INDUCIBILITY OF LATENT HIV-1 IN RESTING CD4$^+$ MEMORY T-CELL SUBSETS

by

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ABSTRACT

The latent reservoir (LR) for HIV-1 in resting memory CD4+ T-cells harbors integrated, replication-competent proviruses that are not actively transcribed while the T-cell remains in a resting state. Recent work has shown that proliferation of infected cells is a major factor in the generation, persistence, and stability of the latent reservoir. Given that the latent reservoir is the major barrier to HIV-1 cure, it is important to understand the proliferative process that contributes to the persistence of the LR. Stimuli that drive T-cell proliferation can also reactivate latent HIV-1, but productively infected cells have a short half-life. Several groups have shown that latently infected cells that clonally expand in vivo can be reactivated in vitro without producing virus. One hypothesis to explain this observation is that certain latently infected memory CD4+ subsets may be in a deeper state of latency and therefore may be able to proliferate without producing virus.

To evaluate this possibility, we cultured resting naïve (TN), central memory (TCM), transitional memory (TTM), and effector memory (TEM) CD4+ T-cells from 10 HIV patients on suppressive ART in a multiple stimulation viral outgrowth assay (MSVOA). The frequencies of viral outgrowth calculated from p24 ELISAs were compared to the frequencies of intact proviral DNA copies calculated by the droplet digital PCR-based Intact Proviral DNA Assay (IPDA), and on average, only 1.5% of intact proviruses across all subsets were induced by multiple rounds of global T-cell activation. In addition, there was no enrichment of intact proviruses in any specific subset nor any correlation between the inducibility of intact proviruses and memory subset phenotype. Furthermore, we observed significant plasticity among the canonical memory subset surface markers during culture of the cells and saw significant patient-to-patient variability in inducibility patterns

that complicates the vision for a targeted cure approach based on T-cell subsets.

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PREFACE

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INTRODUCTION

There are 37 million people in the world infected with HIV-1, of whom 15.8 million are effectively receiving antiretroviral therapy (ART), which prevents new infection events and reduces viral copies to undetectable levels[10]. However, ART is not curative in that the virus can establish latency by integrating into the host genomes of activated CD4+ T cells as a provirus[1]. Reversion of these activated CD4+ T cells into resting memory CD4+ T cells allows the virus to evade transcriptional expression, immune clearance, and ART, thus forming the HIV-1 latent reservoir (LR)[11]. The long half-life of the LR implicates that it would take more than a patient's lifetime of ART to cure HIV[12]. Thus, elimination of the persistent latent reservoir is the key approach to HIV cure.

Although the reservoir consists mostly of defective virus, about 2% of it contains intact virus[13]. The standard viral outgrowth assay (VOA) was developed to measure the frequency of latently infected cells containing replication-competent virus by culturing resting memory patient CD4+ T cells after dilution at a Poisson distribution such that only one virus is present in each culture well, and then activating these cells to proliferate and induce viral replication[14,15]. Previous studies have shown that the standard VOA is a minimal estimate of the size of the LR as additional virus has been shown to grow out after repeated stimulation[2]. This additional virus was previously thought to be intact but noninducible virus. In one of the studies presented in this work, resting CD4+ T cells of chronically infected HIV patients were repeatedly activated in a multiple stimulation VOA (MSVOA) to induce latent virus to replicate. After observing additional induced viruses after multiple stimulations, we sequenced the viral RNA and found identical sequences from different wells. These findings suggested that the replication competent viruses arose

from in vivo clonal expansion of latently infected cells containing replication-competent virus as multiple identical clones were found in each patient from a single blood draw. This implies that the LR persists by clonal expansion of these resting memory cells containing replication-competent virus. There are two possible mechanisms for clonal expansion: 1) one virus infects multiple precursor memory cells which then proliferate, or 2) one virus establishes latency in one precursor memory cell which then proliferates. The latter is most likely given the identical sequences among clones and the large number of clones sampled in a single blood draw from a patient. The lack of mutations between the sequences suggests expansion by cellular proliferation rather than viral replication followed by cellular proliferation. The specifics of the mechanism of clonal expansion are unknown. From this study, we concluded that intact, replication-competent proviruses persist through *in vivo* clonal expansion of these infected cells that can be activated without producing virus.

Several groups have now shown that latently infected cells that clonally expand in vivo can be activated in vitro without producing virus. One hypothesis to explain this observation is that certain subpopulations of reservoir cells, such as particular CD4+ memory subsets, are predisposed to delay reactivation of the latent virus or promote a deeper state of latency. To evaluate this possibility, we cultured resting naïve (TN), central memory (TCM), transitional memory (TTM), and effector memory (TEM) CD4+ T-cells from 10 HIV patients on suppressive ART in the MSVOA. The frequencies of viral outgrowth calculated from p24 ELISAs were compared to the frequencies of intact proviral DNA copies calculated by the droplet digital PCR-based Intact Proviral DNA Assay (IPDA), allowing us to estimate that on average only 1.5% of intact proviruses can be

induced by multiple rounds of global T-cell activation. There was no enrichment of intact proviruses in any specific subset nor any correlation between the inducibility of intact proviruses and memory subset phenotype. Furthermore, we observed significant plasticity among the canonical memory subset surface markers and saw significant patient-to-patient variability that complicates the vision for a targeted cure approach based on T-cell subsets.

Overall, we have concluded that the LR persists through clonal expansion of infected cells, among other mechanisms. We have determined that replication-competent proviruses in these clonally expanded cells can be reactivated without producing virus, thus contributing to the persistence of the latent reservoir. Lastly, we have determined that the differential inducibility of these replication-competent proviruses is not related to the memory subset phenotype of the cell they are harbored in, and of these cells that contain intact proviruses, only a small fraction can be induced to replicate in culture. Next steps for the field would include investigation of these noninduced intact proviruses to determine whether they actually can be induced to replicate and what role they play in residual viremia and the persistence of the latent reservoir.

CHAPTER 1: The Latent Reservoir for HIV-1: How Immunologic Memory and Clonal Expansion Contribute to HIV-1 Persistence

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Abstract

Combination antiretroviral therapy (ART) for HIV-1 infection reduces plasma virus levels to below the limit of detection of clinical assays. However, even with prolonged suppression of viral replication with ART, viremia rebounds rapidly after treatment interruption; thus ART is not curative. The principal barrier to cure is a remarkably stable reservoir of latent HIV-1 in resting memory $CD4^+$ T cells. Here we consider explanations for the remarkable stability of the latent reservoir. Stability does not appear to reflect replenishment from new infection events but rather normal physiologic processes that provide for immunologic memory. Of particular importance are proliferative processes that drive clonal expansion of infected cells. Recent evidence suggests that in some infected cells, proliferation is a consequence of proviral integration into host genes associated with cell growth. Efforts to cure HIV-1 infection by targeting the latent reservoir may need to consider the potential of latently infected cells to proliferate.

Introduction

In 2014, ~37 million people were living with HIV-1 infection (www.unaids.org). Optimal patient outcomes are achieved by initiating combination antiretroviral therapy (ART) as soon infection is diagnosed, regardless of the $CD4^+$ T cell count (1-3). ART reduces plasma virus levels to below the clinical detection limit (20-50 copies of HIV-1 RNA/ml) and halts disease progression (4-6). Recommended initial regimens consist of two nucleoside analog reverse transcriptase inhibitors and a third drug, either an integrase inhibitor or the protease inhibitor darunavir (3). Although ART effectively suppresses viremia, it is not curative, and viremia rebounds upon ART cessation (7, 8). Therefore, lifelong treatment is required. Providing lifelong treatment for all infected individuals poses a major economic and logistical challenge. Only 15 million people currently receive ART. The tolerability of ART regimens has improved dramatically, but long term drug toxicity is also a concern. Other problems include the emergence of resistance with suboptimal treatment and the stigma associated with the infection. For these reasons, there is great current interest in a cure (9, 10).

The principal barrier to cure is a stable reservoir of latent HIV-1 in resting $CD4^+$ T cells (11, 12). The reservoir persists even in patients on long term ART who have no detectable viremia (13-18). The cells comprising this reservoir have a memory phenotype (12, 19-23). Direct measurements of the latent reservoir in patients on ART show a very slow decay rate ($t_{1/2}$=3.7 years) (16, 17). At this rate, eradication of a reservoir of 10^6 cells would require 73 years, making cure unlikely even with lifelong ART. Thus, research towards a cure focuses on eliminating this reservoir. Recent reviews have discussed

molecular mechanisms of HIV-1 latency (24-27) and approaches for eliminating the reservoir (10, 28-30). Here we consider explanations for its remarkable stability.

Why does HIV-1 establish latent infection?

Viral latency is a reversibly nonproductive state of infection of individual cells (31). Latently infected cells contain a stable form of the viral genome, either as a circular plasmid in the case of herpesviruses or as a linear provirus stably integrated into host cell DNA in the case of HIV-1. During latency, there is highly restricted expression of viral genes (31). For some herpesviruses, latency evolved as an essential mechanism of immune evasion and viral persistence (31, 32). For HIV-1, latency is not necessary for persistence as active viral replication occurs throughout the course of infection in untreated patients (33). Escape from immune responses is through rapid evolution of variants not recognized by cytolytic T lymphocytes (CTL) or neutralizing antibodies (34-41). Nevertheless, a latent reservoir is established rapidly in all HIV-1-infected individuals (42). Latently infected cells can be detected in the rare individuals who spontaneously control HIV-1 infection without ART (43). Early ART restricts the size of the reservoir (22, 44) but does not block its establishment (42). In rhesus macaques infected with simian immunodeficiency virus (SIV), which also establishes a latent reservoir in resting $CD4^+$ T cells (45, 46), initiation of ART on day 3 post infection prevents detectable viremia but not the establishment of a latent reservoir (47). Thus, it is difficult to prevent the establishment of the latent reservoir.

A recent theory suggests that HIV-1 evolved a mechanism for rapid establishment of latent infection to facilitate transmission across mucosal barriers (48, 49). Latency is proposed to serve as a "bet-hedging strategy" that allows some infected cells to survive

long enough to transit the mucosa. However, as is discussed below, infected cells can remain in a latent state for years, and a long time interval between mucosal exposure and viremia has never been documented.

Latency is most simply explained as a consequence of viral tropism for activated $CD4^+$ T cells which can transition to a resting memory state that is non-permissive for replication (Fig. 1). HIV-1 has a strong propensity to infect activated $CD4^+$ T cells (50, 51). CCR5, a critical co-receptor for entry of the commonly transmitted forms of HIV-1 (52-57), is upregulated on $CD4^+$ T cell activation (58). Following entry, reverse transcription of the viral RNA genome into DNA and integration of the resulting provirus into host cell DNA occur within hours (59). Transcription of the integrated provirus then begins because active nuclear forms of key host factors needed for the initiation and elongation of viral transcription, including NFκB, NFAT, and pTEFb, are present in activated cells (60-67). In contrast, resting $CD4^+$ T cells mostly lack CCR5 expression (58), and other factors interfere with HIV-1 replication even when the virus has successfully entered. The cellular protein SAMHD1, a deoxynucleoside triphosphate triphosphohydrolase, depletes dNTP levels, thus impeding reverse transcription (68-70). It is expressed at high levels in myeloid cells and resting $CD4^+$ T cells (52-55). Interestingly, SIV and HIV-2 encodes a protein, Vpx, that promotes SAMHD1 degradation (68, 71). However, HIV-1 lacks Vpx, and thus reverse transcription in resting $CD4^+$ T cells is inefficient, taking as long as 3 days (72-74). The static nature of the actin cytoskeleton in resting cells inhibits delivery of the reverse transcribed viral genome to the nucleus (75). These delays facilitate recognition of DNA intermediates generated during reverse transcription by a host innate DNA sensor, IFI16, leading to caspase-1 activation and a

proinflammatory form of cell death known as pyroptosis (76-78). Additional barriers to replication in resting $CD4^+$ T cells include the lack of active forms of NFκB, NFAT, and pTEFb needed for transcription of the provirus (60-63, 65, 66).

Although activated $CD4^+$ T cells are the principle target for HIV-1, they die quickly after infection. Classic studies of viral dynamics revealed a rapid decay in viremia when new infection events are blocked with ART (6, 79-81). This decay reflects the short half-life of plasma virions ($t_{1/2}$ ~ minutes) and of the infected cells that produce most of the plasma virus ($t_{1/2}$ ~ 1 day). Activated T cells are prone to die in the contraction phase of immune responses due to activation-induced cell death (82). In addition, productively infected cells may die from other cell death pathways triggered by viral proteins or by integration of the provirus into the host cell genome (83, 84). Infected $CD4^+$ T lymphoblasts may also be lysed by $CD8^+$ CTL (34, 85-87). Surprisingly CTL do not appear to shorten the $t_{1/2}$ of productively infected cells (88, 89). Nevertheless, it appears that most productively infected $CD4^+$ T lymphoblasts are short-lived.

Given that resting $CD4^+$ T cells are resistant to infection and that activated $CD4^+$ T cells die quickly after infection, how is the latent reservoir established? Some infected $CD4^+$ T lymphoblasts may survive long enough to revert to a resting memory state that is non-permissive for viral gene expression (11), particularly if they are infected within a narrow time window when still permissive for steps in the life cycle up through integration, but not for high level gene expression (Fig. 1). Thus, establishment of latent infection is a rare event, consistent with the low frequency of latently infected cells *in vivo* ($1/10^6$) (13, 16-18). Latency may be further enforced by silencing epigenetic modifications of the integrated provirus (90-92). In this latent state, the virus persists essentially as genetic

information. When antigen or cytokines subsequently activate the cell, the provirus is transcribed, viral proteins are produced, and virus particles are released. Given the long $t_{1/2}$ of memory T cell responses and the fact that the latent proviruses in these cells are not detected by the immune system or targeted by ART, stable persistence of HIV-1 is not surprising. This simple model views latency in the context of the normal physiology of immunologic memory, thereby explaining all clinical observations regarding HIV-1 persistence without requiring the evolution of special viral mechanisms for latency.

Residual viremia, the latent reservoir, and viral rebound

Trace levels of free virus (~1 copy/ml) are present in the plasma of most patients on ART (93-95). Sequence analysis of the residual viremia (RV) reveals that these viruses resemble viremia present earlier in infection, are sensitive to the patient's current ART regimen, and generally do not show evidence of ongoing evolution (96-100). These features all suggest RV originates from a stable reservoir (101). In situations where evolution has been detected, suboptimal ART may be the cause (102). Importantly, intensification of standard three drug ART with additional antiretroviral drugs from a different class does not reduce RV (103-105), indicating that it originates from long-lived cells infected prior to ART. Latently infected resting $CD4^+$ T cells are at least one source of RV. The presence of RV suggests that multiple latently infected cells are become activated every day. While patients remain on ART, the viruses released do not infect additional cells. However, if ART is interrupted, viral rebound occurs. Rebound is typically seen within 2 weeks (7, 8), the time required for washout of antiretroviral drugs and growth of the recently released viruses to detectable levels. The rebound virus is archival in character, consistent with the conclusion

that it originates from a stable latent reservoir (106). The limited variation in time to rebound, despite a two log variation in reservoir size, also suggests that multiple cells are activated per day (107). This conclusion is consistent with a recent analysis of viral rebound which detected multiple viral lineages emerging in multiple sites (lymph node, ileum, and rectum) (8).

Evidence for latent infection of resting memory CD4$^+$ T cells.

This model for latent HIV-1 infection as a barrier to cure is supported by several lines of evidence: 1) Replication-competent HIV-1 can be readily recovered from highly purified resting CD4$^+$ T cells from essentially all infected individuals, regardless of the duration of ART (12, 13, 15, 16, 18, 108). Recovery requires activating the cells to reverse latency, as predicted by the model. As is discussed below, recovery only fails when the size of the latent reservoir is substantially reduced (44, 109-111). Controversy remains over the question of whether other cell types including macrophages serve as stable HIV-1 reservoirs (112-123). To date, long term persistence of replication-competent HIV-1 in the setting of optimal ART has only been demonstrated for resting CD4$^+$ T cells (124). This may in part reflect the difficulty of sampling tissue macrophages, particularly in sites such as the central nervous system. Persistence in tissue macrophages can in principle be studied in novel humanized mouse models (123) and in the SIV model, but only through the use of animals on fully suppressive, long term ART and with the caveat that restriction by SAMHD1 is counteracted by SIV Vpx (68, 69). 2) Latent HIV-1 is found in resting memory CD4$^+$ T cells but only to a limited extent in naïve CD4$^+$ T cells (12, 19-23). 3) The generation of latently infected cells can be reproduced *in vitro* in

primary $CD4^+$ T cells that have been activated in some manner, infected, and then cultured to allow reversion to a resting state (125-129). Restimulation of these cells through the T cell receptor (TCR) leads to HIV-1 gene expression. Together, these results support persistence of latent HIV-1 in resting $CD4^+$ T cells that have been previously infected while in an activated state.

The general concept of HIV-1 latency is also strongly supported by the cure and "near cure" cases of the "Berlin patient" (109), the two "Boston patients" (110, 111), and the "Mississippi baby" (44). The Boston and Berlin patients were HIV-1-infected individuals who developed malignancies requiring hematopoietic stem cell transplantation (HSCT) resulting in immune reconstitution with donor cells. The Berlin patient received HSCT from a donor whose cells were homozygous for a deletion in CCR5 and was cured as the reconstituting T cells were not permissive for entry of R5-tropic HIV-1 (109). Attempts to reproduce this cure have thus far been unsuccessful due in large part to progression of the malignancy. In one case, the appearance of viral variants that utilize the alternative HIV-1 coreceptor, CXCR4, has been noted (130). The Boston patients received HSCT from donors with wild-type CCR5, and ART was continued throughout the transplant period to protect donor cells from infection. When apparently complete reconstitution with donor T cells had occurred, and HIV-1 was no longer detectable by standard assays, ART was interrupted. The patients maintained suppression of viremia for 3 and 8 months before sudden and dramatic rebounds.

The Mississippi baby, born to an infected mother who had no prenatal care, had a plasma HIV-1 RNA level of ~20,000 copies/ml shortly after birth and was immediately started on ART. Plasma HIV-1 RNA declined to below the limit of detection and remained

there even after treatment was interrupted against medical advice between 15 and 18 months of age. Treatment was not restarted, and viremia remained undetectable for over 2 years before suddenly rebounding. Importantly, HIV-1-specific T cell responses were absent in all three subjects because of the transplant process or early treatment. Antibodies to HIV-1 were not detected in the Mississippi baby and were markedly decreased in the Boston patients. Because HIV-1 replication is exponential in the absence of immune responses and ART, HIV-1 persistence for months or years in these patients can be best explained by the non-replicating or latent form of the virus. In these cases, HSCT or early ART delayed rebound by reducing the number of latently infected cells to the point where stochastic reactivation was a rare event (107).

Explanations for the long $t_{1/2}$ of the latent reservoir

The decay rate of the latent reservoir was originally measured using a viral outgrowth assay (VOA), which quantifies viral outgrowth from limiting dilutions of mitogen-stimulated resting $CD4^+$ T cells from patients on ART (13, 131-133). The original VOA-based measurements of the reservoir decay, published in 1999 and 2003, indicated a $t_{1/2}$ of 3.7 years (16, 17). This value was confirmed in a more recent study ($t_{1/2}$ =3.6 years), indicating that despite the development of newer, less toxic, and more convenient ART regimens, the fundamental problem of the reservoir as a barrier to cure has not been overcome (18). A critical issue is whether the remarkable stability of the reservoir is the result of normal homeostatic mechanisms that maintain immunologic memory or other factors.

One controversial explanation for stability is that the reservoir is constantly replenished by a low level of *de novo* infection that continues despite ART (134). This replication may reflect inadequate drug levels in certain anatomical sites including the lymph nodes (135, 136) or cell-to-cell spread which is more difficult to block with ART (137). However, multiple lines of evidence indicate that ART effectively curtails new infection of susceptible cells. Because HIV-1 replication is invariably accompanied by the progressive accumulation of mutations (138) reflecting the error prone nature of reverse transcriptase (139) and possibly hypermutation by the host restriction factor APOBEC3G (40, 140-143), the lack of sequence evolution in the viral reservoir (22, 97, 100, 144, 145) indicates that ART blocks ongoing cycles of viral replication. A recent report claiming evolution is confounded by sampling only in the first 6 months of ART, a period during short-lived populations of infected cells not representative of the stable reservoir are dominant (146). Prior to the development of effective ART, a dominant clinical issue was the evolution of drug resistance (147-151), but the incidence of resistance is now decreasing (152-154). Indeed, there are overwhelming clinical data that ART is effective, and treated patients can expect a near normal life expectancy (3, 155-158). As mentioned above, the failure of ART intensification to reduce RV indicates that current ART regimens stop new infection events (103-105). Finally, in the delayed rebound cases mentioned above, HIV-1 persistence during ART cannot be explained by ongoing replication as this would have led to immediate rebound. Therefore, the stability of the latent reservoir is most likely due to the long $t_{1/2}$ of memory T cells and their renewal through proliferation.

Functional studies have shown that memory $CD4^+$ T cell responses in humans can provide lifelong immunity. In individuals who received the smallpox vaccine or cleared

Hepatitis C infection, virus-specific $CD4^+$ T cell responses persist for decades despite the absence of further antigen exposure (159, 160). Early studies of the memory cell lifespan in humans examined radiation-induced chromosomal abnormalities that preclude cell proliferation. This allowed estimates of the intermitotic $t_{1/2}$ of lymphocytes (161, 162). The measured $t_{1/2}$ of 22 weeks for memory T cells is roughly consistent with subsequent *in vivo* measurements using glucose or deuterium labeling which indicate a $t_{1/2}$ on the order of months for human memory $CD4^+$ T cells (163, 164; also reviewed in 165). This is substantially shorter than the $t_{1/2}$ of individual naïve T cells (1-8 years). Importantly, it is shorter than the $t_{1/2}$ of the HIV-1 reservoir (3.7 years) and of functional memory T cell responses (8-12 years). The discrepancy between the half-life of individual memory T cells and the overall memory immune response suggests that proliferation of memory cells must contribute to the stability of the latent reservoir. However, as discussed above, productively infected cells have a very short $t_{1/2}$, and therefore the concept that infected cells can proliferate is not well appreciated. The HIV-1 Vpr protein induces cell cycle arrest at G_2 by interacting with a host E3 ubiquitin ligase (166, 167) and stimulating the degradation of host proteins including the DNA replication factor MCM10 (168). For cells in a latent state of infection, this block to proliferation is not operative, and latently infected cells can, in principle, proliferate if the driving stimulus does not strongly upregulate HIV-1 gene expression (169-172).

