments. Points that fall on or near the black line are likely to represent samples without clonally expanded genomes. Points that fall far below the black line (green shaded area) indicate patients with a high likelihood of true clonality. **(B)** The time the patient was on ART until the time of blood draw was plotted against the percent of replication-competent clones present after the MS-VOA; the coefficient of determination was calculated ($R^2 = 0.07106$). **(C)** The change from initial to final IUPM as calculated by the MS-VOA was plotted against the percent of replication-competent clones present after the MS-VOA; the coefficient of determination was calculated ($R^2 = 0.01874$).



Supplemental Figure 3.1. Analysis of CD4$^+$ T cell proliferation and cell death in MS-VOA cultures.

(A) Total CD4$^+$ T cell numbers in MS-VOA wells. The total number of cells in individual MS-VOA transwells were counted one week after each PHA stimulation. By this point, the lethally irradiated allogeneic PBMC used in the PHA stimulation had all died. Therefore, cell counts reflect the number of patient CD4$^+$ T cells remaining in the culture. The input value for each well was $2x10^5$ cells. Total cell numbers were corrected for the effect of splitting the wells during each PHA stimulation. **(B)** Cell viability assessed using an amine-reactive fluorescent dye to which viable cells are impermeable.



Supplemental Figure 3.2. Additional patients show cells divide and express activation markers after each round of activation.

(A) Expression of activation markers following each round of PHA stimulation. Cells were stained with antibodies to CD4 (x-axis), and the activation markers CD25, CD69, and HLA-DR (y-axis) one week after each round of PHA stimulation (red dot plots). Cells that did not receive the relevant round of PHA stimulation were analyzed in parallel (blue dot plots). Results are shown for patient 11. **(B)** Results shown for patient 10. Effect of each round of PHA stimulation on proliferation of patient $CD4^+$ T cells. Immediately prior to PHA stimulation, patient cells were stained with CFSE. One week later, dilution of CFSE was analyzed by flow cytometry and used to determine the percentage of cells that had undergone division (red histograms). Cells were sampled a day after addition of PHA as an internal gating control for the decrease in CFSE (orange histograms). Cells that did not receive an additional round of PHA stimulation served as controls (blue histograms).

Supplemental Table 3.1. Patient characteristics.

Patient	Sex	Duration of Infection (months)[1]	Time on ART (months)	CD4 count (cells/ul)[2]	Viral load (c/ml)[3]	ART at time of draw[4]
1	M	205	205	682	<20	ABC/3TC, DRV/r
2	F	190	185	1,115	<20	DRV/r, ETR, RAL
3	F	116	36	624	<20	ATV/r, FTC/TDF
4	F	133	116	450	<20	DRV/r, MCV, RAL
5	F	184	183	800	<20	ABC/3TC, NVP
6	M	250	208	1,478	<20	EFV/FTC/TDF
7	M	192	167	706	<20	FTC/RPV/TDF, DTG
8	M	350	56	865	<20	EFV/FTC/TDF
9	M	278	181	1,156	<20	ABC, ETR, 3TC, RAL
10	F	208	100	771	<20	FTC, LPV/r, RAL
11	M	101	49	962	<20	TDF/FTC, DTG
12	F	304	220	980	<20	RAL, DRV/r,MVC

[1] Time of initial diagnosis
[2] Time at sampling
[3] Time at sampling
[4] **ABC/3TC** = abacavir sulfate/lamivudine; **DRV/r** = darunavir/ritonavir; **ETR** = etravirine; **RAL** = raltegravir; **ATV/r** = atazanavir/ritonavir; **FTC/TDF** = tenofovir/emtricitabine; **MVC** = maraviroc; **NVP** = nevirapine; **EFV** = efavirenz; **DTG** = dolutegravir; **LPV/r** = lopinavir/ritonavir

Chapter 4

Significance and future directions

The importance of accurately measuring the HIV-1 latent reservoir

Latently infected resting CD4+ T cells are not targeted by the immune system or ART, making the latent reservoir (LR) the major barrier to cure. In addition, the LR is extremely stable and would take the entire lifetime of a patient to eliminate[6,7]. Current eradication clinical trials aim to reverse latency by using pharmacological agents that employ the 'shock and kill' strategy, through which latency is reversed such that now the infected cell can then recognized and 'killed' by the immune system[15]. Even if the field finds promising pharmacological agents and other therapeutic interventions to reverse latency[15,32,148], there needs to be an assay that can accurately detect a decrease in the LR in patients. To date, PCR- and culture-based assays are used to measure the LR[17], but don't correlate with one another[16,153]. PCR-based assays overestimate the size of the LR and fail to distinguish between replication-competent and defective proviruses. Culture-based assays that use a single round of maximum T cell activation do not activate all latently infected cells with replication-competent viruses[17,26]. For these reasons, it is critical to develop an assay that can accurately measure the size of the LR.

The MS-VOA provides a more accurate measure of the size of the latent reservoir

Not all proviruses in the LR are induced by a single round of maximal T cell activation. Previous work has shown additional viral outgrowth after a second round of maximal T cell activation[26]. My graduate thesis work has aimed at taking these previous findings further to develop and modify the quantitative viral outgrowth assay (QVOA) to include multiple stimulations (MS-VOA), specifically four rounds of maximal T cell activation. The MS-VOA was performed on resting cells isolated from 12 patients on suppressive ART treated during the chronic phase of infection. Results from my work showed that two rounds of maximal T cell activation were insufficient to induce all replication-competent viruses in 11/12 patients. In addition, the frequency of latently infected cells, as measured by IUPM (infectious units per

million), was two-fold larger than the initial IUPM value calculated after the first round of activation. The development of the MS-VOA has been important in advancing the field and may assist in interpreting eradication clinical trials since it gives a larger estimate may be closer to the true frequency of latently infected cells *in vivo*.

Clonal expansion of latently infected cells may add an additional barrier to curing HIV-1

In addition to providing a more accurate measurement of the size of the LR, the MS-VOA revealed the presence of large numbers of clones of replication-competent virus from most patients. Sequencing of the V3/V4 region of the *env* gene in all outgrown virus revealed that 9/12 patients had one or more identical sets of replication-competent viral isolates from different rounds of activation. This result is significant in light of recent work that has shown the presence of clonally expanded resting CD4+ T cells[18,19] and how the majority of these expanded cells contain defective proviruses[21]. However, a very recent study has shown the presence of replication-competent virus in clonally expanded cells in a single patient that had squamous cell carcinoma[20]. My work supports the presence of clonally expanded cells harboring latent HIV-1 and also shows how these expanded cells can release replication-competent in a stochastic manner. This work is significant in advancing the field since it not only highlights the presence of clonally expanded latently infected cells, but provides evidence of these cells harboring replication-competent viruses that can be released in stochastic manner after maximal T cell activation. This suggests the possibility of the LR increasing in size due to the presence of these clonally expanded cells and, thus, would add an additional barrier to eradication of HIV-1.

