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INVESTIGATING THE ROLE OF T CELL ACTIVATION ON THE
ESTABLISHMENT OF HIV-1 LATENCY IN PRIMARY CD4+ T
CELLS USING A DUAL-REPORTER VIRUS

by

Leonard Chavez

DISSERTATION

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I dedicate this work to Mi Familia: my Mom and my Dad; my brothers, David and Isaac; and my sister, Taressa. As the youngest member of Mi Familia, I have had the privilege of learning about life from each and every one of them. Without their guidance, none of this would be possible.

I also dedicate this work to my girlfriend, Helen. From the first day I met her, she has embodied the spirit and values of home that I thought only Mi Familia could offer. And, even though I had to leave Mi Familia and continue this journey on my own, Helen was put in their place to remind me of where I come from and why I was on this journey in this first place. Though not official yet, Helen is very much a part of Mi Familia.

CONTRIBUTIONS OF CO-AUTHORS TO THE PRESENTED WORK

Chapter 2 of this dissertation is a reprint of the following reference: Vincenzo Calvanese[1,3], Leonard Chavez[1,3,5], Timothy Laurent[2,4], Sheng Ding[2,4], Eric Verdin[1,3,5]. Dual-color HIV reporters trace a population of latently infected cells and enable their purification. Virology. 2013 Nov;446(1-2):283-92.

Author contributions:

Vincenzo Calvanese conceived and designed experiments, and generated the HIV dual-reporter viruses. Leonard Chavez performed experiments in primary CD4^{+} T cells. Timothy Laurent performed the Tocris drug screen. Shen Ding conceived and designed experiments. Eric Verdin conceived and designed experiments, and supervised the work.

Chapter 3 of this dissertation is based on the following manuscript in preparation: Leonard Chavez[1,3,5], Vincenzo Calvanese[1,3], Eric Verdin[1,3,5]. Activated and resting primary CD4^{+} T cells can both become latently infected by an HIV dual-reporter virus.

Author contributions:

Leonard Chavez conceived and designed experiments, and performed all experiments. Vincenzo Calvanese generated the HIV dual-reporter virus. Eric Verdin designed and conceived experiments, and supervised the work.

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ABSTRACT

Acquired Immunodeficiency virus (AIDS) has killed 36 million people since the first cases were reported in 1981, and is considered a worldwide pandemic. AIDS is caused by the human immunodeficiency virus (HIV), which first originated in Africa in the early 1900s, and by the 1980s had spread around the globe. HIV is a lentivirus that slowly destroys the immune system by infecting and killing CD4$^+$ T cells. There is currently no cure or vaccine for HIV. However, drug therapy exists that is capable of controlling HIV infection, and prevents the progression to AIDS. Drug therapy cannot eradicate HIV because the virus can become transcriptionally silent after integrating its genetic material into the host cell's genome, resulting in latent infection. HIV latency is a reversible state, and allows the virus to remain within an infected individual for the entirety of their life-span. The study of HIV latency has been ongoing since 1986, but has been hindered by two major problems: 1) latently infected cells are extremely rare, *in vivo*; and 2) latently infected cells are indistinguishable from uninfected cells. The work of this dissertation focused on the development and utilization of an HIV dual-reporter virus that could identify latently infected cells, and distinguish them from productively infected and uninfected cells. The HIV dual-reporter viruses utilize two different fluorescent proteins. One fluorescent protein is under the control of the HIV LTR promoter, and identifies cells that are productively infected, while the other fluorescent protein is under the control of a constitutive promoter, independent of the viral promoter, and identifies cells that have integrated provirus. Infection of Jurkat cells and primary CD4$^+$ T cells with these dual-reporter viruses allows for the identification and purification of latently infected cells. This isolated latent population which only

expresses the fluorescent protein that is under the control of the constitutive promoter, is devoid of viral transcripts and viral proteins. One of these dual-reporter viruses, HIV Duo-Fluo I, was used to study the role of T cell activation on the establishment of HIV latency in primary CD4$^+$ T cells. Resting primary CD4$^+$ T cells are capable of supporting productive HIV infection, but show a propensity towards latent infection, while activated primary CD4$^+$ T cells are capable of supporting latent infection, but show a propensity towards productive infection. Further, activated primary CD4$^+$ T cells are capable of supporting latent infection without the need to return to a resting state. Our findings suggest that HIV latency is not completely dependent on T cell activation and that infection of both resting and activated primary CD4$^+$ T cells can contribute to the HIV latent reservoir.

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CHAPTER 1: Introduction

HIV/AIDS pandemic

Human immunodeficiency virus (HIV) is a lentivirus that causes acquired immunodeficiency syndrome (AIDS) (1). HIV originated in west-central Africa at the turn of the twentieth century via multiple zoonotic transmission events between non-human primates and humans (2). Based on these zoonotic transmission events, HIV can be divided into two major types, HIV-1 and HIV-2. HIV-1 is related to simian immunodeficiency viruses (SIVs) found in chimpanzees and gorillas, while HIV-2 is related to SIV found in the African sooty mangabey (2). Whereas, both viruses originated in Africa, HIV-1 is responsible for the AIDS pandemic.

Despite HIV-1 infection originating in Africa during the early 1900s, AIDS wasn't clinically observed until 1981 in the United States (3). AIDS is characterized by a weakening of the host immune system that allows opportunistic infections and cancers to thrive within an infected individual, and if left untreated, has a mortality rate of 100%, with the average survival time after HIV-1 infection estimated at ~10 years (4). Since its discovery, HIV/AIDS has developed into a worldwide pandemic that has claimed the lives of an estimated 36 million people (as of 2012) (4). Today, approximately 35.2 million people are living with HIV-1, with 6,300 new infections occurring every day (4). The biggest burden of the HIV/AIDS pandemic is shouldered by low and middle-income countries, as they account for 95% of new infections worldwide. Of these low and middle-income countries, those residing in Sub-Saharan Africa are hit the hardest, with

the region being home to 71% of people living with HIV-1 worldwide, and 88% of the world's HIV-positive children (4).

HIV-1 can be transmitted through contact with bodily fluids, specifically, blood, semen, vaginal fluid, and breast milk (5). The most likely routes of infection occur via unprotected sexual intercourse, contaminated blood transfusions, sharing of contaminated hypodermic needles during intravenous drug use, and from mother to child during pregnancy, delivery, or breastfeeding. After infection occurs, HIV-1 starts to attack the immune system, and causes the depletion of CD4$^+$ T cells. An infected individual has progressed to AIDS once their CD4$^+$ T cell count drops below 200 cells/μl (5). At this point the immune system becomes too weak to fight off opportunistic infections that an, otherwise, healthy individual would easily combat. These opportunistic infections include, but are not limited to fungal induced pneumocystis pneumonia and esophageal candidiasis, as well as viral induced Kaposi's sarcoma and Burkitt's lymphoma (6). AIDS patients can also suffer from caxechia, also known as wasting syndrome (6).

There is no cure or vaccine for HIV-1 infection. However, in 1995 combination drug treatment known as highly active antiretroviral therapy (HAART) became widely available in the developed world, and transformed HIV-1 infection from a deadly disease into a manageable chronic condition (7). The basis of HAART involves the administration of several different drugs that target multiple stages of the viral life cycle, and consists of a daily regime that must be maintained for the entirety of an HIV-1 infected individual's lifetime. Despite the development of this revolutionary treatment, countries that bear the largest burden of the pandemic still don't have full access to the

drugs (4). Recent efforts by the Bush administration of the United States in the form of the Presidents Emergency Plan for AIDS Relief (PEPFAR), and its continuation by the Obama administration, has allowed 9.7 million HIV-1 infected individuals residing in low and middle-income countries access to HAART (8). However, within these regions, an estimated 20 million infected individuals still don't have access to the drugs (4). Because of this, the main strategy to combat HIV-1 within these regions is prevention through education.

HIV-1 genome and virion structure

HIV-1 is a lentivirus that belongs to the Retroviridae family (9). The genus, lentivirus, derives its name from the latin, lente, which means "slow", and lentiviral infections are characterized by a long incubation period, followed by a long-duration illness. All lentiviruses share common characteristics related to biological properties and morphology.

The lentiviral genome consists of an RNA genome that ranges from 7-12 kb in length and encodes three genes common to all retroviruses, gag, pol, and env. In addition, lentiviruses encode two regulatory genes, tat and rev, as well as several accessory genes not found in other retroviruses. All the open reading frames are flanked by two long terminal repeats (LTRs). HIV-1 contains two copies of a single-stranded, positive-sense, RNA genome that is ~9.7 kb and contains nine genes that are flanked by two LTRs of ~640 bps (Figure 1.1) (9). The 5' LTR contains the HIV-1 promoter that initiates the transcription of the nine genes which encode 15 different proteins.

Lentiviral virions are spherical in shape and range from 80-120nm in diameter. They consist of an RNA genome and viral enzymes that are encased within a viral protein core, that is enclosed within a host cell derived membrane spiked with viral envelope glycoproteins. The nucleiod is usually rod-shaped or shaped like a truncated cone, and distinguishes lentiviruses from other retroviruses. HIV-1 is roughly spherical in shape with a diameter of 120nm, making it a very large lentivirus (9). The structure of the HIV-1 virion is set-up as follows (10): Its outer membrane is composed of a lipid bilayer taken from the host cell during viral budding (Figure 1.2). Embedded within the lipid membrane are host proteins, as well as the viral Env protein, which consists of glycoprotein 120 (gp120) and glycoprotein 41 (gp41). Three molecules of gp41 anchor into the lipid membrane and act as a stem to support three molecules of gp120. The surface glycoprotein, gp120, mediates virus attachment to target cells by binding to host cell surface receptors, while the transmembrane glycoprotein, gp41, contains a fusion peptide that mediates fusion with and entry into the target cell.

Attached to the inner surface of the lipid membrane is the viral matrix protein, p17, which is encoded by the gag gene. p17 serves as a structural protein that stabilizes the virion and also aids in virion packaging. Enclosed within the matrix protein is an assembly of the viral structural protein, p6 (encoded by gag), that plays an important role in viral budding. Enclosed within the p6 matrix is a matrix of the viral capsid protein, p24, which is also encoded by the gag gene. Approximately, 5,000 p24 molecules assemble within the matrix lattice to form the cone-shaped nucleoid that encompasses the viral genomic RNA (11).

Within the p24 nucleoid, resides two copies of genomic RNA that are tightly bound to the viral nucleocapsid protein, p7. p7 is encoded by the gag gene and is responsible for packaging the viral RNA into the virion. Also, within the viral nucleoid are two viral enzymes encoded by the pol gene, reverse transcriptase and integrase. Reverse transcriptase is responsible for converting the viral RNA genome into a double-stranded cDNA, while integrase catalyzes the integration of the cDNA into the host genome. The HIV-1 virion also contains a third enzyme encoded by the pol gene, protease, which plays a major role in the maturation of the virion after it has budded from an infected cell.

Finally, several accessory viral proteins are also packaged within the HIV-1 virion, including the virion infectivity factor (Vif), viral protein R (Vpr), and negative factor (Nef). Vif counteracts a host restriction factor, APOBEC, upon viral entry into cells; Vpr arrests the host cell in the G2/M cell-cycle; and Nef has several important functions which includes down-regulating the expression of the cell surface molecules, CD4 and the major histocompatability complex (MHC). Down-regulation of CD4 aids in env being incorporated into the budding virion, and MHC down-regulation aids in the infected cell avoiding detection by the immune system.

Not packaged into the virion, are the two regulatory proteins, Tat and Rev. Both proteins are the first to be expressed from the newly integrated viral DNA (provirus); Tat is a transcriptional transactivator that exponentially enhances the transcriptional activity of the viral promoter, and Rev is an RNA binding protein that exports viral RNA from the nucleus to the cytoplasm. Lastly, the accessory viral protein, Vpu, is also not packaged

into the virion, but is expressed from the provirus after integration. Vpu plays a role in CD4 down-regulation, as well as virion budding.

HIV-1 life cycle

Upon infection, HIV-1 targets several different cell types including dendritic cells, macrophages, and its main target, CD4$^+$ T cells. When infecting a CD4$^+$ T cell, HIV-1 uses gp120 to bind to the host cell (12) (Figure 1.3). gp120 first binds to the host cell's CD4 receptor and then undergoes a structural change that exposes its chemokine binding domains. These chemokine binding domains are used by gp120 to bind to one of two chemokine co-receptors, CCR5 or CXCR4. Binding of gp120 to both CD4 and either co-receptor is required for viral binding and fusion, as cells lacking CCR5 and CXCR4 are resistant to HIV-1 infection. Once both receptor and co-receptor have been engaged by gp120, the envelope protein undergoes another conformational change that exposes the fusion peptide within gp41. This fusion peptide penetrates the host cell membrane and forms a hairpin structure that allows fusion of the virus with the host cell. After HIV-1 has fused with the host cell, the viral core is released into the cell.

Inside the cell, the viral core starts to disassemble, and the viral reverse transcriptase uses cellular dNTPs to convert the viral genomic RNA into a double-stranded cDNA (13). This process of reverse transcription is extremely error prone, resulting in multiple mutations that can be advantageous to the virus. After reverse transcription is complete, the viral cDNA is incorporated into a pre-integration complex (PIC) consisting of both viral proteins (Vpr, p17 and integrase) and host proteins (14). The PIC then enters the cell nucleus via a nuclear pore complex. Once in the nucleus,

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the HIV-1 integrase protein, in combination with several host proteins, inserts the viral cDNA into the host cell genome. The proviral DNA then comes under the control of the host cellular transcriptional machinery.

Within the HIV-1 LTR promoter are several transcription factor binding sites that the virus uses for the initial transcription of its mRNA (15). The most important of these transcription factors is NF-κB, which is upregulated in activated CD4$^+$ T cells, meaning that viral transcription is most efficient in activated cells. Using NF-κB to initiate viral transcription, the provirus transcribes mRNA that gets spliced, as well as unspliced mRNA. The spliced mRNA exists the nucleus and gets translated by the cellular machinery into the two regulatory proteins, Tat and Rev. Tat and Rev both have a nuclear localization sequence that then allows them to re-enter the nucleus through a nuclear pore complex. Inside the nucleus, Tat binds to a short stem-loop structure called the transactivation response element (TAR) that is located at the 5' terminus of HIV-1 RNAs. Once bound to TAR, Tat recruits several host proteins that work in conjunction to increase transcription from the HIV-1 LTR promoter by at least 1000-fold.

Once Rev has accumulated inside the nucleus it binds to the rev responsive element (RRE) of unspliced viral mRNAs, and exports them to the cytoplasm where they can be translated into viral proteins (15). Without Rev, the unspliced viral mRNAs would remain in the nucleus until spliced.   The unspliced viral mRNAs are processed into proteins originating from the Gag, Pol, and Env genes. Full-length unspliced viral mRNA serves as the viral genomic RNA and also requires Rev for nuclear export. The Gag, Pol, and Env proteins are all translated into polyproteins that require further processing.

The envelope polyprotein (gp160) goes through the endoplasmic reticulum and the gogli apparatus where it is cleaved into gp41 and gp120, which are then transported to the cell membrane via the cell secretory pathway. The Pol polyprotein is transcribed and translated as a Gag-Pol polyprotein (p160) that is generated by a ribosomal frame-shift occurring in the distal region of the Gag RNA. This frame-shift allows the read-through of a stop codon at the gag-pol junction, and occurs 5% of the time resulting in the production of the Gag polyprotein (p55) and the Gag-Pol polyprotein at a 20:1 ratio. p55 and p160 are then targeted to the cell membrane via the myristoylation of Gag, which allows the polyproteins to associate with the innner surface of the plasma membrane, where they start to polymerize. All other viral components, including accessory proteins and viral RNA are transported to the cell membrane by direct protein-protein and protein-RNA interactions with Gag. Once all viral components have assembled at the cell membrane, gp41 and the myristoylated Gag interact within the cell membrane to ensure that the envelope proteins are incorporated into the budding virion.

The virion buds from the host cell by hijacking the endosomal sorting complex required for transport (ESCRT) pathway (16). This process is mediated the p6 protein which directly binds ESCRT proteins and initiates the termination of Gag polymerization at the cell membrane, and catalyzes the release of the budding virion. Once the virion has budded, the viral protease cleaves p55 and p160 at ten different sites to produce fully functional p17, p24, p7, p6, protease, reverse transcriptase and integrase proteins.

HIV-1 latency

Clinical significance

One of the major barriers to the eradication of HIV-1 within infected individuals is HIV-1 latency. HIV-1 latency occurs when the virus has integrated into the host genome but fails to transcribe its viral genes (17), resulting in an infected cell that can't be recognized by the immunes system, or affected by HAART. However, the latent state is reversible and viral transcription can be activated via different stimuli, leading to the completion of the viral life cycle and the generation of new virions (18, 19).

HIV-1 latency was first observed in the laboratory within transformed T-cell lines in 1986 (17), ~3 years after HIV-1 was identified as the virus that causes AIDS. In this initial study, it was observed that not all cells infected with HIV-1 would die. The small number of cells that did survive did not produce virus, but could be induced to do so with various stimuli, including those that cause T-cell activation, suggesting that these cells harbored latent virus. The significance of this finding wasn't fully appreciated for several years as it wasn't until 1995 that HIV-1 latency was first observed in infected patients when inverse PCR was used to detect integrated HIV-1 DNA in highly purified resting CD4^{+} T cells from infected individuals (20). Just like in the transformed T-cell lines, these resting cells did not express virus until treated with activating stimuli.

Shortly after resting CD4^{+} T cells from infected patients were shown to harbor latent HIV-1, HAART became widely available in developed countries like the United States. In 1997, a study published in Nature showed that HAART was effective enough to lower the viral load in infected patients down to undetectable levels, and suggested that HAART could completely eliminate HIV-1 from the body within 2-3 years of

treatment (21). However, published in that same Nature issue was a study that showed the majority of latent virus found in patients resides in resting memory CD4^{+} T cells (22), which immediately, called into question the ability of HAART to cure patients, since latently infected memory CD4^{+} T cells could potentially survive, *in vivo*, for decades or longer (22, 23). In fact, only six months later, three studies published, simultaneously, showed that latently infected CD4^{+} T cells could still be found within patients who had successfully been treated with HAART (18, 24, 25), proving that HAART has little to no effect on the latent reservoir. Finally, in 1999, it was shown that cessation of HAART after viremia has been lowered to undetectable levels leads to a viral rebound within weeks of stopping the treatment (26), further dampening the hopes that HAART could cure HIV-1. At this point, it was widely accepted that HIV-1 latency is a major barrier to viral eradication, and that the study of the mechanisms that lead to the establishment and maintenance of HIV-1 latency are imperative.

Theory of HIV-1 latency establishment

As discussed above, latent HIV-1 is primarily found in memory CD4^{+} T cells. Additionally, it is widely accepted that HIV-1 can replicate efficiently only within activated CD4^{+} T cells (27-30), as resting CD4^{+} T cells present several early post-entry blocks to viral replication (31). Studies have shown that resting CD4^{+} T cells do not efficiently support reverse transcription or viral integration (30, 32-34). Because of this, HIV-1 latency is thought to be established within memory CD4^{+} T cells through the exploitation of the natural physiology of CD4^{+} T cells (35). Upon encountering an antigen, resting CD4^{+} T cells undergo proliferation and differentiation into effector cells that will fight off

the specific antigen. As the infection begins to subside, the majority of effector CD4$^+$ T

cells die off, but a small subset survives and returns to a resting state as memory CD4$^+$

T cells that serve as immunological memory. These memory CD4$^+$ T cells are long-

lived, as they are required to for the rapid response to fight infection if the antigen is

ever encountered in the future. Because activated CD4$^+$ T cells are highly susceptible

to HIV-1 infection, it is thought that they become infected and then are able to survive

long enough to revert back to a memory state with an integrated provirus. Since HIV-1

transcription is tightly coupled to the state of activation of T cells, this return to

quiescence is thought to *lead* to a suppression of HIV-1 transcription and , ultimately,

latency.

Alternatively, resting CD4+ T cells can also be directly infected by HIV-1, resulting

in integration but no viral transcription (36-38). The latter was noted in both naïve and

memory CD4$^+$ T cells, but infection of such quiescent cells is far less efficient than

infection of activated CD4$^+$ T cells. Nonetheless, HIV-1 latency models have been

developed in both activated and resting CD4$^+$ T cells.

In vitro models of HIV-1 latency

Transformed Cell Lines. The first *in vitro* HIV-1 latency models were

established in transformed cell lines by infecting these cell lines with HIV-1 and then

clonally expanding individual cells that survived the infection. Three of the earliest

transformed cell lines of HIV-1 latency were U1 (39), ACH-2 (40, 41) and JΔK (42). U1

was cloned from a monocytic cell line, U937, after HIV-1 infection, while ACH-2 and JΔK

were cloned from the T cell lines, A301, and Jurkat, respectively. Each of these cell

lines provided important insight into HIV-1 latency and HIV-1 transcription, in general. However, all three of these cell lines contained mutated HIV-1. U1 and ACH-2 both, inadvertently, contained mutations in the Tat gene, and Tar element, respectively, while JΔK was generated by, purposefully, infecting Jurkat cells with an HIV-1 isolate that contained mutations in the two NFκB binding sites that reside in the HIV-1 LTR promoter. Because of these mutations, it was thought that these cell lines represented an artificial form of HIV-1 latency, and left unanswered, whether replication-competent HIV-1 could establish latency.