Memory $CD4^+$ T cell proliferation can be driven by antigen, cross-reactive recognition of other self or foreign peptides presented with MHC class II, or cytokines. In the murine system, neither antigen nor class II MHC is required for memory T cell persistence (173), although memory $CD4^+$ T cells that persist in the absence of MHC class

II are functionally impaired (174). The requirements for maintenance of human memory $CD4^+$ T cell responses is less clear and could include interactions with cognate signals or cytokines. Little is currently known about the antigen specificity of cells harboring latent HIV-1, although a small subset of them may be HIV-1-specific (175). However, human memory $CD4^+$ T cells exhibit cross-reactivity, and specificities for antigens never encountered can be detected among memory $CD4^+$ T cells in peripheral blood (176). As discussed above, it is expected that stimuli acting through the TCR will upregulate expression of latent HIV-1, but this may not be the case for cytokine-driven proliferation. The cytokines implicated in memory T cell homeostasis and survival are IL-7 and IL-15 (reviewed in 177). IL-7 is required for stimulating homeostatic proliferation of memory $CD4^+$ T cells. Mice deficient in IL-7 (or IL-7R) have severely reduced total T lymphocyte levels and reduced splenic size and cellularity (178). IL-15 also plays a role in homeostatic proliferation of memory $CD4^+$ T cells (179). Early *in vitro* studies indicated that IL-7 can actually induce expression of latent HIV-1 (180, 181). However, in patients on ART, infusion of IL-7 leads to the proliferation of memory $CD4^+$ T cells, including latently infected cells, with little or no induction of HIV-1 gene expression (182, 183). *In vitro* studies in a primary cell model of HIV-1 latency confirm that latently infected cells can proliferate in response to IL-7 (plus IL-2) without upregulation of HIV-1 gene expression (169). These studies suggest that the latent reservoir can be maintained within memory T cells undergoing homeostatic turnover. Analysis of memory T cell subsets has provided additional insight into this issue.

Memory $CD4^+$ T cell subsets

Memory T cells can be divided into two main subsets, central memory (T_{CM}) and effector memory cells (T_{EM}), based on expression of homing and chemokine receptors involved in preferential trafficking to secondary lymphoid organs or peripheral sites, respectively (184). HIV-1 DNA is preferentially harbored in T_{CM} and another subset of memory T cells, transitional memory T cells (T_{TM}) (21). T_{TM} have a phenotype ($CD45RA^-$, $CD27^+$, $CCR7^-$) intermediate between T_{CM} and T_{EM}. The two main subsets of $CD4^+$ memory T cells that harbor latent HIV-1, T_{CM} and T_{TM}, may provide a more stable reservoir for HIV-1 than T_{EM} cells, which have a higher proliferative index and are more susceptible to programmed cell death (21, 185). A recent study using the viral outgrowth assay rather than PCR demonstrated replication-competent HIV-1 persisting in T_{CM} but to a much lesser extent in T_{TM}, indicating that T_{CM} may represent the major source of persistent HIV-1 in most patients (186).

Another recently defined subset of memory $CD4^+$ T cells that may contribute to HIV-1 persistence is the stem cell-like memory T cell subset (T_{SCM}) (187). T_{SCM} are phenotypically similar to naïve T cells (T_N) in that they are $CD45RO^-$, $CD45RA^+$, and $CCR7^+$. However, they also express surface markers characteristic of memory cells, such as CD95 and IL-2Rβ (187). T_{SCM} rapidly respond to antigen and secrete IFN-γ, IL-2, and TNF. They are also stimulated to proliferate by IL-7. A stepwise progression from T_N to T_{SCM} to T_{CM} to T_{EM} has been proposed, with T_{SCM} potentially able to give rise to other types of memory T cells and self-renew upon stimulation. T_{SCM} can be infected with HIV-1 *in vitro*, and in patients on ART, HIV-1 DNA is present in T_{SCM} at higher levels than in other memory subsets (188). Although latently infected T_{SCM} represent only a small fraction of the total reservoir, they may be of particular importance because of their stability and

capacity for self-renewal (23, 188).

In summary, analysis of memory subsets reveals HIV-1 genomes distributed in multiple memory cell subsets, with higher frequencies in subsets with greater potential to survive. Several issues remain. One concern is that many studies of the distribution of HIV-1 genomes in T cell subsets rely primarily on PCR-based measures of the proviral DNA. This is problematic in that the vast majority of proviruses in resting $CD4^+$ T cells from treated patients are highly defective (189). There is also substantial patient-to-patient variability in the distribution of viral genomes within these subsets. Finally, these subsets are not static and can interconvert in ways that are not yet fully understood, and it is therefore unclear whether latent HIV-1 stably persists in a given subset.

Anatomical distribution of the latent reservoir

Most studies of the latent reservoir sample $CD4^+$ T cells from peripheral blood. Given the continuous recirculation and wide tissue distribution of memory T cells, it is generally presumed that latently infected resting $CD4^+$ T cells will be present in most secondary lymphoid organs and in non–lymphoid tissues (190-192). Early studies demonstrated latently infected cells at roughly equal frequency in blood and lymph nodes (12). In the SIV model, latently infected resting $CD4^+$ T cells were demonstrated in blood, lymph node, and spleen (45, 46). Interestingly, as is discussed below, some recently described memory cell populations that are not present in the blood may also contribute to HIV-1 persistence.

HIV-1 can infect follicular helper T cells (T_{FH}) (193-196), and this population has received considerable attention because $CD8^+$ CTL lack chemokine receptors needed for

migrating into B cell follicles (196), thus making the follicles a site of "immune privilege". In the subset of rhesus macaques that spontaneously control SIV, viral replication is restricted to T_{FH}, presumably because $CD8^+$ CTL lyse infected cells elsewhere in the node (196). The extent to which T_{FH} serve as a long term reservoir for HIV-1 in the setting of optimal ART remains to be determined. If latently infected T_{FH} persist, HIV-1 eradication strategies may need to include not only latency reversing agents and stimuli to enhance the $CD8^+$ CTL response (197), but also interventions to disrupt B cell follicles to permit access by CTL (196).

Another population of memory T cells that could potentially harbor latent HIV-1 is the tissue resident memory T cell (T_{RM}) populations (198, 199). Pioneering studies in the murine system demonstrated wide distribution of memory $CD4^+$ T cells, including in non-lymphoid tissues such as liver and lung (190). Subpopulations of memory cells may be generated in or recruited to particular non-lymphoid tissues where they reside for long periods of time (192, 199). These T_{RM} lack expression of CCR7 and share phenotypic and functional properties with T_{EM}. However, unlike other memory subsets, they express CD69, a cell surface lectin that is upregulated at early times following T cell activation. In humans, the majority of T_{EM} cells in lymphoid and mucosal tissues, including lungs and intestines, express CD69 and therefore may be retained in these sites as T_{RM} (191, 200). Human skin also contains significant T_{RM} populations (198). Thus far, T_{RM} have not been directly examined for the presence of latent HIV-1. However, persistent HIV-1 has been detected in gut-associated lymphoid tissue of individuals on ART (201, 202). T_{RM} are prominent in the lamina propria and among intraepithelial lymphocytes, and it is possible that T_{RM} harbor HIV-1. Many $CD4^+$ T_{RM} exhibit activated phenotypes, with reduced surface

expression of CD28 (191), and therefore it is unclear whether latent infection can be established in these cells. Further characterization of tissue-specific reservoirs for HIV-1 is an important research priority.

Evidence for clonal expansion of infected cells

Consideration of the mechanism of memory cell homeostasis suggests that the stability of the latent reservoir is at least partially dependent upon the ability of infected cells to proliferate. Several studies have provided direct evidence for clonal expansion of infected cells, beginning with studies RV (100, 102). Although patients starting ART during chronic infection harbor diverse viral quasispecies (138), the RV is often dominated by identical sequences detected on independent sampling over months to years. The origin of these sequences is unknown but may reflect infection of cells that then proliferate giving rise to multiple progeny cells carrying identical proviruses (100, 102, 203). The fraction of identical HIV-1 sequences within samples from patients on ART increases with time, consistent with proliferation of infected cells (145). More recent studies have used integration site analysis to provide definitive evidence for the proliferation of infected cells. The sites of integration in different cells are generally different and are distributed widely throughout the human genome. Early studies in cell lines infected *in vitro* with HIV-1 (204) and in resting $CD4^+$ T cells from patients on ART (205) revealed a strong preference for integration within active transcriptional units. However, integration occurs in either orientation with respect to the host gene, and there is no consensus sequence at the integration site. Therefore, the precise human sequence at the junction between host and HIV-1 DNA uniquely identifies individual infection events and thus all the clonal progeny

of a single infected cell. In addition, novel deep sequencing analysis allows enumeration of the clonal progeny of a single infected cell within a sample by detection of differences in the random break points in fragments of sheared DNA containing the same integration site (170, 206). Application of this and related approaches to $CD4^+$ T cells from patients on ART has provided dramatic evidence for clonal expansion (170, 171, 207). Maldarelli et al. showed 43% of 2410 integration sites in $CD4^+$ T cells from 5 patients were in clonally expanded cells (170). The finding that multiple cells with the same integration site can be captured in a single blood sample reflects dramatic clonal expansion *in vivo*.

Interestingly, some expanded clones had proviruses integrated in human genes associated with cell growth, and some of these genes have been observed to contain integrated proviruses in multiple independent studies (170, 171, 205, 207, 208). These include myocardin-like protein 2 (*MKL2)*, a transcription factor, and basic leucine zipper transcription factor 2 (*BACH2*), a transcription regulator affecting lymphocyte growth, activation, senescence, and cytokine homeostasis. For these genes, integration events were found in a specific region of the gene and in the same transcriptional orientation as the host gene. This skewed pattern reflects a post-integration selection process that favors the *in vivo* growth and survival of cells with those integration events since these patterns were not seen in *in vitro* infections (170, 171, 204). These results raise the interesting possibility that integration into certain host genes contributes to HIV-1 persistence by stimulating infected cells to proliferate in a manner distinct from homeostatic proliferation. The molecular mechanisms are currently unclear.

A caveat to these studies is that the methods used do not capture the full sequence of the integrated provirus. Some methods capture only the junction between host and HIV-

1 DNA. Given that the vast majority of proviruses are defective, as a result of large internal deletions or APOBEC3G-mediated hypermutation (189, 207), it must be assumed that most expanded clones carry defective proviruses. There is no selective pressure against cells carrying defective proviruses that do not produce viral proteins. Previous studies have described expanded clones carrying defective proviruses, some of which persist for many years (22, 209). However, a recent report has described dramatic *in vivo* expansion of an infected $CD4^+$ T cell clone in a treated patient who also had squamous cell carcinoma (210). Importantly, this clone was capable of producing replication-competent virus. The integration site could not be precisely localized because it was in a region of repetitive sequence. The clone was found widely distributed in sites of metastatic tumor throughout the body, raising the possibility that the clonal expansion occurred in response to tumor antigen. A current issue of great importance is the extent to which expanded cellular clones harbor replication-competent HIV-1.

Implications

The stability of the latent reservoir is the principal reason that HIV-1 infection cannot be cured. The normal mechanisms that maintain immunologic memory provide a simple explanation for this stability. However, the pool of latently infected cells is not static. While the total pool size decreases only very slowly, cells in the reservoir are continually being activated to produce virus that is evident as residual viremia. These cells may die, but homeostatic proliferation of memory cells helps to balance the loss. In addition, a more cell autonomous process of proliferation driven by integration-site dependent alterations in host gene expression may allow some infected cells to undergo dramatic clonal expansion.

Efforts to target the latent reservoir have generally assumed that intervention-dependent reductions in the frequency of latently infected cells will be stable so that repeated interventions will ultimately allow cure. The possibility that subpopulations of infected cells can continue to proliferate may further complicate eradication efforts.

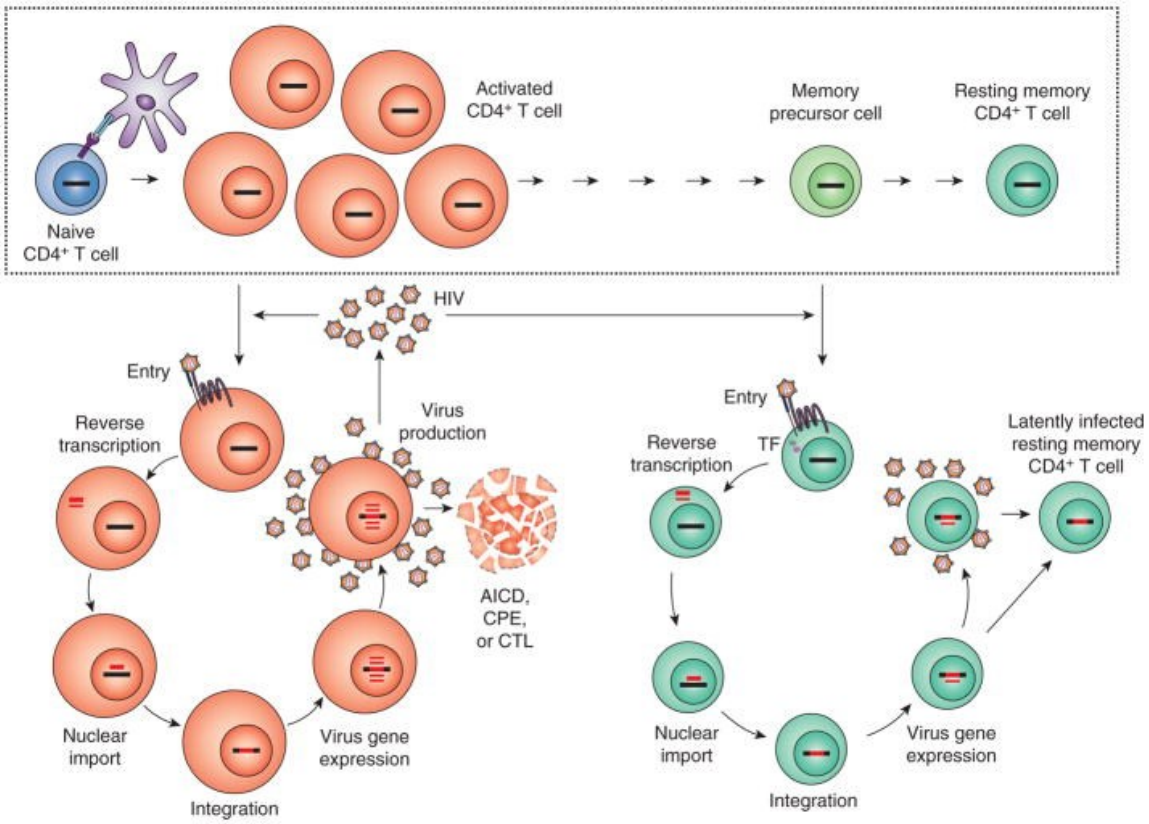

Figure 1.1. Model for the establishment of latent HIV-1 infection in resting memory CD4+ T cells. The normal process of memory cell generation (boxed) involves the exposure of a resting $CD4^+$ T cells to antigen which leads to blast transformation, proliferation, and differentiation into effector cells. Many effector cells die during the contraction phase of the immune response, but a fraction survive and gradually return to a quiescent state as long-lived resting memory cells. Most resting $CD4^+$ T cells lack expression of CCR5, a critical coreceptor for HIV-1 entry. Activation of resting cells by antigen (Ag) upregulates CCR5 expression and reverses other blocks to HIV-1 replication in resting $CD4^+$ T cells, allowing productive infection of these cells. Most productively infected $CD4^+$ T lymphoblasts die rapidly from activation-induced cells death (AICD), viral cytopathic effects (CPE), or lysis by CTL. As activated cells transition back to a resting state, active forms of key host transcription factors needed for HIV-1 gene expression are sequestered. Infection at this stage may lead to latent infection rather than cell death. Other models posit direct infection of resting cells. Please see text for references.

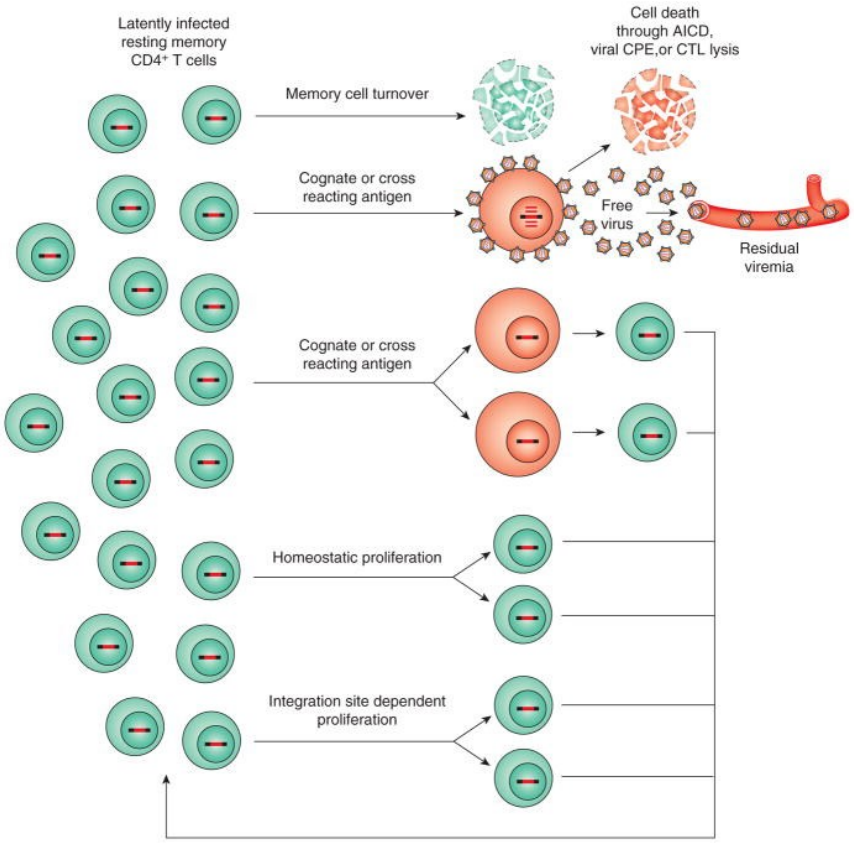

Fig. 1.2. Dynamics of the latent reservoir. ART largely blocks new infection of susceptible cells. In patients on long term ART, the pool of latently infected cells is extremely stable ($t_{½}$ = 3.7 years) so that memory cell turnover must be largely balanced by proliferation of previously infected cells. Latently infected resting memory $CD4^+$ T cells occasionally encounter the relevant cognate antigen (or a cross-reacting antigen) and become activated. Activation reverses latency, allowing viral gene expression and virus production. In patients on ART, the released viruses do not successfully infect new cells, but may be detected at very low levels in the plasma where they constitute the residual viremia (RV). Most productively infected cells die quickly from AICD, CPE, or lysis by CTL. It is possible that some degree of antigen-driven proliferation may occur without activation of viral gene expression. Homeostatic proliferation of memory cells may also occur without reactivating viral gene expression. For some infected cells, integration of the provirus into genes associated with cell growth may also stimulate proliferation. See text for references.

CHAPTER 2: HIV Persistence: Clonal Expansion of Cells in the Latent Reservoir

Kyungyoon J. Kwon and Robert F. Siliciano. HIV Persistence: Clonal Expansion of Cells in the Latent Reservoir. *Journal of Clinical Investigation 2017 Jun 30;127(7):2536-2538.*

In response to:

Lee GQ, Orlova-Fink N, Einkauf K, et al. Clonal expansion of genome-intact HIV-1 in functionally polarized Th1 CD4+ T cells. *J Clin Invest*. 2017;127(7):2689-2696.

Targeting the latent HIV-1 reservoir: challenge for a cure

Despite the efficacy of antiretroviral therapy (ART) in suppressing HIV-1 replication, there is still no cure for HIV-1 infection due to the presence of a latent reservoir for the virus (1). This latent reservoir consists mainly of resting memory $CD4^+$ T cells harboring integrated HIV-1 proviruses. The reservoir has an extremely slow decay rate such that viral eradication by ART alone is not possible (1). Recent studies have shown that one of the major mechanisms of reservoir persistence is the clonal expansion of these latently infected cells. Initial evidence for this route of persistence came from the independent detection of multiple proviruses with exactly the same viral sequence or integration site within host cells (2–6). Full genome sequencing studies have established that most proviruses present in resting $CD4^+$ T cells are defective (6–8), and thus many of these proviruses are unlikely part of the latent reservoir. Nevertheless, recent work from several groups has demonstrated that cells carrying replication-competent proviruses can also clonally expand in vivo (9–12). The proliferation of cells carrying intact, replication-competent proviruses is a troubling finding that helps explain the stability of the HIV-1 reservoir and raises concerns about the feasibility of eradication.

Growing evidence for clonal expansion

Clonal expansion is a basic aspect of normal T cell biology. Antigen-driven proliferation and cytokine-driven homeostatic proliferation are well established as mechanisms by which T cell populations expand(13). However, it has been less clear as to whether or not infected $CD4^+$ T cells can proliferate, especially as productively infected cells have a very

short in vivo half-life (14,15). Early evidence for clonal expansion of HIV-1-infected cells came from studies of residual viremia, the trace level of free virus detectable in the plasma of treated patients with special methods. Despite the extensive viral sequence diversification that occurs over time in untreated patients, the residual viremia observed once patients start a suppressive ART regimen is often surprisingly oligoclonal, suggesting that it is produced by expanded cellular clones carrying exactly the same proviruses(16,17). However, an alternative explanation for viral sequence identity is that multiple cells are infected by a single dominant viral variant. Definitive proof that clonal expansion of infected cells has occurred can be obtained by demonstrating that these cells carry the same proviral sequence integrated at the same exact position in the human genome. After the development of next-generation sequencing technologies that allowed for efficient integration site sequencing, several groups reported the detection of expanded clones based on the presence of identical integration sites (3-5); however, integration site analysis captures only the very end of the viral genome. Given that the majority of proviruses are defective, these studies establish the clonal expansion of infected cells but do not necessarily identify those carrying intact viral genomes. One interesting concept to come out of these studies was the idea that proviral integration into particular host genes might alter expression of those genes in a way that promotes cell proliferation and/or survival (2,3).

Recently, several groups have provided evidence for the clonal expansion of cells carrying replication-competent proviruses (9–12). Simonetti and colleagues identified an integration site associated with a single, dominant $CD4^+$ T cell clone carrying a replication-competent provirus in an HIV-1-infected patient with squamous cell carcinoma (9).