Future work will need to thoroughly determine the contribution of these clonally expanded cells to the size of the LR. In addition, it will be important to analyze where HIV-1 integrates in clonally expanded cells. Knowing if certain integration sites are more likely to contain inducible proviruses would enable better strategies to specifically target the replication-

competent viruses in these clonally expanded cells. For example, an elegant study has shown that specific integration sites in MKL2 and BACH2 lead to clonal expansion of infected cells[18]. However, it has not been shown if proviruses integrated into either MKL2 or BACH2 are replication-competent. Further studies will need to be done to identify the integration sites of the outgrowth viruses in the MS-VOA to determine whether this can account for stochastic activation of latently infected, clonally expanded CD4+ T cells.

The development of the MS-VOA was initially developed to serve as a more accurate measure of the LR, but in addition has revealed populations of latently infected cells, including clonally expanded cells, that release replication-competent virus in a stochastic manner after multiple rounds of maximal T cell activation. This work strongly suggests that multiple rounds of maximal activation may be required to fully purge the LR. The work presented in this thesis highlights the need for the development of additional assays that accurately measure the size of the LR, but also readdresses current strategies to eliminate the LR, which should include targeting of clonally expanded cells.

References

1. Chun TW, Carruth L, Finzi D, et al. Quantification of latent tissue reservoirs and total body viral load in HIV-1 infection. *Nature*. 1997;387(6629):183-188. doi:10.1038/387183a0.

2. Wong JK, Hezareh M, Günthard HF, et al. Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. 1997;278(5341):1291-1295.

3. Finzi D, Blankson J, Siliciano JD, et al. Latent infection of CD4+ T cells provides a mechanism for lifelong persistence of HIV-1, even in patients on effective combination therapy. *Nat Med*. 1999;5(5):512-517. doi:10.1038/8394.

4. Chun TW, Finzi D, Margolick J, Chadwick K, Schwartz D, Siliciano RF. In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. *Nat Med*. 1995;1(12):1284-1290.

5. Chun TW, Stuyver L, Mizell SB, et al. Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. *Proc Natl Acad Sci USA*. 1997;94(24):13193-13197.

6. Crooks AM, Bateson R, Cope AB, et al. Precise Quantitation of the Latent HIV-1 Reservoir: Implications for Eradication Strategies. *J Infect Dis*. 2015;212(9):1361-1365. doi:10.1093/infdis/jiv218.

7. Siliciano JD, Kajdas J, Finzi D, et al. Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. *Nat Med*. 2003;9(6):727-728. doi:10.1038/nm880.

8. Siliciano RF, Greene WC. HIV latency. *Cold Spring Harb Perspect Med*. 2011;1(1):a007096-a007096. doi:10.1101/cshperspect.a007096.

9. Durand CM, Blankson JN, Siliciano RF. Developing strategies for HIV-1 eradication. *Trends Immunol*. 2012;33(11):554-562. doi:10.1016/j.it.2012.07.001.

10. Brenchley JM, Hill BJ, Ambrozak DR, et al. T-cell subsets that harbor human immunodeficiency virus (HIV) in vivo: implications for HIV pathogenesis. *J Virol*. 2004;78(3):1160-1168. doi:10.1128/JVI.78.3.1160-1168.2004.

11. Chomont N, El-Far M, Ancuta P, et al. HIV reservoir size and persistence are driven by T cell survival and homeostatic proliferation. *Nat Med*. 2009;15(8):893-900. doi:10.1038/nm.1972.

12. Buzon MJ, Sun H, Li C, et al. HIV-1 persistence in CD4+ T cells with stem cell-like properties. *Nat Med*. 2014;20(2):139-142. doi:10.1038/nm.3445.

13. Ruelas DS, Greene WC. An integrated overview of HIV-1 latency. *Cell*. 2013;155(3):519-529. doi:10.1016/j.cell.2013.09.044.

14. Hütter G, Nowak D, Mossner M, et al. Long-term control of HIV by CCR5 Delta32/Delta32 stem-cell transplantation. *N Engl J Med*. 2009;360(7):692-698.

doi:10.1056/NEJMoa0802905.

15. Deeks SG. HIV: Shock and kill. *Nature*. 2012;487(7408):439-440. doi:10.1038/487439a.

16. Eriksson S, Graf EH, Dahl V, et al. Comparative analysis of measures of viral reservoirs in HIV-1 eradication studies. Douek DC, ed. *PLoS Pathog*. 2013;9(2):e1003174. doi:10.1371/journal.ppat.1003174.

17. Bruner KM, Hosmane NN, Siliciano RF. Towards an HIV-1 cure: measuring the latent reservoir. *Trends Microbiol*. 2015;23(4):192-203. doi:10.1016/j.tim.2015.01.013.

18. Maldarelli F, Wu X, Su L, et al. HIV latency. Specific HIV integration sites are linked to clonal expansion and persistence of infected cells. 2014;345(6193):179-183. doi:10.1126/science.1254194.

19. Wagner TA, McLaughlin S, Garg K, et al. HIV latency. Proliferation of cells with HIV integrated into cancer genes contributes to persistent infection. 2014;345(6196):570-573. doi:10.1126/science.1256304.

20. Simonetti FR, Sobolewski MD, Fyne E, et al. Clonally expanded CD4+ T cells can produce infectious HIV-1 in vivo. *Proc Natl Acad Sci USA*. February 2016:201522675. doi:10.1073/pnas.1522675113.

21. Cohn LB, Silva IT, Oliveira TY, et al. HIV-1 integration landscape during latent and active infection. *Cell*. 2015;160(3):420-432. doi:10.1016/j.cell.2015.01.020.

22. Siliciano JD, Siliciano RF. Enhanced culture assay for detection and quantitation of latently infected, resting CD4+ T-cells carrying replication-competent virus in HIV-1-infected individuals. *Methods Mol Biol*. 2005;304:3-15. doi:10.1385/1-59259-907-9:003.

23. Rouzioux C, Mélard A, Avéttand-Fénoël V. Quantification of total HIV1-DNA in peripheral blood mononuclear cells. *Methods Mol Biol*. 2014;1087(Chapter 21):261-270. doi:10.1007/978-1-62703-670-2_21.

24. O'Doherty U, Swiggard WJ, Jeyakumar D, McGain D, Malim MH. A sensitive, quantitative assay for human immunodeficiency virus type 1 integration. *J Virol*. 2002;76(21):10942-10950. doi:10.1128/JVI.76.21.10942-10950.2002.