This question was answered, in part, by the generation of the J-Lat cell line, which was cloned from Jurkat cells after infection with a GFP-reporter HIV-1 (43). The GFP-reporter, under the control of the HIV-1 promoter, was used to distinguish productively infected cells from latently infected and uninfected cells. After sorting the cells based on GFP-expression, the latently infected cells could then be separated from the uninfected cells by reactivating any latent virus with activating stimuli such as TNF-α. Reactivatable provirus would express GFP, and these cells could then be sorted and clonally expanded, giving rise to several latently infected cell lines that did not contain any mutations within the HIV-1 genome. The generation of the J-Lat cell lines helped to further our understanding of HIV-1 latency, but as with all transformed cell lines, concerns were raised about the relevance of such models to how HIV-1 latency was occurring in primary CD4^{+} T cells. This then led to the development of HIV-1 latency models in primary CD4^{+} T cells.

Activated primary CD4^{+} T cells. Several groups have attempted to mimic the infection of activated CD4^{+} T cells *in vitro*, with HIV-1 or HIV-1-derived vectors, followed

by the transition of these infected cells to a resting state to establish latency in memory CD4$^+$ T cells. This is a difficult process due the complicated nature of generating and maintaining memory CD4$^+$ T cells *in vitro* (44). The major problem is that most CD4$^+$ T cells die soon after activation if not continuously cultured in the presence of specific cytokines. This results in too few cells that have transitioned to a quiescent state and contain integrated HIV-1 that can be used for further study. Culturing of CD4$^+$ T cells in the presence of cytokines, such as IL-2 or IL-7, after activation increases their survival and allows time (several weeks) for more cells to transition to a memory state with integrated HIV. However, IL-2 and IL-7 have been implicated in the reactivation of latent HIV-1 (19, 45, 46). Therefore, these cytokines must be used at a concentration that can maintain CD4$^+$ T cells in culture without reactivating latent HIV-1. Alternatively, activated and HIV-1 infected CD4$^+$ T cells can be maintained in culture and allowed to transition back to a resting state in the absence of cytokines by utilizing strategies, such as transduction with anti-apoptotic proteins or co-culture with feeder cell lines (47-49) (Figure 1.4). All these strategies lead to more viable cells that have been infected and returned to a resting state, allowing for HIV-1 latency to be studied in a physiologically relevant setting.

The first model of HIV-1 latency in primary lymphocytes was developed by Sahu et al. in 2006 (47) (Figure 1.4). They isolated CD4$^+$ T cells from blood of uninfected donors and activated them with plate-bound α-CD3 antibody in the presence of IL-2. Activated cells were then infected with a replication-competent virus at a multiplicity of infection (moi) of 1–10 and allowed to transition to a quiescent, memory state. Cells were allowed to transition to a resting state by being co-cultured with a brain tumor-

derived cell line, H80. This feeder cell line promoted cell survival in the absence of any

cytokines. The exact mechanism by which the H80 feeder cell line works is still

unknown, but it allowed the production of a long-lived, mostly central memory CD4$^+$ T

cell population. However, despite being phenotypically similar to resting cells in many

respects, a significant fraction of this population continued to express the early

activation marker, CD69, suggesting that these cells were not completely resting.

Interestingly, a small fraction of quiescent cells also continuously expressed low levels

of p24, suggesting that low-level viral replication is ongoing in resting cells. Notably, this

low-level virus production came from resting cells that were CD69$^-$ and CD69$^+$.

Reactivation of this population of cells by the protein kinase C-activator prostratin, led to

~twofold increase in p24-positive cells, showing that latently infected cells were also

present within the resting CD4$^+$ T cell population, accounting for >5% of the cell

population.

Tyagi et al. published a study in which they used the H80 feeder cell line to

transition activated CD4$^+$ T cells that had been infected with a HIV-1 vector back to a

resting state (49) (Figure 1.4). Freshly isolated CD4$^+$ T cells were isolated from both

uninfected donor blood or uninfected tonsils and activated and expanded with αCD3/

αCD28 antibodies in the presence of IL-2. Activated cells where then infected with a

Δgag HIV-1 vector that contained a mutated tat and encoded a fluorescent marker in

place of nef. Infected cells were enriched by fluorescence-activated cell sorting (FACS)

and expanded via the αCD3/αCD28/IL-2 cocktail. These cells where then co-cultured

with the H80 feeder cells and allowed to return to a resting state, producing a mostly

central memory CD4$^+$ T cell population that continued to express significant levels of the

late activation marker, CD25 (CD69 expression was not measured). However, a decrease in viral production, as measured by fluorescence, coincided with the cells returning to a mostly resting state. Still, low-level GFP expression was observed within the resting cell population showing, again, that low-level viral expression is ongoing in this primary latency model. Latently infected cells were also present within the central memory CD4+ T cell population as demonstrated by the significant increase in GFP expression upon CD3/CD28 stimulation as compared to infection with a wild-type Tat. This model of HIV-1 latency produced enough cells for further biochemical characterization that implicates epigenetic silencing and low levels of the transcription factor, P-TEFb, in HIV-1 latency.

A primary latency model in CD4+ T cells utilizing more physiologically relevant parameters has been developed by Marini and coworkers (50) (Figure 1.4). First, freshly isolated CD4+ T cells from peripheral blood of uninfected donors were activated by co-culture with antigen-loaded monocyte-derived dendritic cells (Ag-MDDCs). Activated cells where then infected with a replication competent HIV-1 at an moi of 0.002. Ongoing infection, as measured by p24, increased over the course of 15 days within the activated CD4+ T cell population. After the 15-day infection period, CD4+ T cells that remained activated were enriched for a cell population that was activated and infected by HIV-1, and eliminating uninfected cells as well as resting cells that may be infected. These activated cells were then allowed to transition to a resting state in the presence of IL-7. After 4 weeks, ~20% of the cells remained alive with the majority exhibiting a central memory phenotype. These quiescent cells were negative for CD25 (CD69 expression not shown) but were larger and more granular than freshly isolated

CD4$^+$ T cells suggesting that they may not be completely resting. Reactivation of this resting central memory CD4$^+$ T cell population indicated that ~2% of the cells were latently infected.

In 2009, Bosque et al. developed a primary latency model in activated CD4$^+$ T cells that allowed for the study of both central memory and effector memory populations (51) (Figure 1.4). Their system also shortened the time period from activation of freshly isolated CD4$^+$ T cells to reactivation of latent virus down to less than a month. Isolated CD4$^+$ T cells were first activated with αCD3/αCD28 antibodies in the presence of IL-2 and then were cultured for several days in three different conditions that produced Th1-helper, Th2-helper and non-polarized CD4$^+$ T cells (52). Biochemical analysis indicated that the Th1 and Th2 populations closely resembled both effector memory and central memory CD4$^+$ T cells, *in vivo*, while the non-polarized population more closely resembled central memory CD4$^+$ T cells. These three different populations of CD4$^+$ T cells were then infected with an envelope-deficient HIV virus that was pseudotyped with HIV-1 env, thus limiting infection to a single round. After infection these cells were allowed to transition back to a resting state over the course of 7 days in the presence of IL-2. Three days post-infection cells were analyzed for productive infection by intracellular staining for the viral p24^{gag} protein. Cells expressing high levels of p24^{gag} were considered to be productively infected and die in culture within 3–5 days after infection due to virus-induced apoptosis, leaving both uninfected and latently infected cells alive in culture. Seven days post-infection, the remaining cells were stimulated with αCD3/αCD28 antibodies, and p24^{gag} levels were determined to assess the latently infected population. Cells expressing p24^{gag} after reactivation were considered the

latently infected population. However, activation markers such as CD25 and CD69 were not assessed before reactivation, giving no indication if these cells were resting or active. Additionally, it is unclear if reactivated virus arose completely from post-integration latency. Since integration was not assessed, it is possible that a fraction of the reactivated virus came from pre-integration latency. Also of note, mechanistic studies performed using this primary latency model showed that NFκB was not critical for latent reactivation.

A system allowing the screening of drugs that reactivate latent provirus in CD4$^+$ T cells has been described by Yang and colleagues (48) (Figure 1.4). Freshly isolated CD4$^+$ T cells from uninfected donor blood were first activated and expanded using a αCD3/αCD28/IL-2 cocktail and then transduced with a lentiviral vector carrying the Bcl-2 gene. In this system, Bcl-2, a downstream target of IL-7, acts as an anti-apoptotic protein that allows CD4$^+$ T cells to remain long-lived in culture without the need for feeder cells or cytokine stimulation. Bcl-2-transduced cells were carried in culture for several weeks, allowing them to return to a resting state. During this time, non-transduced cells eventually died off in the absence of any cytokine signaling leaving only Bcl-2-transduced cells alive in culture. Characterization of these cells showed that they more closely resembled effector memory cells. Bcl-2-transduced cells were reactivated and expanded with a αCD3/αCD28/IL-2 cocktail and then infected with a HIV-derived vector (moi<0.1). This HIV-1-vector lacked several genes, including gag, vif, vpr, vpu and env, and encoded GFP in place of nef. Infected cells were allowed to transition back to a resting state over the course of several weeks in the absence of cytokines. To enrich for latently infected cells, GFP-negative cells were isolated via

FACS and then used to test more than 4000 compounds for their ability to reactivate latent provirus. Before reactivation, however, cells were first assessed for activation markers to prove that they were truly in a resting state. A small fraction of cells still expressed CD69 and CD25. To look at only latent infection in resting cells, CD69$^-$ and CD25$^-$ cells were purified and used for the reactivation studies. In this primary latency model, 1–3% of GFP-negative cells could be induced to express latent provirus.

Resting primary CD4$^+$ T cells. Direct infection of resting CD4$^+$ T cells is an inefficient process due to several blocks imposed by the cellular environment of resting T cells (28, 34, 53). Lack of dNTPs needed for reverse transcription, and a lack of ATP needed for nuclear import of the viral DNA, as well as a restrictive cortical actin barrier, all make infection of resting CD4$^+$ T cells difficult but not impossible.

The direct infection of resting CD4$^+$ T cells with HIV-1 in the absence of any activating stimuli has been described by Swiggard and coworkers (36) (Figure 1.4). Freshly isolated CD4$^+$ T cells that included a mixture of naive, T$_{CM}$ and T$_{EM}$ were infected with a replication-competent HIV-1 (moi~22–150) by centrifugation of the virus with the target cells in a minimal volume. Integration occurred in resting cells, albeit at a much lower frequency than activated CD4$^+$ T cells. Integration of the virus took up to 3 days to complete and produced a productive infection in which only 0.3% of cells expressed HIV-Gag. However, reactivation of the cells 3 days post-infection with αCD3/αCD28 antibodies induced 4.5% of the cells to express HIV-Gag showing that a fraction of these cells contained latent provirus.

Saleh et al. developed a primary latency model in resting CD4$^+$ T cells based on the observation that the majority of HIV-1 infected resting CD4$^+$ T cells express CCR7, a

lymphoid organ homing receptor (54) (Figure 1.4). Freshly isolated resting CD4^{+} T cells

from uninfected donor blood were first stimulated with the CCR7 ligands, CCL19 and

CCL21. These chemokines did not induce either CD69 or CD25 expression, but did

increase the susceptibility of resting CD4^{+} T cells to infection by a replication competent

HIV-1 virus (moi=1). Both reverse transcripts and integrated provirus were detected in

these cells, and the production of reverse transcripts after αCD3/αCD28 antibody

stimulation increased, showing that latent virus was present in these cells.

Figures
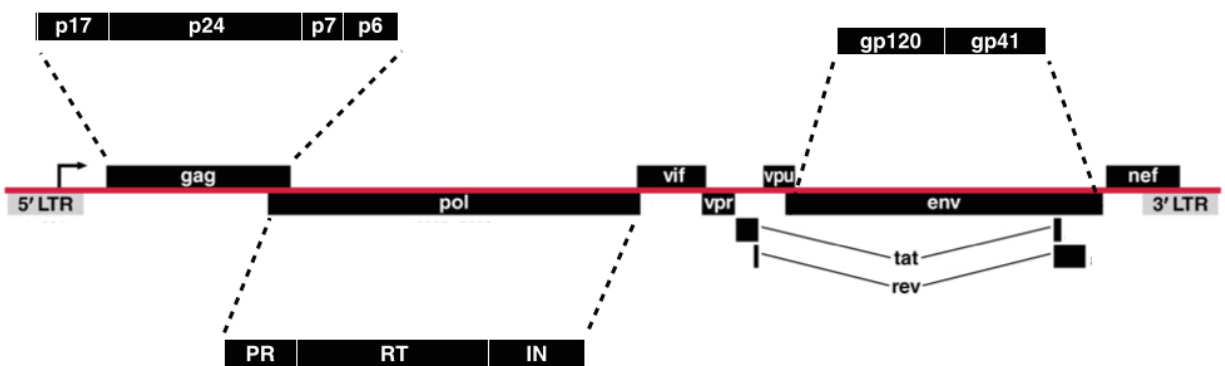
Figure 1.1. HIV-1 genome. The organization of the 9.7 kb HIV-1 genome. HIV-1

contains nine genes flanked by two LTRs. The nine genes encode 15 different proteins,

and transcription is initiated at the 5' LTR. The gag gene encodes four different

structural proteins; the pol gene encodes three enzymes; and the env gene encodes

two proteins that combine to form the viral receptor. The tat gene and rev gene are

regulatory genes. The vif, vpr, vpu, and nef genes are all accessory genes.
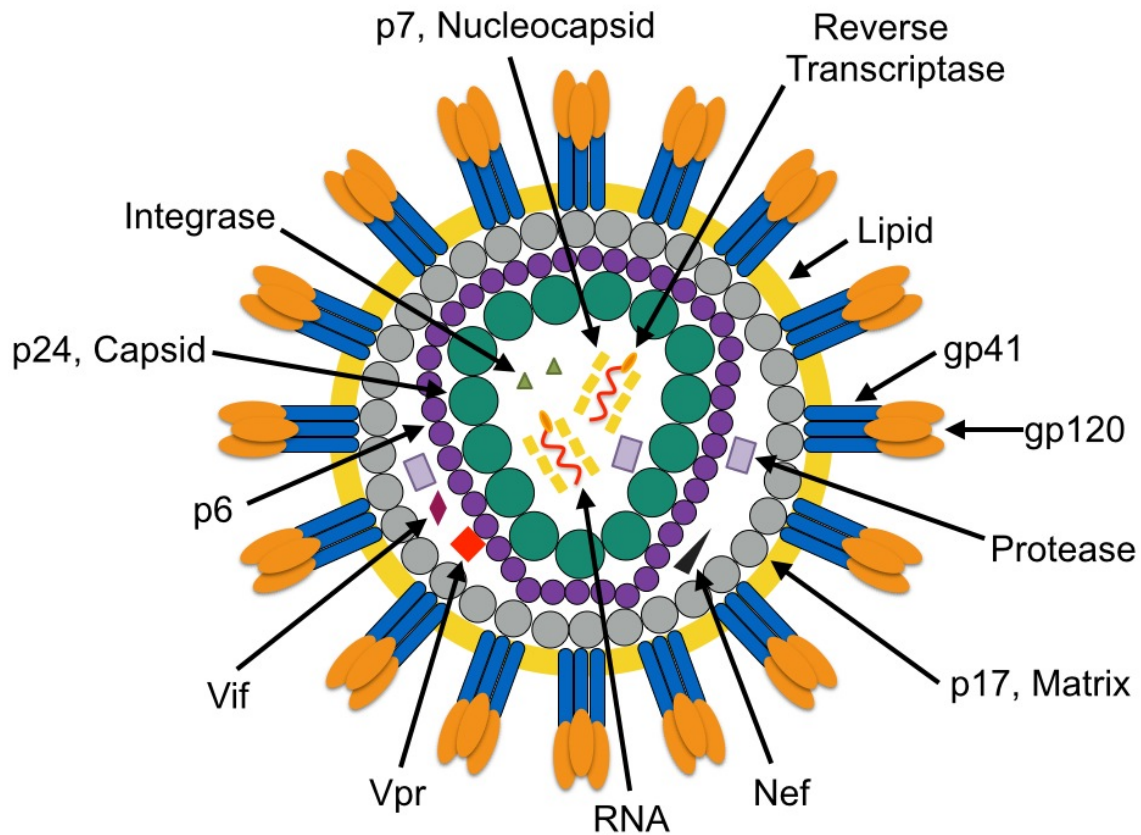
Figure 1.2. HIV-1 virion structure. The HIV-1 virion has a diameter of 120 nm. The viral envelope receptor spikes the lipid membrane, while a matrix of p17 lines the inner surface of the lipid bilayer. A matrix of the p6 structural protein surrounds a matrix of p24 Capsid protein. Within the p24 matrix is two copies of viral genomic RNA that are encapsidated by the p7 nucleocapsid. Also found within the p24 matrix are several molecules of reverse transcriptase, integrase and protease. Vif, vpr, nef are also packaged into the HIV-1 virion along with several host cell proteins. Tat and rev are not packaged in the virion, but are the first genes transcribed after integration of the viral cDNA.
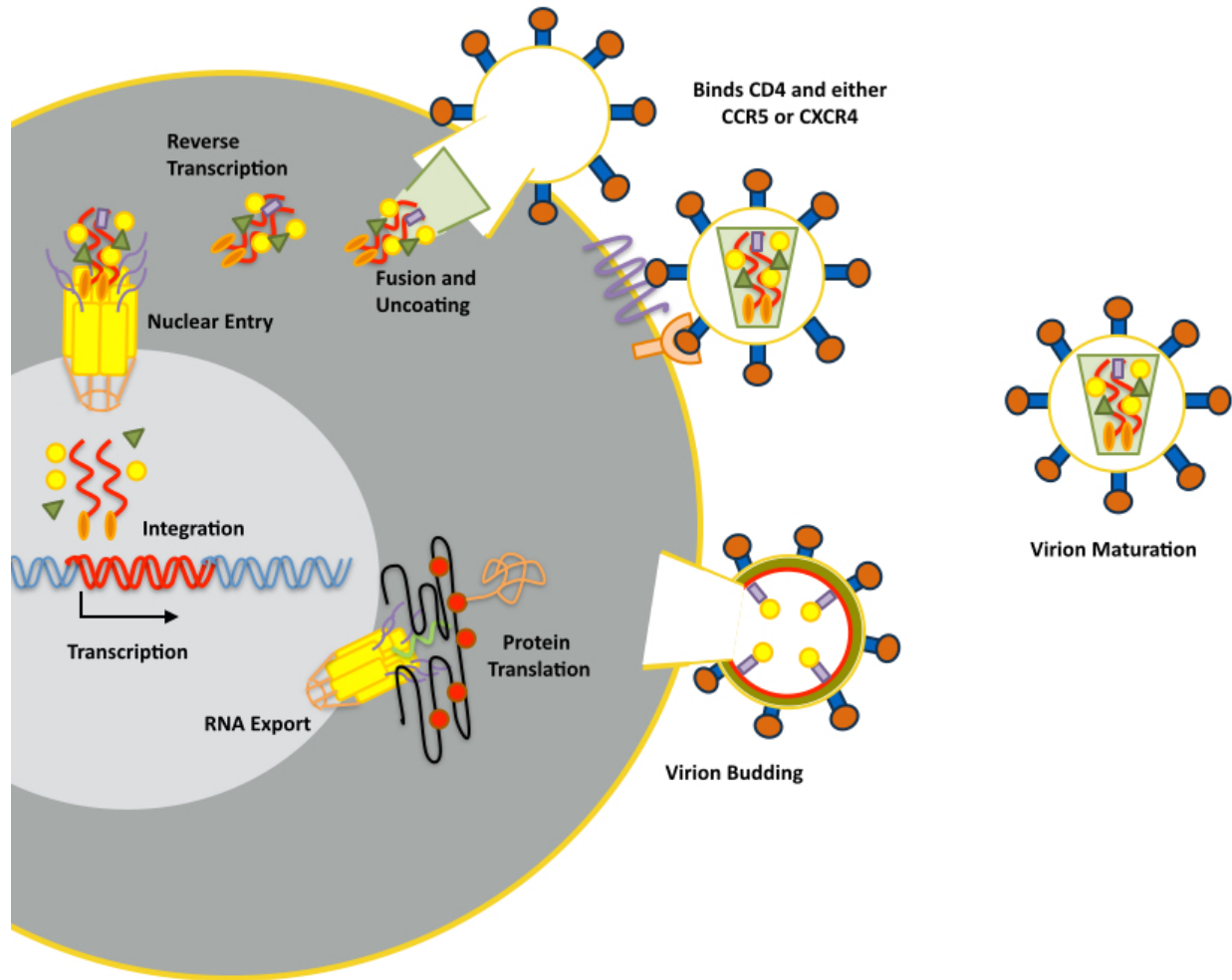
Figure 1.3. HIV-1 life cycle. HIV-1 binds to target cells using its gp120 protein. gp120 binds to the CD4 receptor of the target cell, as well a co-receptor. Once bound to primary and co-receptors, HIV-1 fuses with the target cell via gp41. After fusion, uncoating of the p24 matrix occurs, and reverse transcriptase generates a double stranded viral cDNA that enters the nucleus and integrates into the host genome via integrase. Once integrated, the provirus transcribes viral genes that are exported to the cytoplasm, where they are translated by host proteins. Viral proteins assemble at the cell membrane and immature virions bud from the host cell. Protease then cleaves

several viral poly-proteins that leads to maturation of the HIV-1 virion. HIV-1 latency is

defined as integration of the viral cDNA that results in a lack of viral transcription.