Interestingly, the clonally expanded T cells were found at sites of the disseminated malignancy in this patient, raising the possibility of antigen-driven expansion. In addition, three groups recently showed that multiple $CD4^+$ T cells carrying identical, replication-competent proviruses are often present in a single blood sample from treated patients (10–12). In all three studies, the fraction of viral isolates that had sequence exactly matching another independent isolate from the same sample was over 50%. With additional sampling, it is likely that matching sequences could be found for most if not all of these isolates. Together, these findings suggest that the majority of cells comprising the latent reservoir are generated by the proliferation of a smaller number of previously infected cells rather than by direct infection. These results do not support the idea that de novo infection events are a major factor in reservoir stability as has been recently suggested (18). Rather it appears that the remarkable stability of the latent reservoir is largely due to cellular proliferation.

How can we reconcile the strong evidence for proliferation with the short in vivo half-life of productively infected cells? In the studies described above, the clonal T cell populations carrying replication-competent proviruses were demonstrated using variations of the viral outgrowth assay in which limiting dilutions of resting $CD4^+$ T cells are activated with a mitogen and replication-competent viruses are allowed to grow out. In one study, restimulation of cultures that were negative for viral outgrowth, despite uniform T cell activation with mitogen, resulted in additional outgrowth of replication-competent viruses[12]. These findings show that $CD4^+$ T cells carrying replication-competent proviruses can proliferate without producing virus while retaining the ability to do so upon subsequent stimulation[12]. Thus, proliferation need not be limited by the short half-life of productively

infected cells. It is unclear what prevents some latent proviruses from being induced upon cellular activation. The fact that cells carrying replication-competent proviruses can multiply while evading immune recognition presents a major challenge to current cure strategies and attempts to eradicate the latent reservoir.

The role of proliferation in persistence of the reservoir

The normal decay of cells in the latent reservoir may be balanced by proliferation of cells with latent provirus, thus resulting in the long observed half-life of the reservoir (19,20). However, the dynamics of various subpopulations of $CD4^+$ T cells in the context of HIV-1 infection are not well understood, and most studies are limited to peripheral blood samples. More studies on the proliferation of various populations of infected cells in tissue compartments would be beneficial in determining the natural dynamics of the latent reservoir. Latent proviruses are present in various $CD4^+$ T cells subsets (13,21,22). For example, previous studies from Lichterfeld and colleagues have described the presence of proviral DNA in a small, long-lived population of $CD4^+$ memory T cells with stem cell properties (21). In this issue, Lee et al. apply a full genome sequencing approach to evaluate functional subsets of $CD4^+$ T cells (23). Cells were sorted based on the production of signature, subset-defining cytokines, and cellular DNA was subjected to full genome sequencing of the proviruses present. Consistent with previous studies, Lee et al found that the vast majority of proviruses are defective. They also determined that independent sequences that were identical to other sequences from the same patient comprise over half of all intact sequences detected, consistent with previous studies of replication-competent isolates[23]. Importantly, Lee et al. report that there is an enrichment of clonally expanded

intact proviruses in functionally-polarized Th1 cells (23) as depicted in Figure 1, adding to Lichterfeld and colleagues' previous findings of enriched integration in Th1 cells (22). While clonal expansion of cells harboring defective proviruses was also observed, intact proviruses were enriched in circulating Th1-polarized cells. Lee and colleagues propose that the virus may exploit the relatively high proliferation rate of Th1 cells to propagate via the division of infected host cells (23). It would be of interest to compare the proliferation and dynamics of infected and uninfected Th1 cells to further assess the role of proliferation of specific functional subsets on viral persistence. If the reservoir is largely maintained by natural host physiological mechanisms, it may be difficult to develop cure strategies that block proliferation without affecting normal T cell homeostasis.

Concluding remarks

Overall, the study by Lee et al. adds to the growing consensus that persistence of the latent reservoir of HIV-1 may be due in large part to the clonal expansion of cells harboring intact, replication-competent virus. Both homeostatic, cytokine-driven proliferation and antigen-stimulated proliferation may contribute to viral persistence, and both forms of proliferation have been shown to occur without reactivation of latent proviruses (12,24). In either case, it may be difficult to block proliferation and reduce the size of the latent reservoir without creating negative consequences for patients. Here, methods to selectively block proliferation may be key. A growing number of studies and several clinical trials are evaluating immunosupressive agents that may block proliferation and decrease the number of cells carrying potentially infectious proviruses. Recent clinical studies involve immunosuppressants such as tacrolimus or sirolimus that are usually administered in the

setting of organ transplantation. There is evidence for decreased levels of HIV-1 DNA in patients treated with some immunosuppressants (25). Further investigation of the direct effects of immunosuppressants on the reservoir are needed to determine whether blocking proliferation might be a possible adjunct strategy in the search for a cure.

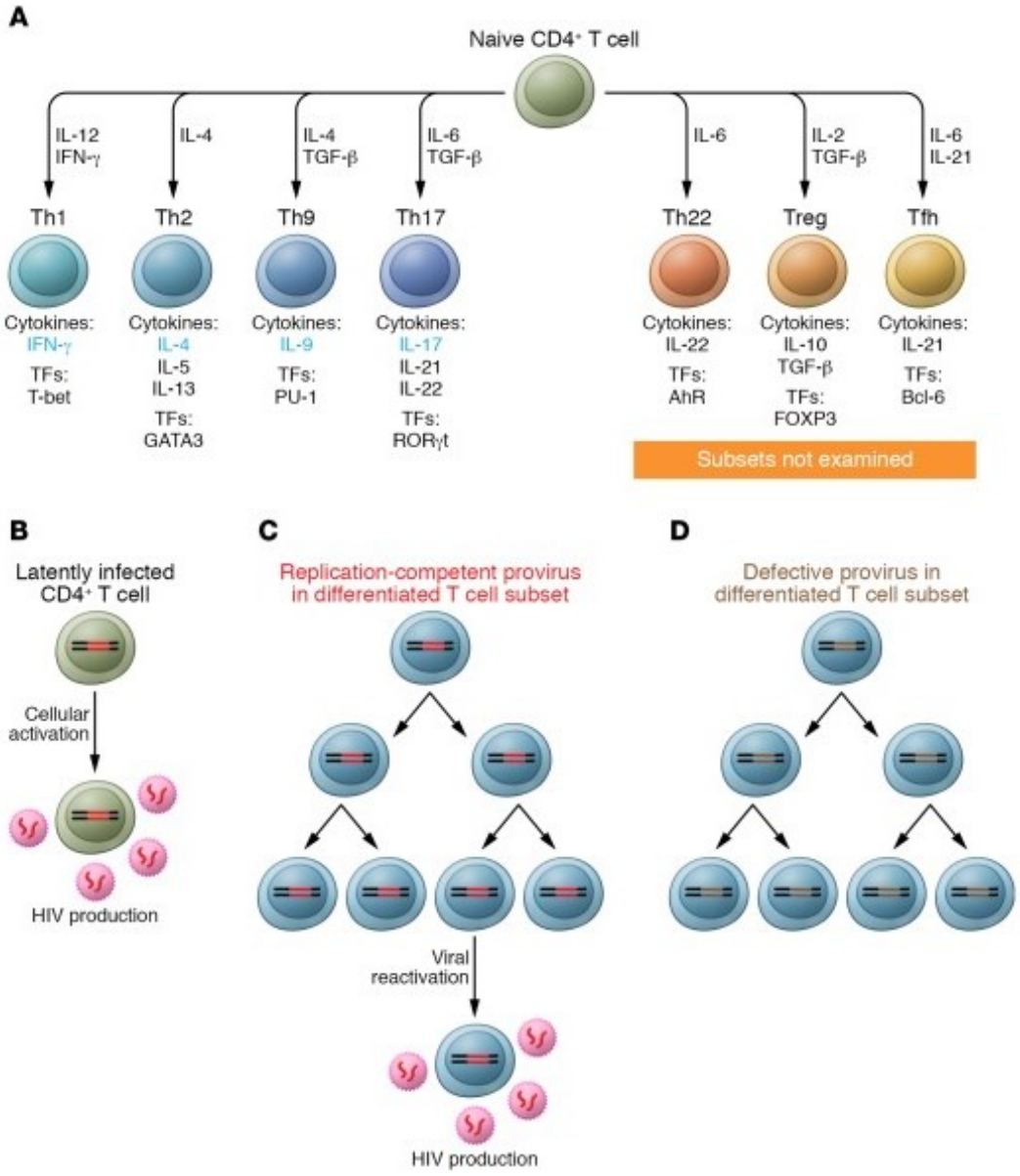

Fig. 2.1. Intact, clonally expanded HIV-1 proviruses in Th1 subpopulation. (A) CD4+ T cells differentiate into functional subsets, each of which is characterized by a defining transcription factor (TF) and a set of cytokines produced following activation. In this issue, Lee et al. sorted peripheral blood mononuclear cells from 3 cART-treated patients into CD4+ T cell subpopulations based on production of 4 signature cytokines (blue). Subsets not examined are labeled as such. Cellular activation induces virus production from latently infected CD4+ T cells (B). Differentiated T cell subsets carrying replication-competent (C) or defective (D) proviruses can also be stimulated to proliferate. In the patients studied by Lee et al., clonally expanded, intact proviral sequences were predominantly in the largest subset, Th1 cells. See Supplemental Table 3 in ref. 23. Tfh, T follicular helper.

CHAPTER 3: Proliferation of Latently Infected CD4+ T cells Carrying Replication-competent HIV-1: Potential Role in Latent Reservoir Dynamics

Hosmane NN, **Kwon KJ,** Bruner KM, Capoferri AA, Beg S, Rosenbloom DI, Keele BF, Ho YC, Siliciano JD, Siliciano RF. Proliferation of latently infected CD4+ T cells carrying replication-competent HIV-1: Potential role in latent reservoir dynamics. *Journal of Experimental Medicine, 2017 Apr 3;214(4):959-972.*

Abstract

A latent reservoir for HIV-1 in resting $CD4^+$ T-lymphocytes precludes cure. Mechanisms underlying reservoir stability are unclear. Recent studies suggest an unexpected degree of infected cell proliferation *in vivo*. T-cell activation drives proliferation but also reverses latency resulting in productive infection that generally leads to cell death. We show that latently infected cells can proliferate in response to mitogens without producing virus, generating progeny cells that can release infectious virus. Thus, assays relying on one round of activation underestimate reservoir size. Sequencing of independent clonal isolates of replication-competent virus revealed that 57% had *env* sequences identical to other isolates from the same patient. Identity was confirmed by full genome sequencing and was not attributable to limited viral diversity. Phylogenetic and statistical analysis suggested that identical sequences arose from *in vivo* proliferation of infected cells rather than infection of multiple cells by a dominant viral species. The possibility that much of the reservoir arises by cell proliferation presents challenges to cure.

Introduction

A stable latent reservoir for HIV-1 in resting memory $CD4^+$ T cells persists despite antiretroviral therapy (ART)[1-9]. The extremely long half-life of this reservoir is a major barrier to cure[6-9]. This reservoir of latent but replication-competent HIV-1 was originally identified in resting $CD4^+$ T cells in the blood and lymph nodes[1,2], but known patterns of circulation, activation, and differentiation of memory T cells predict that persistent HIV-1 resides in multiple memory cell subsets in multiple tissues[10-14]. The latent reservoir is a major target of cure efforts, some of which focus on reversing latency so that infected cells can be eliminated by immune mechanisms[15-18].

One potential explanation for the remarkable stability of the latent reservoir involves the proliferation of infected cells[10,19-25]. Proliferation of infected cells is to some extent unexpected. Some stimuli that drive T cell proliferation also drive latently infected cells into a productively infected state, and productively infected cells have a very short half-life (1 day)[26,27]. In addition, the HIV-1 Vpr protein causes cell cycle arrest[28-35]. In some model systems, cytokines including IL-7 and IL-15 can drive homeostatic proliferation of $CD4^+$ T cells without inducing virus gene expression[21,36]. However, IL-7 can also reverse latency in some systems[37,38].

Despite the issues raised above, there is considerable evidence that infected cells can proliferate *in vivo*. The evidence comes in two forms. In patients who start antiretroviral therapy during chronic infection, extensive viral sequence diversification takes place prior to treatment[39,40], making it unlikely that multiple independently sampled viral sequences from a single patient will be identical. Therefore, repeated isolation of identical viral sequences from individual patients can be most readily explained by

assuming that an initially infected cell carrying the sequence subsequently proliferated, copying the integrated viral genome without error into progeny cells. Sequencing of trace levels of plasma virus present in treated patients initially provided the surprising result that this residual viremia was often dominated by a single frequently isolated sequence[19,20]. Subsequent studies of proviral DNA also revealed independent identical sequences[20,25,41,42]. Although these studies strongly suggest *in vivo* proliferation of infected cells, there are caveats. Isolates that are identical in the sequenced part of the genome may differ elsewhere and not be clonal[43] or may represent separate infection events with an identical virus. Furthermore, the vast majority of proviruses are defective[42,44,45], and without full genome sequencing[44] or viral outgrowth assays[25], it remains unclear whether the identical sequences represent replication-competent virus. An important recent study by Lorenzi et al. has examined a large number of independent isolates of replication-competent virus from treated patients and found that over 50% share sequence identity in the *env* gene with other isolates from the same patients[25].

Definitive evidence for the proliferation of infected cells' virus has come from an experimental approach involving the analysis of HIV-1 integration sites. Recent studies have demonstrated that within a given patient, a surprisingly large fraction of infected $CD4^+$ T cells shows proviral integration into precisely the same position in the human genome[22,23,46]. Given the relatively non-specific integration of HIV-1 into expressed genes throughout the genome[47,48], this result can only be explained by the proliferation of infected cells after integration. Interestingly, some of the expanded clones showed integration into cellular genes associated with cell survival and/or proliferation, raising the possibility that altered host gene expression could drive proliferation[22,23]. However, integration site

analysis captures only the ends of the viral genome, and it is likely that most of the expanded cellular clones detected by integration site analysis are replication defective, as has been shown by Cohn et al. [46]. Unfortunately, with current methods, it is difficult to simultaneously and efficiently obtain both the integration site and the full proviral sequence. Interestingly, a recent report by Simonetti et al. has described a massively expanded $CD4^+$ T cell clone carrying replication-competent virus in a patient with a complex disease course[24].

Taken together, the above results raise the interesting possibility that the proliferation of infected cells may contribute to the stability of the latent reservoir. To explore this issue, we have carried out *ex vivo* stimulations and single genome analysis to look for evidence of clonal expansion in infected individuals on ART.

Materials and Methods

Study Subjects. Subjects were HIV-1 infected adults who met the inclusion criteria of suppression of viremia to <20 copies HIV-1 RNA/ml of plasma on ART for >6 months. This study was approved by the Johns Hopkins Institutional Review Board. Written informed consent was obtained from all subjects.

Multiple Stimulation Viral Outgrowth Assay (MS-VOA). The MS-VOA was performed on purified resting $CD4^+$ T cells as described previously[44,50,51] with modifications to allow 4 consecutive stimulations with PHA (Fig. 1a,b). Resting $CD4^+$ T cells were isolated from PBMC using a two-step negative selection protocol[51] with monoclonal antibodies and magnetic beads (Miltenyi Biotec) as previously described and plated in the upper chambers of 12-well transwell plates (Corning) at a predetermined limiting dilution for viral outgrowth (200,000 cells per well). Cells were activated with PHA (0.5 ug/mL) and

irradiated allogeneic PBMC from uninfected donors as previously described[1-3]. The following day, half the media from each transwell was removed and replaced with fresh media lacking PHA, and then 10^6 MOLT-4/CCR5 cells[3] were added to the bottom chamber of the transwell to allow robust replication of virus released from infected cells. Previous studies have shown that viral outgrowth in this transwell system is equivalent to that seen with direct co-culture[44]. MOLT-4/CCR5 cells were obtained from the NIH AIDS Reagent Program and were maintained in G418 until use in the assay. The cells tested for CCR5 expression by flow cytometry and were negative for mycoplasma. Eight days after the initial PHA stimulation, half of the volume from both the top and bottom chambers of each transwell was transferred to a new set of transwell plates for a second round of PHA stimulation. The initial plates were cultured without further stimulation for a total of 21 days. The restimulated plates were cultured for 8 days following the second round of PHA stimulation and then split as above to generate a third set of plates, which received a third round of stimulation. Similarly, these plates were split 8 days later to generate the fourth set of plates, which received a fourth round of stimulation. A p24 ELISA (PerkinElmer) was performed on the supernatant 21 days after each respective round of PHA stimulation (Fig. 1b).

CFSE dilution and activation/exhaustion marker staining. An aliquot of resting $CD4^+$ T cells was stained with 5 uM CFSE prior to the initial PHA stimulation. The dilution of CFSE was analyzed 1 week later by flow cytometry using a 488 nm laser on a BD FACSCANTO II cytometer (BD Biosciences) with an emission of 492⁄517 nm. Unstimulated cells served as a control. Expression of activation markers was analyzed 1 week after each round of PHA stimulation. An aliquot of cells was stained with anti-CD4

(FITC), anti-CD25 (APC), anti-CD69 (APC), and anti-HLA-DR (APC) antibodies (BioLegend, San Diego, CA) at 4º C for 15-30 minutes and analyzed by flow cytometry on BD FACSCANTO II cytometer (BD Biosciences). Cells that did not receive the most recent round of PHA stimulation served as controls. To assess changes in expression of inhibitory receptors, cells were stained a week after each round of PHA activation with anti-PD1 (FITC), anti-CTLA-4 (PE), and anti-Tim-3 (P3/Cy7) antibodies (BioLegend, San Diego, CA) and analyzed by flow cytometry.

RNA isolation, cDNA synthesis and amplification of the env gene. Viral RNA was isolated from 200 µL of the supernatant from each $p24^+$ well using a ZR-96 Viral RNA Kit™ (Zymo Research Corporation, Irvine, CA). RNA was then treated with DNase (Life Technologies, Carlsbad, CA) and reverse transcribed using the qScript cDNA Supermix kit (Quanta Biosciences, Gaithersburg, MD). Since the cultures were seeded at limiting dilution for viral outgrowth, we ran a nested PCR on undiluted cDNA from each well. A nested PCR for the V3/V4 region of *env* was performed using 600 ng of cDNA and primers ES7 (CTGTTAAATGGCAGTCTAGC) and ES8 (CACTTCTCCAATTGTCCCTCA) for the outer reaction. The outer PCR products were diluted 1:50 and 5 µL of this dilution were used for the inner PCR reaction with primers Nesty8 (CATACATTGCTTTTCCTACT) and DLoop (GTCTAGCAGAAGAAGAGG). Primers were obtained from Integrated DNA Technologies (Coralville, IA). Amplification conditions were as follows: denaturation at 94ºC for 3 min, followed by 40 cycles of denaturation at 94ºC for 30 sec annealing at 55ºC for 30 sec, and extension at 68ºC for 5 min. PCR products were run on a 1% agarose gel, and bands were extracted using the QIAquick Gel Extraction Kit (Qiagen,

Hilden, Germany). Extracted DNA was analyzed directly by Sanger sequencing at Genewiz, Inc. (Frederick, MD).

Amplification of the env gene from proviral DNA. Nested PCR carried out at limiting dilution was used to analyze the *env* gene of proviruses in resting $CD4^+$ T cells from study subjects. Amplification was carried out as previously described[20], except the outer PCR primers were the ES7/ES8 described above. 1 μL of the outer PCR product was used for the inner PCR reaction, with the Nesty8/DLoop primers described above. PCR products were directly sequenced as described above.

Full genome sequencing. For representative isolates, viral RNA was sequenced by single genome amplification as previously described[54]. Viral RNA in the supernatants of $p24^+$ wells was extracted and reverse transcribed. cDNA was serially diluted and amplified with nested PCRs in two overlapping half genome reactions as described[705]. For each isolate, 6-12 amplicons obtained at the limiting dilution were directly sequenced and a full genome consensus sequence was generated as described in the text.

Phylogenetic analysis. Forward and reverse sequences for each sample were aligned into a single consensus contig per sample using default assembly parameters on CodonCode Aligner software (CodonCode Corporation). Rare sequences that did not appear clonal were discarded. Each sample consensus sequence was aligned with reference sequences of catalogued viruses from the Los Alamos National Laboratory 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. Genetic distances were calculated and neighbor joining trees[71] were generated using a maximum composite likelihood algorithm and default parameters

using MEGA7 software (Molecular Evolutionary Genetics Analysis Program)[72] Maximum likelihood trees were also generated. The conclusions were not sensitive to the method of tree generation, and neighbor joining trees are shown in the figures.

Data availability. Sequences are available through Genbank (Accession numbers pending).

Statistical test for clonal proliferation. We designed a statistical analysis based on coalescent theory to explore the possibility that the high degree of sequence identity observed could be explained merely by rounds of viral replication in which no mutation occurred. The test was applied to a subset of study participant (01, 03, 10, 11, and 12) for whom a single large set of identical isolates that were phylogenetically distinct from smaller sets and unique individual sequences (singletons) from the same participant (Figure 3). For the other participants, the test could not be readily applied because there were either no singletons (02), no gap between small and large sets (05, 06), or no unique large clone after the gap (09). For each patient, the mutation parameter estimate $\hat{\theta}$ was chosen to maximize the likelihood of observing the configuration according to Ewens' sampling formula for the coalescent[58]. This calculation provides the nested model likelihood for the statistical test, (i.e., likelihood without clonal proliferation). Clonal proliferation is assumed to alter the isolate set configuration by taking one sequence and increasing its multiplicity, by a mechanism other not present in the standard coalescent model. To model this behavior, we suppose that the proliferating sequence is (one of) the most frequent sequences observed, and that it effectively replaced a number of singleton sequences that would have otherwise been observed in the sample. To maximize the likelihood, there are now two parameters: the mutation parameter (as in the nested model), and the number of sequences that this largest clone effectively replaced. We again use Ewens' sampling

formula, but on an isolate set configuration that is modified to reverse the effect of this supposed replacement. In other words, the largest clone is shrunk, and singleton sequences are added to keep the total sample size the same. The mutation parameter estimate $\hat{\theta}$ and log-likelihood are computed for this modified configuration. A p-value is obtained using the likelihood ratio test with one degree of freedom.