25. Strain MC, Lada SM, Luong T, et al. Highly precise measurement of HIV DNA by droplet digital PCR. Wu Y, ed. *PLoS ONE*. 2013;8(4):e55943. doi:10.1371/journal.pone.0055943.

26. Ho Y-C, Shan L, Hosmane NN, et al. Replication-competent noninduced proviruses in the latent reservoir increase barrier to HIV-1 cure. *Cell*. 2013;155(3):540-551. doi:10.1016/j.cell.2013.09.020.

27. Sanchez G, Xu X, Chermann JC, Hirsch I. Accumulation of defective viral genomes in peripheral blood mononuclear cells of human immunodeficiency virus type 1-infected individuals. *J Virol*. 1997;71(3):2233-2240.

28. Simon-Loriere E, Holmes EC. Why do RNA viruses recombine? *Nat Rev Microbiol*. 2011;9(8):617-626. doi:10.1038/nrmicro2614.

29. Yu Q, König R, Pillai S, et al. Single-strand specificity of APOBEC3G accounts for minus-strand deamination of the HIV genome. *Nat Struct Mol Biol*. 2004;11(5):435-442. doi:10.1038/nsmb758.

30. Kieffer TL, Kwon P, Nettles RE, Han Y, Ray SC, Siliciano RF. G-->A hypermutation in protease and reverse transcriptase regions of human immunodeficiency virus type 1 residing in resting CD4+ T cells in vivo. *J Virol*. 2005;79(3):1975-1980. doi:10.1128/JVI.79.3.1975-1980.2005.

31. Stopak K, de Noronha C, Yonemoto W, Greene WC. HIV-1 Vif blocks the antiviral activity of APOBEC3G by impairing both its translation and intracellular stability. *Mol Cell*. 2003;12(3):591-601. doi:10.1038/nri1236.

32. Archin NM, Margolis DM. Emerging strategies to deplete the HIV reservoir. *Curr Opin Infect Dis*. 2014;27(1):29-35. doi:10.1097/QCO.0000000000000026.

33. Margolis DM. How Might We Cure HIV? *Curr Infect Dis Rep*. 2014;16(3):392-395. doi:10.1007/s11908-014-0392-2.

34. Wren LH, Stratov I, Kent SJ, Parsons MS. Obstacles to ideal anti-HIV antibody-dependent cellular cytotoxicity responses. *Vaccine*. 2013;31(47):5506-5517. doi:10.1016/j.vaccine.2013.08.035.

35. Archin NM, Liberty AL, Kashuba AD, et al. Administration of vorinostat disrupts HIV-1 latency in patients on antiretroviral therapy. *Nature*. 2012;487(7408):482-485. doi:10.1038/nature11286.

36. Shirakawa K, Chavez L, Hakre S, Calvanese V, Verdin E. Reactivation of latent HIV by histone deacetylase inhibitors. *Trends Microbiol*. 2013;21(6):277-285. doi:10.1016/j.tim.2013.02.005.

37. Ylisastigui L, Archin NM, Lehrman G, Bosch RJ, Margolis DM. Coaxing HIV-1 from resting CD4 T cells: histone deacetylase inhibition allows latent viral expression. *AIDS*. 2004;18(8):1101-1108.

38. Archin NM, Bateson R, Tripathy MK, et al. HIV-1 expression within resting CD4+ T cells after multiple doses of vorinostat. *J Infect Dis*. 2014;210(5):728-735. doi:10.1093/infdis/jiu155.

39. Archin NM, Espeseth A, Parker D, Cheema M, Hazuda D, Margolis DM. Expression of latent HIV induced by the potent HDAC inhibitor suberoylanilide hydroxamic acid. *AIDS Res Hum Retroviruses*. 2009;25(2):207-212. doi:10.1089/aid.2008.0191.

40. Wei DG, Chiang V, Fyne E, et al. Histone deacetylase inhibitor romidepsin induces HIV expression in CD4 T cells from patients on suppressive antiretroviral therapy at concentrations achieved by clinical dosing. Desrosiers RC, ed. *PLoS Pathog*. 2014;10(4):e1004071. doi:10.1371/journal.ppat.1004071.

41. Rasmussen TA, Tolstrup M, Brinkmann CR, et al. Panobinostat, a histone deacetylase inhibitor, for latent-virus reactivation in HIV-infected patients on suppressive antiretroviral therapy: a phase 1/2, single group, clinical trial. *Lancet HIV*. 2014;1(1):e13-e21. doi:10.1016/S2352-3018(14)70014-1.

42. Bullen CK, Laird GM, Durand CM, Siliciano JD, Siliciano RF. New ex vivo approaches distinguish effective and ineffective single agents for reversing HIV-1 latency in vivo. *Nat Med*. 2014;20(4):425-429. doi:10.1038/nm.3489.

43. Cillo AR, Sobolewski MD, Bosch RJ, et al. Quantification of HIV-1 latency reversal in resting CD4+ T cells from patients on suppressive antiretroviral therapy. *Proc Natl Acad Sci USA*. 2014;111(19):7078-7083. doi:10.1073/pnas.1402873111.

44. Blazkova J, Chun T-W, Belay BW, et al. Effect of histone deacetylase inhibitors on HIV production in latently infected, resting CD4(+) T cells from infected individuals receiving effective antiretroviral therapy. *J Infect Dis*. 2012;206(5):765-769. doi:10.1093/infdis/jis412.

45. Hermankova M, Siliciano JD, Zhou Y, et al. Analysis of human immunodeficiency virus type 1 gene expression in latently infected resting CD4+ T lymphocytes in vivo. *J Virol*. 2003;77(13):7383-7392.

46. Laird GM, Eisele EE, Rabi SA, et al. Rapid quantification of the latent reservoir for HIV-1 using a viral outgrowth assay. Silvestri G, ed. *PLoS Pathog*. 2013;9(5):e1003398. doi:10.1371/journal.ppat.1003398.

47. Han Y, Lassen K, Monie D, et al. Resting CD4+ T cells from human immunodeficiency virus type 1 (HIV-1)-infected individuals carry integrated HIV-1 genomes within actively transcribed host genes. *J Virol*. 2004;78(12):6122-6133. doi:10.1128/JVI.78.12.6122-6133.2004.

48. Besson GJ, McMahon D, Maldarelli F, Mellors JW. Short-course raltegravir intensification does not increase 2 long terminal repeat episomal HIV-1 DNA in patients on effective antiretroviral therapy. *Clin Infect Dis*. 2012;54(3):451-453. doi:10.1093/cid/cir721.

49. Zhu W, Jiao Y, Lei R, et al. Rapid turnover of 2-LTR HIV-1 DNA during early stage of highly active antiretroviral therapy. Jiang S, ed. *PLoS ONE*. 2011;6(6):e21081. doi:10.1371/journal.pone.0021081.