Latency can be reversed, and viral transcription can be initiated via cell stimulation.
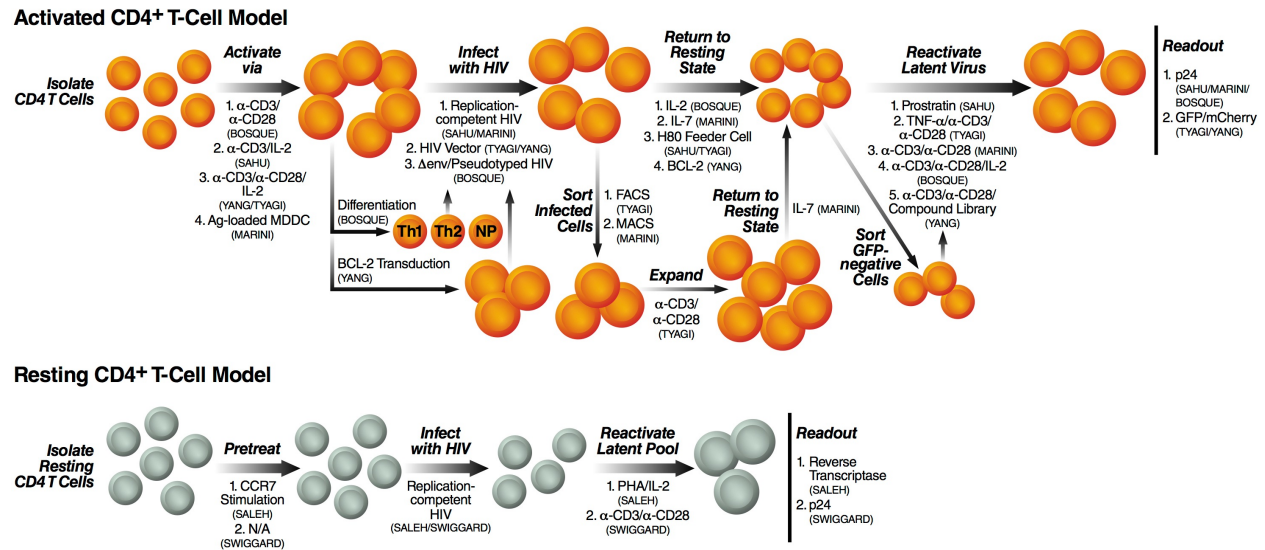
Figure 1.4. Models of HIV-1 latency in primary CD4+ T cells. HIV-1 latency can be

established in primary human CD4+ T cells using the procedures illustrated on this

diagram. The different models are defined by whether activated CD4+ T cells or resting

CD4+ T cells are used. If the cells are activated, the mechanism of activation is shown,

as well as the type of HIV-1 vector (full length viral vector or not) used. The procedure

used to return cells to a resting state, the reagent used to reactivate the latent pool (i.e.

the stimulating agent) and the type of assay used to assess viral reactivation are also

shown. Each primary latency model system is named by the first author of the

publication.

CHAPTER 2: Dual-color HIV reporters trace a population of latently infected cells and enable their purification

Abstract

HIV-1 latency constitutes the main barrier for clearing HIV-1 infection from patients. Our inability to recognize and isolate latently infected cells hinders the study of latent HIV-1. We engineered two HIV-1-based viral reporters expressing different fluorescent markers: one HIV-1 promoter-dependent marker for productive HIV-1 infection, and a second marker under a constitutive promoter independent of HIV-1 promoter activity. Infection of cells with these viruses allows the identification and separation of latently-infected cells from uninfected and productively infected cells. These reporters are sufficiently sensitive and robust for high-throughput screening to identify drugs that reactivate latent HIV-1. These reporters can be used in primary CD4 T lymphocytes and reveal a rare population of latently infected cells responsive to physiological stimuli. In summary, our HIV-1 reporters enable visualization and purification of latent cell populations and open up new perspectives for studies of latent HIV-1 infection.

Introduction

The discovery of effective therapies against HIV-1, called highly active antiretroviral therapy (HAART), has transformed a once deadly disease into a life-long chronic condition (7). By hijacking the viral machinery during infection, HAART effectively reduces infection to undetectable levels. Nonetheless, complete suppression of viral replication by HAART cannot clear HIV-1 infection and the virus reappears rapidly upon treatment interruption (55). HIV-1 persists under HAART in a rare population of long-lived, latently infected cells. In addition, the persistence of latent reservoirs feeds an inflammatory-like state that contributes to the development of accelerated aging phenotypes and accompanying age-related diseases in the HAART-treated HIV-positive population (56).

HIV-1 latency is defined as a state in which proviral DNA is integrated irreversibly in the cell genome, but expression of the viral genes is silenced due to a repressed state of the viral LTR promoter (57). Alternatively, HIV-1 latency can be due to blockade of virus production at any step (58).

Latently-infected cells are extremely rare in patients and carry silent integrated HIV-1 genomes that produce no detectable virus. Current technologies do not allow the identification and purification of live infected cells that do not express any HIV-1 proteins. Latency has therefore been studied "a posteriori" via reactivation of expression of the latent provirus. This type of analysis has allowed the quantification of latent cells in different lymphoid populations and the testing of different drugs that reactivate latent HIV-1. However, our inability to identify latently infected cells before reactivation has precluded a full understanding of the latency process.

In this study, we present a novel tool that uses fluorescent reporter–based HIV-1 constructs expressing two fluorescent proteins, one dependent and one independent of HIV-1 promoter activation state. This reporter system allows the detection and purification of a cell population of latently infected cells that can be reactivated by physiological and pharmacological stimulation. We show that this system can be used in various cell lines to identify and purify polyclonal populations of latently infected cells. Such populations can be used in high-throughput assays while overcoming the clonal-derived biases of current in vitro systems. We also show that these viral constructs are suitable for the study of latency in human primary CD4$^+$ T cells.

Materials and Methods

Plasmid Construction. HIV-1 clones 89.6-DNE-SFG (donated by Dr. Kathleen Collins's laboratory) and R7/E-/GFP were linearized by PCR using opposing primers (see Supplementary Table 2, #A and B) in the position selected for insertion (Figure 2.1). mApple and the EF1a-mCherry sequences were cloned in the respective plasmids by a modified SLIC technique (59). Briefly, inserts were PCR-amplified from plasmid templates with primers that have a 20 bp 5'-overhang complementary to the end sequences of the blunted plasmids (see Supplementary Table 2, #C and D). DNA from all amplifications was gel-purified, digested with 3'-5' exonuclease (T4-polymerase, Novagen, 30 min, 25°C) and heat-inactivated (20 min, 75°C). Digested inserts and vectors for each construct were mixed at a 2:1 molar ratio for complementary-end sequence annealing (20 min, 37°C) and transformed in chemically competent Stbl-2 E. coli (Life Technologies). Positive clones were resequenced entirely.

Virus Production. HEK293T cells were cotransfected with the viral plasmid clone of interest and the env-encoding plasmid pSVIII-92HT593.1 (NARP), using Lipofectamine 2000 (Invitrogen). Medium (DMEM, 10% FBS) was changed 8 h post-transfection and collected at 72 h. Virus supernatant was filtered through a 0.45 µm-pore membrane, concentrated by ultracentrifugation and stored at -80°C. Virus concentration was estimated by p24 titration (HIV-1 alliance p24 ELISA kit, PerkinElmer).

Cells and Treatments. HEK293T, Jurkat, A301 and SupT1 cell lines were obtained from ATCC. Suspension cells were cultured in RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (PSG) (37°C, 5% CO_2). Peripheral blood from healthy human donors was obtained from the Stanford Blood Center and processed the day of collection. $CD4^+$ T cells were isolated with RosetteSep Human $CD4^+$ T cell Enrichment Cocktail (Stem Cell Technologies) and cultured in human cell medium (HCM, RPMI, 10% human AB pooled serum, PSG). Resting $CD4^+$ T cells were activated in U-bottom 96-well TC plates with 25 µl human anti-CD3 and anti-CD28-coated dynabeads (Life technologies)/10^6 cells (48 h). Dynabeads were removed by magnetic separation and cells seeded in HCM with 30 IU/ml rhIL-2 (NIH AIDS Reagents Program). Cell lines and activated $CD4^+$ cells were infected by spinoculation (60) with a virus amount equivalent to 20 ng p24 or as indicated. Viral infection was assessed by FACS analysis after 72 h. At 5 days post-infection, cells were sorted to separate negative, single- or double-positive cells. Sorted cell lines were treated after re-expansion for 5-15 days. Cells were seeded in 96-well U plates and treated for 24 h before analysis. $CD4^+$ T cells were reseeded after sorting in

96-well U plates with HCM in the absence of IL-2 and treated after 24 h with drugs or human T-activator Dynabeads (48 h). Raltegravir (NARP), TNF-α (LT), prostratin, SAHA, bryostatin-1, PMA, HMBA, PHA-M (all from Sigma) were used in culture at indicated concentrations. This study was conducted according to the principles expressed in the Declaration of Helsinki. All individuals provided written informed consent for the collection of samples and subsequent analysis, as approved by the Institutional Review Board of the Stanford University Blood Bank.

Flow Cytometry and Cell Sorting. EGFP, mApple and mCherry fluorescence were measured in a MACSQuant VYB FACS analyzer (Miltenyi Biotech GmbH), FACSCalibur, LSRII, and sorted in a FACSAriaII (all BD). Data were analyzed using FlowJo 9.4 (TreeStar).

DNA, RNA and Protein Extraction, qPCR and Western Blot. DNA and RNA were extracted with Dneasy and RNeasy kits, respectively (Qiagen). RNA was retrotranscribed with the high capacity kit cDNA (Life technologies) and qPCR was performed in the AB 7900HT Fast Real-Time PCR System, using TaqMan 2x Master Mix and the appropriate primer-probe combinations (Supplementary Table 2, #E, F, and G). Quantification for each qPCR reaction was assessed by interpolation on a template dilution curve. As normalization controls TaqMan Copy Number Reference Assay, RNase P was used for genomic DNA and TaqMan assay GAPDH Hs99999905_m1 for cDNA. Protein was extracted from freshly sorted cells in RIPA buffer, followed by SDS-PAGE. Bands were detected by chemiluminescence (ECL Hyperfilm Amersham) or

fluorescence (Licor Odyssey) detection with anti-vif, HIV-p24 and -α-actin (Sigma) primary antibodies.

Fluorescence Microscopy. Cells were analyzed with an Axio observer Z1 microscope (Zeiss) equipped with EC Plan Neofluar 20X/0.5 PHM27, EC Plan Neofluar 40X/0.75 PH, and Plan Apo 63X/1.4 Oil DIC M27 objectives, filter sets 38HE, 43HE, 45, and 50, Optovar 1.25X and 1.6X magnification, and an Axiocam MRM REV 3.

Drug Screening. Sorted GFP-only 89mASG cells-infected Jurkat cells were expanded in culture and seeded at a 5×10^3 cells/well density in 384-well plates (black, clear bottom, Greiner), coated with Cell-Tak (BD). Tocriscreen total drug screening library, containing 1120 biologically active compounds dissolved in DMSO, was dispensed in the plate at a concentration of 30 µM, using the automated Beckman and Coulter Biomek FXP robot and 50 nl VP 384 pin tool. After 24h culture in standard conditions, Hoechst 33342 was automatically dispensed with Thermo multodrop 384 to each well to evaluate total cell number of cell; plates were imaged in the InCell analyzer and analyzed with InCell Developer image analysis software. We quantified the total area masked by the nuclear stain and the total area of cells expressing mApple as proxies for cell number and number of cells which had reactivated viral transcription, respectively. On negative control wells, we calculated the median and standard deviation and used these statistics to select significant conditions that differed from these expected values by more than 2SD.

Results

Dual-Color Viruses Allow Direct Labeling of Live HIV-1 Latently Infected Cells.

To identify latently infected cells prior to reactivation, we designed two HIV-1-based lentiviral constructs in which LTR expression is monitored by production of a fluorescent protein while another independent transcriptional unit expresses a spectrally distinct fluorescent protein under the control of an independent promoter. To construct the first clone, we used the 89.6/DNE/SFG reporter in which nef is replaced by a Spleen Focus Forming Virus (SFFV)-promoter-driven enhanced green fluorescent protein (EGFP; Figure 2.1B) (61). This clone has an env deletion, which limits infection to a single round. We used a sequence- and ligation-independent cloning (SLIC) (59) strategy to reconstitute the nef ATG sequence and replace the remainder of the nef open reading frame with the sequence of a red fluorescent protein, mApple (62) (Figure 2.1B, D); this construct was named 89.6/DNE/mApple/SFG (89mASG).

The second construct was derived from the HXB2-based R7/3 clone bearing EGFP in place of nef and an env deletion (43) (Figure 2.1C). We used the SLIC technique to insert the whole EF1α-mCherry transcriptional unit between the EGFP and the virus 3' LTR (Figure 2.1C, D). We named this construct R7/E-/GFP/EF1a-mCherry (R7GEmC).

To test the potential of these viral reporters to differentiate latent from actively infected and uninfected cell populations, we infected Jurkat T cells with our new constructs (Figure 2.1E, F). After infection, a double-positive population expressing the HIV-1 promoter-dependent reporter and the HIV-1 promoter-independent reporter (SFFV or EF1α promoter) emerged for both viruses (Figure 2.1E, F). In addition, we

detected a single-positive population expressing only the HIV-1 promoter-independent reporter, green for 89mASG (Figure 2.1E) and red for R7GEmC (Figure 2.1F).

To confirm that the single-positive cells expressing only the non-HIV-1 promoter-driven reporter were latently infected cells, we sorted the GFP-only-positive and mCherry-only-positive cells from the 89mASG-infected and R7GEmC-infected pools, respectively, as well as the double-positive (productively infected) and the double-negative (uninfected) cells for each pool (Figure 2.1G, H; left). The single-positive and double-negative cells were expanded *in vitro* after sorting, whereas the double-positive cells did not expand efficiently and largely died after sorting (especially the 89mASG-infected; not shown), as predicted by the cytotoxic properties of active viral infection (63). The different sorted populations were analyzed for viral DNA, RNA and protein content. Whereas the double-negative cells had low HIV-1 DNA levels, the single-positive cells had DNA amounts comparable to those of double-positive cells (Figure 2.1I) but expressed no detectable viral mRNA (Figure 2.1J) or protein (Figure 2.1K). Only the double-positive population expressed viral transcripts and proteins. These results are consistent with our model that cells expressing only the non-HIV-1 promoter-driven reporter were latently infected cells.

Cells Enriched for HIV-1-Promoter Independent Expression Contains an HIV-1 Genome That Can Be Reactivated.

To estimate the fraction of single-positive cells that can be reactivated in terms of HIV-1 expression, we treated the purified cells with drugs reported to reactivate latent HIV, including tumor necrosis factor (TNF)–α (43); prostratin, a protein kinase C (PKC)

activator (64); and suberoylanilide hydroxamic acid (SAHA) (65), a non selective histone

deacetylase (HDAC) inhibitor. All drugs increased expression of HIV-1 promoter-

dependent reporter (Figure 2.2A, B top, C, D). Testing of drug combinations that act on

different pathways showed maximal reactivation in response to combined SAHA and

prostratin treatment, as previously reported by others (66). In both 89mASG- and

R7GEmC-infected latent populations, we observed spontaneous reactivation of latency

in the absence of activating molecule, likely reflecting the bi-stable nature of HIV-1

expression (67).

Due to the integration site of the provirus or the cell state, it is possible that some

latently infected cells were classified as double negative because of silencing of both

the HIV-1 promoter and the SFFV or EF1α promoter. To address this possibility, we

treated the double-negative populations with the same drugs (Figure 2.2A and B,

bottom) and found only very limited reactivation, affecting <1% of the population. This

implies that the silent HIV-1 provirus in the double-negative population has little to no

ability to reactivate in response to stimulation of latent HIV-1-reactivating cell pathways.

This two-color latency model, which relies on de novo cell infection, can easily be

transferred to study the biology of latency in a wide variety of cell types. As an example,

we infected and sorted two additional T cell lines commonly used in HIV-1 biology,

SupT1 (Figure 2.2E) and A301 (Figure 2.2F). Comparison of the sorted latent

populations from those cell lines and Jurkat T cells with an extensive panel of HIV-1-

reactivating drugs—including TNFα, prostratin, SAHA, phorbol myristate acetate (PMA),

bryostatin, hexamethylbisacetamide (HMBA), and phytohemagglutinin type-M (PHA-M)

(Figure 2.2G)—revealed significant differences in drug-independent and drug-

dependent reactivation rates. This observation supports the concept that use of a non-clonal, non-stimulus-biased experimental system to study HIV-1 latency, as exemplified by these new viruses, adds significance and robustness to the study of mechanisms for reactivation from latency.

Two-Color HIV-1 Latency Model Can Be Used For High-Throughput Screening.

Next, we tested the power of this novel HIV-1 latency experimental system to identify novel drugs that reactivate latent HIV-1 in a high-throughput format. We plated 5,000 GFP-only-positive cells of 89mASG-infected Jurkat T cells in each well of 384-well plates, coated with a protein solution (Cell-tak) to induce cell attachment and efficient imaging. Preliminary experiments indicated that this protocol yielded the lowest background reactivation and the highest dynamic range (up to 75% cell reactivation with the most effective drug combinations) (data not shown). Cells were treated with 1,120 individual compounds from the Tocriscreen biologically active compound library at 30 μM for 24 hr (drug combinations are shown in Figure 2.2A, B) before they were stained with Hoechst. Next, we imaged each well and quantified total red fluorescence (indicative of reactivated virus) and total blue fluorescence (Hoechst-stained nuclei) (Figure 2.3A).

For most compounds in each plate, we found a uniform distribution of signal around the median of the negative controls, while a notably higher red signal for positive controls that were added in control wells on each plate (TNF, SAHA, Prostratin and their combinations as described in the previous section) (Figure 2.3A). We also identified a group of drugs that reactivated latent HIV-1, although to a lesser extent than the positive

controls SAHA and prostratin (Supplementary Table 1). Some, such as resveratrol (68),

and genistein (69), have been reported previously to reactivate latent HIV-1, thereby

validating this experimental system. In addition, a number of unanticipated and novel

drug classes emerged from the analysis, such as those that act on epidermal growth

factor receptor (EGFR), dopamine and serotonin receptors.

Next, using the 89mASG and R7GEmC constructs and FACS analysis, we

further tested a small group of commercially available drugs and analogs not present in

the Tocris library that act on similar pathways, to assess their involvement in HIV-1

latency maintenance. Cells were treated with the dopamine receptor agonist

apomorphine, used clinically as an emetic and for male impotence, and the D2-

receptor-specific analog R-(-)-propylnorapomorphine. In addition, we tested the

serotonin receptor antagonists ritanserin and the antipsychotic clozapine, the EGFR

receptor antagonists AG555 and AG18 (candidates for specific cancer treatments), the

resveratrol analogs piceatannol and pterostilbene (antioxidants with broad and

incompletely understood mechanisms of action), the adenosine reuptake inhibitors

dilazep and dipyridamole (used as vasodilators and platelet anti-aggregants), the cdc25

dual-phosphatase inhibitor NC95397, the selective BTK inhibitor (-)-terreic acid, and the

HMG-CoA synthase inhibitor simvastatin (Figure 2.3B, C). Among these, AG555,

piceatannol, dilazep and terreic acid produced slight but significant reactivation activity

in the 89mASG (Figure 2.3B, C) and R7GEmC (Figure 2.3B, D) single-positive latent

populations. Other molecules were less effective (simvastatin) or had distinct effects in

the two HIV-1 clones (apomorphin). In some cases, the intrinsic fluorescence of the

molecule produced clear artifacts or false positives (see NSC 95397; Figure 2.3B).

A recent study showed that the approved HDAC inhibitor SAHA can reactivate HIV-1 RNA expression in a subset of patients (70). Thus, it might be of interest to identify compounds that can potentiate HIV-1 reactivation by SAHA or prostratin or TNFα. Sorted latent cell populations were co-treated for 24 h with low levels of SAHA, prostratin and TNFα, and several effective, non-toxic concentrations of the most promising compounds (Figure 2.3E). Interestingly, combining SAHA with terreic acid, dilazep or piceatannol (10 μM) strongly enhanced the effect of low SAHA concentrations on HIV-1 latency on these cells; the same drugs were also synergistic with prostratin, as indicated by the fact that their combined activity when present together was greater than the sum of their individual activities. TNFα combinations showed synergistic interaction in most combinations except terreic acid, possibly indicating that the drugs act on the same pathway.

Two-Color HIV-1 Constructs Detect Latency in Primary T Cells.

Studies of the basic biology of HIV-1 latency have recently emphasized the use of primary cell–based models in an effort to more closely mimic the situation in patients. The use of primary CD4$^+$ T cells from human donors nonetheless remains a challenge, due to low latency establishment rates and limited expansion potential. Resting peripheral CD4$^+$ T cells are generally refractory to HIV-1 infection and require pre-stimulation to achieve efficient infection. Furthermore, T-cell-receptor stimulation and IL2 and/or IL7 supplementation (71), which is needed for expansion of these cells in culture and for permissive HIV-1 infection, activate the latent HIV-1 provirus, thereby

making expansion of latently infected cells unfeasible unless a transformed-like phenotype is induced (48).

Despite these limitations, a number of experimental model system for HIV-1 latency have been established in primary CD4+ T cells (72). We therefore tested our viral constructs for their potential to generate a latent infection in primary CD4+ T cells. Cells were activated with anti-CD3 and anti-CD28-coated dynabeads for 48 hours and infected with both viruses. We found that both viruses infected activated CD4$^+$ T cells efficiently, generating an active infection as determined by expression of the HIV-1 promoter-dependent reporters mApple (for 89mASG) (Supplementary Figure 2.1), and GFP (for R7GEmC)(Figure 2.4A, B). A very small population of cells expressing only the latency-associated fluorescent marker was also generated in approximately 1 of 100 active infection events. As the GFP signal in 89mASG-infected primary cells was dim and difficult to distinguish from uninfected cells, the R7GEmC construct was used for additional experiments in primary CD4+ cells.