Results

Analysis of infected cell proliferation using a multiple stimulation viral outgrowth assay

Given that productively infected cells have a short *in vivo* half-life (1 day)[26,27], we hypothesized that proliferation of latently infected cells carrying replication-competent HIV-1 could take place without release of infectious virus. To test this hypothesis, we subjected resting $CD4^+$ T cells from patients on long term ART to multiple rounds of mitogen stimulation in order to detect virus release from cells that had proliferated in response to a previous stimulation without producing infectious virus. This assay, modified from the standard quantitative viral outgrowth assay (QVOA)[2,3,6,8,49-51] used to measure the frequency of latently infected cells, is described in Figure 1. Limiting dilutions of purified resting $CD4^+$ T-cells were maximally stimulated with the potent T cell mitogen phytohemagglutinin (PHA) and irradiated allogeneic peripheral blood mononuclear cells (PBMC) from uninfected donors. CCR5-transfected MOLT4 cells (MOLT4/CCR5)[50] were added to expand virus released from cells in which latency was reversed. Patient cells and MOLT4/CCR5 cells were plated in separate chambers of transwell plates. This allows separate manipulation of patient cells while giving T-cell activation and viral outgrowth equivalent to standard co-cultures[44]. After 8 days, half the

contents of each well were transferred to the corresponding chambers of new plates which received an additional stimulation. The original plates were cultured without the additional stimulation for a total of 21 days (Fig. 1b). A total of four sequential stimulations were performed in this manner. Each well was cultured for 21 days after the most recent stimulation, sufficient time to allow virus released from a single cell to grow exponentially in MOLT4/CCR5 cells to levels readily detectable by p24 ELISA[50]. Viral RNA in the supernatants of $p24^+$ wells was then subjected to sequence analysis. This protocol allows assessment of whether cells that have previously proliferated in response to T cell activation without producing infectious virus can release virus with additional stimulation (Fig. 1b).

The initial stimulation caused >99% of patient resting $CD4^+$ T cells to proliferate (Fig. 1c). Cell number increased 8-fold during the first week with smaller increases after the 2nd, 3rd, and 4th stimulations (3.5-, 2.5-, and 2-fold respectively; Fig. 1d). The initial stimulation also caused >99% of the cells to express T cell activation markers (Fig. 1e). Although cells did not return to a fully quiescent state before the 2nd, 3rd, and 4th stimulations, each stimulation increased activation marker expression relative to cells that did not receive the most recent stimulation (Fig. 1e).

This multiple stimulation viral outgrowth assay (MS-VOA) was performed on purified resting $CD4^+$ T cells from 12 participants (01-12) on long-term suppressive ART (see Supplementary Table 1 for patient characteristics). The mean frequency of latently infected cells detected after with the first stimulation was 0.83 infectious units per million (IUPM) resting $CD4^+$ T cells, not significantly different from that observed in studies using the standard QVOA[6,8,52]. However, for every subject, the additional rounds of stimulation

caused viral outgrowth in cultures split from wells that remained negative for viral outgrowth without the additional stimulation (Fig. 2a). Because all cells proliferate in response to PHA (Fig. 1c), this result indicates that some latently infected cells proliferated without releasing infectious virus but retained the capacity to do so following subsequent stimulation. These results are consistent with previous work showing that a single round of stimulation in the standard QVOA does not detect all the latent virus present[44] and with studies in a primary cell model showing that cytokine-driven homeostatic proliferation of latently infected cells can occur without upregulation of HIV-1 gene expression[21].

These studies provided insight into the relationship between T cell activation, proliferation, and latency reversal. By splitting cultures of proliferating $CD4^+$ T cells, it is possible to determine whether additional stimulation can induce virus production when other cells from the same clonal population fail to produce virus after the initial stimulation. Of all cultures that eventually become positive for outgrowth, only an average of 60% were detected after the first stimulation (Fig. 2b). In 11 of 12 patients, two rounds of stimulation were insufficient to induce all of the proviruses that were ultimately induced with the third or fourth rounds (Fig. 2a, b). These results illustrate the difficulty in purging the latent reservoir even with maximum T cell activation. Multiple rounds of maximal stimulation are likely to be required. However, in 4 of 12 participants, 70-80% of isolates were obtained in the first round of stimulation. Interestingly, because cell number increased with each stimulation (Fig. 1d), the per-cell probability of outgrowth fell after the first stimulation (Fig. 2c). This may reflect differences in proviral inducibility, with a readily induced population and a generally smaller population that is more difficult to induce. Alternatively, changes in the transcriptional environment with repetitive stimulation during

long term *in vitro* culture might prevent induction of some intact proviruses that could be induced *in vivo*. To address this possibility, we examined $CD4^+$ T cell expression of surface proteins associated with functional inhibition or immune exhaustion[53], including PD-1, CTLA-4, and Tim-3. We observed increased Tim-3 expression after multiple stimulations (Fig. 2d). Exhaustion of the cells in culture may contribute to the consecutive decreases in the probability of outgrowth. Thus, the MS-VOA provides only a minimal estimate of latent reservoir size because immune exhaustion developing with repetitive stimulation and/or long term *in vitro* culture may not allow outgrowth from all potentially inducible replication-competent proviruses. The frequency of latently infected cells detected with the MS-VOA was ~2 fold greater than the standard QVOA value measured after one round of stimulation (Fig. 2e). The actual frequency of latently infected cells is likely to lie between the MS-VOA measurement and the total number of intact (non-defective) proviruses (Fig. 2f). Together, these results demonstrate that latently infected cells carrying replication-competent HIV-1 can proliferate in response to *ex vivo* stimulation without producing infectious virus while retaining the ability to do so subsequently.

Independent isolation of identical sequences of replication-competent virus from the latent reservoir

The ability of latently infected cells to proliferate *ex vivo* without releasing virus suggests that *in vivo* clonal expansion of infected cells could maintain the latent reservoir. If expanded cellular clones comprise a significant fraction of the latent reservoir, then it should be possible to obtain from individual patients independent isolates of replication-

competent virus with identical sequence throughout the viral genome. We were able to directly test this prediction in a unique way because of the large number of independent isolates of replication-competent HIV-1 obtained at limiting dilution following different rounds of stimulation in the MS-VOA. We first amplified by RT-PCR the highly variable V3-V4 region of the *env* gene from viral RNA in supernatants of all p24+ wells from the MS-VOA from all 12 study subjects. Because the cells were initially plated at limiting dilution for viral outgrowth, most positive wells contained only a single sequence, and wells with multiple sequences were discarded. Sequences from each subject clustered together and separately from other subjects in phylogenetic analysis (Supplementary Fig. 1). Although all participants started ART during chronic infection (Supplementary Table 1), 9 of 12 subjects had one or more sets of independent isolates with identical sequence in the highly variable V3-V4 region of the *env* gene (Fig. 3). Sets of isolates with identical *env* sequences were seen in 9 of 9 patients from whom >10 isolates were obtained, strongly suggesting this phenomenon is general. It is important to note that isolates with identical *env* sequences are not the result of *in vitro* proliferation - all of these isolates originated from different wells of the original tissue culture plates (Fig. 1b), indicating they are derived from different infected cells present *in vivo*. Of 197 independent isolates from 12 subjects, 113 (57%) belonged to sets of isolates with identical *env* sequences while the remaining 43% had unique *env* sequences (Fig. 3).

Isolates with identical *env* sequences are identical throughout the HIV-1 genome

These results are consistent with the idea that the majority of latently infected cells arise from proliferation of a smaller number of previously infected cells. However, alternative

explanations must be excluded. First, it is possible that isolates with identical *env* sequences differ elsewhere in the genome[43]. To address this possibility, we first determined the Clonal Prediction Score (CPS), a measure of the ability of subgenomic amplicons to predict clonality[43]. For this *env* amplicon, the CPS was 96, meaning that 96% of the time sequences identical in this region are identical throughout the entire viral genome based on available full genome sequences from viral outgrowth assays. To provide direct experimental evidence that isolates with identical *env* sequences were identical throughout the entire HIV-1 genome, we used single genome analysis of genomic viral RNA in the supernatants of $p24^+$ wells to obtain the full genome sequences of representative isolates belonging to sets of isolates with identical *env* sequences. The sequencing was carried out on two overlapping half-genome fragments. We were able to produce full genome sequences from the half-genome sequences because the overlap regions were identical, and more importantly, because only a single provirus gave rise to outgrowth in these limiting dilution cultures. Although defective proviruses may have been present in these cultures, the profound nature of the commonly observed defects[42,44,45] would preclude virion release into the supernatant. For each isolate tested, 6-12 single genome sequences were obtained at limiting dilution from RNA in the supernatant. To avoid PCR errors, PCR products were sequenced directly without cloning[54]. Sequences for each isolate were identical or very similar (Fig. 4a), with 1-4 nucleotide differences likely representing the expected variation[55] arising during the three week culture period used to obtain each isolate (Fig. 1b). Importantly, single genome sequences from different isolates belonging to a given set with identical *env* sequences were intermingled in phylogenetic analysis (Fig. 4a). The genetic distances between sequences from the same

isolate and from other isolates belonging to the same set were not significantly different and were much smaller than the genetic distance between sequences from a given set and other isolates from the same subject (Fig. 4b). The consensus half-genome sequences for each isolate were used to construct full genome sequences. Phylogenetic analysis of full genome sequences again showed that isolates with identical *env* sequence clustered together in a monophyletic pattern (Fig. 4c). Together, these results demonstrate that isolates with identical *env* sequence are identical throughout the genome.

Dominant viral sequence vs. clonal expansion

There are two explanations for the presence in infected individuals of many infected cells with an identical viral sequence. Either all of the cells were infected by a predominant uniform virus population without mutation or a single cell carrying a particular viral sequence proliferated extensively after infection, copying the viral genome without error into daughter cells. The first explanation might apply during acute infection before diversification occurs. However, participants in this study initiated ART during chronic infection (Supplementary Table 1) and are thus expected to have extensive viral sequence diversity[39,40,56]. To demonstrate this diversity, we used single genome amplification of DNA from uncultured resting $CD4^+$ T cells to obtain sequences of the *env* genes of proviruses from the same patients. These sequences were displayed on phylogenetic trees together with *env* sequences from the replication-competent isolates describe above. This analysis demonstrated that the replication-competent isolates were part of much more complex populations of proviruses in each subject (Fig. 5). Many of these proviruses may have defects elsewhere in the genome and thus will not necessarily show a close

phylogenetic relationship to the replication-competent isolates. Together these results show that the isolation of multiple independent clones of replication-competent virus with identical sequence is not due to a lack of sequence diversity in these individuals.

To examine the possibility that isolates with identical sequence arose from infection of many cells by a dominant virus population, we first examined the genetic distance between isolates from the same subject (Fig. 6). This was done using the *env* sequences obtained from viral RNA in the supernatants of all clonal $p24^+$ wells from the MS-VOA (Fig. 3). If within a given subject a substantial fraction of the latent reservoir is generated by infection of many cells by a dominant viral species, then sequences close to the dominant species should also be present. Based on the error rate of reverse transcriptase[55,57], a single a base substitution is expected in 15-30% of cells infected by this dominant viral species. Other closely related sequences should be generated as this infection spreads, resulting in many sequences close to the dominant sequence. We therefore examined the distribution of genetic distances between independent isolates to determine whether isolates close in sequence to the dominant viral species were present. As is illustrated in Figure 6, the observed distribution of intra-subject genetic distances does not support this hypothesis of infection of many cells by a dominant viral species. There is in fact a striking paucity of variants close in sequence to the sets of identical sequences.

To further explore the possibility that the high fraction of independent isolates with identical sequence results from infection of many cells by a dominant viral species, we focused on five subjects (S01, S03, S10, S11, and S12) for whom there was is a single large set of identical isolates that was phylogenetically separate from other isolates or sets of

isolates from the same subject. We designed a statistical analysis based on coalescent theory to explore the possibility that the high degree of sequence identity observed could be explained merely by rounds of viral replication in which no mutation occurred. The data for the statistical test is the configuration of sets of identical isolates, that is, the number of sets of a given size containing identical *env* sequences (Table 1). For each patient, the mutation parameter estimate $\hat{\theta}$ is chosen to maximize the likelihood of observing the configuration according to Ewens' sampling formula for the coalescent[58]. This calculation provides the nested model likelihood for the statistical test, (i.e., likelihood without clonal proliferation). Clonal proliferation is assumed to alter the isolate set configuration by taking one sequence and increasing its multiplicity, by a mechanism otherwise not present in the standard coalescent model. To model this behavior, we suppose that the proliferating sequence is (one of) the most frequent sequences observed, and that it effectively replaced a number of singleton sequences that would have otherwise been observed in the sample. To maximize the likelihood, there are now two parameters: the mutation parameter (as in the nested model), and the number of sequences that this largest clone effectively replaced. We again use Ewens' sampling formula, but on an isolate set configuration that is modified to reverse the effect of this supposed replacement. In other words, the largest clone is shrunk, and singleton sequences are added to keep the total sample size the same. The mutation parameter estimate $\hat{\theta}$ and log-likelihood are computed for this modified configuration. A p-value is then obtained using the likelihood ratio test with one degree of freedom (Table 1). The patterns in all five of these subjects significantly violate the distribution of isolate sets expected under a standard evolutionary dynamic without

proliferation (p=0.0003–0.027). For all five participants analyzed, the low p-values indicate that mutation-free viral replication is an insufficient explanation for the data.

Two simplifications may make this test too conservative. First, the test supposes that only one sequence may have been expanded. However, multiple sets of identical isolates were observed in several subjects. For example, subjects S02 and S09 both have two large sets of identical isolates that could have arisen through clonal proliferation. However, the statistical test was not designed to pick up this pattern. Second, by using only the allele configuration and not the full sequence data, the test ignores other potential evidence for clonal proliferation. Note that the test checks for any mechanism that may increase the multiplicity of a sequence. For example, if positive selection prior to administration of ART dramatically increased the frequency of a sequence in the latent reservoir, it could produce a positive result by this test. In the setting of chronic infection, however, archival viral populations are highly diverse and mostly latent, making this sort of dramatic selective sweep unlikely. A final important simplification must be mentioned. The coalescent, used in this test, models a single contemporaneous sample of an evolving population experiencing generational turnover; however, the latent reservoir for HIV-1 is an archive populated over time, more closely resembling a longitudinal sample[59]. Overall, this analysis of the distribution of identical isolates is more consistent with clonal expansion of infected cell than with infection of multiple cells be a dominant viral population without mutation.

Discussion

The extremely stable latent reservoir for HIV-1 in resting $CD4^+$ T cells is a major barrier

to cure[6-9]. There is concern that the reservoir could be maintained or even expanded by the cellular proliferation even when viral replication is fully arrested by ART. Proliferation of infected cells could be driven by antigen, cytokines, or effects related to the site of integration[10,22,23]. Previous sequencing studies have provided evidence for *in vivo* proliferation of infected cells[19,20,22-25,41,46,60]. However, a full understanding of the importance of clonal expansion requires consideration of three limitations of current studies. First, much of the evidence for clonal expansion comes from studies showing that multiple independent sequences from a single patient are identical. However, in most patients on ART, >98% of proviruses are defective[42,44,45], and thus without full length sequencing[10,22,23] or direct demonstration of viral replication[25], it must be assumed that the identical sequences represent defective virus, particularly since cells carrying defective proviruses are less likely to die from viral cytopathic effects or lysis by host effector cells. Second, most studies use subgenomic amplicons, and it is possible that sequences identical in the regions analyzed differ elsewhere in the genome[43]. Most importantly, even complete viral sequence identity does not prove clonal expansion; an alternative explanation is infection of multiple cells by a dominant viral species. Integration site analysis provides direct evidence for clonal expansion but does not establish replication competence.

In light of these issues, two recent studies are of particular importance. Simonetti et al. have described an expanded cellular clone carrying replication-competent provirus integrated into a unique site in the human genome that could not be precisely mapped[24]. This clone was identified in a patient with a concurrent malignancy that may have driven the clonal expansion[24]. Lorenzi et al. recent demonstrated that 54% of replication-competent isolated from four treated patients had *env* sequences identical to other isolates

from the same individual[25]. Together, these studies highlight the disturbing possibility that *in vivo* proliferation of infected cells may be a major factor in determining the composition and stability of the latent reservoir.

The data presented here contribute to our understanding of this issue in several ways.

We directly demonstrate that cells carrying replication-competent HIV-1 can proliferate *ex vivo* without producing infectious virus and then release virus upon subsequent stimulation. Thus, strong mitogenic stimuli do not always reactivate latent HIV-1. In light of the short half-life of productively infected cells[26,27], this finding helps explain how clonal expansion could occur *in vivo*. We also demonstrate the presence of a large number of latently infected cells carrying identical viral sequences such that cells with identical sequences can be routinely captured in a single blood sample from most patients. Overall, 57% of the isolates had *env* sequences identical to other isolates from the same patient. This finding agrees very well with the study of Lorenzi et al.[25] We went on to show that sequences identical in the *env* gene were identical throughout the genome. In addition, through two independent approaches we sought evidence that the identical sequences arose through infection of multiple cells by a dominant viral species. Both approaches failed to support this alternative hypothesis, leading us to favor the explanation that the identical sequences arose through the *in vivo* proliferation of latently infected cells. Definitive proof will require simultaneous identification of the integration site and demonstration of replication-competence for many proviruses from multiple patients, something that cannot be readily achieved with current technology. Nevertheless, the studies presented here raise the possibility that a substantial fraction of the latent reservoir is generated by cell proliferation which our *ex vivo* studies show can occur without release

of infectious virus.

Our results have implications for measurement of the latent reservoir. Although PCR-based assays for proviral DNA are widely used, there is a large discrepancy between culture- and PCR-based assays of the latent reservoir. In comparative studies, PCR assays for proviral DNA give infected cell frequencies that are ~300 fold higher than frequencies measured by the QVOA[52]. This is mainly due to the fact that the vast majority of proviruses are defective[42,44,45]. However, the number of proviruses that appear intact by full length sequencing still exceeds the number induced to release infectious virus in the standard QVOA by 20-60 fold[42,44]. We show here using a multiple stimulation version of the QVOA that additional isolates of replication-competent HIV-1 can be obtained after the 2nd, 3rd, and 4th additional rounds of T cell activation. On average, only 60% of the isolates ultimately obtained in the MS-VOA grew out after a single stimulation. Thus, the standard QVOA and other assays that rely on a single round of T cell activation to induce latent HIV-1 may underestimate the frequency of latently infected cells as previously suggested[44]. It is important to determine what accounts for this difference. Some proviruses that appear intact by full length sequencing may harbor minor missense mutations that decrease replication-capacity. However, when apparently intact proviruses were reconstructed and tested for replication in primary CD4^{+} T lymphoblasts, 6 of 6 proviruses from 4 different infected individuals showed replication equivalent to that of NL4-3 and replication-competent viruses isolated from the same individuals[44]. Another possibility is that some proviruses are permanently silenced by integration into regions non-permissive for viral gene expression[61,62], by transcriptional interference[63-65], or by epigenetic modifications[66-68]. Finally, stochastic features of the Tat transactivation mechanism may prevent induction

of all intact proviruses even in the setting of T cell activation[69]. Identification of successful latency reversing strategies may require a deeper understanding of these issues.

The possibility that a substantial fraction of the latent reservoir arises by proliferation of a smaller number of infected cells presents some obvious challenges to HIV-1 cure efforts. It will be important to determine the factors driving the proliferation. In addition, because the total size of the reservoir does not increase over time[6,8,9], any proliferation must be roughly balanced by cell loss, and it will be important to understand the mechanisms involved and whether they can be enhanced to promote cure.

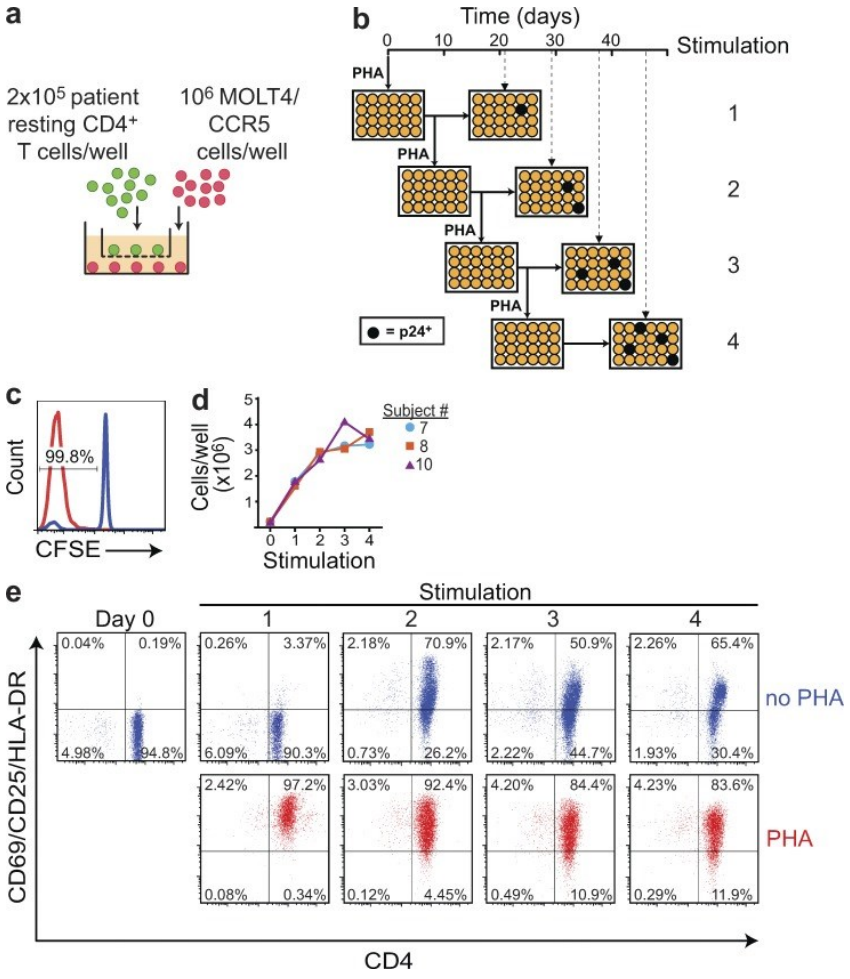

Fig. 3.1. Multiple stimulation viral outgrowth assay (MSVOA). (a) A transwell co-culture system (Ho et al., 2013) was used to allow separate manipulation of patient CD4+ T cells (green) and MOLT4/CCR5 cells (red). Purified resting CD4+ T cells from subjects on ART were plated at a limiting dilution for viral outgrowth and stimulated with PHA and irradiated allogeneic PBMC (not shown) as previously described (Finzi et al., 1997, Finzi et al., 1999, Siliciano et al., 2003, Laird et al., 2016). After 24 h, PHA was removed and 106 MOTL4/CCR5 cells were added. Outgrowth in this system is equivalent to standard co-cultures (Ho et al., 2013). (b) Assay time course. At 8d after the initial PHA stimulation, half the volume from the top and bottom chambers of each transwell is transferred to a new set of transwell plates for a 2nd round of PHA stimulation. The initial plates are cultured without further stimulation for a total of 21 days. The restimulated plates are cultured for 8d following the 2nd round of PHA stimulation and then split as above to generate a 3rd set of plates, which receive a 3rd round of stimulation. Similarly, these plates are split 8d later to generate the 4th set of plates, which receive a 4th round of stimulation. A p24 ELISA was performed 21d after each respective round of PHA stimulation to quantify viral outgrowth (dashed lines). In this hypothetical example, each round of PHA stimulation induced outgrowth from an additional well (black circles). (c) PHA stimulation induces uniform proliferation of resting CD4+ T-cells. Immediately prior to the 1st PHA stimulation, an aliquot of resting CD4+ T-cells was stained with carboxyfluorescein diacetate succinimidyl ester (CFSE). CFSE dilution was quantitated by flow cytometry 7d after stimulation to determine the fraction of cells that had proliferated (red histogram). Cells that did

not receive PHA stimulation served as controls (blue histograms). Histogram is representative of CFSE dilution from 3 subjects. (d) Increase in cell number after each round of stimulation. Results for 3 representative subjects are shown. (e) Activation marker expression induced by 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) 7d after the indicated round of PHA stimulation (red dot plots). Cells that did not receive the most recent round of PHA stimulation were analyzed in parallel (blue dot plots). Results are shown for subject 10 and are representative of 2 other subjects analyzed.