50. Hatano H, Delwart EL, Norris PJ, et al. Evidence for persistent low-level viremia in individuals who control human immunodeficiency virus in the absence of antiretroviral therapy. *J Virol*. 2009;83(1):329-335. doi:10.1128/JVI.01763-08.

51. Gandhi RT, Coombs RW, Chan ES, et al. No effect of raltegravir intensification on viral replication markers in the blood of HIV-1-infected patients receiving antiretroviral therapy. *J Acquir Immune Defic Syndr*. 2012;59(3):229-235. doi:10.1097/QAI.0b013e31823fd1f2.

52. Cillo AR, Krishnan A, Mitsuyasu RT, et al. Plasma viremia and cellular HIV-1 DNA persist despite autologous hematopoietic stem cell transplantation for HIV-related

lymphoma. *J Acquir Immune Defic Syndr*. 2013;63(4):438-441. doi:10.1097/QAI.0b013e31828e6163.

53. Chun T-W, Murray D, Justement JS, et al. Relationship between residual plasma viremia and the size of HIV proviral DNA reservoirs in infected individuals receiving effective antiretroviral therapy. *J Infect Dis*. 2011;204(1):135-138. doi:10.1093/infdis/jir208.

54. Markowitz M, Evering TH, Garmon D, et al. A randomized open-label study of 3- versus 5-drug combination antiretroviral therapy in newly HIV-1-infected individuals. *J Acquir Immune Defic Syndr*. 2014;66(2):140-147. doi:10.1097/QAI.0000000000000111.

55. McBride K, Xu Y, Bailey M, et al. The majority of HIV type 1 DNA in circulating CD4+ T lymphocytes is present in non-gut-homing resting memory CD4+ T cells. *AIDS Res Hum Retroviruses*. 2013;29(10):1330-1339. doi:10.1089/AID.2012.0351.

56. Chun T-W, Nickle DC, Justement JS, et al. Persistence of HIV in gut-associated lymphoid tissue despite long-term antiretroviral therapy. *J Infect Dis*. 2008;197(5):714-720. doi:10.1086/527324.

57. Yukl SA, Gianella S, Sinclair E, et al. Differences in HIV burden and immune activation within the gut of HIV-positive patients receiving suppressive antiretroviral therapy. *J Infect Dis*. 2010;202(10):1553-1561. doi:10.1086/656722.

58. Hatano H, Somsouk M, Sinclair E, et al. Comparison of HIV DNA and RNA in gut-associated lymphoid tissue of HIV-infected controllers and noncontrollers. *AIDS*. 2013;27(14):2255-2260. doi:10.1097/QAD.0b013e328362692f.

59. Durand CM, Ghiaur G, Siliciano JD, et al. 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. *J Infect Dis*. 2012;205(6):1014-1018. doi:10.1093/infdis/jir884.

60. Tebas P, Stein D, Tang WW, et al. Gene editing of CCR5 in autologous CD4 T cells of persons infected with HIV. *N Engl J Med*. 2014;370(10):901-910. doi:10.1056/NEJMoa1300662.

61. Henrich TJ, Gallien S, Li JZ, Pereyra F, Kuritzkes DR. Low-level detection and quantitation of cellular HIV-1 DNA and 2-LTR circles using droplet digital PCR. *J Virol Methods*. 2012;186(1-2):68-72. doi:10.1016/j.jviromet.2012.08.019.

62. Stevenson M, Haggerty S, Lamonica CA, Meier CM, Welch SK, Wasiak AJ. Integration is not necessary for expression of human immunodeficiency virus type 1 protein products. *J Virol*. 1990;64(5):2421-2425.

63. Zack JA, Arrigo SJ, Weitsman SR, Go AS, Haislip A, Chen IS. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell*. 1990;61(2):213-222.

64. Pierson TC, Zhou Y, Kieffer TL, Ruff CT, Buck C, Siliciano RF. Molecular characterization of preintegration latency in human immunodeficiency virus type 1 infection. *J Virol*. 2002;76(17):8518-8531. doi:10.1128/JVI.76.17.8518-8531.2002.

65. Blankson JN, Finzi D, Pierson TC, et al. Biphasic decay of latently infected CD4+ T cells in acute human immunodeficiency virus type 1 infection. *J Infect Dis*. 2000;182(6):1636-1642. doi:10.1086/317615.

66. Brady T, Kelly BJ, Male F, et al. Quantitation of HIV DNA integration: effects of differential integration site distributions on Alu-PCR assays. *J Virol Methods*. 2013;189(1):53-57. doi:10.1016/j.jviromet.2013.01.004.

67. Liszewski MK, Yu JJ, O'Doherty U. Detecting HIV-1 integration by repetitive-sampling Alu-gag PCR. *Methods*. 2009;47(4):254-260. doi:10.1016/j.ymeth.2009.01.002.

68. Mexas AM, Graf EH, Pace MJ, et al. Concurrent measures of total and integrated HIV DNA monitor reservoirs and ongoing replication in eradication trials. *AIDS*. 2012;26(18):2295-2306. doi:10.1097/QAD.0b013e32835a5c2f.

69. De Spiegelaere W, Malatinkova E, Lynch L, et al. Quantification of integrated HIV DNA by repetitive-sampling Alu-HIV PCR on the basis of poisson statistics. *Clin Chem*. 2014;60(6):886-895. doi:10.1373/clinchem.2013.219378.

70. Vandegraaff N, Kumar R, Burrell CJ, Li P. Kinetics of human immunodeficiency virus type 1 (HIV) DNA integration in acutely infected cells as determined using a novel assay for detection of integrated HIV DNA. *J Virol*. 2001;75(22):11253-11260. doi:10.1128/JVI.75.22.11253-11260.2001.

71. Li L, Olvera JM, Yoder KE, et al. Role of the non-homologous DNA end joining pathway in the early steps of retroviral infection. *EMBO J*. 2001;20(12):3272-3281. doi:10.1093/emboj/20.12.3272.

72. Farnet CM, Haseltine WA. Circularization of human immunodeficiency virus type 1 DNA in vitro. *J Virol*. 1991;65(12):6942-6952.

73. Pace MJ, Graf EH, O'Doherty U. HIV 2-long terminal repeat circular DNA is stable in primary CD4+T Cells. *Virology*. 2013;441(1):18-21. doi:10.1016/j.virol.2013.02.028.

74. Murray JM, McBride K, Boesecke C, et al. Integrated HIV DNA accumulates prior to treatment while episomal HIV DNA records ongoing transmission afterwards. *AIDS*. 2012;26(5):543-550. doi:10.1097/QAD.0b013e328350fb3c.