We measured HIV-1 proviral DNA and HIV-1 gag in sorted mCherry-only populations compared to uninfected and actively infected sorted populations. Viral DNA was detected at high levels in double-positive cells and slightly lower levels in mCherry-positive cells (Figure 2.4C). Interestingly, lower but still detectable amounts of proviral DNA were also detected in double-negative cells, indicating that some latent events might be found in this population if EF1α promoter expression is also blocked. As predicted, the HIV-1 gag protein was also detectable in the actively infected population, and absent in latent and uninfected populations (Figure 2.4D).

We next evaluated the extent to which mCherry-only-positive populations could be reactivated by further stimulation. After sorting the three populations of mCherry-only-expressing, GFP-positive, and double-negative cells, cells were treated with anti-CD3/28-coated beads to reactivate the latent provirus (Figure 2.4E). The latent population responded to this treatment with a 15–18% increase in cells expressing GFP. The double-negative population also responded to a lesser extent, with 0.5–2% reactivation. A population of very bright mCherry-only cells did not respond to treatment, suggesting aberrant integration events or non-reactivatable latent provirus. A subset of less-bright mCherry-only positive cells also responded to prostratin and SAHA treatment (Figure 2.4F), suggesting that this population of primary CD4$^+$ T cells contains latently infected, reactivatable cells.

HIV-1 latency *in vivo* is reported to result, in part, from pre-integration latency, whereby unintegrated proviral DNA (after full retrotranscription) is maintained in a stable cytoplasmic form in a quiescent cell, and can respond to cell activation by de novo integration and productive infection (34). To rule out pre-integration latency, we infected activated CD4$^+$ T cells with R7GEmC in the presence of the HIV-1 integrase inhibitor, Raltegravir (Supplementary Figure 2.2A). Under these conditions, we found no integration-independent expression of the fluorescent proteins after 3 days, thereby excluding pre-integration latency as a major mechanism in this primary-cell-infection model. In addition, Raltegravir treatment did not reduce viral reactivation in the mCherry-only-positive population, indicating that all reactivatable latent cells were in a post-integration latency stage (Supplementary Figure 2.2B).

Discussion

A major obstacle in the development of an eradication-based cure for HIV-1 is the virus' ability to establish latent infection (73). As latent viruses are inactive and extremely rare *in vivo*, the ability to detect and enrich for a latently infected cell population would powerfully advance our understanding of the mechanism of HIV-1 latency. Here, we describe two HIV-1-reporter systems that allow for the identification and purification of live, latently infected cells and their separation from actively infected and uninfected cells.

Using Jurkat and other cell lines, we show that purification of latently infected cells from a primary infected pool allows for the study of HIV-1 latency independent of cell clonality, insertion sites in the genome, and reactivating stimuli. The dynamic range of reactivation assays is also increased, due to the separation of homogeneous latently infected from uninfected cells. This permits the detection of novel compounds that reactivate latent HIV-1, providing new possibilities for combination therapies.

A recent paper has reported a similar dual fluorescence HIV construct to study HIV-1 latency. In this construct, a GFP marker was inserted in the Gag gene leading to the synthesis and incorporation of a fusion Gag-GFP protein which is proteolytically released in the mature virion and used to monitor HIV-1 expression. A CMV-mCherry cassette was added in place of nef to monitor integration events (74). This tool has similar applications to the HIV-1 reporter that we describe in this work, although the presence of GFP in the virion limits the possibility of studying the early kinetics of integration and HIV-1 promoter activation. In addition, the use of the CMV promoter

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which shows variable activity in hematopoietic lineages and a high dependence on the degree of CD4+ T cell activation, might not be optimal (75).

Other current cell models that involve enrichment of latently infected cells require prestimulation with specific compounds or cytokines to identify the infected population, and subsequent induction of a latent state (51, 72, 76). This experimental system skews the study of latent HIV-1 populations by restricting analysis to a subset of latent infection events responsive to that compound and able to overcome the cytotoxic effect of HIV-1 reactivation. Our constructs detect a population of latently infected cells regardless of their responsiveness to the reactivation of cellular pathways, and thus more closely approximates the establishment of latent infection early in the viral life cycle prior to initiation of tat-dependent HIV-1 transcription.

Although our system overcomes the issues of clonality and dependence on specific reactivation pathways, it does not detect latent infection events that repress both the HIV-1 and the EF1α or SFFV promoters. Although this limitation could potentially skew analysis of latent infection events, we detect lower levels of proviral DNA in the double-negative population than in the fluorescent cells, indicating that the events that we missed are relatively rare. In addition, a very small number of the potentially latently infected cells in this population are responsive to pharmacological reactivation, which implies that these double-negative latent cells are incapable of, or refractory to, rebound of the viral life cycle and may represents HIV-1 proviruses that are terminally silenced. In contrast, Dahabieh et al. observe a significant and robust of reactivation from latency in the sorted double negative population, possibly due to a subset of latent events in their system that fails to activate the CMV promoter (74).

Our HIV-1 latency model uses a replication-deficient lentiviral system that is easily pseudo-typed and packaged in a number of ways, which facilitates its use for the study of latent infection events in any cell population of interest. Our study focused on the widely used Jurkat T–cell line; however, two other T cell–derived cell lines, A301 and SupT1, can also be infected if the receptors and co-receptors expressed are compatible with the viral envelope of choice (in our experiments, a dual-tropic HIV-1 gp160 derived from a patient isolate (clone 92HT593.1) (77). Our system can thus be potentially used in different cell types and candidate reservoirs of the latent HIV-1 pool.

The use of two viral clones on different cell lines allowed us to observe a phenomenon of variable basal reactivation from latency in the isolated latently infected populations. The vector R7GEmC appears to have higher basal reactivation, which leads to a tendency to double positive cell accumulation upon re-expansion from sorted mCherry-only cells. This basal reactivation as well as the responsiveness to different drugs also varied among the three tested cell lines. This instability might be a result of selecting latent infection events early after infection, before lon-gterm maintenance processes have established, and might reflect the bistable nature of HIV-1 transcription, as described by Weinberger et al. (67).

To demonstrate the potential of these dual-color viruses for high-throughput applications such as drug discovery, we conducted a small-scale high-throughput screen of constructs with a biologically active compound library. Although none of the 1,120 compounds tested showed higher reactivation potential than the known latent HIV-1 reactivators, we identified classes of clinically or experimentally used drugs, not typically associated with the biology of HIV-1 latency, that produced small but significant

reactivation of the provirus, such as drugs acting on neurotransmitter receptors or Tyr kinases. In flow cytometry assays of the sorted latent-cell population, some of the identified drugs showed a dose-dependent reactivation of latency; in combination with low doses of the current leads for latent HIV-1 reactivation (SAHA, prostratin) they also revealed a synergistic effect that might be exploited for research and as a therapeutic tool in current and future clinical protocols.

For a preliminary validation in primary cells, we tested the HIV-1 latency model in activated CD4$^+$ T cells. These cells contain the main constituents of the latent HIV-1 reservoir, such as central and transitional memory CD4$^+$ T cells. As reported (78), resting CD4$^+$ T cells are refractory to HIV-1 infection, with few or no fluorescent cells when viruses are tested in freshly isolated populations (not shown). Although we prestimulated cells with anti-CD3/CD28-coated beads to boost infection levels, latent infection rates remained very low, as is predicted for a fully activated T cell. The mApple-only-expressing cells obtained from infection of primary activated CD4+ cells had proviral DNA levels similar to those of the double-positive population, but did not express viral products such as p24. Only a small but measurable part of the mApple-only population (<20%) responded to αCD3/CD28 stimulation or to molecules that reactivate latent HIV-1 in other systems (SAHA, Prostratin). Importantly, we can detect a small population of latently infected, reactivatable CD4+ T cells after infection of activated T cells.

In conclusion, we present a powerful instrument to study HIV-1 latent infection. These new reporter viruses offer the advantage of enabling detection and purification of live, latent-HIV-1-infected cells and to study the kinetics of latency establishment early

upon cell infection. This tool can further advance the study of HIV-1 latency and provide

new routes to accelerate the quest for a functional cure and eradication of HIV-1

infection.
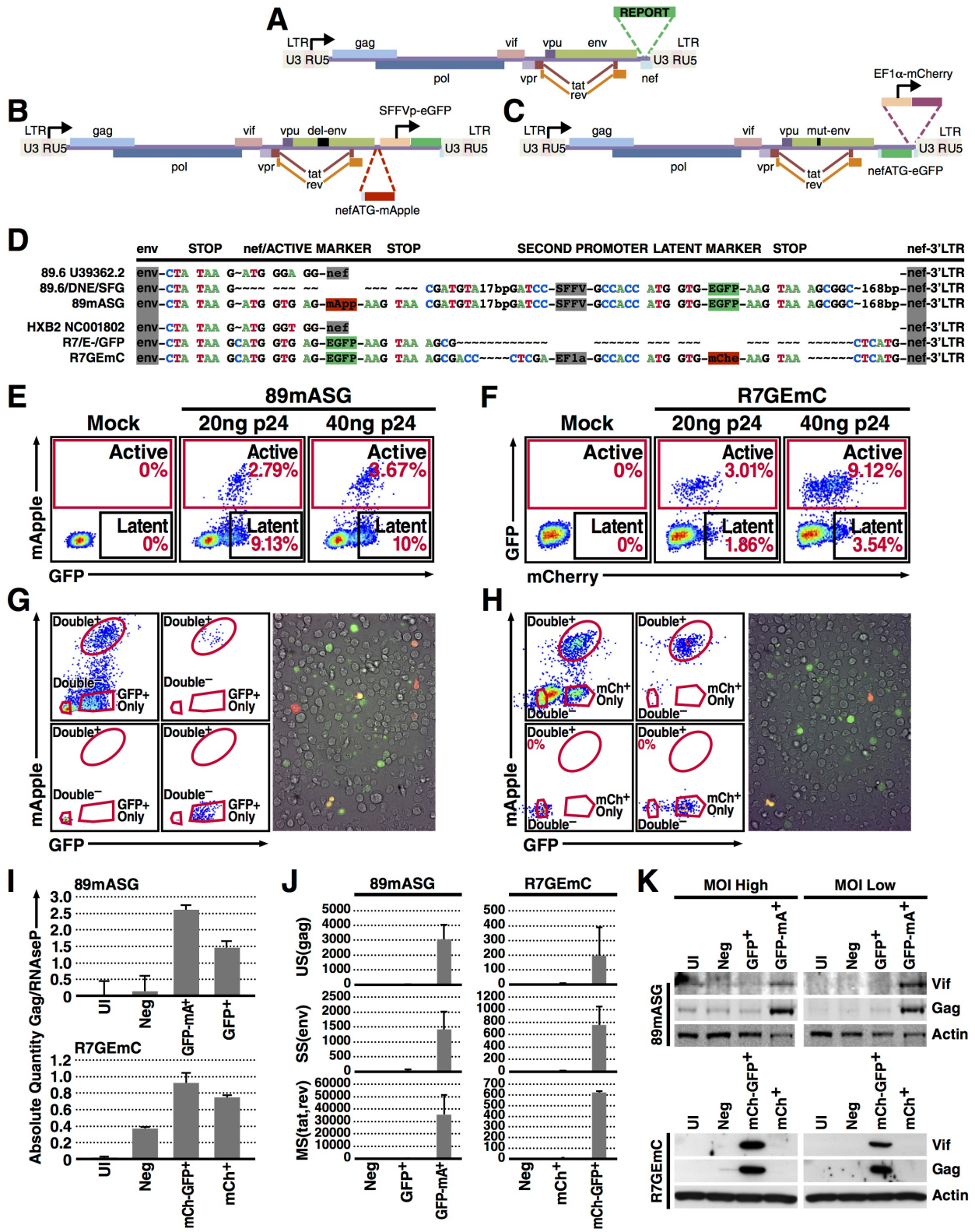

Acknowledgements

Figures

Figure 2.1. Two-color viruses identify a population of latently infected cells.

(a) Diagram of the derivation of two-color viruses from the original strains, with a scheme of a classical single reporter virus bearing the reporter gene in the nef ORF. (b) In the 89mASG construct, EGFP is under the control of the SFFV promoter and mApple was added upstream of the transcriptional unit in the position of the nef ATG (see d). (c) In the R7GEmC construct, GFP replaces nef and a whole transcriptional unit (EF1α-mCherry) was inserted downstream. (d) Alignment of regions of interest (from the end of env to the 3'LTR) of the original 89.6 and HXB2 strains deposited in the NCBI database (U39362.2 and NC001802), the starting plasmid and final constructs for each of the HIV-1 clones 89mASG (d, top) and R7GEmC (d, bottom). Insertion points of the first reporter in the nef ORF, the promoter (SFFV or EF1α, respectively) and the second reporter, followed by the end of nef and 5'LTR. (e,f) Cytometric analysis of Jurkat T cells 3 days post-infection, with the indicated titers of 89mASG (e) and R7GEmC (f). (g,h) FACS analysis of 89mASG (g, left) and R7GEmC (h, left) Jurkat T cells sorted 5 days post-infection. Double-negative (uninfected), double-positive (actively infected) and single-positive (latently infected, expressing GFP- or mCherry-only) cells are shown for both viral strains. Fluorescence microscopy images of 89mASG- (g, right) and R7GEmC- (h, right) infected Jurkat T cells. (i) qPCR quantification of proviral DNA in the sorted Jurkat populations, using the primer-probe combination #E for the HIV-1 gag gene and RNAseP for normalization. (j) qPCR quantification of viral transcripts of unspliced (US) gag, singly- (SS) env and multiply-spliced (MS) tat/rev common regions (#E, F, and G; Supplementary Table 2), normalized

for cell GAPDH. (k) Western blot quantification of the HIV-1 proteins vif and gag and

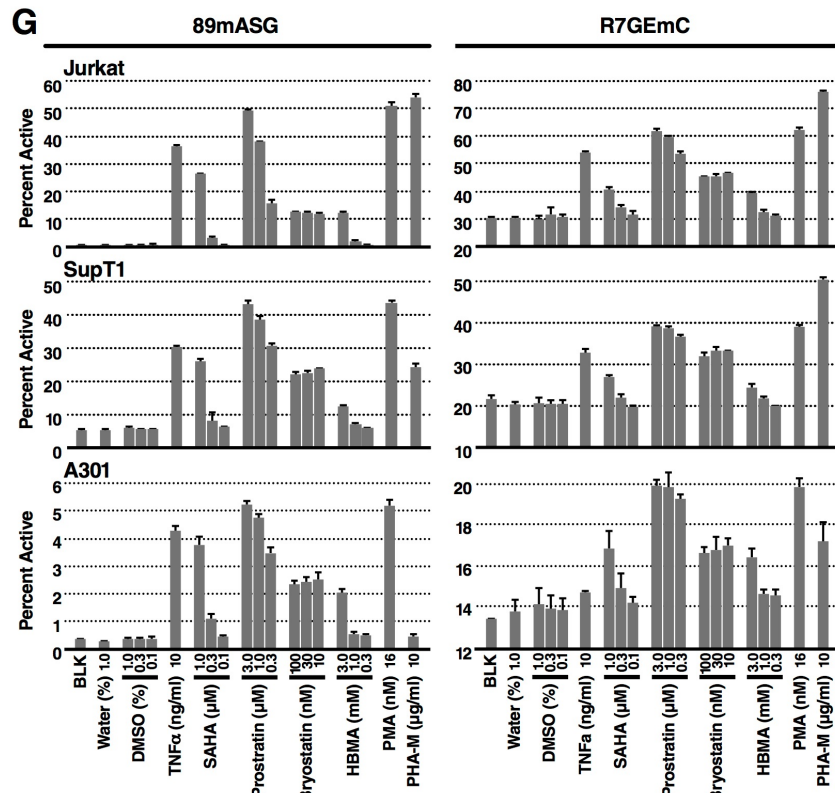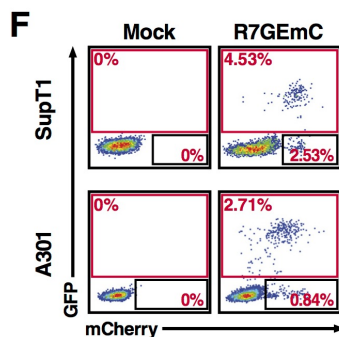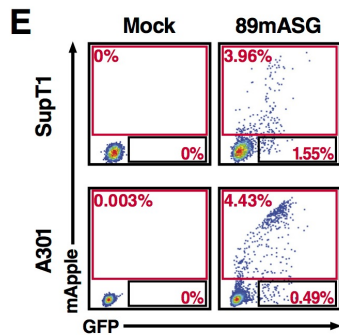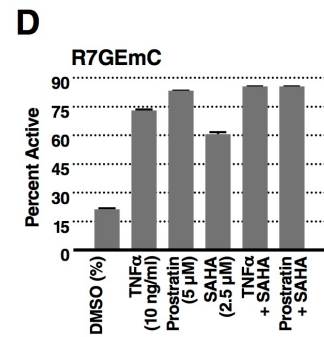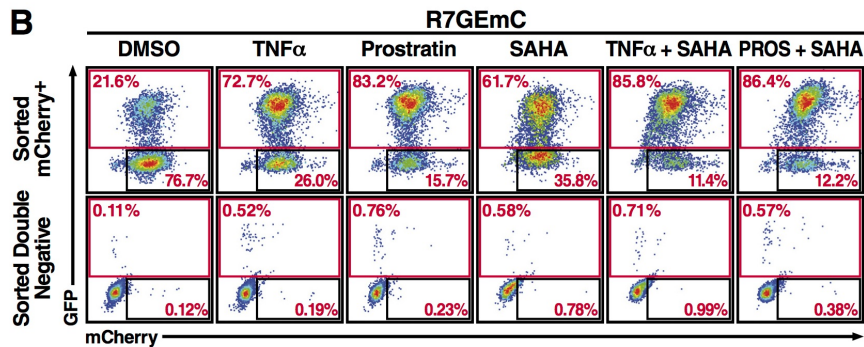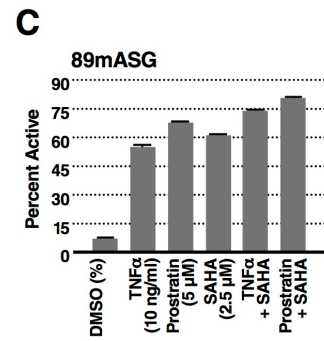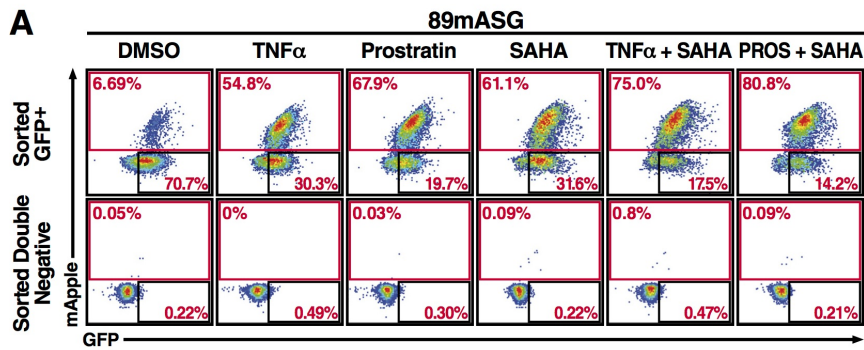the endogenous protein α-actin in the sorted Jurkat populations.

Figure 2.2. Latently infected cells respond to reactivating drugs. (a–d) Five days after sorting, expanded single-positive (latent) and double-negative (uninfected) 89mASG (a, c) and R7GEmC (b, d) Jurkat T cells were treated with 10 ng/ml TNFα, 5 µM prostratin or 2.5 µM SAHA, alone or in combination, as indicated. FACS analysis of a representative treatment (a, b) and histogram quantification of percent population in the active gate for three different experiments (c, d). $P<0.001$, compared to DMSO control for all treatments. $P<0.01$ for combination vs. single treatments, except for R7GEmC prostratin vs. prostratin+SAHA $P<0.05$. (e,f) FACS analysis of SupT1 and A301 cell lines, infected with 20 ng p24 of 89mASG (e) or R7GEmC (f) virus. (g) A larger panel of latent HIV-1-reactivating drugs was used at indicated concentrations on sorted, single positive Jurkat, SupT1 and A301 cells. Histograms show quantification of the percent population in the active gate. Data indicate mean ± SD for three different treatments. $P<0.01$, compared to DMSO 0.1% control for all treatments, except for: SAHA 0.1 not significant (n.s.) in all combinations; SAHA 0.1 n.s. in SupT1 and A301-R7GEmC, $P<0.05$ in Jurkat-R7GEmC; Bryostatin 30 $P<0.05$ in A301-R7GEmC; HMBA 0.3 n.s. in all combinations; HMBA 1 n.s. in A301 and SupT-R7GEmC, $P<0.05$ in Jurkat-R7GEmC and SupT-89mASG; PHA-M n.s in A301-89mASG and $P<0.05$ in A301-R7GEmC.