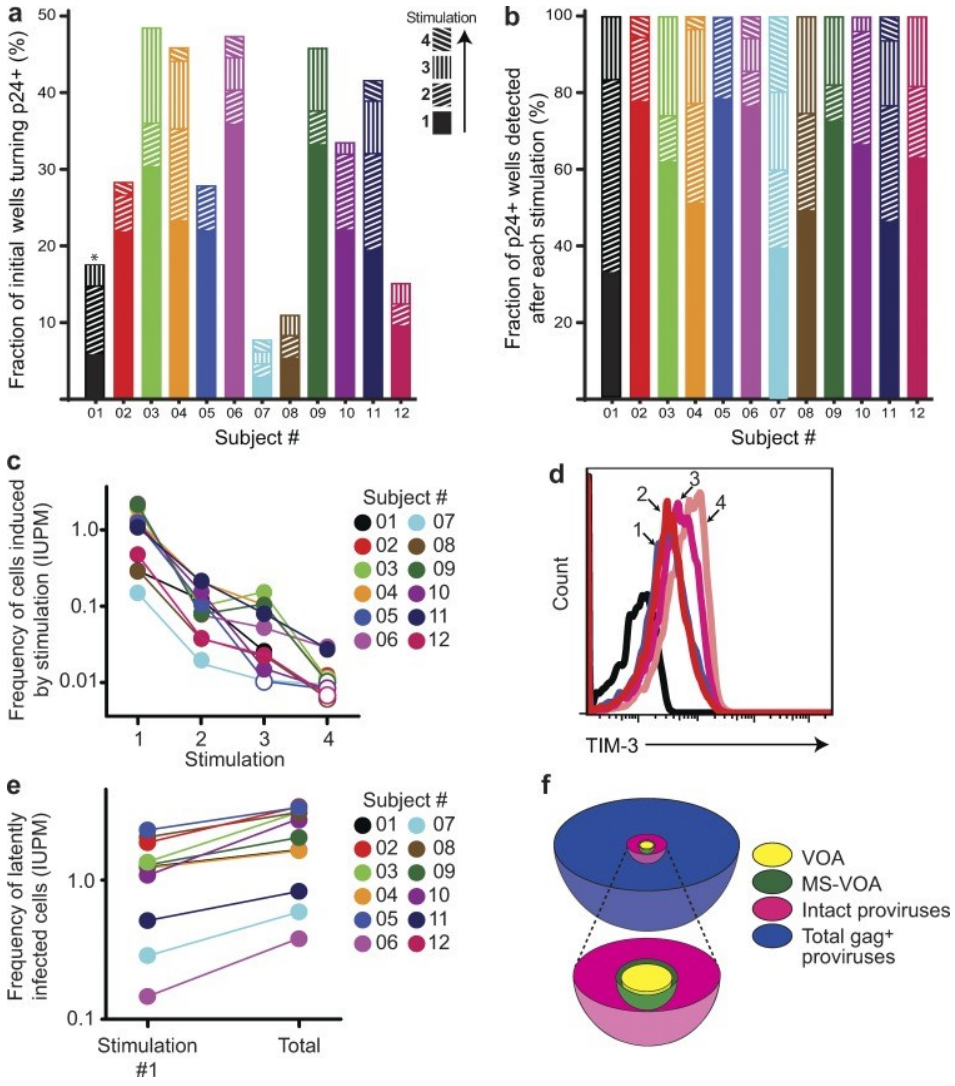

Fig. 3.2. Further rounds of T cell activation induce additional proviruses to produce replication-competent virus. (a) Fraction of initially seeded wells becoming p24+ after each of four consecutive stimulations with PHA. For each subject (n = 12), a total of 64–72 wells were initially seeded. The asterisk indicates that cells from subject 1 only received three rounds of PHA stimulation. (b) Fraction of total p24+ wells from each subject (n = 12) that were detected for each round of PHA stimulation. The denominator is the total number of initially seeded wells. (c) Frequency of cells in the culture at the time of stimulation that are induced to produce replication-competent virus by the indicated round of stimulation. Frequency in infectious units per million (IUPM) cells was determined for each subject (n = 12) from mean cell counts before each stimulation and the number of wells turning p24+ after stimulation using a maximum likelihood estimation of infected cell frequency (Rosenbloom et al., 2015). (d) Expression of TIM-3 before stimulation (black line) and 7 d after the indicated round of stimulation. Results are presentative of exhaustion marker analysis in three subjects. (e) Frequency of latently infected cells among the initially plated cells from each subject (n = 12) as detected after a single round of PHA stimulation and after a total of four consecutive rounds of stimulation. (f) Schematic representation of the relative frequency of infected resting CD4+ T cells detected by different assays. The VOA result presents the mean frequency detected after a single round of PHA

stimulation in cultures from 12 subjects studied here. The MS-VOA result represents the mean frequency among the initially plated cells of latently infected cells as detected by a total of four consecutive rounds of PHA stimulation. The frequencies of cells with intact (nondefective) proviruses and the total frequency of infected cells as measured by PCR with gag primers were estimated from the VOA results and the ratios described by Bruner et al. (2016). The true frequency of latently infected cells is between the frequency measured in the MS-VOA and the frequency of cells with intact proviruses.

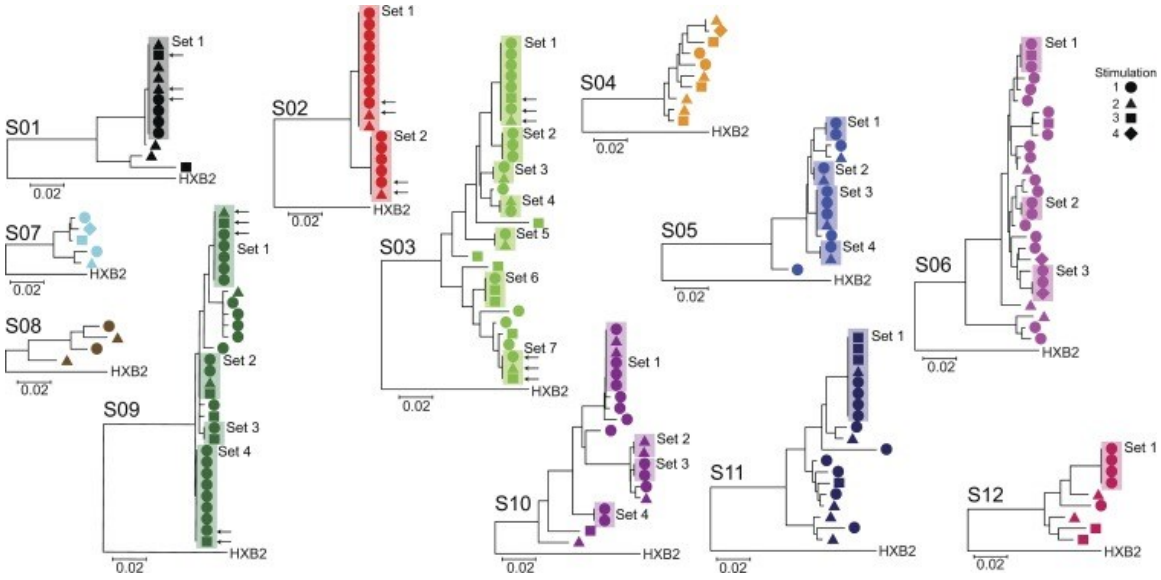

Fig. 3.3. Many independent isolates of replication-competent HIV-1 have identical sequences. Phylogenetic trees of env sequences of independent isolates of replication-competent virus from each subject (n = 12). Sequencing was performed on genomic viral RNA in supernatants of p24+ wells. Cultures were established at limit dilution for viral outgrowth. Isolates containing more than a single sequence as indicated by double peaks at one or more positions in the chromatograms were not further analyzed. Symbols indicate the number of PHA stimulations after which the isolate was obtained. Sets of isolates with identical env sequences are boxed. Representative isolates obtained after different numbers of stimulations (arrows) from randomly selected sets were subjected to full-length sequencing at the single-genome level (see Fig. 4).

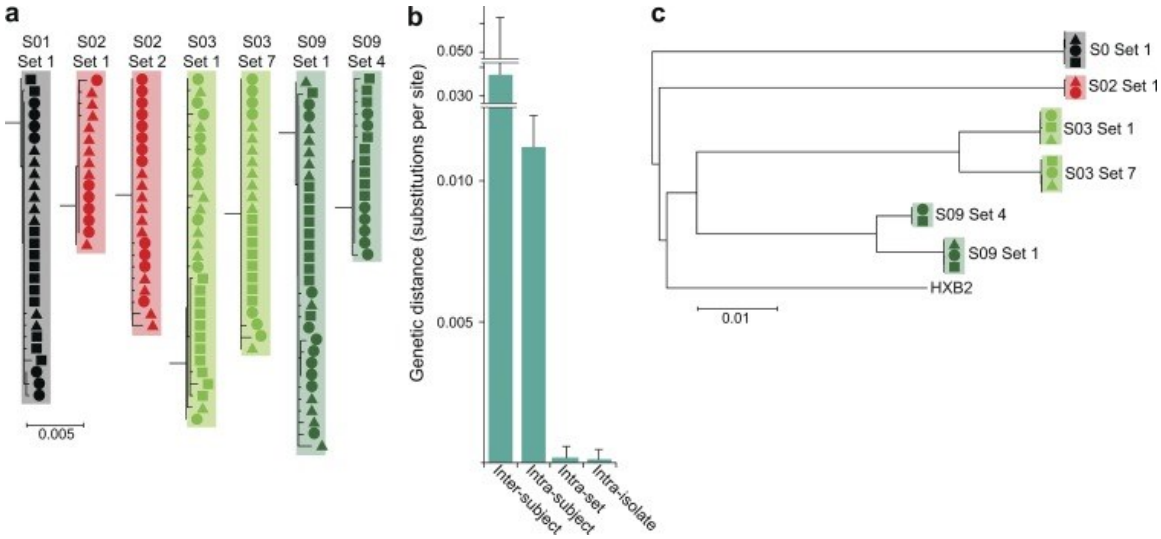

Fig. 3.4. Full-genome analysis of isolates with identical env sequences. (a) Full-genome sequences of isolates with identical env sequences. Randomly selected isolates (arrows in Fig. 3) belonging to sets of isolates with identical env sequence were subjected to full-length sequencing. Sequencing was performed on single HIV-1 genomic RNA molecules present in the supernatants of p24+ cultures after reverse transcription and nested PCR amplification performed at limiting dilution. Two overlapping half-genome fragments were amplified. To minimize errors, PCR products were directly sequenced without cloning. For each isolate, 6–12 limiting 5′ sequences and 6–12 3′ sequences were obtained. Neighbor joining phylogenetic trees demonstrated that all sequences from a given set of isolates with identical env sequences co-clustered. The minimal differences (one to four nucleotides) likely reflect mutations expected to arise during the 3-wk outgrowth culture. Symbols indicate the PHA stimulation after which the isolates were detected, as in Fig. 3. Results are shown for the 5′ half-genome sequences. Similar results were obtained for the 3′ half-genome sequences. (b) Genetic distance (mean ± SD) between single-genome sequences from a single isolate (intra-isolate), between single-genome sequences from different isolates belonging to a set of isolates with identical env sequences (intra-set), between single-genome sequences from different sets from the same study subject (intra-subject), and between single genome sequences from different patients. For each set of two to three isolates from a given subject, 6–12 sequences were used for intra-isolate and intra-set comparisons. Then, all sequences were used for intra-subject and inter-subject comparisons. (c) Phylogenetic tree of full-genome consensus sequences. The 6–12 single half-genome sequences from each isolate were condensed to half-genome consensus sequences, which were joined to produce full-genome sequences. Set 2 from subject 2 is not depicted because of failure of the 3′ half-genome reaction as a result of primer mismatch.

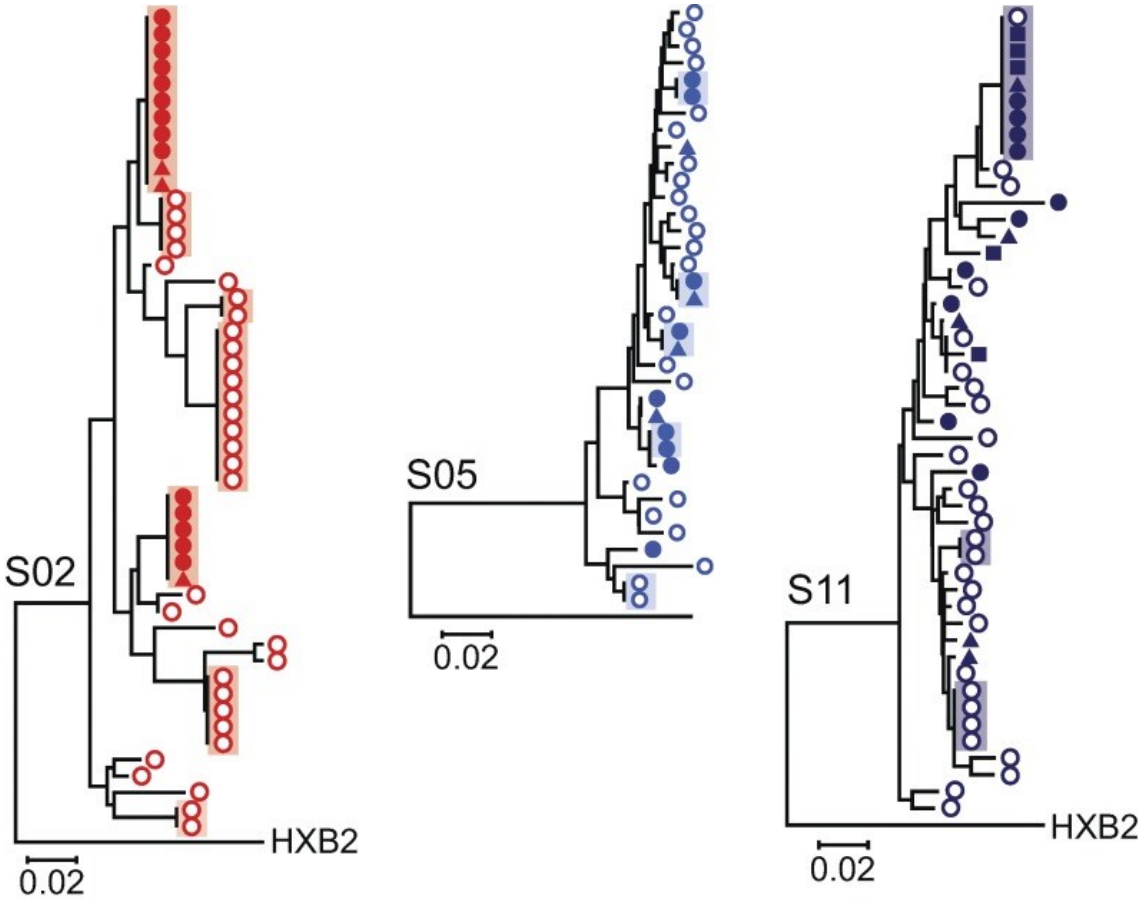

Fig. 3.5. Isolates with identical sequence are part of more complex proviral populations reflecting diversification over time during chronic infection. Limiting dilution analysis of proviruses present in resting CD4+ T cells was performed on cells from three representative patients from whom sets of identical isolates of replication-competent virus were obtained. The V3-V4 region of env was sequenced, and the resulting sequences (open circles) were used in phylogenetic analysis along with the replication-competent isolates (closed symbol; shapes reflect the number of PHA stimulation as in Fig. 3). Sets of identical independent sequences are boxed.

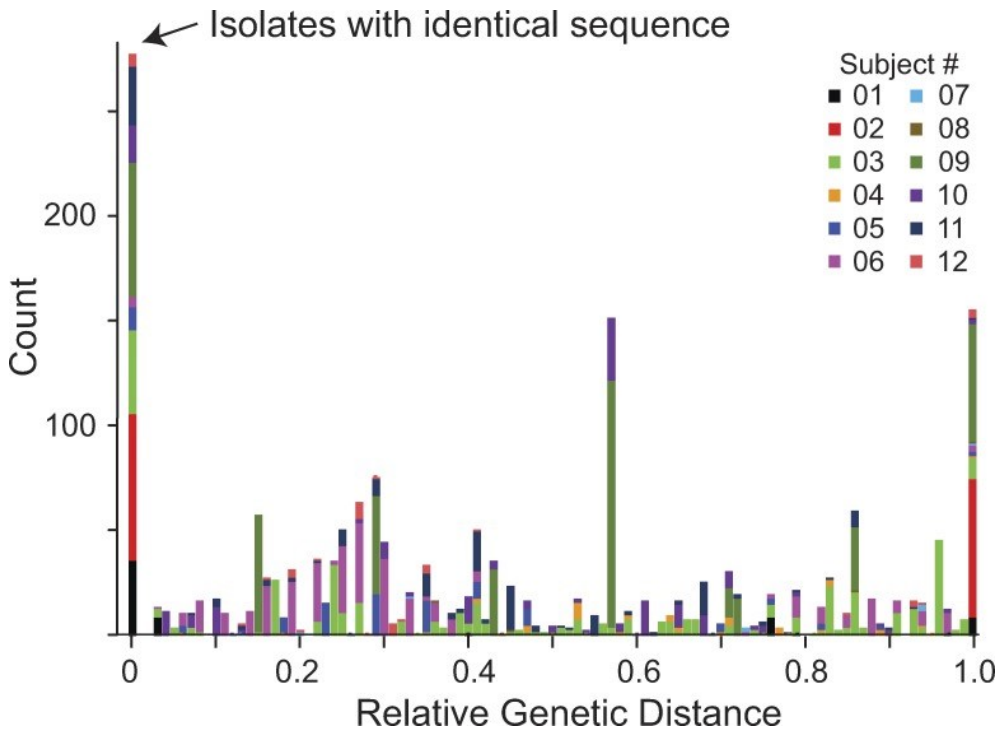

Fig 3.6. Distribution of genetic distances between isolates of replication-competent virus from individual subjects (n = 12) does not support infection of a large number of cells by a dominant viral population. Composite histogram showing the distribution of intra-subject genetic distances between isolates of replication-competent virus. To account for differing degrees of genetic divergence over time in different subjects, distances are normalized by the largest observed intra-subject distance. Analysis is based on the highly variable V3-V4 region of the env gene obtained as described in Fig. 3. The large peak at the origin reflects the zero branch lengths between independent identical isolates of replication-competent virus obtained from 9 of 12 patients.

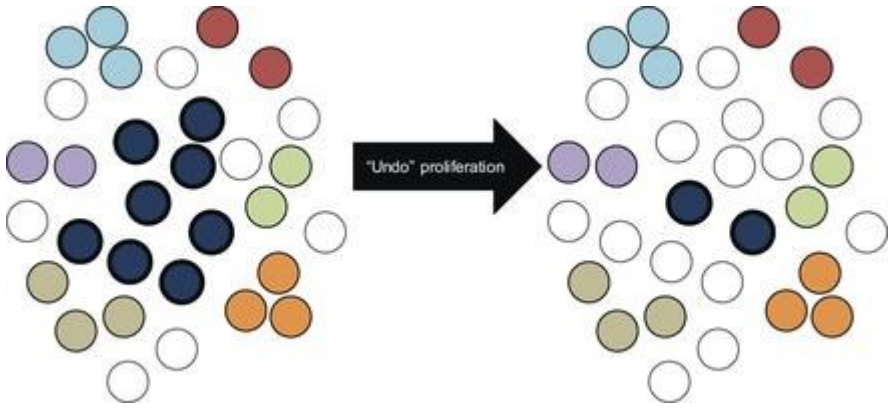

Fig. 3.7. Schematic illustration of a statistical test based on coalescent theory to explore the possibility that the high degree of sequence identity observed could be explained merely by rounds of viral replication in which no mutation occurred. The left side of the figure represents the sample from participant 03 as analyzed by sequencing of the env gene in supernatant HIV-1 RNA from clonal p24+ wells (see Fig. 3). A total of 31 independent isolates were obtained, including one set with eight isolates (dark blue cells), six smaller sets (other colors), and eight unique isolates (singletons). The right side imagines a sample of the same size that would have been collected had no clonal proliferation occurred. The large clone is reduced to the size that maximizes likelihood under a neutral coalescent, and singletons are added to keep the total sample the same size. Because reducing the large clone essentially adds a parameter, a likelihood ratio test can be done. As shown in Table 1, p-values for all subjects tested in this manner were <0.05, indicating that mutation-free viral replication is an insufficient explanation for the data.

ID[a]	Actual isolate set configuration[b]			Modified isolate set configuration[f]			χ^2 statistic (df=1)	p-value[h]
	Size of isolate set: # sets of this size[c]	$\widehat{\theta}$[d]	LL[e]	Size of isolate set: # sets of this size[g]	$\widehat{\theta}$[d]	LL[e]		
01	1 : 3 **9 : 1**	1.67	-3.75	**1 : 12**	100	-0.64	6.23	*0.013*
03	1 : 8 2 : 3 3 : 3 **8 : 1**	10.81	-6.51	1 : 14 **2 : 4** 3 : 3	27.38	-4.06	4.91	*0.027*
10	1 : 8 2 : 3 **6 : 1**	11.74	-4.70	**1 : 14** 2 : 3	50.68	-1.85	5.71	*0.017*
11	1 : 11 **8 : 1**	12.96	-7.57	1 : 17 **2 : 1**	100	-1.08	12.98	*0.0003*
12	1 : 5 **4 : 1**	6.69	-3.01	**1 : 9**	100	-0.35	5.33	*0.021*

Table 3.1. a Subjects with a single large set of identical isolate were included in the analysis.

b The nested model (i.e., model without clonal proliferation) uses the isolate set configuration observed for each subject.

c Clonal proliferation alters the configuration of sets of identical isolates by taking one sequence and increasing its multiplicity by a mechanism not present in the standard coalescent model. To model this behavior, we suppose that the proliferating sequence is the most frequent sequence observed and that it effectively replaces several singleton sequences that would have otherwise been observed in the sample. To maximize the likelihood, there are now two parameters: the mutation parameter (as in the nested model) and the number of sequences that the largest clone effectively replaced. We again use Ewens' sampling formula but on an isolate set configuration that is modified to reverse the effect of this supposed replacement.

d P-value obtained using the likelihood ratio test with one degree of freedom. A value <0.05 (italicized) indicates that mutation-free viral replication is an insufficient explanation for the data.

e The largest set of isolates from each subject is shown in bold.

f For each subject, the mutation parameter estimate θ^^ is chosen to maximize the log-likelihood of observing the isolate set configuration according to Ewens' sampling formula for the coalescent (Ewens, 1972). The maximum permitted value for θ^^ is 100.

g Log-likelihood.

h The size of the reduced isolate set that is created to reverse the effect of clonal proliferation is shown in bold.