75. Pierson TC, Kieffer TL, Ruff CT, Buck C, Gange SJ, Siliciano RF. Intrinsic stability of episomal circles formed during human immunodeficiency virus type 1 replication. *J Virol*. 2002;76(8):4138-4144. doi:10.1128/JVI.76.8.4138-4144.2002.

76. Buzon MJ, Massanella M, Llibre JM, et al. HIV-1 replication and immune dynamics are affected by raltegravir intensification of HAART-suppressed subjects. *Nat Med*. 2010;16(4):460-465. doi:10.1038/nm.2111.

77. Yoder KE, Fishel R. PCR-based detection is unable to consistently distinguish HIV 1LTR circles. *J Virol Methods*. 2006;138(1-2):201-206. doi:10.1016/j.jviromet.2006.07.022.

78. Mbisa JL, Delviks-Frankenberry KA, Thomas JA, Gorelick RJ, Pathak VK. Real-time

PCR analysis of HIV-1 replication post-entry events. *Methods Mol Biol*. 2009;485(Chapter 5):55-72. doi:10.1007/978-1-59745-170-3_5.

79. Sahu GK, Sarria JC, Cloyd MW. Recovery of replication-competent residual HIV-1 from plasma of a patient receiving prolonged, suppressive highly active antiretroviral therapy. *J Virol*. 2010;84(16):8348-8352. doi:10.1128/JVI.00362-10.

80. Palmer S, Wiegand AP, Maldarelli F, et al. New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma. *J Clin Microbiol*. 2003;41(10):4531-4536. doi:10.1128/JCM.41.10.4531-4536.2003.

81. Palmer S. Advances in detection and monitoring of plasma viremia in HIV-infected individuals receiving antiretroviral therapy. *Curr Opin HIV AIDS*. 2013;8(2):87-92. doi:10.1097/COH.0b013e32835d80af.

82. Dahl V, Peterson J, Spudich S, et al. Single-copy assay quantification of HIV-1 RNA in paired cerebrospinal fluid and plasma samples from elite controllers. *AIDS*. 2013;27(7):1145-1149. doi:10.1097/QAD.0b013e32835cf235.

83. Bailey JR, Sedaghat AR, Kieffer T, et al. Residual human immunodeficiency virus type 1 viremia in some patients on antiretroviral therapy is dominated by a small number of invariant clones rarely found in circulating CD4+ T cells. *J Virol*. 2006;80(13):6441-6457. doi:10.1128/JVI.00591-06.

84. Cillo AR, Vagratian D, Bedison MA, et al. Improved single-copy assays for quantification of persistent HIV-1 viremia in patients on suppressive antiretroviral therapy. *J Clin Microbiol*. 2014;52(11):3944-3951. doi:10.1128/JCM.02060-14.

85. Hermankova M, Ray SC, Ruff C, et al. HIV-1 drug resistance profiles in children and adults with viral load of <50 copies/ml receiving combination therapy. *JAMA*. 2001;286(2):196-207.

86. Kieffer TL, Finucane MM, Nettles RE, et al. Genotypic analysis of HIV-1 drug resistance at the limit of detection: virus production without evolution in treated adults with undetectable HIV loads. *J Infect Dis*. 2004;189(8):1452-1465. doi:10.1086/382488.

87. Dinoso JB, Kim SY, Wiegand AM, et al. Treatment intensification does not reduce residual HIV-1 viremia in patients on highly active antiretroviral therapy. *Proc Natl Acad Sci USA*. 2009;106(23):9403-9408. doi:10.1073/pnas.0903107106.

88. Llibre JM, Buzon MJ, Massanella M, et al. Treatment intensification with raltegravir in subjects with sustained HIV-1 viraemia suppression: a randomized 48-week study. *Antivir Ther (Lond)*. 2012;17(2):355-364. doi:10.3851/IMP1917.

89. Hatano H, Hayes TL, Dahl V, et al. A randomized, controlled trial of raltegravir intensification in antiretroviral-treated, HIV-infected patients with a suboptimal CD4+ T cell response. *J Infect Dis*. 2011;203(7):960-968. doi:10.1093/infdis/jiq138.

90. Gandhi RT, Zheng L, Bosch RJ, et al. The effect of raltegravir intensification on low-level residual viremia in HIV-infected patients on antiretroviral therapy: a randomized

controlled trial. *PLoS Med*. 2010;7(8):e1000321. doi:10.1371/journal.pmed.1000321.

91. Archin NM, Cheema M, Parker D, et al. Antiretroviral intensification and valproic acid lack sustained effect on residual HIV-1 viremia or resting CD4+ cell infection. Sandberg JK, ed. *PLoS ONE*. 2010;5(2):e9390. doi:10.1371/journal.pone.0009390.

92. Tobin NH, Learn GH, Holte SE, et al. Evidence that low-level viremias during effective highly active antiretroviral therapy result from two processes: expression of archival virus and replication of virus. *J Virol*. 2005;79(15):9625-9634. doi:10.1128/JVI.79.15.9625-9634.2005.

93. Anderson JA, Archin NM, Ince W, et al. Clonal sequences recovered from plasma from patients with residual HIV-1 viremia and on intensified antiretroviral therapy are identical to replicating viral RNAs recovered from circulating resting CD4+ T cells. *J Virol*. 2011;85(10):5220-5223. doi:10.1128/JVI.00284-11.

94. Perelson AS, Essunger P, Cao Y, et al. Decay characteristics of HIV-1-infected compartments during combination therapy. *Nature*. 1997;387(6629):188-191. doi:10.1038/387188a0.

95. Davey RT, Bhat N, Yoder C, et al. HIV-1 and T cell dynamics after interruption of highly active antiretroviral therapy (HAART) in patients with a history of sustained viral suppression. *Proc Natl Acad Sci USA*. 1999;96(26):15109-15114.

96. Yukl SA, Boritz E, Busch M, et al. Challenges in detecting HIV persistence during potentially curative interventions: a study of the Berlin patient. Cullen BR, ed. *PLoS Pathog*. 2013;9(5):e1003347. doi:10.1371/journal.ppat.1003347.

97. Henrich TJ, Hu Z, Li JZ, et al. Long-term reduction in peripheral blood HIV type 1 reservoirs following reduced-intensity conditioning allogeneic stem cell transplantation. *J Infect Dis*. 2013;207(11):1694-1702. doi:10.1093/infdis/jit086.

98. Henrich TJ, Hanhauser E, Marty FM, et al. Antiretroviral-free HIV-1 remission and viral rebound after allogeneic stem cell transplantation: report of 2 cases. *Ann Intern Med*. 2014;161(5):319-327. doi:10.7326/M14-1027.

99. Persaud D, Gay H, Ziemniak C, et al. Absence of detectable HIV-1 viremia after treatment cessation in an infant. *N Engl J Med*. 2013;369(19):1828-1835. doi:10.1056/NEJMoa1302976.