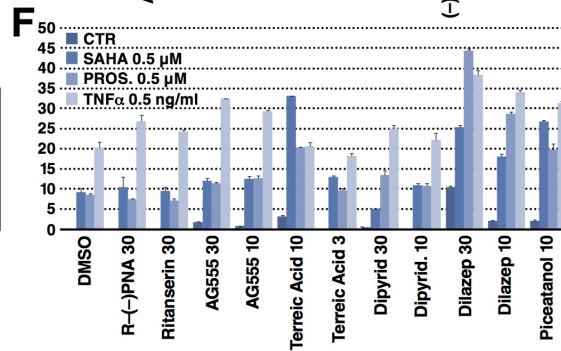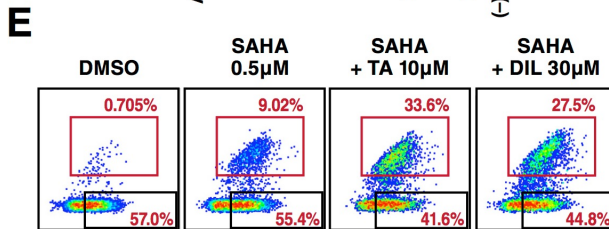**A** 89mASG

B DMSO | AG555 10μM | Terreic Acid 10μM | Dilazep 30μM | Dilazep 10μM | Piceatannol 30μM | Piceatannol 10μm | NSC 95397 30μm

C 89mASG

D R7GEmC

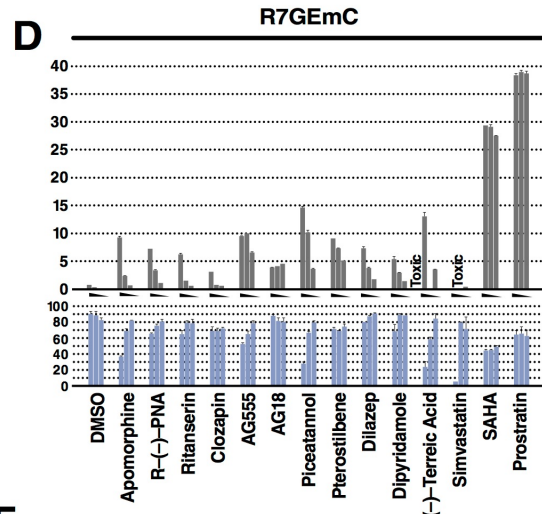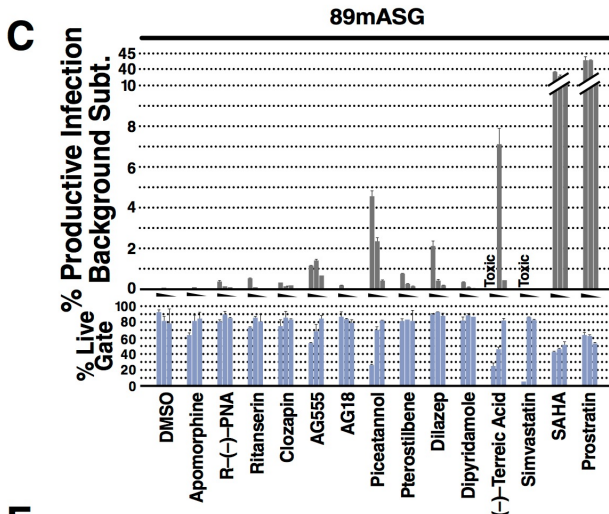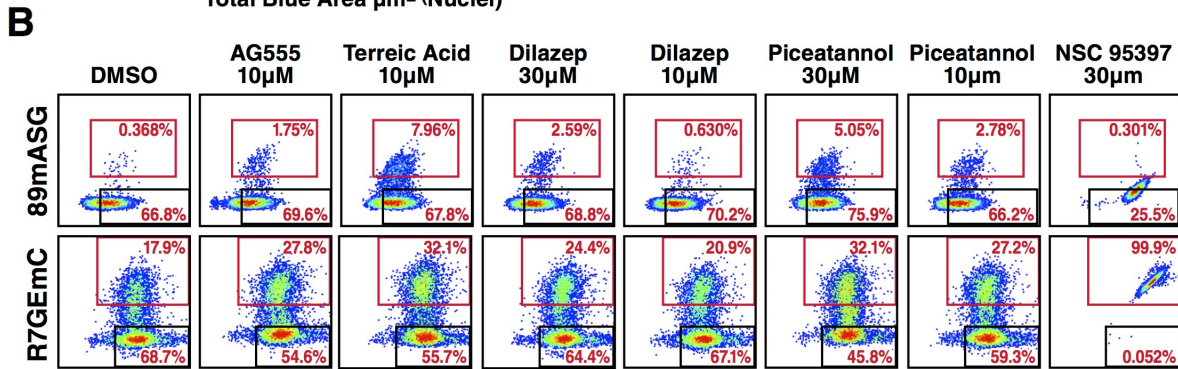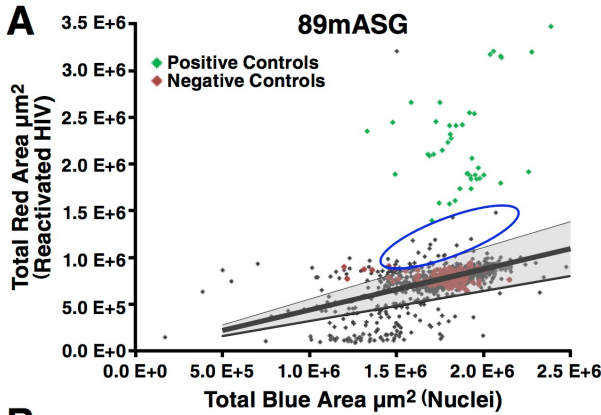**E** DMSO | SAHA 0.5μM | SAHA + TA 10μM | SAHA + DIL 30μM

F

Figure 2.3. High-throughput screening identifies new reactivator drugs using two-color HIV-1 constructs. Sorted 89mASG-infected, GFP-only-positive Jurkat cells were used to test the Tocriscreen drug library for latent HIV-1 reactivation (see Methods). (a) Dot plot showing quantification of total blue area (Hoechst 33342 signal, total cell) against total red area (mApple signal, reactivated HIV-1). Red dots indicate the position of untreated wells; the shaded area indicates the median ± 2SD of the red/blue area ratio for negative control wells. Green dots indicate positive control wells, treated with 10 ng/ml TNFα, 5 µM prostratin or 2.5 µM SAHA and their combinations. Blue circle shows novel reactivators identified in the screening (see Supplementary Table 1). (b) Validation of representative hits by FACS analysis. Apomorphine and its analog R-(-)-propylnorapomorphine (PNA), ritanserin, clozapin, AG555, AG18, piceatannol, pterostilbene, dilazep, dipyridamole, terreic acid (TA), simvastatin and NSC95397 were used to validate hits and pathways identified by drug screening. Representative FACS plots of sorted single-positive Jurkat T cells for both viruses. The productive gate contains the reactivated cells. In the last plot on the right, NSC95397 treatment shows alteration of the fluorescent profile by the drug fluorescence. (c,d) Histogram plot of percent population in the productive gate (grey) for each treatment and percentage of cells in the live gate (light blue) for sorted single-positive Jurkat T cells for 89mASG (c) and R7GEmC (d) reporters. Data shown as mean ± SD for three different treatments. For each compound, a grey triangle between the histograms indicates decreasing drug concentration (30, 10 and 3 µM). Treatments for which cell death was predominant are labeled TOXIC. Ritanserin 30µM, clozapin 30µM, AG555 30, 10 and 3µM, piceatannol 30 and 10µM, pterostilbene 30µM, dilazep 30µM, TA 10 and 3µM (P<0.01) and PNA

30µM, piceatannol 3µM, pterostilbene 10µM, dipyridamole 30µM (P<0.05) show

significant reactivation of the latent 89mASG virus compared to the DMSO control.

Apomorphin 30µM, PNA 30µM, ritanserin 30µM, clozapin 30µM, AG555 30, 10 and

3µM, AG18 30, 10 and 3µM, piceatannol 30 and 10µM, pterostilbene 30 10 and 3 µM,

TA 10µM (P<0.01) and PNA 10µM, ritanserin 10µM , piceatannol 3µM, dilazep 30, 10

and 3µM, TA 3µM (P<0.05) show significant reactivation of the latent R7GEmC virus

compared to the DMSO control. (e) FACS analysis of drug treatments combined with

0.5 µM SAHA, 0.5 µM prostratin and 0.5 ng/ml TNFα. Representative FACS plots of

sorted GFP-only-positive Jurkat T cells for 89mASG treated with DMSO (control), SAHA

(0.5 µM) alone or with terreic acid (10 µM) and dilazep (30 µM). (f) Histogram plot of

percent population of the sorted cells in the productive gate for each treatment at

indicated concentrations (µM). Dilazep 30, 10µM and piceatannol 10µM show

significant (P<0.01) increase in reactivation 0.5 µM SAHA, 0.5 µM prostratin and 0.5 ng/

ml TNFα as compared to the reactivation obtained with these three drugs alone. TA also

shows positive interaction with SAHA and prostratin at 10µM (P<0.01) and with SAHA at

3µM(P<0.05). AG555 interact enhances reactivation mediated by prostratin and TNFα

(P<0.01), whilst dipyridamole 30 and 10µM shows significant (P<0.05) positive

interaction with prostratin only.

Figure 2.4. Two-color viruses label a population of latently infected primary T cells. (a) FACS analysis of activated CD4$^+$ T cells from two human donors (#1, #2) infected with R7GEmC at indicated virus amounts. (b) Fluorescence microscopy image of infected CD4$^+$ T cells showing mCherry-positive (latent, red) and GFP-positive (active, green) cells. (c) qPCR quantification of proviral DNA in the sorted populations, using primer-probe combinations for the HIV-1 gag gene and the RNAseP gene for normalization. Data represent mean ± SEM for three different donors.

(d) Western blot quantification of HIV-1 gag and cell α-actin in sorted CD4$^+$ T cell populations from pooled donors. (e) FACS analysis of sorted CD4$^+$ T cell populations from donors #1 and #2 after 48 h with anti-CD3/CD28-coated Dynabeads (αCD3/28) or left untreated (CTR). Right, histogram plot for the mCherry-only population of each donor in which the treated profile (green line) is overlaid on the untreated (grey shaded).

(f) Dot plot of mCherry-only population from three pooled donors treated with 10 ng/ml, TNFα, 1 µM prostratin, 1 µM SAHA or anti-CD3/CD28-coated Dynabeads (1 bead/cell).

CHAPTER 3: Activated and resting primary CD4$^+$ T cells can both become latently infected by an HIV-1 dual-reporter virus.

Abstract

Highly active antiretroviral therapy (HAART) can suppress human immunodeficiency virus (HIV-1) replication to undetectable levels, but cannot fully eradicate the virus from infected individuals because of a small reservoir of cells that are latently infected. Although it is generally accepted that HIV-1 can only efficiently infect activated CD4$^+$ T cells, the most characterized latent reservoir resides within resting CD4$^+$ T cells. In this study, we use a dual-reporter virus, HIV Duo-Fluo I, which is capable of identifying latently infected cells immediately after infection, to investigate the role of T cell activation on the establishment of HIV-1 latency. We show that HIV-1 latency can arise from the direct infection of both resting and activated CD4$^+$ T cells isolated from peripheral blood of uninfected donors. Latent infection is more likely to occur in resting CD4$^+$ T cells and can be enhanced by pretreatment of the cells with the chemokine, CCL19, or the cytokine, IL-7. Infection of activated CD4$^+$ T cells is more likely to result in productive infection, but can also give rise to latently infected cells immediately after infection, or after productively infected cells return to a resting state. We further show, that resting CD4$^+$ T cells isolated from lymphoid tissue are more susceptible to latent infection. Our findings suggest that infection of both resting and activated primary CD4$^+$ T cells can result in latently infected cells that contribute to the reservoir.

Introduction

       With the advent of highly active antiretroviral therapy (HAART) in 1995, HIV-1 infection was transformed from a deadly disease into a life-long chronic condition (7). By targeting multiple stages of the viral life-cycle, antiretroviral drugs are capable of reducing patient viremia to undetectable levels (21, 79, 80). However, HAART is unable to fully eradicate HIV-1 (26) because of a small reservoir of latently infected cells that contain a transcriptionally silent, yet reactivatable provirus (81). This latent reservoir serves as a major barrier to viral eradication and requires a better understanding at the mechanistic level.

       The major target of HIV-1 infection is CD4$^+$ T cells, and it is within resting CD4$^+$ T cells that the most extensively studied latent reservoir resides (18, 24, 25). During the course of infection, HIV-1 enters a target cell and reverse-transcribes its genomic viral RNA into a double-stranded cDNA that then enters the nucleus and integrates into the host genome where it comes under the control of the host transcriptional machinery. In most cases, integration of the viral cDNA leads to productive infection, characterized by transcription of viral genes and subsequent virion production and budding. However, in rare instances, latency is established and is characterized by a provirus that produces little to no viral transcripts (17). With no viral proteins being produced, the infected cell is not subjected to viral cytopathic effects, and is also not recognized or targeted by the immune system. In addition, antiretroviral drugs only target active viral replication and are, thus, rendered ineffective against latent proviruses. Finally, the largest latent reservoir is found within memory CD4$^+$ T cells which have a long half-life *in vivo* (22, 23), allowing latent virus to persist within infected individuals for decades or more (82).

However, when these latently infected memory CD4$^+$ T cells encounter an antigen or are exposed to certain cytokines or chemokines, proviral transcription is activated leading to productive infection (18, 19). This is likely the cause of viral rebound following HAART cessation, and the reason why infected individuals require a daily regime of antiretroviral drug therapy for life.

The study of HIV-1 latency has been hindered by the rarity of the pool of latent cells, *in vivo* (~1 in 1x10^6 cells) (22), and the inability to distinguish latently infected cells from uninfected cells (83). Despite these challenges, several *in vitro* latency models have been developed, leading to important observations about the maintenance and reactivation of latently infected cells (reviewed in references (72, 84)). However, how the latent reservoir is established is still unclear because current technologies only allow for the quantification of latently infected cells via reactivation after latency has already been established. Our lab has recently overcome some of the major problems associated with studying HIV-1 latency by developing a dual-reporter virus, HIV Duo-Fluo I, that is capable of distinguishing between cells that are productively infected, latently infected or uninfected, and allows for the purification of each population (85). HIV Duo-Fluo I allows for the study of the kinetics of HIV-1 latency immediately after infection by employing two separate fluorescent markers: an LTR-driven eGFP marker (productive infection), and an LTR-independent mCherry marker (latent infection), driven by an EF1α promoter (Figure 3.1A). Using this dual-reporter virus, we can now begin to gain a better understanding of how HIV-1 latency is established.

One major question that still remains, is what role does T cell activation play in the establishment of the HIV-1 latent reservoir? Based primarily on *in vitro* evidence, it

is generally accepted that HIV-1 can only replicate in activated CD4^{+} T cells (27-30).

Resting CD4^{+} T cells present several barriers to HIV-1 infection (reviewed in reference

(31)), as they do not support nuclear import (86) or integration of the viral cDNA (30,

34). However, the most notable blockade to infection in resting CD4^{+} T cells occurs at

the stage of reverse transcription (32, 33). Resting CD4^{+} T cells cannot support reverse

transcription, or do so at a much lower efficiency than activated cells, and this is due, at

least in part, to the restriction factor, SAMHD1 (78, 87). Additionally, the majority of

HIV-1 infected resting CD4^{+} T cells, *in vivo*, exhibit a memory phenotype, suggesting

that they arose from infection of previously activated CD4^{+} T cells. Based on this

evidence, one leading theory for the establishment of latency is that activated CD4^{+} T

cells become infected and survive long enough to revert back to a resting memory state

(35). In order for this type of latency to occur, the infected cell would have to survive the

viral-induced cytopathic effects and the host immune response that are responsible

for the short life span (about 1 day) of productively infected cells (88, 89). Alternatively,

activated CD4^{+} T cells could potentially become infected during their transition back to a

resting memory state, resulting in the establishment of latency in a cell that can support

reverse transcription and viral integration, but not proviral transcription (90).

However, evidence also exists in support of direct infection of resting CD4^{+} T

cells, with the strongest evidence coming from *in vivo* and *ex vivo* studies of both SIV

and HIV-1 infection (91-96). One early study demonstrated that both naive and memory

CD4^{+} T cells from patients contain integrated viral DNA (91). The CD4^{+} T cells analyzed

in this study came from patient blood, but the majority of evidence that supports direct

infection of resting CD4^{+} T cells comes from studies conducted on cells within lymphoid

tissue. Viral RNA can be detected in resting CD4$^+$ T cells residing in lymphoid tissue, *in vivo* (92), and direct infection of resting CD4$^+$ T cells resulting in productive infection was demonstrated in lymphoid tissue, ex vivo (97). Interestingly, a subsequent study found that resting CD4$^+$ T cells within lymphoid tissue, ex vivo, could support HIV-1 infection, whereas purified CD4$^+$ T cells isolated from that same lymphoid tissue could not (98), suggesting that the microenvironment of lymphoid tissue plays a critical role in rendering resting CD4$^+$ T cells permissive to HIV-1 infection. Indeed, several lymphoid tissue-associated factors, including cytokines (99-102), chemokines (54, 101, 103, 104), extracellular matrixes (105-107), and cell surface markers (108), have been shown to enhance HIV-1 infection in resting CD4$^+$ T cells . Therefore, it is possible that HIV-1 latency is established by the direct infection of resting CD4$^+$ T cells via the exposure to soluble factors that don't induce classic T cell activation.

In this study, we use HIV Duo-Fluo I to investigate the role of T cell activation on the establishment of HIV-1 latency in primary CD4$^+$ T cells. In addition, we use HIV Duo-Fluo I to test the different theories of how HIV-1 latency is established, whether it be through infection of activated CD4$^+$ T cells that return to a resting state or through the direct infection of resting CD4$^+$ T cells. Utilizing this dual-reporter virus that is capable of distinguishing latently infected cells from productively infected cells immediately after infection, we show that both resting and activated primary CD4$^+$ T cells are capable of supporting both productive and latent infection. We further show, that while activated and resting CD4$^+$ T cells can both support HIV-1 infection, resting CD4$^+$ T cells are more likely to support latent infection than activated CD4$^+$ T cells.

Materials and Methods

Virus production. Pseudotyped HIV Duo-Fluo I viral stocks were generated by co-transfection of HEK293T cells with a plasmid encoding HIV Duo-Fluo I and a plasmid encoding HIV-1 envelope (pSVIII-92HT593.1) using the standard calcium phosphate transfection method. Vpx-containing HIV Duo-Fluo I pseudotyped virus was generated by co-transfection of HEK293T cells with the HIV Duo-Fluo I plasmid, the pSVIII-92HT593.1 plasmid, and a plasmid encoding a Vpr-Vpx fusion protein (pSIV3+, generously donated by Warner Greene). Supernatants were collected after 72hrs and filtered through a .45μM membrane to clear cell debris, and concentrated by ultracentrifugation for 2hrs at 4°C. Concentrated virions were resuspended in complete media and stored at -80°C. Virus concentration was estimated by p24 titration (HIV-1 alliance p24 ELISA kit, Perkin-Elmer).

Primary cell isolation and cell culture. Primary CD4+ T cells and PBMCs were purified from healthy donor blood (Blood Centers of the Pacific, San Francisco, CA, USA and Stanford Blood Center). CD4+ T cells were isolated by negative selection using the RosetteSep Human CD4+ T Cell Enrichment Cocktail (StemCell Technologies). PBMCs were purified by Histopaque®-1077 density gradient. Purified resting CD4+ T cells and PBMCs from peripheral blood were cultured in RPMI 1640 medium supplemented with 10% FBS, L-glutamine (2 mM), penicillin (50 U/ml) and streptomycin (50 mg/ml). HLACs were purified using tonsillar or splenic tissue from uninfected donors (Cooperative Human Tissue Network) as previously described (109). CD4+ T cells were isolated from HLACs by negative selection using the EasySep™

Human CD4+ T Cell Enrichment Kit (StemCell Technologies). HLACs and CD4$^+$ T cells isolated from splenic and tonsillar tissues were cultured in medium consisting of RPMI 1640 supplemented with 20% heat-inactivated FBS, 100 mg/ml gentamicin, 200 mg/ml ampicillin, 1 mM sodium pyruvate, 1% nonessential amino acids (Mediatech, Manassas, VA, USA), 2mM L-glutamine and 1% fungizone (Invitrogen, Indianapolis, IN, USA).

Cell treatment and infection. Purified resting CD4$^+$ T cells were either left untreated or treated for three days with 20 ng/ml IL-7 (R&D Systems) or 100 µM CCL19 (R&D Systems). Purified CD4$^+$ T cells isolated from peripheral blood and tonsillar and splenic tissues, as well as PBMCs and HLACs were stimulated with αCD3/αCD28 activating beads (Life Technologies) at a concentration of 1 bead/cell in the presence of 30U/ml IL-2 (PeproTech) for 3 days. All cells were spinoculated with either HIV Duo-Fluo I alone or HIV Duo-Fluo I containing vpx at a concentration of 100 ng of p24 per 1x10^6 cells for 2hr at 1,200 X g at 37°C. After spinoculation all cells were returned to culture in the presence of 30 U/ml IL-2, except for CD4$^+$ T cells pre-stimulated with αCD3/αCD28 activating beads, which placed back in culture with the αCD3/αCD28 activating beads in the presence of 30 U/ml IL-2.

Flow cytometry and cell sorting. Uninfected cells were stained in FACS buffer (phosphate buffered saline supplemented with 2 mM EDTA and 2% FBS) with αCD69-PE and αCD25-APC (eBioscience) and fixed in 1% paraformaldehyde. Infected cells were stained in FACS buffer with αCD69-V450 and αCD25-APC/Cy7 (BD Biosciences) and fixed in 1% paraformaldehyde. Data were collected on a FACS Caliber and a

FACS LSRII (BD Biosciences), and analysis was performed using FlowJo software (TreeStar). Untreated and treated CD4+ T cells from Figure 3.2 were sorted based on GFP and mCherry fluorescence 6 days post infection using a FACS AriaII (BD Biosciences) and placed back in culture with or without 30 µM Raltegravir (National AIDS Reagent Program). CD4+ T cells stimulated with αCD3/αCD28 activating beads in the presence of 30 U/ml IL-2 from Figure 3.4 were sorted based on GFP and mCherry fluorescence 4 days post infection.