CHAPTER 4: Inducibility of Latent HIV-1 in Resting CD4+ Memory T-cell Subsets

Kwon KJ, Timmons AE, Sengupta S, Simonetti FR, Zhang H, Hoh R, Deeks SG, Sekaly RP, Siliciano JD, Siliciano RF. Inducibility of Latent HIV-1 in Resting CD4+ Memory T-cell Subsets. *In progress*.

Abstract

The latent reservoir (LR) for HIV-1 in resting memory CD4+ T-cells harbors integrated, replication-competent proviruses that are not actively transcribed while the T-cells remain in a resting state. Given that the LR is the major barrier to HIV-1 cure, it is important to understand the proliferative process that contributes to the persistence of the LR. Recent work has shown that proliferation of infected cells is a major factor in the generation, persistence, and stability of the LR, and that latently infected cells that clonally expand in vivo can be reactivated in vitro without producing virus. One hypothesis to explain this observation is that certain latently infected memory CD4+ subsets may be in a deeper state of latency and therefore may be able to proliferate without producing virus. To evaluate this possibility, we cultured resting naïve (TN), central memory (TCM), transitional memory (TTM), and effector memory (TEM) CD4+ T-cells from 10 HIV patients on suppressive ART in a multiple stimulation viral outgrowth assay (MSVOA). Based on the frequencies of viral outgrowth and intact proviral copy numbers quantitated by the intact proviral DNA assay (IPDA), only 1.6% of intact proviruses across all subsets were induced by the MSVOA. Additionally, there was no enrichment of intact proviruses in any specific subset and no correlation between inducibility and subset. Furthermore, we observed significant plasticity among the canonical memory subset markers in vitro and saw

significant patient-to-patient variability in inducibility that complicates the vision for a targeted cure approach based on T-cell subsets.

Introduction

The major barrier to curing HIV-1 is the latent reservoir,[1,26] which is mainly comprised of latently infected resting memory CD4+ T-cells that harbor stably integrated replication-competent proviruses[27–31]. The latent reservoir in resting $CD4^+$ T cells was first demonstrated using a viral outgrowth assay in which virus production was induced in latently infected resting CD4+ T cells through T cell activation[28,31–34]. The slow decay rate of the latent reservoir necessitates lifelong antiretroviral therapy (ART) to prevent viral rebound and disease progression[26,35]. The slow decay reflects normal memory cell turnover as well as various forms of cell death following cellular activation and the concomitant reversal of HIV-1 latency. This includes death from viral cytopathic effects or immune clearance as well as activation-induced cell death[14,36–38]. The loss of infected cells is largely balanced by clonal expansion driven by antigen, homeostatic proliferation, and effects possibly related to the site of proviral integration[2,3,9–12,16,39–48]. Recent studies have identified proliferation of infected cells as a mechanism for HIV-1 persistence[9–12]. Multiple mechanisms may account for proliferation of latently infected cells including: cytokine-driven homeostatic proliferation, antigen-driven expansion, and proliferation driven by effects related to the site of proviral integration[2,3,39–44,46–48]. Several groups have recently shown that latently infected cells carrying replication-competent proviruses can persist through clonal expansion *in vivo*[7,10–12].

To purge the reservoir, many groups have looked for cellular markers of latent infection[49–51] that would allow these infected cells to be targeted for elimination as well as

identify specific subpopulations of $CD4^+$ T-cells that may be enriched in cells carrying latent, replication-competent proviruses or cells that may differ in propensity for clonal expansion or latency reversal[8–16]. Specific markers of latent infection have not been identified[57–62] and persistent HIV-1 has been found in several different $CD4^+$ memory subsets and at lower levels in naïve CD4+ T cells (TN)[5,21,49,52–56].

The cell surface proteins CD45, CCR7 and CD27 are commonly used to define memory T cell subsets[63–65]. CD45 is a transmembrane protein of the protein tyrosine phosphatase family expressed extracellularly in alternatively spliced isoforms that are differentially glycosylated, most importantly CD45RA and CD45RO[66]. Naïve T-cells express CD45RA and upon TCR recognition of a cognate antigen, undergo blast transformation and proliferate[67]. At the conclusion of the immune response, most of these activated cells die but some survive and return back to a resting memory state, losing CD45RA expression and upregulating CD45RO expression[68–70]. Thus, CD45RO is a marker of memory CD4+ T cells. CCR7 is a chemokine receptor that mediates lymph node homing through interaction with its ligands CCL19 and CCL21, which are highly expressed in lymph tissues. CCR7 facilitates homing of cells to secondary lymphoid tissue but its expression is lost upon secondary stimulation[63]. CD27 is a costimulatory TNF-receptor that binds the ligand CD70, promotes clonal expansion of cells after activation, survival of the activated cell through anti-apoptic pathways, and is irreversibly cleaved off the surface after antigenic stimulation is removed[71].

Antigenic stimulation is responsible for the differentiation of naïve $CD4^+$ T-cells (TN:$CD45RO^-CCR7^+CD27^+$) into memory $CD4^+$ T-cells. There are three main subsets of memory $CD4^+$ T-cells: central memory (TCM: $CD45RO^+CCR7^+CD27^+$), transitional

memory (TTM: $CD45RO^{+}CCR7^{-}CD27^{+}$), and effector memory cells (TEM: $CD45RO^{+}CCR7^{-}CD27^{-}$)[19,72]. TN home to secondary lymphoid organs where they may encounter antigen and eventually differentiate into cells with a memory phenotype[19,68–70,72–74,48,50]. TCM are long-lived cells whose main function is to mediate a recall response to antigen after homing to secondary lymphoid organs[39-41,43-46,48-50]. TTM are cells transitioning from a TCM phenotype to a TEM state[43,44,65,75]. TEM cells express β integrins which allow for homing to inflammatory sites and tissues for rapid effector function by producing IFNγ, IL-4, and IL-5[43-45,48-50]. Based on these functions, CCR7 and CD27 have been adopted as canonical surface markers to delineate the different memory subsets.

There is considerable interest in determining the frequency of latent HIV distribution within various $CD4^{+}$ T cell subsets. Early studies demonstrated higher frequencies of HIV-1 in memory CD4+ T cells than in naïve $CD4^{+}$ T cells[28,77], consistent with models of reservoir formation[28,78]. Subsequent studies have provided conflicting regarding the levels of proviral DNA in various $CD4^{+}$ T cells subsets including TCM, TTM, and TEM[52–54] as well as Th1 cells[79,80], T follicular helper cells (TfH)[81], and stem cell-like memory cells (TSCM)[21,55]. Persistence of latent proviruses is clearly influenced by the dynamics of the T cell subsets that harbor latent HIV. Several studies have shown that TCM (and TTM in some studies) harbor the majority of latent HIV-1 proviruses, consistent with the observation that TCM have the longest half-life of the memory subsets [20,24,52,54,67,82–85]. However, another study has suggested that TEM harbor latent HIV-1 at higher frequency[86]. One factor that may contribute to differences observed by different groups is the assay used to measure the frequency of infected cells in the subsets. There are several different methods to quantify different types of HIV-1 in host cells[87]. The standard QVOA, used for

measuring the frequency of replication-competent virus induced to outgrowth in culture, is an underestimate of LR size due to noninducible intact proviruses[7,28,32–34]. HIV-1 DNA measurements using qPCR are a large overestimate of LR size since they do not discriminate between intact and defective proviruses[88,89]. Near-full-length sequencing (NFLS) is a better estimate of LR size as it can distinguish between intact and defective proviruses but is extremely laborious [86,90,91]. The ddPCR-based IPDA, recently developed in our lab, can distinguish between intact, defective/hypermutated provirus, can sample more proviruses than NFLS, and is significantly less labor intensive[92]. In studies where HIV-1 was quantified in different subsets, different methods were used by each group, mainly the QVOA, qPCR, or NFLS. These methodical differences may account for some of the discrepancies between subset distributions observed for the above reasons.

In addition to the amount of latent HIV-1 present within T cell subsets, it is important to understand the relative inducibility of proviruses in different subsets. Several groups have reported differences across memory subsets in regard to permissibility of HIV-1 infection depending on coreceptor usage and CCR7 expression[93–96], accessibility of chromatin modifiers to genes involved in subset differentiation[97–99], and cytokine responsiveness[52,73,74]. TNs are more susceptible to infection by CXCR4-tropic viruses, and memory cells (especially EMs) are more susceptible to infection by CCR5-trophic viruses[93,95] *in vitro*. Furthermore, the presence of the CCR7 ligands CCL19 and CCL21 increases susceptibility of TNs and TCMs to infection[94,96]. In regard to epigenetic differences, some groups have shown that inducible transcription-related genes are more poised (in an open chromatin state with no active transcription occurring) in resting memory cells than in naïve cells[97,99] and DNA methylation decreases within

differentiation-controlling genes as naïve cells differentiate[98]. Possibly because of the poised nature of these transcription-related genes, several groups have shown that EMs display a higher proliferative response to IL-7 and IL-15 than less differentiated memory subsets[52,73,74,100].

Initial evidence for differential inducibility of latent HIV-1 proviruses came from a multiple stimulation viral outgrowth assay (MSVOA) which was developed to study proviruses that were not induced by a single round of T cell activation. 60% of all replication-competent proviruses assayed required multiple rounds of T-cell activation to reactivate latent virus. One hypothesis to explain this observation is that proviral inducibility is dependent on the subset of memory $CD4^+$ T-cell harboring the latent virus. Cells that produce virus after multiple rather than a single stimulation may have required additional reactivations to differentiate into an effector memory phenotype with a transcriptional landscape that facilitates proviral transcription and expression. To determine whether the memory subset phenotype of cells harboring replication-competent provirus is indicative of proviral inducibility, we performed the MSVOA on sorted resting $CD4^+$ T cells from the TN, TCM, TTM, and TEM subsets from patients on suppressive antiretroviral therapy (ART). We analyzed virus from viral outgrowth in culture, quantified intact and defective HIV DNA using the IPDA[92], and sequenced induced replication-competent viruses to look for correlations between memory subset, proviral enrichment, and viral inducibility. The results provide novel insight on the inducibility of latent proviruses in memory CD4+ T-cell subsets and targeted cure strategy.

Methods

Study Subjects.. Leukopaks were obtained from 10 HIV+ patients. Study subjects were from the UCSF SCOPE cohort who met the inclusion criteria of HIV+ adults who had chronic ART-suppression of viremia <20 copies/ml and CD4 count > 400. This study was approved by the Institutional Review Board at the University of California San Francisco. Written informed consent was obtained from all participants.

Resting CD4+ T-cell isolation and subset sorting. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density centrifugation. CD4+ cells were isolated from PBMCs using the negative depletion EasySep™ Human CD4+ T-cell Isolation Kit (Stemcell Technologies). Resting CD4+ cells were then isolated by negative depletion of cells expressing CD69, CD25, or HLA-DR (CD69 MicroBead Kit II, CD25 MicroBeads, Anti-HLA-DR MicroBeads; Miltenyi Biotec). Cells were stained with CD27-BV421, CD3-BV510, CCR7-PE, CD45RO-APC, CD4-PECY7 (Biolegend). Stained cells were sorted on the MoFlo Legacy at the Johns Hopkins School of Public Health Flow Cytometry and Cell Sorting Core Facility. Viability was determined using a propidium iodide stain. Cells were sorted based on the following expression combinations from gated live singlet CD3+CD4+ lymphocytes: CD45RO-/CCR7+ /CD27+ (TN), CD45RO+/CCR7+/CD27+ (TCM), CD45RO+/CCR7-/CD27+ (TTM), CD45RO+/CCR7-/CD27- (TEM).

IPDA. Digital droplet PCR was performed on resting subset cells after sorting as previously described in the intact proviral DNA assay[92]. Multiplex ddPCR was performed on the QX200 Droplet Digital PCR System (BioRad) using primers and probes that distinguish between 5'-deleted, 3'deleted and hypermutated, and intact proviruses.

MSVOA. Each sorted subset was cultured in separate transwell plates, where $2x10^5$ sorted cells were plated into each top well. The MSVOA was set up as previously described[12], with irradiated allogeneic PBMCs and phytohemagglutinin added to the top wells to stimulate the sorted subset cells, and MOLT-4 cells in the bottom wells to perpetuate outgrown virus. The irradiated PBMCs were stained with CellTrace Violet to distinguish patient cells from feeders in downstream flow cytometry analysis. The cultures involved 4 rounds of global T-cell activation every 9 days, then readout by p24 ELISA (Perkin Elmer) 21 days after each stimulation.

Flow cytometry. Cells from each subset were stained with Live/Dead Fixable Violet Dead Cell Stain Kit (Invitrogen), CD3-BV510, CCR7-PE, CD45RO-APC, CD4-PECy7, and CD27-BV785 (Biolegend). Activation levels were determined using CD25-APCCy7, CD69-APCCy7, and HLA-DR-APCCy7 (Biolegend). CFSE (Invitrogen) staining was done separately on different wells to prevent spillover. Stained and washed cells were analyzed on the iQue Screener Plus (Intellicyt) at 4d, 9d, 14d, and 21d for each subset and each stimulated group. Data was analyzed on FlowJo.

Viral RNA sequencing. Culture supernatants were saved after 21d for each stimulation group for viral RNA isolation from supernatant. cDNA was synthesized from the isolated RNA using the SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific) and gene-specific primer ES8. The V3-V4 region of env was amplified using primers ES7 (5′-CTG TTA AAT GGC AGT CTA GC-3′), ES8 (5′-CAC TTC TCC AAT TGT CCC TCA-3′), and Platinum SuperFi DNA Polymerase (Invitrogen). PCR products were run on a 1% agarose gel, extracted (Qiagen QIAquick Gel Extraction Kit), then submitted for Sanger sequencing to Genewiz using sequencing primers Nesty8 (5′-CAT ACA TTG CTT

TTC CTA CT-3′) and DLoop (5′-GTC TAG CAG AAG AAG AGG-3′). Sequences were analyzed in BioEdit, aligned by ClustalW, trimmed to equal lengths, then used to generate neighbor-joining trees in MEGA6.

Results

Intact proviruses are similar distributed across memory subsets

To understand the distribution and inducibility of latent HIV-1 proviruses in different T cell subsets, we developed a subset isolation method that focuses on the resting $CD4^+$ T cells that represent a stable reservoir for HIV-1 *in vivo*. $CD4^+$ T-cells were isolated from patient PBMCs, and then resting $CD4^+$ T-cells were purified by negative depletion of CD25, CD69, and HLA-DR-expressing cells in order to obtain latently infected cells that were not actively transcribing integrated proviruses. Resting CD4+ T cells were then stained for the subset-defining surface markers (CD27, CCR7, and CD45RO) and also for CD3 and CD4. The stained resting $CD4^+$ T-cells were then sorted using the gating strategy shown in Figure 1a. Because the subset markers change upon T cell activation (see below), this cell purification process ensures that the cells analyzed will accurately reflect the distribution of latent HIV-1 *in vivo*. A fraction of the sorted resting cells was saved for IPDA analysis while the remainder of cells were cultured in the MSVOA as shown in Fig. 1b.

The IPDA was used to quantitate intact and defective proviruses from genomic DNA isolated from sorted resting $CD4^+$ T-cell subsets from 10 patients. The housekeeping gene RPP30 was used as a control to correct for shearing that may have occurred during DNA preparation and as an internal control for cell equivalents. The number of intact, 5'-deleted and 3'deleted/hypermutated proviral copies per 10^6 cell equivalents were calculated for each subset within each patient. Naïve cells contained nearly 10-fold fewer

proviral copies (both intact and defective) than memory cells (Fig. 2a). The mean copy numbers of intact proviruses per 10^6 cells were similar across the subsets (527, 475, 800 for TCM, TTM, and TEM, respectively), suggesting there is no enrichment of intact proviruses in any particular subset (Fig. 2a). To confirm the lack of intact provirus enrichment across subsets, we performed paired t-tests comparing each subset and observed no significant difference among the different subsets ($p>0.17$). Similar mean copy numbers of 5' and 3' deleted proviruses were also found across the subsets for all patients (Fig. 2a).

To determine the contribution of each subset to the total pool of intact proviruses found in each patient, we calculated the frequency of each subset in peripheral blood (Fig 2b). The relative frequencies of each subset were as expected, with TN being the most abundant, followed by CM, EM, and TM. The contribution of each subset to the pool of intact proviral DNA copies for each patient was then calculated by considering both the intact copy values per 10^6 cells and the frequency of each subset. After normalization, we observed a wide distribution of intact proviruses across the subsets (Fig. 2c). Overall, no particular subset consistently contributed most to the total pool of intact proviruses. In different patients, different subsets made the largest contribution to the total population of intact proviruses.

Subsets differentiate toward effector phenotype in MSVOA

In order to understand the apparent lack of a relationship between conventional $CD4^+$ T cell subsets and the distribution of intact proviruses, we examined changes in expression of markers used to define the subsets upon T cell activation. Following sorting, $4.8x10^6$

cells of each subset (resting CD4+ TN, TCM, TTM, and TEM) were seeded into the MSVOA at $2x10^5$ cells/well in a transwell system as previously described[12]. This system allows for repetitive stimulation of cells in wells that are initially negative for viral outgrowth. Cells were then activated with the mitogen phytohemagglutinin (PHA) and irradiated allogeneic PBMCs for twenty-four hours, then co-cultured with MOLT-4 cells to expand induced replication competent virus (Fig. 1b). Cultures were split every 9 days at which time half of each culture was restimulated while the remaining half of the cells were cultured without further stimulation for 21 days. At 21 days after the last stimulation, p24 antigen was measured in the supernatant to detect viral outgrowth, and viral RNA from culture supernatants was saved for sequencing.

We measured markers of activation and proliferative activity at different time points throughout the course of the experiment. Regardless of the number of stimulations received, cells remained at least 70% activated throughout the duration of the culture, as determined by CD25 and CD69 expression (Fig. 3a). CFSE dilution observed in each subset after initial seeding and activation demonstrated that all cells proliferated in the culture system (Fig. 3b).

Phenotypic $CD4^+$ T-cell subset markers were assessed every 5-7 days by flow cytometry, including after each stimulation. Notable changes in phenotype were observed throughout the course of these cultures. TN gained CD45RO expression by 4 days after the initial activation (Fig. 3c). In Figure 3d, we observed that CCR7 expression on TN and TCM remained high after initial activation, then decreased over time as cells approached an EM phenotype in culture by day 21. Interestingly, TTM and TEM strongly upregulated CCR7 expression after initial activation from their CCR7- state. CD27 expression also

showed dramatic changes upon T cell activation, with a more complex pattern that varied between individuals and subsets. In TN and TCM, CD27 expression fluctuated and showed a variety of phenotypes among patients on day 21 (Fig. 3d). In general, CCR7 and CD27 expression demonstrated changes consistent with memory subset differentiation from TN → TCM → TTM → TEM with repeated stimulations, as expected and confirmed by a previous study of *in vitro* subset differentiation in non-HIV-infected individuals[65,75] (Supp Fig. 1). The finding that the markers used to define memory subsets can change dramatically upon T cell activation suggests that studies of memory cell subsets should be performed on purified resting $CD4^+$ T cells.

Inducibility of provirus expression is not dependent on memory subset

To test the hypothesis that inducibility of provirus expression varies based on memory subset, we performed the MSVOA on each sorted subset and p24 ELISAs 21 days after each stimulation in order to determine the amount of outgrowth induced after each stimulation. Resting $CD4^+$ TN, TCM, TTM, and TEM were sorted and separately seeded into the MSVOA as previously described[12] (Fig. 1b). The frequency of cells with replication competent virus was determined as previously described (IUPMStats v1.0)[101] based on the initial number of cells seeded at the beginning of the culture. IUPM values from cultures that received only one stimulation showed no induction of viral outgrowth from TN in most patients while we observed the highest IUPMs in TM and EM (Fig. 4a). When these frequencies were normalized to the frequency of each subset found in the peripheral blood, we observed similar contributions of each subset to the total pool of replication-competent viruses induced after one stimulation (Fig. 4b). To determine the

number of intact proviruses that were replication-competent after one stimulation, we calculated the ratio of IUPM to intact copies per million cells (ICPM). We observed less inducibility in TN due to lower copies of intact provirus quantified in TN; however, in the three memory subsets, we observed on average 0.5% of intact proviruses induced by one stimulation (Fig. 4c).

To test for differential inducibility of the resting CD4+ subsets, we continued our cultures in the MSVOA with 4 consecutive rounds of stimulation. As we have previously shown[12], viral outgrowth after each stimulation varied from patient to patient, with additional outgrowth being observed after multiple stimulations for some patients but not others (Fig. 5a). Outgrowth from the TN cultures was lower relative to outgrowth from the other subsets for all patients, but there were no clear patterns in either outgrowth or the number of stimulations required for outgrowth among the 4 subsets studied (Fig. 5b). For example, TM cells required multiple stimulations for outgrowth in some patients but only one stimulation for maximum outgrowth in other patients, and this trend was seen in other subsets (Fig. 5b). IUPM values calculated after four stimulations versus after one stimulation were on average 10-fold higher, signifying the robustness of the MSVOA in inducing additional viral outgrowth compared to a single round of maximum T cell activation (Fig. 5c). To determine if a particular subset contributed more to the total pool of replication-competent viruses induced after four stimulations, we looked at IUPM values normalized to the frequency of subsets present in peripheral blood as calculated above and found that no particular subset has a significant contribution (Fig 5e). The calculated inducibility factor only rose slightly to an average of 1.56% induction of all intact proviruses four stimulations (Fig. 5f).

In order to understand the lack of a relationship between conventional $CD4^+$ T cell subsets and the distribution of replication-competent proviruses, we examined the phenotypes of the cells in culture at the timepoints when outgrowth was measured (Fig 5g). Some TN only differentiated to TCM or TTM and do not reach TEM phenotype by day 21 in culture (Fig. 5g, Supp Fig. 1a). This suggests that memory CD4+ T-cells do not prefer nor are required to have an effector phenotype to produce virus (Supp Fig. 1b).