100. Siliciano JD, Siliciano RF. AIDS/HIV. Rekindled HIV infection. 2014;345(6200):1005-1006. doi:10.1126/science.1259452.

101. Arnedo-Valero M, Garcia F, Gil C, et al. Risk of selecting de novo drug-resistance mutations during structured treatment interruptions in patients with chronic HIV infection. *Clin Infect Dis*. 2005;41(6):883-890. doi:10.1086/432881.

102. Luebbert J, Tweya H, Phiri S, et al. Virological failure and drug resistance in patients on antiretroviral therapy after treatment interruption in Lilongwe, Malawi. *Clin Infect Dis*. 2012;55(3):441-448. doi:10.1093/cid/cis438.

103. Pace M, Frater J. A cure for HIV: is it in sight? *Expert Rev Anti Infect Ther*. 2014;12(7):783-791. doi:10.1586/14787210.2014.910112.

104. Routy JP, Boulassel MR, Nicolette CA, Jacobson JM. Assessing risk of a short-term antiretroviral therapy discontinuation as a read-out of viral control in immune-based therapy. *J Med Virol*. 2012;84(6):885-889. doi:10.1002/jmv.23297.

105. Strategies for Management of Antiretroviral Therapy (SMART) Study Group, El-Sadr WM, Lundgren JD, et al. CD4+ count-guided interruption of antiretroviral treatment. *N Engl J Med*. 2006;355(22):2283-2296. doi:10.1056/NEJMoa062360.

106. Silverberg MJ, Neuhaus J, Bower M, et al. Risk of cancers during interrupted antiretroviral therapy in the SMART study. *AIDS*. 2007;21(14):1957-1963. doi:10.1097/QAD.0b013e3282ed6338.

107. Hill AL, Rosenbloom DIS, Fu F, Nowak MA, Siliciano RF. Predicting the outcomes of treatment to eradicate the latent reservoir for HIV-1. *Proc Natl Acad Sci USA*. 2014;111(37):13475-13480. doi:10.1073/pnas.1406663111.

108. Jones M, Williams J, Gärtner K, Phillips R, Hurst J, Frater J. Low copy target detection by Droplet Digital PCR through application of a novel open access bioinformatic pipeline, 'definetherain'. *J Virol Methods*. 2014;202:46-53. doi:10.1016/j.jviromet.2014.02.020.

109. Shan L, Deng K, Shroff NS, et al. Stimulation of HIV-1-specific cytolytic T lymphocytes facilitates elimination of latent viral reservoir after virus reactivation. *Immunity*. 2012;36(3):491-501. doi:10.1016/j.immuni.2012.01.014.

110. Puertas MC, Massanella M, Llibre JM, et al. Intensification of a raltegravir-based regimen with maraviroc in early HIV-1 infection. *AIDS*. 2014;28(3):325-334. doi:10.1097/QAD.0000000000000066.

111. International AIDS Society Scientific Working Group on HIV Cure, Deeks SG, Autran B, et al. Towards an HIV cure: a global scientific strategy. *Nat Rev Immunol*. 2012;12(8):607-614. doi:10.1038/nri3262.

112. Zhang Z, Schuler T, Zupancic M, et al. Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. *Science*. 1999;286(5443):1353-1357.

113. Margolick JB, Volkman DJ, Folks TM, Fauci AS. Amplification of HTLV-III/LAV infection by antigen-induced activation of T cells and direct suppression by virus of lymphocyte blastogenic responses. *J Immunol*. 1987;138(6):1719-1723.

114. Bleul CC, Wu L, Hoxie JA, Springer TA, Mackay CR. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. *Proc Natl Acad Sci USA*. 1997;94(5):1925-1930.

115. Baldauf H-M, Pan X, Erikson E, et al. SAMHD1 restricts HIV-1 infection in resting CD4(+) T cells. *Nat Med*. 2012;18(11):1682-1687. doi:10.1038/nm.2964.

116. Yoder A, Yu D, Dong L, et al. HIV envelope-CXCR4 signaling activates cofilin to

overcome cortical actin restriction in resting CD4 T cells. *Cell*. 2008;134(5):782-792. doi:10.1016/j.cell.2008.06.036.

117. Nabel G, Baltimore D. An inducible transcription factor activates expression of human immunodeficiency virus in T cells. *Nature*. 1987;326(6114):711-713. doi:10.1038/326711a0.

118. Böhnlein E, Lowenthal JW, Siekevitz M, Ballard DW, Franza BR, Greene WC. The same inducible nuclear proteins regulates mitogen activation of both the interleukin-2 receptor-alpha gene and type 1 HIV. *Cell*. 1988;53(5):827-836.

119. Duh EJ, Maury WJ, Folks TM, Fauci AS, Rabson AB. Tumor necrosis factor alpha activates human immunodeficiency virus type 1 through induction of nuclear factor binding to the NF-kappa B sites in the long terminal repeat. *Proc Natl Acad Sci USA*. 1989;86(15):5974-5978.

120. Adams M, Sharmeen L, Kimpton J, et al. Cellular latency in human immunodeficiency virus-infected individuals with high CD4 levels can be detected by the presence of promoter-proximal transcripts. *Proc Natl Acad Sci USA*. 1994;91(9):3862-3866.

121. Lin X, Irwin D, Kanazawa S, et al. Transcriptional profiles of latent human immunodeficiency virus in infected individuals: effects of Tat on the host and reservoir. *J Virol*. 2003;77(15):8227-8236. doi:10.1128/JVI.77.15.8227-8236.2003.

122. Dahabieh MS, Battivelli E, Verdin E. Understanding HIV Latency: The Road to an HIV Cure. *Annu Rev Med*. 2015;66(1):407-421. doi:10.1146/annurev-med-092112-152941.

123. Chavez L, Calvanese V, Verdin E. HIV Latency Is Established Directly and Early in Both Resting and Activated Primary CD4 T Cells. Emerman M, ed. *PLoS Pathog*. 2015;11(6):e1004955. doi:10.1371/journal.ppat.1004955.

124. Pace MJ, Graf EH, Agosto LM, et al. Directly infected resting CD4+T cells can produce HIV Gag without spreading infection in a model of HIV latency. Silvestri G, ed. *PLoS Pathog*. 2012;8(7):e1002818. doi:10.1371/journal.ppat.1002818.

125. Williams SA, Greene WC. Regulation of HIV-1 latency by T-cell activation. *Cytokine*. 2007;39(1):63-74. doi:10.1016/j.cyto.2007.05.017.

126. Patel SS, Duby AD, Thiele DL, Lipsky PE. Phenotypic and functional characterization of human T cell clones. *J Immunol*. 1988;141(11):3726-3736.