SAMHD1 protein analysis. Untreated resting primary CD4+ T cells infected with either HIV-Duo-Fluo I alone or HIV Duo-Fluo I containing Vpx were lysed 6 days post infection in radioimmunoprecipitation assay buffer (150 mm NaCl, 1% Nonidet P-40 (vol/vol), 0.5% AB-deoxycholate (vol/vol), 0.1% SDS (vol/vol), 50 mm Tris-HCl (pH 8), 1 mm DTT) and EDTA-free Protease Inhibitor (Calbiochem®). Cell lysates were used for SDS-PAGE immunoblotting analysis. The primary antibodies used were rabbit polyclonal anti-SAMHD1 (Sigma-Aldrich, Cat# SAB2102077) and monoclonal anti-β-actin (A5316, Sigma-Aldrich).

Results

Resting primary CD4+ T cells support both productive and latent infection, but are biased towards latent infection.

There has been conflicting reports as to whether resting CD4+ T cells can be infected by HIV-1, either productively or latently (31). Studies suggest that resting CD4+ T cells are, by themselves, refractory to HIV-1 infection, but can become permissive to

infection following treatment with certain cytokines or chemokines that do not induce classic T cell activation (54, 101). To test the permissibility of resting CD4^{+} T cells to our HIV Duo-Fluo I virus, we first isolated total CD4^{+} T cells from peripheral blood of uninfected donors using a negative selection method. Upon isolation, CD4^{+} T cells were checked for expression of activation markers, CD69 and CD25. Freshly isolated CD4^{+} T cells did not express CD69 or CD25 (Figure 3.1B) and were, therefore, considered to be resting CD4^{+} T cells. Resting CD4^{+} T cells were either left untreated or were treated with the cytokine, IL-7, or the chemokine, CCL19, for 72hrs prior to infection. Stimulation with IL-7 or CCL19 led to a slight increase in CD25 expression, with 0.58% and 1.38% of cells expressing CD25, respectively (Figure 3.1B). As a positive control, resting CD4^{+} T cells were also stimulated with αCD3/αCD28 activating beads in the presence of IL-2 for 72hrs prior to infection, which led to significant expression of both CD69 and CD25 activation markers (Figure 3.1B).

Both untreated and treated cells were spinoculated with HIV Duo-Fluo I for 2hrs at 37°C and then returned to culture in the presence of IL-2. Productive infection (GFP+ and mCherry+/GFP+) and latent infection (mCherry+/GFP-) were monitored by flow cytometry everyday for six days following infection (data not shown). Compared to αCD3/αCD28 stimulated cells at six days post-infection, untreated resting CD4^{+} T cells showed significantly lower levels of HIV-1 infection, but nonetheless, were permissive to both productive and latent infection (Figures 3.2B & 3.2C). Of note, untreated resting CD4^{+} T cells not cultured in the presence of IL-2 after infection were not permissive to either productive or latent infection by HIV Duo-Fluo I (data not shown). Despite having minimal effects on T cell activation, both IL-7 and CCL19 treatment led to an increase in

HIV-1 infection compared to untreated resting CD4$^+$ T cells (Figure 3.1C), suggesting that the permissibility of resting CD4$^+$ T cells can be enhanced without the need of classic T cell activation, which is in agreement with previous studies (54, 100). However, for the first time, we show the distribution of productive and latent infection within resting CD4$^+$ T cells following such treatment (Figure 3.1D). CCL19 treatment led to a 2-fold increase in productive infection compared to untreated resting CD4$^+$ T cells, while IL-7 treatment led to a 5-fold increase. Latent infection was increased by 3-fold after treatment with each.

In addition to cytokine and chemokine treatment, we also investigated the role of SAMHD1 in restricting HIV-1 infection within resting CD4$^+$ T cells by infecting resting CD4$^+$ T cells with HIV Duo-Fluo I containing Vpx which was provided in trans. Vpx is a lentiviral accessory protein encoded by HIV-2 that allows the virus to overcome SAMHD1-mediated post-entry restriction in resting CD4$^+$ T cells (78), as well as dendritic cells, monocytes and macrophages (110, 111). Infection of untreated resting CD4$^+$ T cells with the Vpx-containing HIV Duo-Fluo I virus increased the levels of infection within these cells as compared to resting untreated cells infected with HIV Duo-Fluo I alone (Figures 3.1C & 3.1D). This increase in infection was due to a reduction of SAMHD1 levels (Supplementary Figure 3.1A) and not T cell activation (Supplementary Figure 3.1B). Levels of productive infection increased significantly and were almost comparable to that of activated CD4$^+$ T cells, whereas the levels of latent infection were more than 2-fold greater compared to resting untreated cells infected with HIV Duo-Fluo I alone, but were lower than the levels of latent infection observed in both IL-7 and CCL19 treated cells. This increase in infection suggests that SAMHD1

knockdown in resting CD4$^+$ T cells biases the cells towards productive infection. Calculation of the ratios of latently infected cells to productively infected cells (Figure 3.1E) confirms this observation. Along with untreated resting CD4$^+$ T cells infected with the vpx-containing HIV Duo-Fluo I, IL-7 treated resting CD4$^+$ T cells and activated CD4$^+$ T cells also show a propensity towards productive infection. Alternatively, untreated and CCL19 treated CD4$^+$ T cells show a propensity towards latent infection.

In order to assure that infection of resting CD4$^+$ T with HIV Duo-Fluo I didn't lead to any silent infection events in which viral integration occurred but failed to produce transcription of either fluorescent marker, we sorted the uninfected populations (GFP-/mCherry-) of both untreated and treated CD4$^+$ T cells six-days post-infection via FACS. These cells were then stimulated with αCD3/αCD28 activating beads in the presence or absence of the integrase inhibitor, Raltegravir, in order to distinguish between the reactivation of any pre-integration latent virus and post-integration latent provirus that might reside within these cell populations (Figure 3.1F). As a control, latently infected cells (mCherry+/GFP-) were also isolated via FACS from infected cells pre-treated with αCD3/αCD28 activating beads and subjected to the same treatments as the uninfected cells. Reactivation of latent virus was analyzed by flow cytometry 48hrs post-stimulation. All cell populations contained some level of reactivatable pre-integration latent virus (Figure 3.1G), with the highest levels observed in CCL19 and IL-7 treated populations, as well as the untreated population infected with a Vpx-containing HIV Duo-Fluo I virus. Untreated resting CD4$^+$ T cells infected with the HIV Duo-Fluo I virus alone, showed the lowest levels of reactivatable pre-integration latency, followed closely by activated CD4$^+$ T cells.

Analysis of the reactivatable post-integration latent provirus shows that the uninfected cell population isolated from activated CD4$^+$ T cells contains very little reactivatable provirus (0.26%, Figure 3.1G) compared to the initial latent population identified after infection (1.01%, Figure 3.1D), suggesting that HIV Duo-Fluo I is capable of efficiently identifying latently infected cells within activated CD4$^+$ T cells. Conversely, uninfected cells isolated from untreated resting CD4$^+$ T cells contained 0.4% reactivatable provirus, while only 0.14% latently infected cells where identified after the initial infection, suggesting that HIV Duo-Fluo I is inefficient at identifying latently infected cells within resting CD4$^+$ T cells. Similarly, IL-7 treated resting CD4$^+$ T cells, and untreated resting CD4$^+$ T cells infected with the Vpx-containing HIV Duo-Fluo I, both contained over 1% reactivatable provirus within their isolated uninfected cell populations, which was more than twice the size of the latent cell populations identified after the initial infection (Figure 3.1D), suggesting that IL-7 treatment and SAMHD1 knockdown both lead to silent infection events in resting CD4$^+$ T cells, and, thus, an underestimation of the latently infected cell population. Interestingly, CCL19 treatment of resting CD4$^+$ T cells produced 0.2% reactivatable provirus from the isolated uninfected cell population, while the initial latent cell population following infection was 0.47%. Taken as a whole, these data suggest that HIV Duo-Fluo I is capable of identifying some latently infected cells in resting CD4$^+$ T cells via the mCherry fluorescent marker, but resting CD4$^+$ T cells are also more likely to contain silent infection events that result from the silencing of both fluorescent markers.

Primary CD4+ T cells become less permissive to HIV-1 infection as they transition from an activated state to a resting state, but exhibit a higher propensity towards latent infection.

Based on evidence that HIV-1 replication is most efficient in activated CD4+ T cells (31), and that the largest latent reservoir, *in vivo*, is found within resting memory CD4+ T cells, we next investigated the possibility that HIV-1 latency is preferentially established in CD4+ T cells that become infected as they transition from an activated state to a resting state. To do this, we isolated total CD4+ T cells from peripheral blood of uninfected donors and stimulated the cells with αCD3/αCD28 activating beads in the presence of IL-2 for three days (Figure 3.2A). We then removed the αCD3/αCD28 activating beads and allowed the cells to return to a resting state over the course of 20 days in the presence of IL-2. We infected the CD4+ T cells with HIV Duo-Fluo I at peak activation (day 4) and every five days thereafter as they transitioned back to a resting state. As indicated by the expression levels of the activation markers, CD69 and CD25, the CD4+ T cells transitioned from an activated state to a resting state over the course of 20 days, while remaining over 60% viable throughout the experiment (Figure 3.2B). Maximal activation occurred at day 4 with 79% of cells expressing both CD69 and CD25 (Figure 3.2C). By day 9, however, 31% of the cells no longer expressed CD69 or CD25, and by day 24, 92% of cells no longer expressed either activation marker. Despite the majority of cells losing CD69 expression by day 14 (<1% CD69+), a small fraction of cells continued to express CD25 throughout the entire experiment, with 8% of cells still being CD25+ at day 24, suggesting that while the majority of CD4+ T cells returned to a resting state by day 24, there remained a small population of cells that were still

transitioning back to a resting state. Others have observed similar expression profiles while trying to return activated CD4$^+$ T cells to a resting state (48, 49).

Productive infection and latent infection were analyzed by flow cytometry, three days post-infection for each time-point (Figure 3.2D), and the average of three donors was quantified for each population (Figure 3.2E). Infection at day 4, when the cells were maximally activated, produced the highest levels of both productive and latent infection within CD4$^+$ T cells. As the cells returned to a resting state over the course of 20 days, the levels of both productive infection and latent infection decreased accordingly, suggesting that peak infection for HIV-1 within CD4$^+$ T cells occurs when the cells are most activated, and as the cells lose expression of the activation markers, CD69 and CD25, they become less permissive. However, the ratio of latent infection to productive infection increases progressively as the cells transition from an activated state to a resting state (Figure 3.2F), suggesting that while activated CD4$^+$ T cells are optimal for peak infection, latent infection is more likely to occur in cells that are resting or are transitioning back to a resting state.

Productively and latently infected activated primary CD4$^+$ T cells lose expression of both fluorescent markers as they return to a resting state.

As an alternative to HIV-1 latency being established by the infection of activated CD4$^+$ T cells during their transition to a resting state, we next wanted to investigate whether productively infected activated primary CD4$^+$ T cells could return to a resting state and contribute to the latent reservoir. We isolated CD4$^+$ T cells from uninfected donor blood and activated the cells with αCD3/αCD28 activating beads in the presence

of IL-2 for three days and then spinoculated them with HIV Duo-Fluo I for 2hrs at 37°C (Figure 3.3A). After infection cells were kept in an activated state by returning them to culture in the presence of activating beads and IL-2. Four days post infection CD4$^+$ T cells were sorted in order to isolate the three distinct populations: uninfected (GFP-/mCherry-), productively infected (GFP+/mCherry- & GFP+/mCherry+) and latently infected (mCherry+/GFP-). After sorting the infected cells, each distinct population was placed back in culture in the presence of IL-2 and allowed to return to a resting state over the course of 11 days.

Expression of the activation markers, CD69 and CD25, was highest at day 4 when the primary CD4$^+$ T cells were infected with HIV Duo-Fluo I (Figure 3.3B). After sorting, each population of cells began to lose both CD69 and CD25 expression, but never fully returned to a resting state during the course of our experiments. The uninfected cell population contained the largest percentage of CD69 and CD25 double negative cells at 11 days post activation, suggesting that they were capable of returning to resting state more quickly than either of the infected cell populations. By 11 days post activation, the latently infected cell population contained less CD69 and CD25 double negative cells than the uninfected population, but more than the productively infected cell population, suggesting that latently infected cells are more easily capable of returning to a resting state than are productively infected cells.

In addition to the changes in expression of activation makers, the infection profiles also changed over the course of the 11 days that the cells were allowed to return to a resting state (Figure 3.3C). A small amount of uninfected cell population became productively infected, and an even smaller amount became latently infected,

which is probably reflective of pre-integration latency (85). More interestingly, the vast majority of the productively infected cell population continued to express GFP throughout the 11 days, but during this time two distinct GFP-positive populations began to form: one that remained GFP-high, and one that shifted towards GFP-low. These results indicate that the cells are shifting from a productive state to a latent state, but due to the high levels of cell death (<2% Live Gate, data not shown), we could not continue with the experiment to conclusively prove this. However, gating on the two distinct populations reveals that the GFP-low population contained more CD69/CD25-double negative cells than the GFP-high population, indicating that the GFP-low population is closer to a resting state (Supplementary Figure 3.2A).

The latently infected cell population also shifted as the cells returned to a resting state. Over the course of the 11 days, latently infected cells began to lose mCherry expression, with more than half of the cell population no longer expressing mCherry by the end of the experiment (Figure 3.3C). Analysis of the expression of activation makers for these cells reveals that latently infected cells that lost mCherry expression also had higher levels of CD69/CD25 double-negative cells than latently infected cells that continued to express mCherry (Supplementary Figure 3.2B), suggesting that they are more quickly returning to a resting state.

After allowing the sorted cell populations to return to a resting state for 11 days, we stimulated each population with αCD3/αCD28 activating beads in order to reactivate any latent provirus (Figure 3.3D). Stimulation of the uninfected cell population produced a small amount of reactivatable provirus, but, again, this is likely a result of pre-integration latency. More intriguing, stimulation of the productively infected cell

population led to a shift of GFP-low cells back to GFP-high cells, further suggesting that the GFP-low population was returning to a resting state. Lastly, stimulation of the isolated latently infected cell population produced only a small amount of reactivatable provirus (~2%). However, there seemed to be a large shift from latently infected cells that no longer expressed mCherry to latently infected cells that do express mCherry, as there was a 20% increase in mCherry+ cells after CD3/CD28 stimulation (Figure 3.3D). These results suggest that latently infected cells that lose mCherry expression over time are not dying but instead returning to a resting state.

Primary CD4+ T cells within lymphoid tissue, ex vivo, are biased toward latent HIV-1 infection, but can also support productive infection.

Lastly, based on evidence that resting CD4+ T cells within lymphoid tissue can support HIV-1 replication (91-96), we wanted to investigate the establishment of latency within lymphoid tissue, ex vivo, using our HIV Duo-Fluo I virus. For this purpose, we isolated CD4+ T cells by negative selection from tonsillar and splenic tissues, as well as from peripheral blood from uninfected donors. In addition, we wanted to preserve the biology of the human lymphoid tissue, so we isolated total lymphoid cells from tonsillar and splenic tissues from uninfected donors in the form of human lymphoid aggregate cultures (HLACs) (93). We also isolated total peripheral blood mononuclear cells (PBMCs) from uninfected donors. CD4+ T cells, PBMCs and HLACs were either left untreated or stimulated with αCD3/αCD28 activating beads in the presence of IL-2 for three days and then spinoculated with HIV Duo-Fluo I for 2hrs at 37°C. Expression of the activation markers, CD69 and CD25 were checked before and after stimulation

(Figure 3.4A). CD4$^+$ T cells isolated from peripheral blood, as well as PBMCs expressed very little CD69 or CD25 and thus were considered to be resting cells. However, CD4$^+$ T cells isolated from tonsillar and splenic tissues, as well as HLACs from these tissues had high expression levels of the early activation marker, CD69 (38% and 42%, respectively), while expressing low levels of the intermediate activation marker, CD25. Thus CD4$^+$ T cells isolated from lymphoid tissue cannot be characterized as resting cells, but are also not classically activated. After stimulation with αCD3/αCD28 activating beads in the presence of IL-2 for three days, CD4$^+$ T cells from all three tissues expressed high levels of both activation markers leading to classic T cell activation. However, CD4$^+$ T cells isolated from peripheral blood achieved higher activation levels than those isolated from either lymphoid tissue. Lastly, expression of CD69 and CD25 among CD3/CD28 stimulated PBMCs and HLACs was consistently lower than that of purified CD4$^+$ T cells.

Levels of productive infection and latent infection were analyzed by flow cytometry 72hrs post-infection (Figure 3.4B). Untreated CD4$^+$ T cells isolated from peripheral blood, which expressed no activation markers, produced very little productive infection (0.47%). Despite expressing moderately high levels of CD69, untreated CD4$^+$ T cells isolated from tonsillar tissue did not give rise to significantly higher levels of productive infection (0.68%) compared to CD4$^+$ T cells isolated from peripheral blood. However, untreated CD4$^+$ T cells isolated from splenic tissue, which expressed CD69 at levels comparable to CD4$^+$ T cells isolated from tonsillar tissue, did show an increase in productive infection (1.8%). Levels of latent infection in untreated CD4$^+$ T cells isolated from both lymphoid tissues were at least 2-fold greater than that observed in untreated

CD4$^+$ T cells isolated from peripheral blood (Figure 3.4C), suggesting that CD4$^+$ T cells within lymphoid tissue are more likely to become latently infection.

Overall, untreated PBMCs and untreated HLACs from both lymphoid tissues displayed lower levels of both productive and latent infection than untreated CD4$^+$ T cells isolated from each tissue. Despite differences in activation levels, untreated PBMCs and untreated HLACs from both lymphoid tissues showed similar levels of productive infection, while levels of latent infection were similar in untreated PBMCs and HLACs from splenic tissue, with untreated HLACs from tonsillar tissue showed slightly higher levels than both. Compared to untreated CD4$^+$ T cells and total lymphoid cells from all three tissues, CD3/CD28-stimulated CD4$^+$ T cells, PBMCs and HLACs all showed significantly higher levels of productive infection, as expected.

When analyzing the ratio of latent infection to productive infection (Figure 3.4D), HIV Duo-Fluo I infection of all untreated cells from the three different tissues were at least 5-times more likely to result in latent infection compared to their CD3/CD28-stimulated counterparts, suggesting that activated cells exhibit a higher propensity for productive infection while resting cells exhibit a higher propensity for latent infection. Additionally, infection of untreated total lymphoid cell populations is more likely to result in latent infection compared with purified untreated CD4$^+$ T cells, suggesting that co-culture of CD4$^+$ T cells with other lymphoid cells biases the cells towards latent infection.

Discussion

The role of T cell activation in the establishment of HIV-1 latency within CD4$^+$ T cells is still not fully understood. It is clear that HIV-1 replication is most efficient in

activated CD4$^+$ T cells (27-30), and that the largest latent reservoir, *in vivo*, is found within memory CD4$^+$ T cells (22, 23).   Taken together, this evidence suggests that HIV-1 latency is established in one of two ways: 1) Activated CD4$^+$ T cells become productively infected but survive viral cytopathic effects while evading elimination by the immune system long enough for the cell to transition to a resting memory state; or 2) CD4$^+$ T cells transitioning from an activated state to a resting memory state are infected by HIV-1 while the cellular environment is still capable of supporting integration of the viral cDNA, but unable to support proviral transcription.   Data demonstrating that both naive and memory CD4$^+$ T cells contain integrated viral DNA (91), as well as the demonstration that direct infection of resting CD4$^+$ T cells in lymphoid tissue results in productive infection (97), suggests that HIV-1 latency could also be established by an alternative route: direct infection of resting CD4$^+$ T cells. In this study, we show that all three scenarios can give rise to latent HIV-1 infection. We further show that HIV-1 latency can also be established in activated CD4$^+$ T cells without the requirement of returning to a resting state. Additionally, infection of activated CD4$^+$ T cells is more likely to result in productive infection, while infection of resting CD4$^+$ T cells is more likely to result in latent infection. Finally, HIV-1 latency is more likely to occur in resting lymphoid cell aggregates as opposed to resting CD4$^+$ T cells cultured alone.

Using primary CD4$^+$ T cells isolated from uninfected donor blood, we demonstrate that infection of resting and activated CD4$^+$ T cells with our HIV Duo-Fluo I virus results in both productive and latent infection in the two populations. In activated CD4$^+$ T cells, HIV-1 latency is established within the first few days of infection and doesn't require the cell to return to a resting state. This has been shown previously by

us (85), as well as by another group that has developed a similar dual-reporter virus (74). This construct utilizes a different LTR-independent promoter (CMV) than our EF1α promoter, and places the LTR-driven eGFP cassette in the Gag region, while ours is in place of the Nef open reading frame. Despite these differences, both dual-reporter viruses are capable of detecting latent infection events in activated CD4$^+$ T cells early after initial infection, and these latently infected cells can be reactivated by different stimuli. Additionally, we have sorted these latently infected cells and shown that they still express significant amounts of both CD69 and CD25 activation markers, and only begin to lose expression of these markers as they are allowed to return to a resting state. As they return to a resting state, latently infected CD4$^+$ T cells also begin to lose expression of the EF1α-driven mCherry marker, suggesting that as these cells return to a resting state, both promoters become silenced, perhaps by packaging into heterochromatin (112). This raises the possibility that HIV-1 latency can be established after the initial infection of activated CD4$^+$ T cells, and it is, potentially, these cells that survive and return to a resting memory state, and are a major contributor to the latent pool. How HIV-1 latency is established in activated CD4$^+$ T cells immediately after infection is still unknown, but could be the result of stochastic viral gene expression (67, 113-115).