Only a small fraction of intact proviruses are induced to replicate despite multiple stimulations

To confirm the lack of correlation between inducibility and subsets, we tested several different methods of interpretation. We quantified inducibility by comparing the fold-changes in IUPM after one or multiple stimulations within each patient, and within each subset (Fig. 6b). The lower the fold-change, the higher inducibility due to most outgrowth being observed after a single stimulation. We observed varying ranges of IUPM fold-change across patients; for example, patients 2006, 3147, 2013, and 2274 did not see substantial additional outgrowth in any of the subsets compared to the other patients. When looking at IUPM fold-change by subset, we did not observe any pattern of inducibility within an individual subset. The differences in numbers of stimulations required for outgrowth for the different subsets further show that the inducibility of a provirus is not dependent on the particular subset of the host cell.

To determine the number of intact proviruses that were replication-competent in culture, we calculated the ratio of IUPM to ICPM. This inducibility factor was on average 1.56% across all subsets (Fig 6a). This suggests that only 1.56% of the intact proviruses

present in these cells are induced to replicate in culture. The MSVOA is currently the most robust method of inducing outgrowth; however, it is only able to induce 1 more log of infectious units per million cells compared to the standard QVOA (Fig. 5c), and even then can only induce 1.56% of intact proviruses to outgrowth. We also observed no correlation between IUPM and ICPM, suggesting that not all intact proviruses are equally inducible (Fig. 6c).

Replication-competent viral clones are stochastically distributed across different subsets

The V3-V4 region of *env* was sequenced from viral RNA from the supernatants of p24+ positive culture wells (Fig. 7a). Based on the clonal prediction score calculated for this region[102], we used sequences from this region to assess clonality of the viruses that replicated from each subset. As shown by our group and others, we observed identical viruses growing out from different wells at different timepoints, confirming clonal expansion of these replication-competent proviruses *in vivo*. Interestingly, from the 157 sequences we obtained from the different subsets from 10 patients, we observed identical clones that replicated from different subsets after different numbers of stimulations, suggesting that the subset phenotype plays no role in preferential infection or differential inducibility of proviral expression. To determine if there were any trends in sequence variability between subsets, we calculated the genetic distances of each viral sequence from each subset to the reference genome hxb2 (Fig 7b). We found no significant differences in the sequences from one subset compared to another, in consensus with findings from Heeregrave et al[103]. Overall, we saw no compartmentalization of viruses or clones within

a single subset, confirming that there are no distinguishing viral characteristics within certain subsets.

Discussion

In this study, we looked at the distribution and inducibility of intact, replication-competent proviruses in resting CD4+ TN, TCM, TTM, and TEM. Intact proviruses quantified by ddPCR were measured in all CD4 + T cell subsets and were unevenly distributed in all 10 patients. We calculated similar mean ICPMs across the 3 memory subsets. Although we observed 10-fold less copies of intact and defective proviral DNA in TNs, 0.01% of TNs still contained HIV-1 DNA. Even more compelling, we discovered a measurable number of replication-competent virions from TNs that were induced in the MSVOA. However, in 5/10 patients, we detected no intact proviral DNA in TNs and no viral outgrowth after all 4 stimulations (Supp Table 2). Normalized to the relative frequencies of each subset present in the peripheral blood, there was a wide distribution of each subset's contribution to the total pool of intact proviruses within each patient. This further confirms that there is no enrichment of intact proviruses within any particular subset and highlights the patient-to-patient variability that prevents any generalization about the role of different memory subsets in the latent reservoir.

Despite not finding enrichment of intact proviruses in any particular CD4+ memory T-cell subset, it was possible that there might be differential inducibility of replication-competent provirus in a particular memory subset. We initially hypothesized that subset-specific differences in transcriptional and translational activity may result in differential inducibility. Characterization of the epigenetic landscapes and cytokine responsiveness of each subset suggested that TEMs would be easiest to induce transcription and translation

in, followed by TTMs, TCMs, then TNs[52,73,74,85,97–99]. If so, TCMs would be the most difficult subset to induce viral transcription and latency reversal. To test this, we subjected sorted subset cells to 4 consecutive rounds of global T-cell activation to experimentally test whether TEMs would require the least number of stimulations. However, we found that there was no trend or correlation between the number of stimulations needed for viral outgrowth and the subset phenotype. Several groups have looked at the inducibility of HIV-1 in primary cell models, where some groups observed differential inducibility and some observed no differences between subsets[104].

To assess the frequency of intact proviruses that were induced to replicate in the MSVOA, we calculated the inducibility factor from the ratio of IUPM to ICPM. On average, we observed 1.6% of all intact proviruses were induced to replicate in this *in vitro* culture assay. This value is similar to that seen after a single round of maximum T cell stimulation in the standard QVOA (~1%) (ref), despite seeing IUPM values 10-fold higher in the MSVOA than QVOA. Fascinatingly, this number is in exact agreement with similar inducibility measurements performed by Cillo et al, in which they detected virion-associated RNA from 1.5% of proviruses in culture using anti-CD3/CD28 antibodies and a maximum likelihood estimate to calculate the total number of proviruses (intact and defective)[105]. The alarmingly high 98.44% of intact uninducible proviruses must be further investigated to determine how and whether they contribute to the latent reservoir and residual viremia. Of many possibilities, they a) may either have small defects not detected by the IPDA that render them replication-incompetent, b) may be permanently silenced thus never transcribed or translated, c) may in fact be replication-competent *in vivo* but not able to be induced *in vitro* by global T-cell activating methods, or d) may also be integrated

in a genetic location that affects inducibility. However, if a large proportion of these are eventually inducible and replication-competent but were not inducible *in vitro*, it implies an enormous hurdle to eradicating the reservoir. Therefore, further investigation will be needed to characterize these intact proviruses.

This study also highlights the importance of distinguishing resting vs activated CD4+ T-cells when sorting memory subsets based on the canonical subset surface markers. As expected, we induced differentiation of the subset cells in culture after activating them with PHA. However, TMs and EMs, which are characterized as $CCR7^-$, demonstrated CCR7 expression a few days after activation before downregulating CCR7. Figure 8 highlights the differences in surface marker expression in resting vs activated states. Activated TMs have the surface phenotype of a CM, and activated EMs have a $CCR7^+CD27^-$ phenotype, which would exclude them from being sorted as $CCR7^-CD27^-$ EMs. The plasticity of these surface markers is important to consider when performing a surface marker-based sort of the memory subsets. Functional characteristics of these cells based on cytokine production or transcription factor activity may more accurately reflect the specific subsets[106]. In this study, we did not observe any enrichment or differential inducibility of latent intact proviruses in any particular subset of memory CD4+ T-cells. This suggests that targeting a particular subset may not significantly reduce the latent reservoir. It is not possible to know the exact memory phenotype of the cell at the time of infection *in vivo*; based on our env sequences, 95% of the viruses were predicted R5-tropic[107] but most were from largely expanded clones. Some groups have looked at the different levels of susceptibility of each subset to infection but this is independent of the differentiation timeline of the cell[93,103,108].

Recently, many cure strategies involve identifying markers of latently infected cells which would allow this population to be selectively targeted. Overall, our results demonstrate that a specific memory CD4+ T-cell subset cannot be targeted, as latent replication-competent proviruses seem evenly distributed among the memory subsets with no significant differences in the inducibility of proviruses within any particular subset.

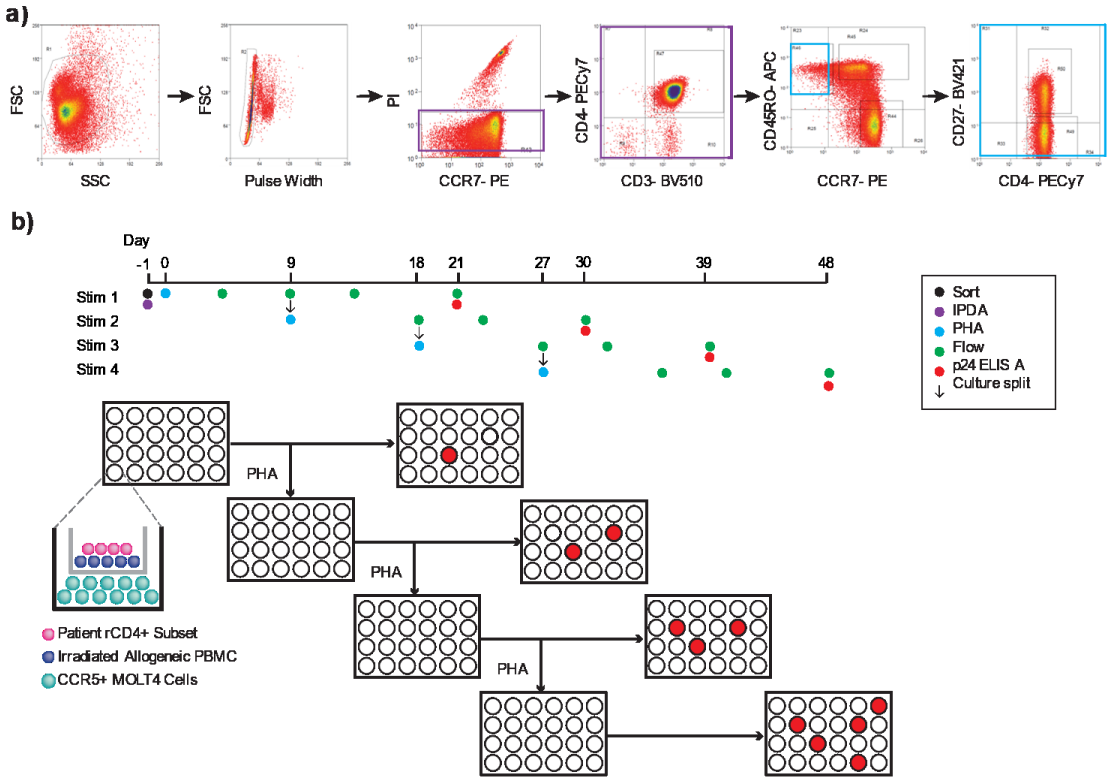

Fig. 4.1. Subset sorting strategy and MSVOA culture schematic. (a) Resting CD4+ T-cells were isolated from leukopaks from 10 patients on ART, stained for CD3, CD4, CD45RO, CCR7, and CD27. Cells were sorted by lymphocytes, singlets, live cells (based on propidium iodide stain), CD3+ CD4+, and then TN (CD45RO- CCR7+ CD27+), TCM (CD45RO+ CCR7+ CD27+), TTM (CD45RO+ CCR7- CD27+), and TEM (CD45RO+ CCR7- CD27-). Sorted cell numbers were used to calculate frequency of each subset present in peripheral blood in Fig. 2b. **(b)** A small aliquot of the sorted resting cells were set aside for IPDA analysis and calculations of intact and defective proviruses (Fig. 2a). Of the remainder, $2x10^5$ cells from each subset were seeded into separate plates in a transwell system, with irradiated allogeneic PBMCs and subset cells in the top chamber and MOLT-4 cells in the bottom chamber. Cells were activated with PHA on day 0, then cultures were split in half on day 9, with half remaining incubated until day 21 for p24 ELISA (outgrowth data in Fig. 4) and the other half activated again with PHA. This was repeated for a total of 4 PHA stimulations every 9 days, and p24 data are shown in Fig. 5. Flow cytometry was run every 5-7 days on cultures to collect marker expression data at those timepoints, shown in Fig. 3.

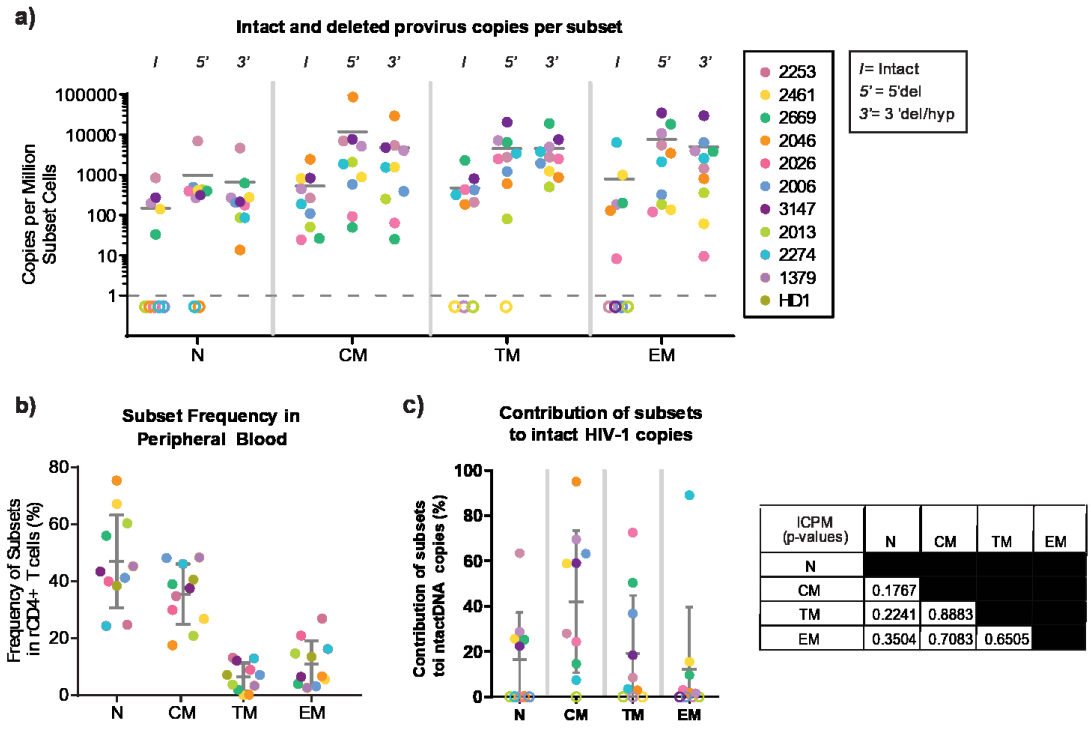

Fig. 4.2. Frequencies of subsets in periphery and proviral DNA copies. (a) IPDA data from resting cells of each subset. There are always ~10-fold more defective (5' half deleted, 3' half deleted, hypermutated) copies than intact in any patient sample. Less naïve cells harbor proviruses, but on average, each memory subset contains roughly equivalent numbers (mean=600) of intact proviral copies per million cells. For some subsets in some patients, the frequency of intact proviruses was below the limit of detection (noted by open circles). Paired t-tests were performed to compare ICPM within each subset to each other to show that there is no significant difference in the ICPM of each subset. **(b)** Frequency of each subset sorted from a single leukapheresis (mean±SD). Naïve CD4+ T-cells are the most prevalent, followed by central memory, then transitional and effector memory cells with mean frequencies less than 12%. **(c)** Percent of intact proviruses found in each subset (b) normalized to frequency of subset in peripheral blood (a). Contribution of each subset to the total pool of intact proviruses varies from patient to patient with no apparent enrichment within any particular subset. Paired t-tests between subsets showed no significant differences. Open circles denote samples in which 0 intact copies were found.

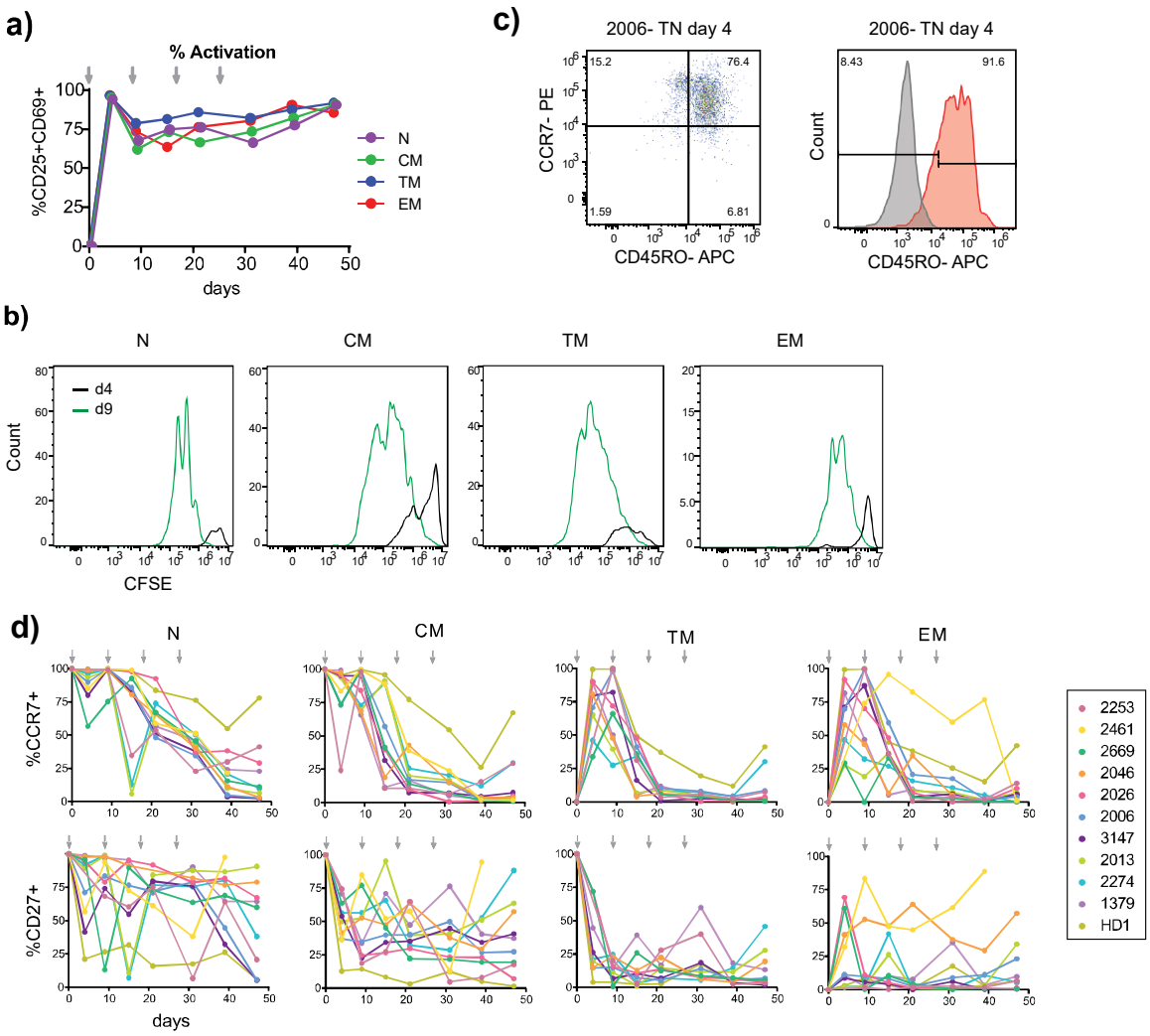

Fig. 4.3. Cell culture maintenance and phenotype marker kinetics. (a) Activation status of CD4+ T cells throughout the MSVOA averaged across 4 representative patients. Arrows denote stimulation times. Graph shows percent of cells co-expressing CD25 and CD69. **(b)** CFSE proliferation of cells in MSVOA after first stimulation. Cells from each subset were stained with CFSE and activated on day 0, then analyzed by flow cytometry at days 4 and 9 to verify proliferation of each subset. **(c)** CD45RO expression of naïve cells from representative patient. Red histogram represents CD45RO+ patient cells, gray represents negative control. Of naïve cells that were originally CD45RO- at time of sort (Supp. Fig. 1a), 91.6% of cells became CD45RO+ by day 4 after initial stimulation. **(d)** Percent of subset cells in culture expressing CCR7 and CD27 at given timepoints. Arrows denote times of additional stimulation. CCR7 expression decreases over time as cells differentiate in culture. In TM and EM, where CCR7 is not normally expressed, activation led to upregulation of CCR7, then downregulation over time. Cultures were activated at days 0, 9, 18, and 27 as denoted by arrows. CD27 expression generally decreases over time in culture in subsets except for naïve cells, in which they maintain surface expression in most patients. All TM lost CD27 expression, and in a few patients, EM seemed to upregulate CD27 after activation.

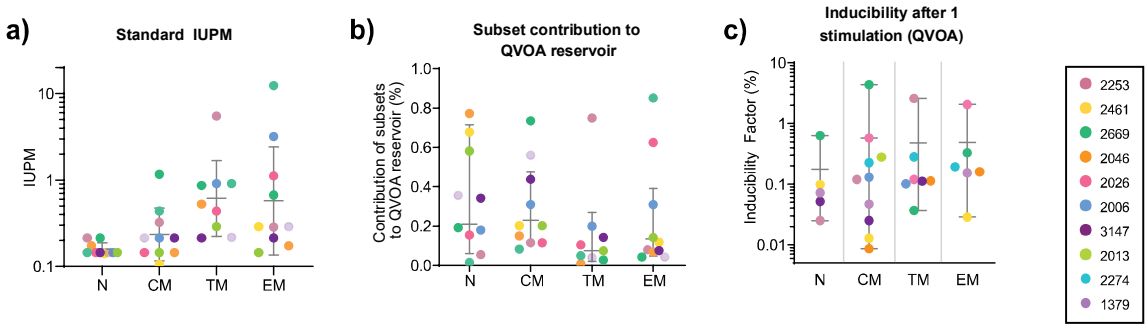

Fig. 4.4. P24 results and outgrowth observed after one stimulation as in traditional QVOA. **(a)** IUPM calculated after one stimulation. **(b)** Contribution of subsets to total pool of replication-competent proviruses induced after one stimulation. **(c)** Percentage of intact proviruses that were induced to replicate after one stimulation.