127. Li JZ, Etemad B, Ahmed H, et al. The size of the expressed HIV reservoir predicts timing of viral rebound after treatment interruption. *AIDS*. 2016;30(3):343-353. doi:10.1097/QAD.0000000000000953.

128. Lyons AB. Divided we stand: tracking cell proliferation with carboxyfluorescein diacetate succinimidyl ester. *Immunol Cell Biol*. 1999;77(6):509-515. doi:10.1046/j.1440-1711.1999.00864.x.

129. Lyons AB. Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. *J Immunol Methods*. 2000;243(1-2):147-154.

130. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*. 2011;28(10):2731-2739. doi:10.1093/molbev/msr121.

131. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol*. 2013;30(12):2725-2729. doi:10.1093/molbev/mst197.

132. Rosenbloom DIS, Elliott O, Hill AL, Henrich TJ, Siliciano JM, Siliciano RF. Designing and Interpreting Limiting Dilution Assays: General Principles and Applications to the Latent Reservoir for Human Immunodeficiency Virus-1. *Open Forum Infect Dis*. 2015;2(4):ofv123. doi:10.1093/ofid/ofv123.

133. Ferris RL, Lu B, Kane LP. Too much of a good thing? Tim-3 and TCR signaling in T cell exhaustion. *J Immunol*. 2014;193(4):1525-1530. doi:10.4049/jimmunol.1400557.

134. Rahman AN. TIM-3 and Its Immunoregulatory Role in HIV Infection. *J Clin Cell Immunol*. 2013;04(01):1-5. doi:10.4172/2155-9899.S7-007.

135. Brooks DG, McGavern DB, Oldstone MBA. Reprogramming of antiviral T cells prevents inactivation and restores T cell activity during persistent viral infection. *J Clin Invest*. 2006;116(6):1675-1685. doi:10.1172/JCI26856.

136. Brooks DG, Teyton L, Oldstone MBA, McGavern DB. Intrinsic functional dysregulation of CD4 T cells occurs rapidly following persistent viral infection. *J Virol*. 2005;79(16):10514-10527. doi:10.1128/JVI.79.16.10514-10527.2005.

137. Oxenius A, Zinkernagel RM, Hengartner H. Comparison of activation versus induction of unresponsiveness of virus-specific CD4+ and CD8+ T cells upon acute versus persistent viral infection. *Immunity*. 1998;9(4):449-457.

138. Iyasere C, Tilton JC, Johnson AJ, et al. Diminished proliferation of human immunodeficiency virus-specific CD4+ T cells is associated with diminished interleukin-2 (IL-2) production and is recovered by exogenous IL-2. *J Virol*. 2003;77(20):10900-10909. doi:10.1128/JVI.77.20.10900-10909.2003.

139. Fuller MJ, Zajac AJ. Ablation of CD8 and CD4 T cell responses by high viral loads. *J Immunol*. 2003;170(1):477-486.

140. Hastings WD, Anderson DE, Kassam N, et al. TIM-3 is expressed on activated human CD4+ T cells and regulates Th1 and Th17 cytokines. *Eur J Immunol*. 2009;39(9):2492-2501. doi:10.1002/eji.200939274.

141. Jones KA, Peterlin BM. Control of RNA initiation and elongation at the HIV-1 promoter. *Annu Rev Biochem*. 1994;63(1):717-743. doi:10.1146/annurev.bi.63.070194.003441.

142. Pereira LA, Bentley K, Peeters A, Churchill MJ, Deacon NJ. A compilation of cellular transcription factor interactions with the HIV-1 LTR promoter. *Nucleic Acids Res*. 2000;28(3):663-668.

143. Jones KA, Kadonaga JT, Luciw PA, Tjian R. Activation of the AIDS retrovirus promoter by the cellular transcription factor, Sp1. *Science*. 1986;232(4751):755-759.

144. Pearson R, Kim YK, Hokello J, et al. Epigenetic silencing of human immunodeficiency virus (HIV) transcription by formation of restrictive chromatin structures at the viral long terminal repeat drives the progressive entry of HIV into latency. *J Virol*. 2008;82(24):12291-12303. doi:10.1128/JVI.01383-08.

145. Shankarappa R, Margolick JB, Gange SJ, et al. Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. *J Virol*. 1999;73(12):10489-10502.

146. Blankson JN, Siliciano JD, Siliciano RF. Finding a cure for human immunodeficiency virus-1 infection. *Infect Dis Clin North Am*. 2014;28(4):633-650. doi:10.1016/j.idc.2014.08.007.

147. Archin NM, Sung JM, Garrido C, Soriano-Sarabia N, Margolis DM. Eradicating HIV-1 infection: seeking to clear a persistent pathogen. *Nat Rev Microbiol*. 2014;12(11):750-764. doi:10.1038/nrmicro3352.

148. Martin AR, Siliciano RF. Progress Toward HIV Eradication: Case Reports, Current Efforts, and the Challenges Associated with Cure. *Annu Rev Med*. 2016;67(1):215-228. doi:10.1146/annurev-med-011514-023043.

149. Siliciano JM, Siliciano RF. The Remarkable Stability of the Latent Reservoir for HIV-1 in Resting Memory CD4+ T Cells. *J Infect Dis*. 2015;212(9):1345-1347. doi:10.1093/infdis/jiv219.

150. Laird GM, Rosenbloom DIS, Lai J, Siliciano RF, Siliciano JD. Measuring the Frequency of Latent HIV-1 in Resting CD4(+) T Cells Using a Limiting Dilution Coculture Assay. *Methods Mol Biol*. 2016;1354(Chapter 16):239-253. doi:10.1007/978-1-4939-3046-3_16.

151. Kim M, Siliciano RF. Reservoir expansion by T-cell proliferation may be another barrier to curing HIV infection. *Proc Natl Acad Sci USA*. 2016;113(7):1692-1694. doi:10.1073/pnas.1600097113.

152. Kearney MF, Wiegand A, Shao W, et al. Origin of Rebound Plasma HIV Includes Cells with Identical Proviruses that are Transcriptionally Active Before Stopping Antiretroviral Therapy. *J Virol*. November 2015:JVI.02139–15. doi:10.1128/JVI.02139-15.

153. Massanella M, Richman DD. Measuring the latent reservoir in vivo. *J Clin Invest*. 2016;126(2):464-472. doi:10.1172/JCI80567.