Our studies also suggest that activated CD4$^+$ T cells that become productively infected can also contribute to the latent pool as they return to a resting state. In our studies, these cells were not able to return to a completely resting state due to a lack of cell survival, but our data clearly indicates that there is a population of productively infected cells that are starting to return to a resting state, and as they do so, they are

losing expression of the LTR-driven GFP marker (GFP-low). Upon CD3/CD28

stimulation of these cells, the GFP-low population shifts back to GFP-high, indicating

that these cells are not losing GFP expression because they are dying, but because

they are returning to a resting state and in the process are shutting down expression

from the LTR promoter. Further studies are needed to definitively prove this, but current

technology makes it extremely difficult to culture primary CD4$^+$ T cells for extended

periods of time. Of interest is the combined application of using the HIV Duo-Fluo I

virus with an *in vitro* primary CD4$^+$ T cell latency model that is capable of sustaining

long-lived CD4$^+$ T cells after infection, such as the recently reported BCL-2 transduced

cell model developed by the Siliciano lab (48).

Lastly, infection of activated CD4$^+$ T cells leads to a higher percentage of

productively infected cells as compared to latently infected cells, while infection of

resting CD4$^+$ T cells leads to a higher percentage of latently infected cells. These data

are in agreement that HIV-1 replicates more efficiently in activated CD4$^+$ T cells, but

also underscores the notion that resting CD4$^+$ T cells can support HIV-1 infection, at

least up to the point of viral integration.

In resting primary CD4$^+$ T cells, we show that both productive and latent HIV-1

infection can be achieved, though at levels much lower than those seen in activated

CD4$^+$ T cells. The kinetics of infection in resting CD4$^+$ T cells seems to be slower than

in activated cells since peak infection wasn't reached until 6-days post-infection, while

activated cells reached peak infection at 3-days post-infection (data not shown). This is

in agreement with others findings (30, 36). Also in agreement with previous findings,

resting CD4$^+$ T cells could be made more permissive to HIV-1 infection by the

chemokine, CCL19, which has been reported to increase the ability of resting CD4$^+$ T cells to support latent infection (54). However, our data demonstrate that CCL19 also increases permissibility to productive infection, although its overall effect on resting CD4$^+$ T cells is an increase in latent infection. Interestingly, the cytokine, IL-7, which has previously been reported to increase permissibility of resting CD4$^+$ T cells to productive HIV-1 infection, also increases both productive and latent infection in resting CD4$^+$ T cells in our study. Lastly, infection of untreated resting CD4$^+$ T cells with a Vpx-containing virus resulted in a significant increase in productive infection while only modestly increase latent infection.

Interestingly, infection of resting CD4$^+$ T cells with our HIV Duo-Fluo I virus resulted in a significant amount of silent infection events where expression of both fluorescent proteins was silenced, leaving latently infected cells within our uninfected population. In fact, resting CD4$^+$ T cells contained more silent infection events within the isolated uninfected cell population than latently infected cells that could be identified via the mCherry fluorescent marker after the initial infection. This was true for all untreated and treated resting CD4$^+$ T cells, with the exception of CCL19-treated cells, and was highest in IL-7 treated cells and untreated resting CD4$^+$ T cells infected with a Vpx-containing virus. The reasons for this are uncertain. It's reported that within resting CD4$^+$ T cells, viral integration occurs in regions of the host genome that are unfavorable for viral gene expression (116), and studies also suggest that latently infected cells are more likely to contain provirus in or near heterochromatin (43, 117). Integration into such regions would not only be unfavorable for LTR-driven gene expression, but for EF1α-driven gene expression as well. The inability of SAMHD1 to inhibit HIV-1 reverse

transcription in the presence of Vpx allows the virus to bypass one of the major viral replicative blocks in resting CD4^{+} T cells, and therefore, may allow for integration of the viral cDNA to occur more readily in these unfavorable heterochromatic regions. IL-7, signaling through the JAK/STAT pathway (118), may work in a similar manner.

Lastly, previous studies have reported that resting CD4^{+} T cells can only be infected by HIV-1 in the context of total lymphoid cell aggregates (98). However, our results show that infection of untreated resting CD4^{+} T cells alone and untreated resting total lymphoid cells from peripheral blood and lymphoid tissue all produced both productive and latent populations. Although, it does appear that latent infection is more likely to occur in total resting lymphoid cell aggregates as opposed to resting CD4^{+} T cells alone. The reasons for this are still unclear, but recent studies have shown that co-culture of resting CD4^{+} T cells with myeloid dendritic cells (119), or co-culture of resting CD4^{+} T cells with endothelial cells (120) enhances HIV-1 latency, further proving that the lymphoid environment plays an important role in the establishment of HIV-1 latency within resting CD4^{+} T cells.

In conclusion, our studies show that HIV-1 infection can occur in both resting and activated CD4^{+} T cells, with infection of the former more likely to result in latent infection, and infection of the latter more likely to result in productive infection. Based on our data, we now have a better understanding of the contribution that each infected cell type contributes to the latent reservoir, and underscores the importance of considering both resting and activated CD4^{+} T cells when investigating the nature of HIV-1 latency.
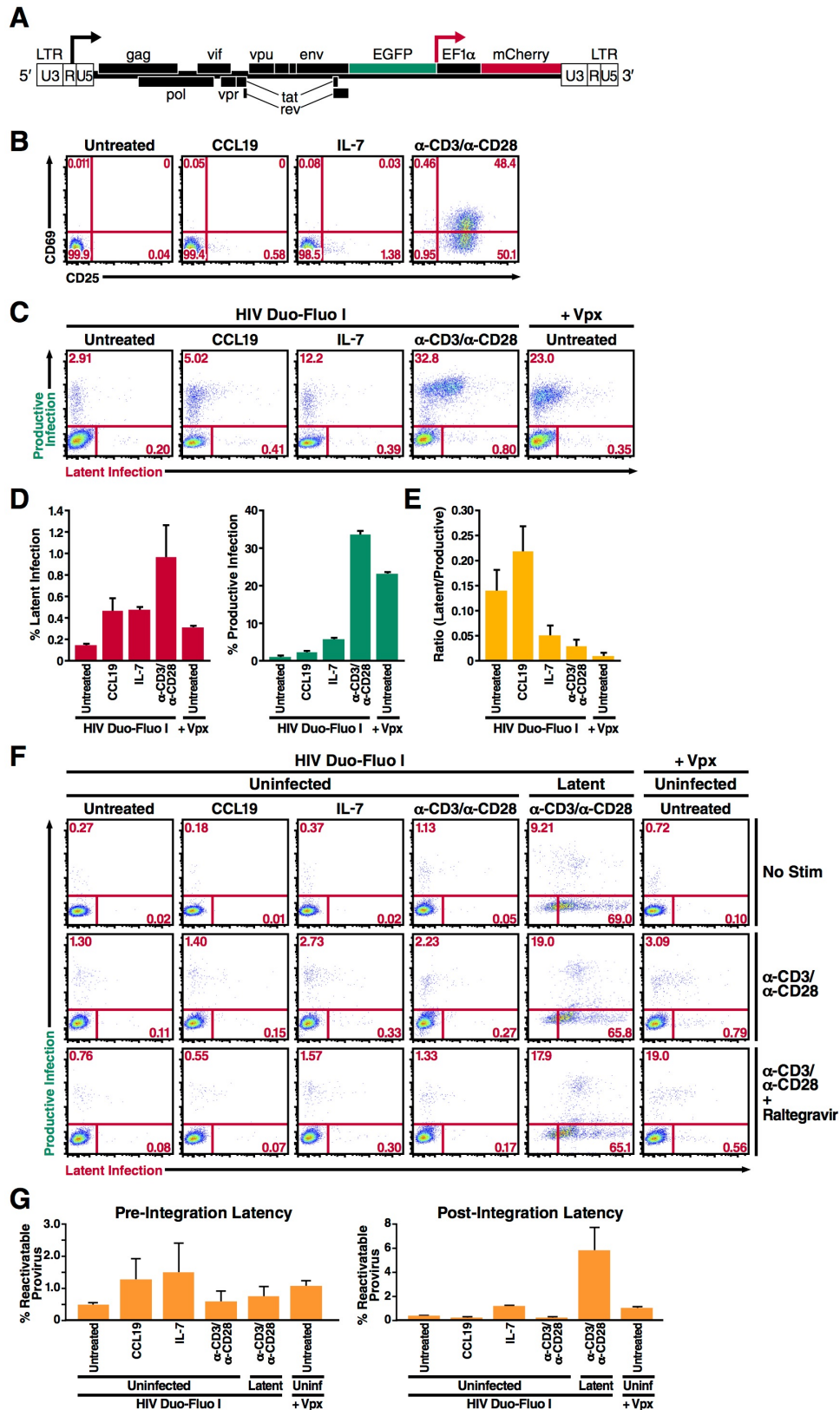
Acknowledgements

Figures

Figure 3.1. Resting primary CD4+ T cells support both productive and latent HIV-1 infection. (A) Diagram of the HIV Duo-Fluo I virus in which eGFP has replaced the nef gene, and a whole transcription unit, consisting of an EF1α promoter driving the expression of an mCherry fluorescent marker, has been inserted downstream. Upon infection with the HIV Duo-Fluo I virus, cells that express GFP alone or GFP and mCherry are considered productively infected; cells that express only mCherry are considered latently infected; and cells that don't express either fluorescent marker are considered uninfected. (B) Expression of activation markers, CD69 and CD25, in resting primary CD4+ T cells either left untreated or stimulated with CCL19, IL-7, or αCD3/αCD28 activating beads for 72hrs. (C) Infection profiles of untreated or stimulated primary CD4+ T cells six days post infection via flow cytometry. Untreated resting CD4+ T cells were infected with either HIV Duo-Fluo I virus alone or HIV Duo-Fluo I containing Vpx. Stimulated cells were infected with HIV Duo-Fluo I alone. Productive infection (GFP+ and GFP/mCherry double-positive) and latent infection (mCherry+) were analyzed by flow cytometry. Data shown is from a single donor, but is representative of three separate donors. (D) Quantification of latent infection and productive infection from panel B. Data represents the average of three donors. (E) Ratios of latent infection to productive infection were calculated using data from panel C. Data represents the average of three donors. (F) Reactivation of latent provirus within the isolated uninfected populations (GFP/mCherry double-negative) of untreated and stimulated primary CD4+ T cells via flow cytometry. Six days post-infection uninfected cells were isolated via FACS and were either left unstimulated or stimulated with αCD3/αCD28 activating beads alone or αCD3/αCD28 activating beads in the

presence of Raltegravir for 48hrs. Data shown is from a single donor, but is representative of three separate donors. (G) Quantification of reactivation of pre-integration latent virus and post-integration provirus calculated from panel E. Reactivatable pre-integration latent virus was calculated by subtracting the amount of productive infection from cells treated with αCD3/αCD28 activating beads alone and cells treated with αCD3/αCD28 activating beads in the presence of Raltegravir. Reactivatable post-integration latent provirus was calculated by subtracting the amount of productive infection from unstimulated cells and cells treated with αCD3/αCD28 activating beads in the presence of Raltegravir. Data represents the average of 3 donors.
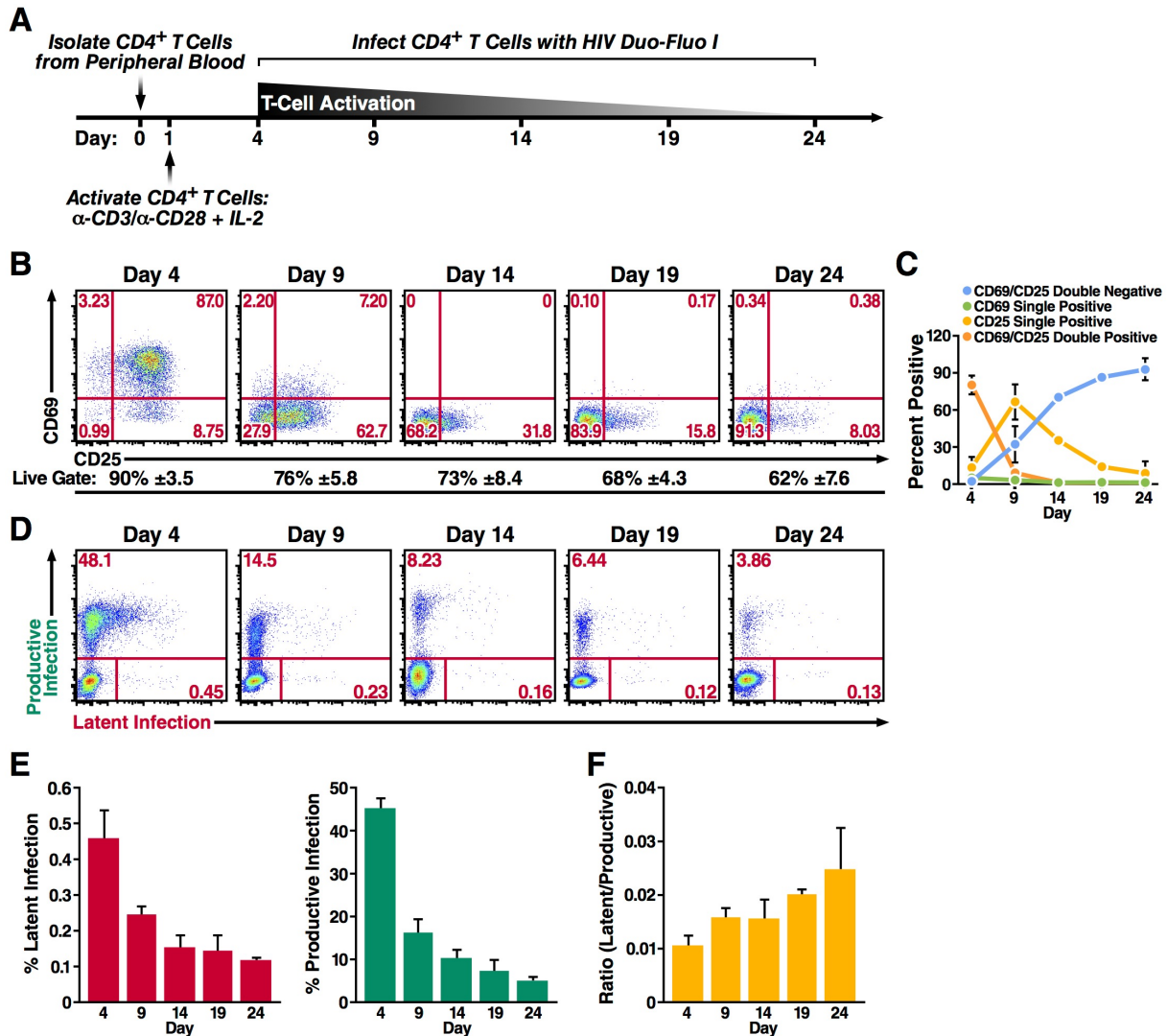
Figure 3.2. Primary CD4+ T cells transitioning from an activated state back to a resting state are more likely to become latently infected. (A) Schematic of experimental procedure. Primary CD4+ T cells were isolated from uninfected donor blood and stimulated with αCD3/αCD28 activating beads in the presence of IL-2 for 72hrs, and then allowed to return to a resting state over the course of 20 days in the presence of IL-2 alone. Cells were infected at peak activation (day 4) and every five days, thereafter, as they returned to a resting state. (B) Expression of activation

markers, CD69 and CD25, as the cells transition from an activated state to a resting state. Flow cytometry was performed 72hrs post activation and every five days following the removal of the activation beads. Data shown is from a single donor, but representative of three separate donors. Percentage of live cells is calculated from the live gate in the FACS analysis and represents the average of three donors. (C) Quantification of the activation status of the cells from panel B. Data represents the average of three donors. (D) Infection profiles for primary CD4$^+$ T cells as they transition back to a resting state. Cells were spinoculated with HIV Duo-Fluo I 72hrs post activation and every five days following the removal of the activation beads. Infection was analyzed by flow cytometry 72hrs post-infection. Data shown is from a single donor, but representative of three separate donors. (E) Quantification of latent infection and productive infection from panel D. Data represents the average of three donors. (F) Ratios of latent infection to productive infection were calculated using data from panel E. Data represents the average of three donors.
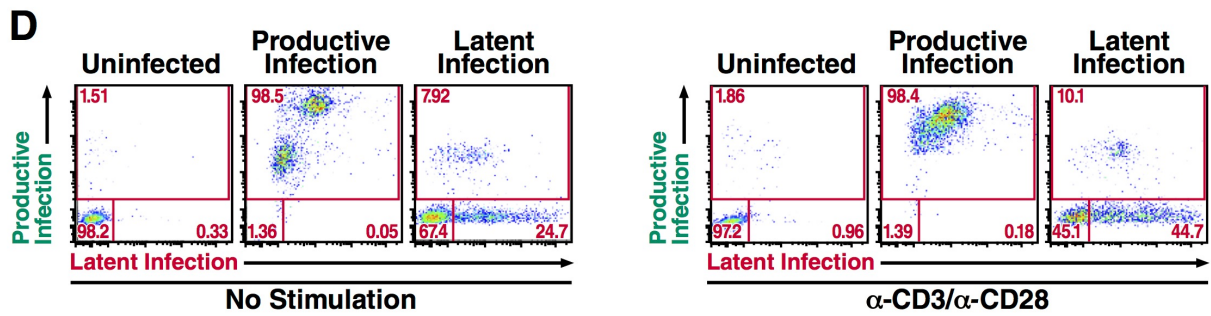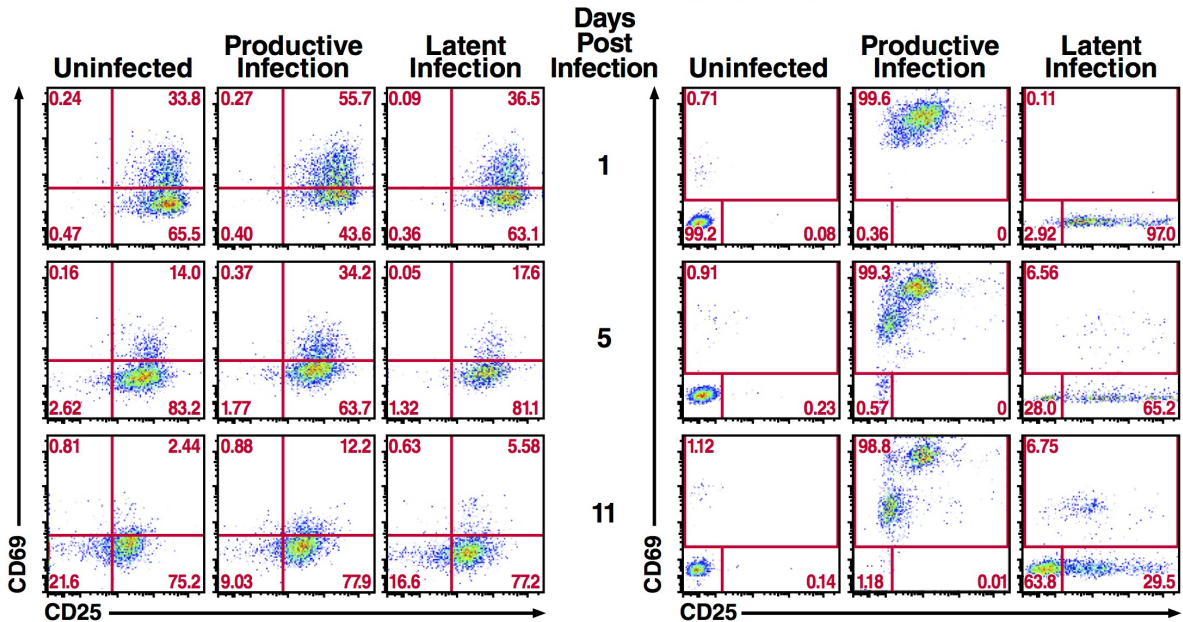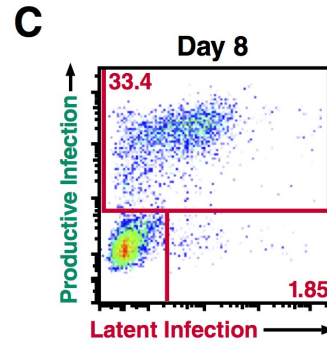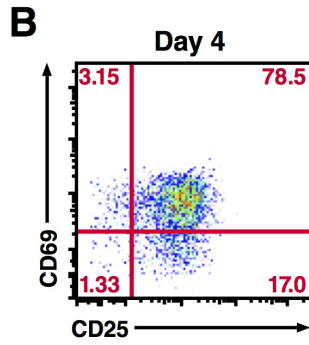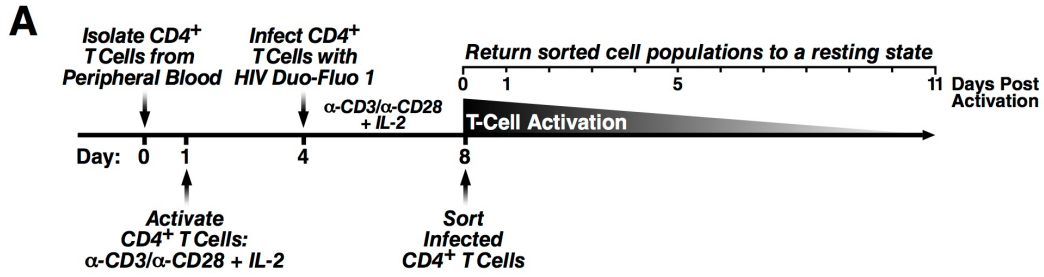
Figure 3.3. Productively infected and latently infected primary CD4⁺ T cells isolated by FACS return to a resting state. (A) Schematic of experimental procedure. Primary CD4⁺ T cells were isolated from uninfected donor blood and stimulated with αCD3/αCD28 activating beads in the presence of IL-2 for 72hrs, and then infected with HIV Duo-Fluo I virus. Productive, latent, and uninfected cell populations were isolated via FACS 4 days post-infection, and allowed to return to a resting state over the course of 11 days in the presence of IL-2. (B) Expression of activation markers, CD69 and CD25, of each isolated population as they return to a resting state. (C) Infection profiles of each isolated population as they return to a resting state as analyzed by flow cytometry. (D) Expression of CD69 and CD25 of specific cell populations from productively infected cells and latently infected cells 11 days post-sorting in panel C. Specific cell populations were gated on and analyzed for CD69 and CD25 expression as in panel B. (E) Reactivation of isolated cell populations after returning to a resting state. Cells were split in half and either left unstimulated or stimulated with αCD3/αCD28 activating beads for 48hrs. All data shown is from a single donor, but representative of three separate donors.