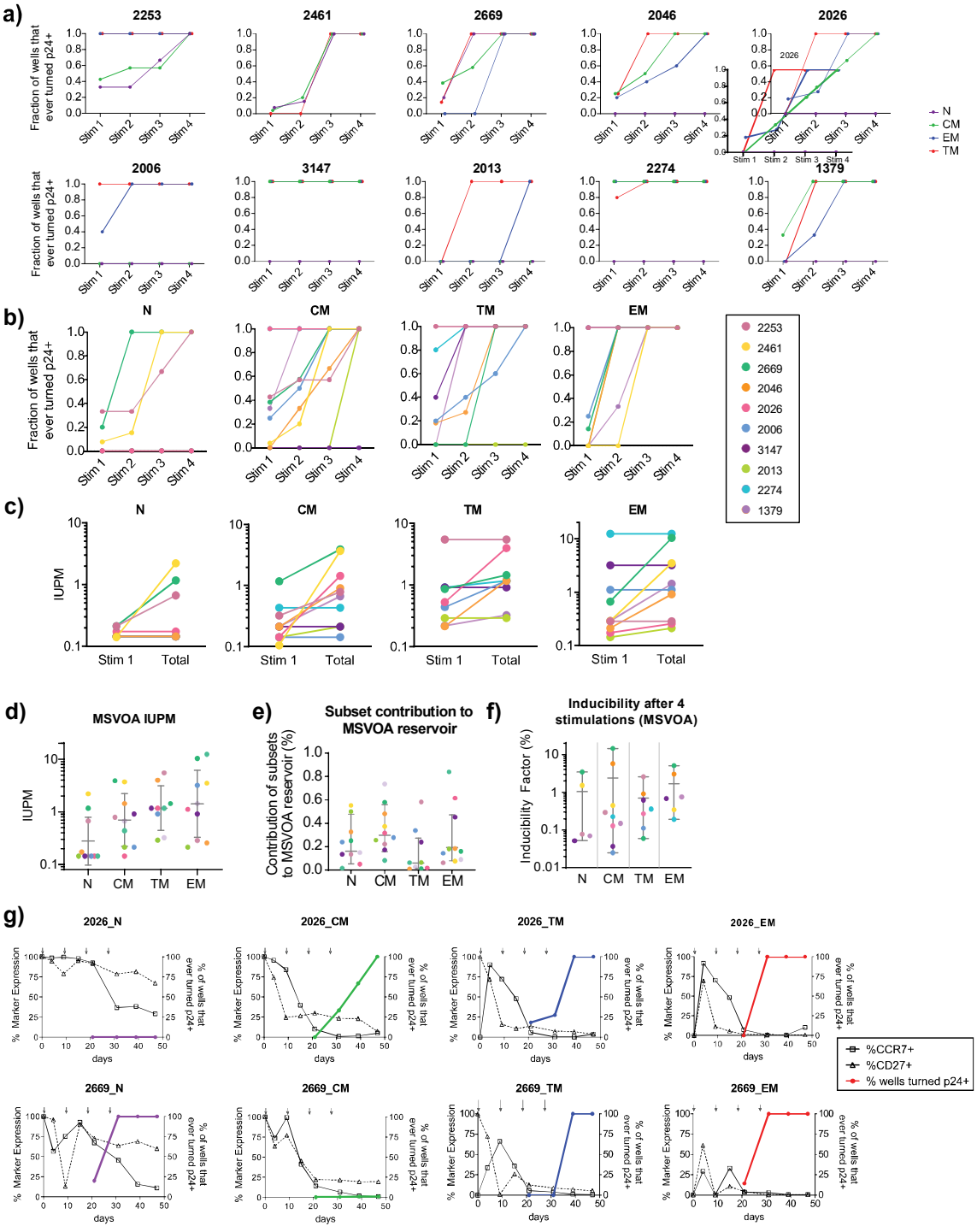

Fig. 4.5. P24 results and additional outgrowth observed after four stimulations in MSVOA. (a) Fractions of all wells that became p24+ after each stimulation for individual patients. **(b)** Fractions of all p24+ culture wells after each stimulation for each subset. **(c)** Infectious units per million subset cells calculated based on initial number of cells seeded and number of wells turned p24+. IUPM after all 4 stimulations was ~1 log higher than IUPM after a single stimulation.

There seems to be no pattern in inducibility of virus in each subset. **(d)** IUPM calculated from total numbers of wells that became p24+ throughout MSVOA based on initial number of cells seeded. **(e)** Contribution of each subset to total pool of replication-competent proviruses (IUPM) based on frequency of subset present in peripheral blood (Fig. 2b). **(f)** Inducibility factor calculated from ratio of MSVOA IUPM to ICPM from each subset. **(g)** Phenotype of cultured cells overlaid with outgrowth data for each subset for one representative patient. Left y-axis shows levels of CCR7 and CD27 expression at each timepoint to represent phenotype. Right y-axis shows percent of wells that became p24+ at each timepoint.

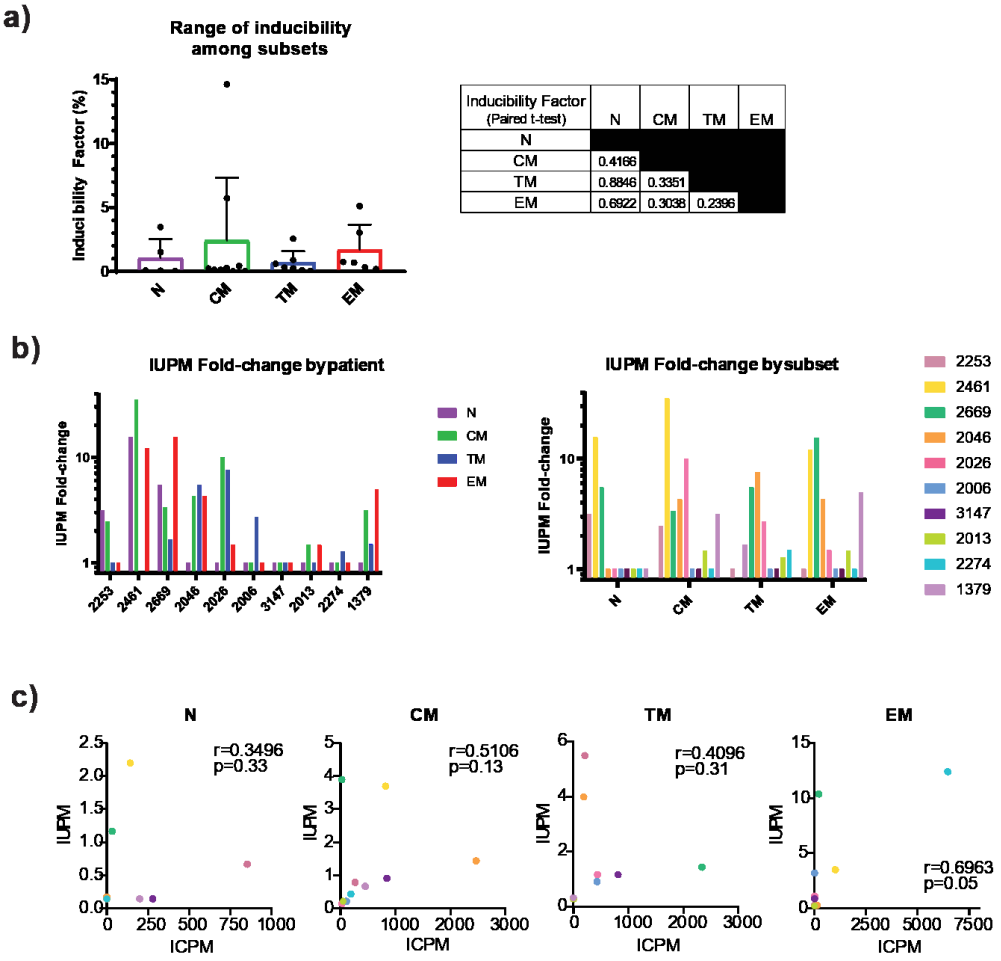

Fig. 4.6. Inducibility of viruses from different subsets. (a) Range of inducibility factors among subsets (mean and SD shown). On average, only 1.56% of intact proviruses measured were induced to replicate in culture. Paired t-tests showed no significant difference in means of inducibility factors across subsets ($p>0.24$). **(b)** Fold-change in IUPM by patient and by subset. There is patient-to-patient variability seen in inducibility across all subsets. For example, patient 3147 did not require additional stimulations for additional outgrowth from any of the subsets, whereas patient 2461 required multiple stimulations for additional outgrowth from all subsets tested. **(c)** Spearman correlation tests between IUPM and ICPM with rho and p values shown.

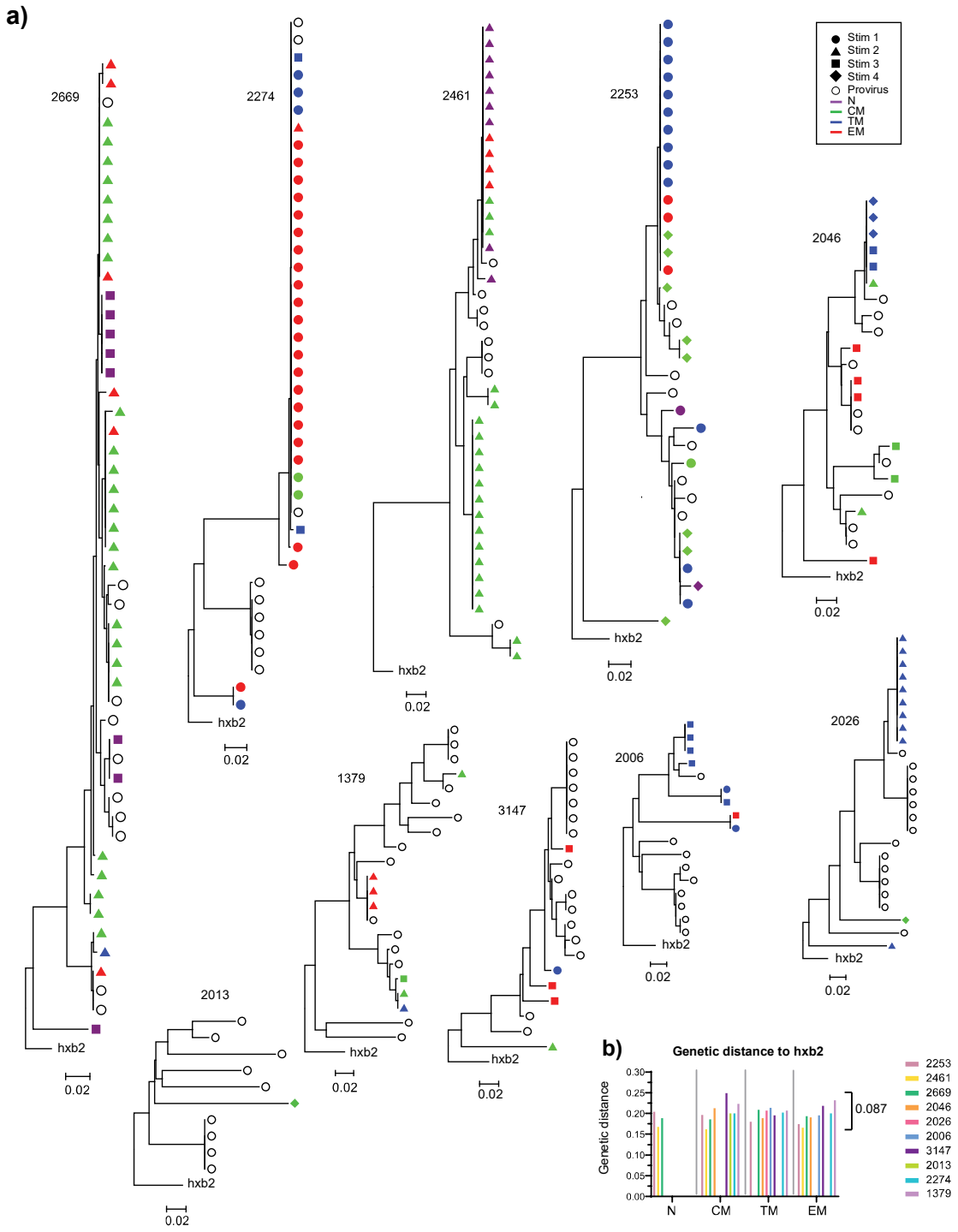

Fig. 4.7. Neighbor-joining env trees of outgrown viruses from subsets. (a) env sequencing of outgrown viruses from 4 different timepoints (21d after each stimulation) from culture supernatants. Clonal viruses from different subsets show that cells carrying these clonal proviruses can differentiate separately from each other, further showing that subset phenotype is not a predictor of inducibility. **(b)** Genetic distances of sequences from each subset were calculated relative to the reference genome hxb2. No significant genetic differences were seen in viral sequences between subsets or within subsets.

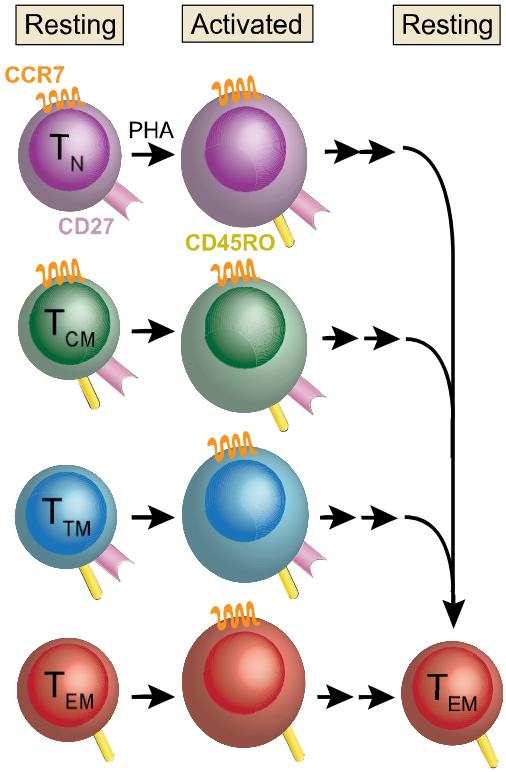

Fig. 4.8. Illustration of surface marker differences on resting vs activated CD4+ subsets. After activation with PHA, expression of the canonical subset-distinguishing surface markers changes. Naïve cells begin expressing CD45RO as they differentiate into memory cells. TMs and EMs, which are CCR7-, start expressing CCR7 after activation. In culture after activation, the initially resting subset cells differentiate toward the effector memory phenotype (CCR7-CD27-). This highlights the importance of distinguishing resting vs total CD4+ T-cells which include activated cells. For example, the activated TM cell exhibits the same surface markers as the activated CM cell; even if they may not be functionally similar, they would be sorted into the same population based on the canonical usage of CCR7 and CD27 to sort memory subsets.

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Chapter 4

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CURRICULUM VITAE

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CAREER HIGHLIGHTS

- **7 years of experience** in wetlab research across -omics, infectious diseases, and immunology disciplines:
  - 1 year of experience in next-generation sequencing pipeline from wetlab library preparation to bioinformatic analysis including genome assembly; 5 years of experience in Sanger sequencing analysis of viral and human DNA
  - 6 years of experience in designing and performing immunological assays including flow cytometry, ELISAs, and tissue culture of cell lines and human primary cells
- **7 publications** from graduate career: 5 published; 1 first-author and 1 second-author manuscripts currently in progress
- Contributed to **multi-center clinical trial** (HOPE in Action) research objectives
- Led **2 collaborations** with other academic institutions that led to a first-author manuscript in progress
- Presented thesis work at the NIH Strategies for an HIV Cure Conference (2018), JHU Center for AIDS Research (CFAR) Symposium (2018), and at the Conference on Retroviruses and Opportunistic Infections (CROI) (2019)

EDUCATION

Johns Hopkins University School of Medicine **Aug 2014 – Apr 2019**
PhD, Department of Pharmacology and Molecular Sciences, Laboratory of Dr. Robert Siliciano

University of California, Berkeley **2009 – 2013**
Bachelor of Arts, Molecular and Cell Biology

EXPERIENCE

AVIDEA TECHNOLOGIES, Baltimore MD **Jan 2019 – Present**
Business Development Intern (through JHU Biomedical Careers Initiative)
- Conducted immuno-oncology (IO)/ immunotherapy market research
- Analyzed potential partnering opportunities with biotech and pharmaceutical companies

JOHNS HOPKINS UNIVERSITY SCHOOL OF MEDICINE **June 2015 – May 2019**
PhD Candidate, Laboratory of Dr. Robert Siliciano
- Investigated inducibility of latent replication-competent HIV-1 in memory T-cell subsets for thesis project; first-author manuscript in progress
  - Led 2 collaborations with faculty at UCSF and Case Western University to gain their expertise and input on project development and obtain patient samples from their cohorts

- Designed and performed novel experiments involving viral outgrowth cultures from memory T-cells from patients and encompassing flow cytometric/ddPCR/ELISA/sequencing assays to find potential cell targets for HIV cure

- Contributed to historical clinical trial as research team member providing pre- and post-operative quantitation of the HIV latent reservoir in organ recipients enrolled in the HOPE (HIV Organ Policy Equity) in Action Clinical Trial (NCT03500315), under which Johns Hopkins was the first hospital in US history to perform HIV+-to-HIV+ organ transplants; research manuscripts in progress
- Demonstrated key finding for the field that latent HIV can clonally expand *in vivo,* resulting in second-author publication (Hosmane et al. 2017) named "Best of the Journal of Experimental Medicine 2017" for being one of their top-requested articles of the year
- Led collaboration with the bioinformatics team in the Bushman Lab at the University of Pennsylvania to analyze integration sites of latent HIV in patient genomic DNA to look for sites of preferential integration within cancer-related genes and help optimize the sequencing/analysis pipeline
- Developed bead-based method to isolate rare copies of proviral DNA integrated in human genomic DNA to streamline sample collection for integration site sequencing and analysis assisted by collaborators in the Bushman Lab

NATIONAL INSTITUTES OF HEALTH, NIAID – Clinical Parasitology Section
Post–baccalaureate Intramural Research Training Award (IRTA) Fellow - Laboratory of Dr. Thomas Nutman **July 2013 – April 2014**

- Optimized clinical diagnostic ELISAs for neurocysticercosis in CSF and serum samples of *T.solium*–infected patients
- Ran anticestode drug screening assays by repurposing cancer drugs for neurocysticercosis treatment

LOS ALAMOS NATIONAL LABORATORY (LANL) **2010 – 2013 (12 months)**
Bioscience Division Undergraduate Student Summer Intern
Genome Sciences Group B–6: Metagenomics team with Dr. Shawn Starkenburg Summer/Winter 2011 – 2013

- Completed *de novo* genome sequencing and annotation, transcriptome analysis, and comparative genomic analysis of the nuclear and organellar genomes of a novel alga *Nannochloropsis salina* that led to second-author publication

Advanced Measurement Sciences Group B–9: Structural Biology team with Dr. Pawel Listwan May – Aug 2010

- Optimized protocol for protein-protein interaction screening using an internally developed split–GFP toolbox

PUBLICATIONS

1) **Kwon KJ**, Timmons AE, Sengupta S, Simonetti FR, Zhang H, Hoh R, Deeks SG, Sekaly RP, Siliciano JD, Siliciano RF. "Inducibility of Latent HIV-1 in Resting CD4+ Memory T-cell Subsets." ***In progress.***
2) Martin AR*, Bender AM*, **Kwon KJ**, Desai N, Brown D, Segev D, Quinn T, Tobian A, Durand C, Redd A, Siliciano RF. "Quantification of inducible and intact HIV-1 provirus in peripheral blood and lymph nodes." ***In progress.***
3) Bruner KM, Wang Z, Simonetti FR, Bender AM, **Kwon KJ**, Sengupta S, Fray EJ, Beg SA, Antar AA, Jenike KM, Kufera JT, Timmons AE, Nobles C, Gregg J, Wada N, Ho YC, Margolick JB, Blankson JN, Deeks SG, Bushman FD, Siliciano JD, Laird GM, Siliciano RF. "A quantitative approach for measuring the reservoir of latent HIV-1 proviruses." ***Nature,*** **Feb 2019**.
4) Wang Z, Gurule EE, Brennan TP, Gerold JM, **Kwon KJ**, Hosmane NN, Kumar MR, Beg SA, Capoferri AA, Ray SC, Ho YC, Hill AL, Siliciano JD and Siliciano RF. "Expanded cellular clones carrying replication–competent HIV–1 persist, wax, and wane." ***Proceedings of the National Academy of Sciences*****, Feb 2018**.
5) **Kyungyoon J. Kwon** and Robert F. Siliciano. "HIV persistence: clonal expansion of cells in the latent reservoir." ***The Journal of Clinical Investigation,*** **Jun 2017.**

6) Hosmane NN, **Kwon KJ**, Bruner KM, Capoferri AA, Beg S, Rosenbloom D, Keele BF, Ho YC, Siliciano JD, Siliciano RF. "Proliferation of latently infected CD4+ T cells carrying replication–competent HIV–1: potential role in latent reservoir dynamics." ***The Journal of Experimental Medicine*****, Mar 2017**.

7) Murray AJ, **Kwon KJ**, Farber DL, Siliciano RF. "The Latent Reservoir for HIV–1: How Immunologic Memory and Clonal Expansion Contribute to HIV–1 Persistence." ***The Journal of Immunology*****, Jul 2016**.

8) Starkenburg SR, **Kwon KJ**, Jha RK, McKay C, Jacobs M, Chertkov O, Rocap G, Cattalico RA. "A Pangenomic Analysis of the *Nannochloropsis* Organellar Genomes Reveals Novel Genetic Variations in Key Metabolic Genes." ***BMC Genomics*****, Mar 2014**.

SKILLS

- **Bioinformatics:** PCR primer design, qPCR primer/probe design, qPCR-based gene expression assays, Sanger sequencing analysis, BioEdit, CodonCode, MEGA, Galaxy, BLAST, MG-RAST, IGV, Artemis, KEGG
- **Molecular Biology:** PCR, qPCR, ddPCR, gel electrophoresis, DNA/RNA/protein extraction and purification, Nanodrop, Qubit, Bioanalyzer, Illumina library prep for gDNA and RNA
- **Immunology/Tissue Culture:** human tissue (lymph node) and blood processing, human primary cell culture, bloodborne pathogen handling (HIV), flow cytometry, ELISA, drug screening culture assays, *in vitro* virus infection assays, Western Blot, parasite handling (tapeworm larvae)
- **Instrumentation:** flow cytometry (Intellicyt iQue Screener, BD FACSCanto, BD LSRFortessa), qPCR (AB systems), ddPCR (BioRad QX200 Auto Droplet Generator, Droplet Reader)
- **Technical:** FlowJo, QuantaSoft (for ddPCR), GraphPad Prism, Adobe Illustrator, EndNote, Mendeley, GlobalData industry database, Microsoft Office

AWARDS

- **New Investigator Scholarship –** International AIDS Society/CROI **2019**
- **Best Articles of the Journal of Experimental Medicine** **2017**
- **SJ Yan and HJ Mao Graduate Fellowship** **2016 – 2019**
- **NIH Postbaccalaureate Intramural Research Training Award** **2013 – 2014**

VOLUNTEER AND EXTRACURRICULAR ACTIVITIES

- Peabody Conducting Student Recitals, violinist **2017 – Present**
- Baltimore Animal Rescue and Care Shelter, dog walker **2016 – Present**
- Tahirih Justice Center, volunteer in career workshops and miscellaneous **2016 – 2017**
- Hopkins Symphony Orchestra, violinist **2015 – 2016**
- Berkeley Food and Housing Project, kitchen volunteer **2010 – 2013**
- St. Mark's Hot Meals for the Hungry, kitchen volunteer **2009 – 2013**