CURRICULUM VITAE

The Johns Hopkins University School of Medicine

Nina Narayan Hosmane March 2016

Educational History

2011-2016 The Johns Hopkins University School of Medicine
Baltimore, MD
Ph.D., Cellular and Molecular Medicine

2007-2011 University of Illinois at Urbana-Champaign
Urbana-Champaign, IL
B.S. Molecular/Cellular Biology with Honors; Chemistry Minor

Research Experience

Thesis Research (Medicine), Lab of Dr. Robert F. Siliciano 2012-2016
Johns Hopkins School of Medicine
Research Rotation (Neuroscience), Lab of Dr. Jeremy Nathans 2012
Johns Hopkins School of Medicine
Research Rotation (Oncology), Lab of Dr. William Matsui 2012
Johns Hopkins School of Medicine
Undergraduate Research (Developmental Biology), Lab of Dr. Phillip Newmark 2008-2011
University of Illinois at Urbana-Champaign

Academic Honors

Young Investigator's Scholarship - CROI Conference in Seattle, WA 2015
NSF Pre-Doctoral Fellowship Honorable Mention 2012
Departmental Distinguished Research in Cell Biology Award 2011
Department of MCB Best Senior Thesis Award 2011
Department of MCB: Highest Distinction in Molecular and Cellular Biology 2011
Procter & Gamble Undergraduate Student Research Award 2011
Dean's List (School of Liberal Arts & Sciences) - University of Illinois 2011
Honors Concentration Track in Department of Molecular and Cellular Biology 2007- 2011

Publications

Hosmane, N.N., Kwon, K.J., Kim, M., Capoferri, A.A., Ho Y.C., Siliciano, J.D., and Siliciano, R.F. Repetitive stimulation reveals stochastic reactivation of latent HIV-1 from infected CD4+ T cells including expanded cellular clones carrying replication-competent virus. 2016. *Manuscript in preparation.*

Hansen, E.C., Ransom, M., Hesselberth, J.R., **Hosmane, N.N**., Capoferri, A.J., Bruner, K.M., Pollack, R.A., Zhang, H., Drummond, B.M., Siliciano, J.M., Siliciano, R.F., and Stivers, J.T. Diverse fates of uracilated HIV DNA during infection of myeloid lineage cells. 2016. *Manuscript in preparation.*

Shang, L., Deng, K., Xing, Sifei, Rabi, S.A., Kim, M., **Hosmane, N.N.,** Durand, C.M., Zhang, H., Margolick, J.B., Yang, H.C., Siliciano, J.D., and Siliciano, R.F. Unique features of effector to memory transition render CD4+ T cells permissive for latent HIV infection. 2016. *Manuscript in preparation.*

Hakre, S., Kampmann, M., **Hosmane, N.N.,** Lim, H.W., Bassik, M.C., Verschueren, E., Battivelli, E., Chan, C., Greene, W., Krogan, N.J., Siliciano, R.F., Weissman, J.S., and

Verdin, E. Human Genome-Wide shRNA Profiling Identifies Roles for TGFβ Signaling and the mTOR Pathway in Regulating HIV latency. 2016. *Manuscript in preparation.*

Bruner, K.B.*, **Hosmane, N.N.*,** and Siliciano, R.F. Towards an HIV-1 cure: measuring the latent reservoir. *Trends Microbiol*. 2015 Apr;4(23):192–203. ***contributed equally**

Kim M, **Hosmane N.N.,** Bullen C.K., Capoferri A., Yang H.C., Siliciano J.D., and Siliciano R.F. A primary CD4+ T cell model of HIV-1 latency established after activation through the T cell receptor and subsequent return to quiescence. *Nat Protoc.* 2014 Dec;9(12):2755-2770.

Dar, R.D., **Hosmane, N.N**., Arkin, M.R., Siliciano, R.F., and Weinberger, L.S. Screening for noise in gene expression identifies drug synergies. *Science.* 2014 Jun 20;344(6190):1392-6.

Ho, Y., Shan, L., **Hosmane, N.N**., Wang J., Laskey S.B., Rosenbloom D.I., Lai J., Blankson J.N., Siliciano J.D., and Siliciano R.F. Replication-Competent Noninduced Proviruses in the Latent Reservoir Increase Barrier to HIV-1 Cure. *Cell.* 2013 Oct 24;155(3):540-51.

Abstracts and Presentations

Hosmane, N.N., Kwon, K.J., Kim, M., Capoferri, A.A., Ho Y.C., Siliciano, J.D., and Siliciano, R.F. "Multiple Rounds of Maximum T Cell Activation Induce Additional Replication-Competent HIV-1 From Resting Memory CD4+ T Cells." JHSOM Department of Medicine Research Retreat. March 10, 2015. Baltimore, MD.

Hosmane, N.N., Capoferri, A.A., and Siliciano, R.F. "Multiple Rounds of T-Cell Activation Induce Additional HIV-1 From the Latent Reservoir." Conference on Retroviruses and Opportunistic Infections, February 25, 2015. Seattle, WA.

Hosmane, N.N. and Siliciano, R.F. "*Repeated Rounds of T Cell Activation Induce Additional Outgrowth of Virus From The Latent Reservoir*", The Collaboratory of AIDS Researchers for Eradication (CARE), June 11-13, 2014. University of North Carolina at Chapel Hill.

Ho Y.C., Shan L., Wang J., **Hosmane N.N.,** Blankson, J.N., Siliciano, R.F. "Replication-competent non-induced HIV-1 proviruses in the latent reservoir increase the barrier to HIV-1 cure." Johns Hopkins Center for AIDS Research (CFAR) Annual Meeting. 2013.

Ho, Y., Shan L., Wang, J., **Hosmane, N.N.,** Blankson J., Siliciano, RF. "Characterization of Non-induced HIV-1 Proviruses Dampens the Hope for HIV-1 Eradication", Conference on Retroviruses and Opportunistic Infections, March 4, 2013. Atlanta, GA.

Hosmane, N.N. "Characterizing the Expression of a kruppel-like Transcription Factor (klf) in Sexual and Asexual *Schmidtea mediterranea".* Senior Thesis Presentation for Distinction. April 22, 2011. University of Illinois at Urbana-Champaign.

Hosmane, N.N. "The Benefits of Undergraduate Research", Illinois Biological Society, November 11, 2010. University of Illinois at Urbana-Champaign.

Chong, T., Stary, J., **Hosmane, N.N.,** and Newmark, PA. "Identification and Characterization of Genes Involved In Spematogenesis in the Planarian *Schmidtea mediterranea*." Cold Spring Harbor Laboratory's Germ Cell Meeting, October 5, 2010. Cold Spring Harbor, NY.

Hosmane, N.N., "Spatial and Temporal Analyses of the Female Reproductive System in the Regenerating Planarian *Schmidtea mediterranea*", Procter & Gamble Undergraduate Student Research Award, April 28, 2010. University of Illinois at Urbana-Champaign.

Hosmane, N.N., "Spatial and Temporal Analyses of the Female Reproductive System in the Regenerating Planarian *Schmidtea mediterranea*", Undergraduate Research Symposium, April 15, 2010: *Abstract, #1, pg 48* (2010). University of Illinois at Urbana-Champaign.