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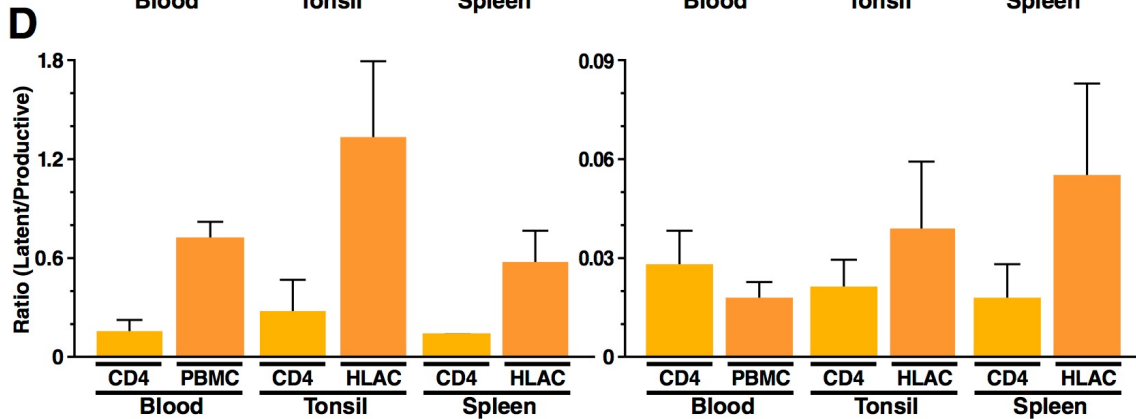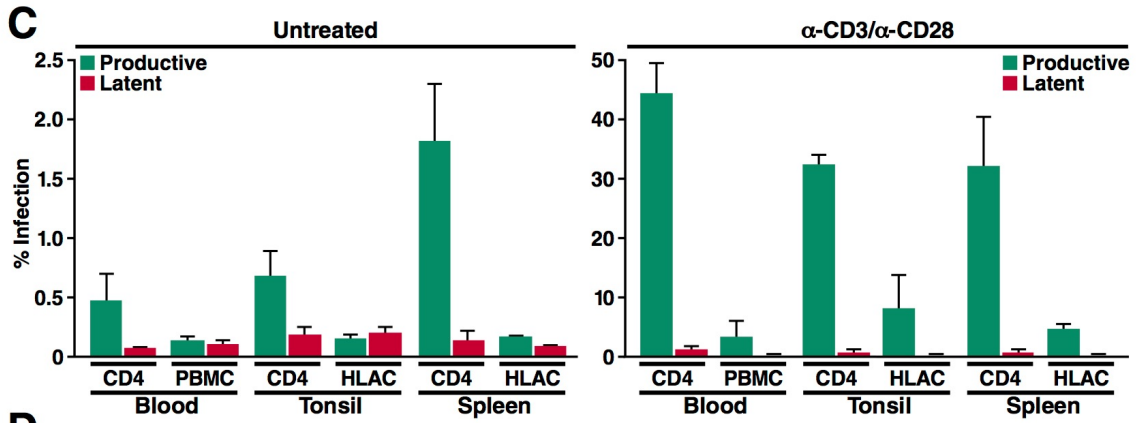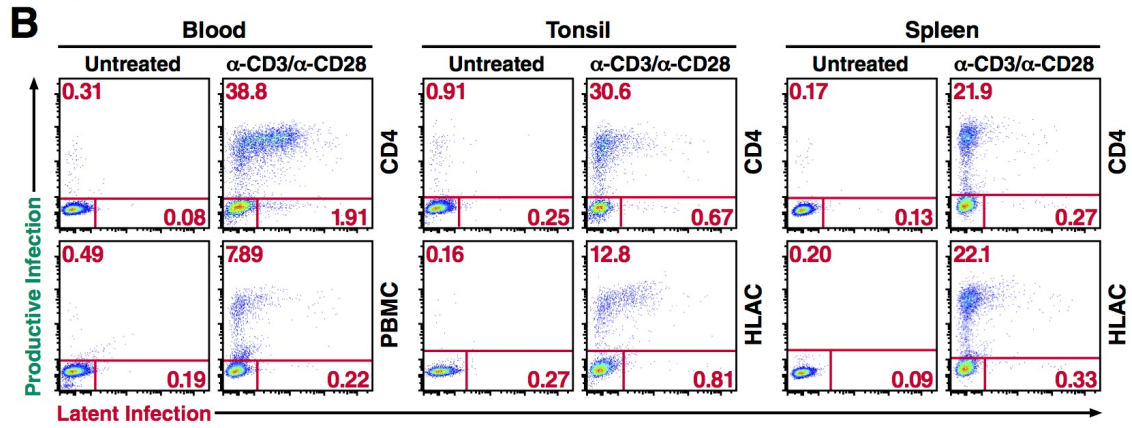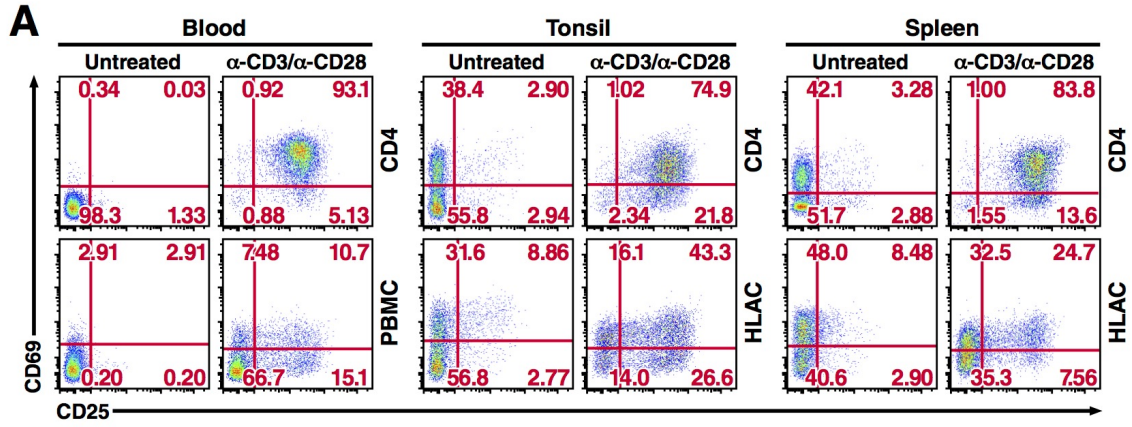
Figure 3.4. Primary CD4$^+$ T cells and total lymphoid cell populations isolated from peripheral blood and tonsillar and splenic tissues are more likely to become latently infected. (A) Expression of activation markers, CD69 and CD25, of CD4$^+$ T cells and total lymphoid cell populations either left untreated or stimulated with αCD3/αCD28 activating beads for 72hrs. Data shown is from a single donor, but representative of three separate donors. (B) Infection profiles of CD4$^+$ T cells and total lymphoid cell populations from panel A. Cells were infected with HIV Duo-Fluo I and analyzed for productive and latent infection 72 hours post-infection. Data shown is from a single donor, but representative of three separate donors. (C) Quantification of latent infection and productive infection from panel B. Data represents the average of three donors. (D) Ratios of latent infection to productive infection were calculated using data from panel E. Data represents the average of three donors.

CHAPTER 4: Conclusions and future directions

Summary

The goal of this thesis work was to gain a better understanding of the mechanisms that lead to the establishment of HIV-1 latency. However, studying HIV-1 latency presents many problems. Specifically, latently infected cells are rare, *in vivo*, with only 1 in 1 million cells harboring latent provirus; and latently infected cells have no known identifiable markers that allow us to distinguish them from uninfected cells. Because of this, it has been extremely difficult to study how HIV-1 latency is established. Current *in vitro* models of HIV-1 latency are elaborate and tedious, with readouts that occur at least one week after the initial infection, which only allows for the quantification of reactivatable provirus after latency has already been established. Thus, all previous studies of HIV-1 latency have only examined the mechanisms that maintain the provirus in a latent state, and not the mechanisms of how latency can be established. In order for the study of the establishment of HIV-1 latency to occur, a model is needed that allows for the identification of latently infected cells immediately after infection without the need to reactivate them. Such a system would allow us to determine the time-line of when latency is established after infection, and how latency is established.

In chapter 2, we present two new HIV dual-reporter constructs that allow for the identification and isolation of latently infected, productively infected, and uninfected cells, immediately after infection. This is made possible by engineering the viruses to express one fluorescent marker only when the HIV-1 LTR promoter is active, and one

fluorescent marker after integration of the viral DNA has occurred, regardless of viral promoter activity. Cells that express only the fluorescent marker of integration are considered to be latently infected. We show, using both jurkat cells and primary CD4+ T cells from healthy donors, that the isolated latent cell population contains integrated viral DNA, but does not express any viral mRNA transcripts or viral proteins. We further show that the latent population can be reactivated, thus proving that these cells are indeed latently infected, and that our HIV dual-reporter viruses work as expected.

In chapter 3, we use one of the HIV dual-reporter viruses, HIV Duo-Fluo I, to investigate the establishment of HIV-1 latency with regards to T cell activation. Importantly, we begin to test the theories of how HIV-1 latency is established. Currently, the most widely accepted theory is that HIV-1 infects activated CD4+ T cells, and then some of these infected activated cells survive the cytopathic effects of infection long enough for the cell to transition to a resting memory state, and in the process shut down the HIV-1 LTR promoter, resulting in a latently infected cell. This theory is based primarily on evidence that the major latent reservoir, *in vivo*, resides in resting memory CD4+ T cells, and that only activated CD4+ T cells can support HIV-1 replication. However, there is evidence that suggests that naive resting CD4+ T cells can support both latent infection and productive infection without the need to be activated first. Using HIV Duo-Fluo I, we show that both scenarios are possible, but that latency is more likely to occur through the infection of resting CD4+ T cells as opposed to activated CD4+ T cells. We further show that activated CD4+ T cells don't need to return to a resting state in order for latency to be established. Instead, activated CD4+ T cells can

support latency immediately after infection. This finding, alone, has major implications on how we study HIV-1 latency, and requires further investigation.

Establishment of HIV-1 latency in activated CD4$^+$ T cells

One of the more interesting discoveries from our research is that activated CD4$^+$ T cells can support latent infection without the need to return to a resting state. Our findings are supported by those from another group who simultaneously created a HIV dual-reporter virus (74). Their virus differs from ours in the placement of the fluorescent markers, but the results are similar. Understanding the mechanism of how HIV-1 latency is established in activated CD4$^+$ T cells could have immense implications on field of HIV-1 latency.

Currently, it is believed that HIV-1 replication is dependent on T cell activation (activated CD4$^+$ T cells support replication/resting CD4$^+$ T cells don't support replication) (27). Because of this, it is also believed that HIV-1 latency arises as a mere consequence of the natural physiology of CD4$^+$ T cells: Resting naive CD4$^+$ T cells become activated in response to an antigen; activated CD4$^+$ T cells become infected with HIV-1; and infected activated CD4$^+$ T cells return to a resting memory state, and in the process, shut down the HIV-1 LTR promoter, inducing a latent state of infection that can be reversed by reactivation of the infected cell. However, our data argue against this model of latency establishment. Using our HIV dual-reporter virus, we show that resting CD4$^+$ T cells can support productive infection, and that activated CD4$^+$ T cells can support latent infection. This breaks down the argument that HIV-1 replication is entirely dependent on T cell activation. And, if HIV-1 replication is not entirely

dependent on T cell activation, then HIV-1 latency may not be just a mere consequence of the CD4$^+$ T cell physiology. This suggests that other mechanisms exist for the establishment of HIV-1 latency.

Perhaps, the establishment of HIV-1 latency is stochastic. The spatial organization of proteins within any given cell is unique and affects the complex T cell signaling cascade, resulting in dynamic and random gene expression amongst individual cells (121). Recent studies suggest that these stochastic fluctuations may also determine viral gene expression of HIV-1 proviruses (67, 113-115). However, it's also possible that host restriction factors that act after viral integration has occurred could induce a state of latency. Recent studies have suggested that certain tripartite motif proteins (TRIMs), such as TRIM22 (122) and TRIM28 (123) can inhibit HIV-1 transcription. The TRIM22 studies were performed in 293T cells and U937 cells, so further work within primary CD4$^+$ T cells is required. Also, no mechanism has been proposed for TRIM22s effect. TRIM28 is thought to inhibit HIV-1 transcription by being recruited to the LTR promoter and initiating heterochromatin formation. However, in another study TRIM28 was shown to inhibit HIV-1 integration (124). Whether TRIM28 is an inhibitor of both stages of the viral life cycle requires further investigation, as does the link between TRIM28 inhibition of HIV-1 transcription and its link to the establishment of HIV-1 latency.

Identification of HIV-1 latency markers

One of the major obstacles that HIV-1 latency researchers face is the inability to distinguish latently infected cells from uninfected cells, *in vivo*. This makes it impossible

to isolate and examine a pure population of latently infected cells from patients. Current

techniques involve isolating resting memory CD4^{+} T cells from patients and then

performing PCR (20) or outgrowth assays (22) to assess whether any latently infected

cells reside in the sampled population. Needless to say, these assays are

cumbersome, with results only telling us that some of the cells harbor latent virus. In

addition, not being able to identify latently infected cells hampers our ability to identify

latent reservoirs, *in vivo*. Currently, memory CD4^{+} T cells are the most notable latent

reservoir (22, 23), but other cell types could harbor latent provirus, and there could be

important anatomical compartments that also harbor latently infected cells. For

instance, it's possible that macrophages could harbor latent provirus. *In vivo* studies

suggest that macrophages are more resistant to viral cytopathic effects of HIV-1

infection than are CD4^{+} T cells (125, 126), suggesting that they could survive and

harbor latent provirus. It's also anticipated that the gut-associated lymphoid tissue

(GALT) (127-129) and the central nervous system (130, 131) serve as two anatomical

compartments that are important to viral persistence. How these different cell types and

anatomical compartments contribute to HIV-1 latency could be better understood if there

existed a marker of latently infected cells.

Using our HIV dual-reporter virus, it may be possible to identify such a marker, or

at least an expression profile unique to latently infected cells. Our system allows, for

the first time, isolation of a pure population of latently infected cells that can be used for

downstream applications such as mass cytometry and RNAseq. Mass cytometry is an

improvement on flow cytometry in that it offers the detection of multiple probes that are

not limited by spectral overlap (132). Due to spectral overlap, flow cytometry only

allows for the detection of, at most, 17 probes, and thus the detection of 17 cell markers. Mass cytometry, using rare Earth metals as probes, allows for the capacity to measure 100 individual probes, thus allowing for the detection of 100 different cell markers. Though current technology only allows for the use of 35 probes, mass cytometry can be used to characterize a cell surface expression pattern of latently infected cells, and potentially, identify important markers that distinguish them from both productively infected cells and uninfected cells. In conjunction with mass cytometry, RNAseq (133), or whole transcriptome deep sequencing, can also be used with HIV Duo-Fluo I to identify individual genes, or whole signaling pathways that are dysregulated in latently infected cells by comparing gene expression profiles of uninfected, productively infected, and latently infected cells. Together, mass cytometry and RNAseq performed on a pure population of latently infected cells isolated after infection with HIV Duo-Fluo I would provide both a cell surface expression and gene expression pattern that could help us to identify latently infected cell, *in vivo*.

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Table 2.1

Position	Compound	Function	% React.	Receptor	Rec. class	II messenger
01-F11	AG 18	EGFR/ PDGFR-kinase inhibitor	17.10	EGFR	TyrK-R	MAPK, Akt, JNK, IP3, Ca++
01-G08	AG 99	EGFR-kinase inhibitor	14.22			
01-M12	(+)-Tyrphostin B44	EGFR-kinase inhibitor	11.21			
01-M04	AG 490	EGFR-kinase inhibitor; JAK2, JAK3 inhibitor	14.50			
01-B17	AG 555	Potent EGFR-kinase inhibitor	16.43			
01-A18	AG 494	Potent EGFR-kinase inhibitor	8.39			
02-L10	Genistein	EGFR kinase inhibitor; ER and PPARγ ligand	9.40			

Table 2.1 continued

Position	Compound	Function	% React.	Receptor	Rec. class	II messenger
02-I18	SB 202190	Potent, selective inhibitor of p38 MAPK	9.66	p38	Kinase	MAPK
02-F24	SB 203580 hydrochloride	Selective inhibitor of p38 MAPK	9.44			
02-C08	NBQX disodium salt	AMPA receptor blocker; Kainate rec. blocker	9.66	Glutamate	ion channel	Ca
02-D14	SIB 1893	mGlu5 antag; mGlu4 positive modulator	5.15		GPCR	DAG, IP3 Ca++
02-M12	LY 294002 hydrochloride	Selective PI3-kinase inhibitor	11.21	PI3K	Kinase	
02-M10	Quercetin	Non-selective PI3-kinase inhibitor	10.60			
02-H20	(-)-Terreic acid	BTK selective inhibitor	26.56	BTK	Kinase	
02-F17	Cyclopiazonic Acid	SERCA ATPase Inhibitor	28.78		ion channel	Ca
03-D04	(±)-Bay K 8644	Ca++-channel activator (L-type)	7.01	Ca	ion channel	Ca
03-D05	(S)-(-)-Bay K 8644		6.08			
03-D16	Linopirdine dihydrochloride	KCNQ channel blocker	7.37	K	ion channel	Ca

Table 2.1 continued

| Position	Compound	Function	% React.	Receptor	Rec. class	II messenger
02-A08	Piribedil dihydrochloride	Dopamine D3 receptor agonist	8.67	DOPA	GPCR	cAMP
02-C12	GR 55562 dihydrochloride	5-HT1B antagonist	8.47	5-HT	GPCR	cAMP
03-O08	Ritanserin	Potent 5-HT2 antagonist	6.58	5-HT	GPCR	DAG, IP3, Ca
03-H17	CI 898 trihydrochloride	Dihydrofolate reductase inhibitor	5.59	DHFR	Enzyme	
01-E09	Dilazep dihydrochloride	Adenosine uptake inhibitor	11.68	Pump	Pump	Adenosin
03-J02	SB 216763	Potent, selective GSK-3 inhibitor	9.79	GSK-3	Kinase	
03-C06	NSC 95397	Selective Cdc25 phosphatase inhhibitor	#100.68	cdc25	Phosphatase	
01-E15	2-(1-Thienyl)ethyl 3,4-dihydroxybenzylidenecyanoacetate	5-,12-,15-Lipoxygenase inhibitor	16.74	LOX	Enzyme	
02-J20	Resveratrol	Cyclooxygenase inhibitor, Sirt1 activator, Egr1	8.09		Enzyme, Kinase	
03-F03	Piceatannol	Syk tyrosine kinase inhibitor, Sirt1 activator	12.91		Enzyme, Kinase	

Supplementary table 2.1. Compounds from the Tocris screen drug library that reactivate latent HIV. Compounds with a total red area higher than the median + 2 SD for negative control wells are grouped by function. Position (plate-well), name, candidate function and pathways targeted by each compounds are indicated. % react. indicates the percent increase of the red signal over the median of negative controls, normalized to the most potent combination of reactivating drugs (SAHA 2.5µM + prostratin 0.5µM, 100%). #, artifact; see figure 3B, right. EGFR, epidermal growth factor rec. ; TyrK-R, Tyrosine kinase rec.; MAPK, Mitogen-activated protein kinase; ER, estrogen rec.; PPARγ, Peroxisome proliferator-activated rec. γ; PI3K, Phosphoinositide 3-kinase; GPCR, G protein-coupled receptor; 5-HT, 5-hydroxytryptamine; DAG, diacylglycerol; cAMP, Cyclic adenosine monophosphate; DHFR, Dihydrofolate reductase.

#	Name	Sequence
A	SLIC_mApple_Nef_F	GGAAAGGGCTTTGCTATAAGATGGTGAGCAAGGGCGA
	SLIC_mApple_Nef_R	TATATCTGGCCCGTACATCGTTACTTGTACAGCTCGTCCA
B	89.6_upNef_R	CTTATAGCAAAGCCCTTTCC
	89.6_downNef_F	CGATGTACGGGCCAGATATA
C	SLIC_R7_R	GCTTTACTTGTACAGCTCGTCCATGC
	SLIC_R7_F	CTCATGAGCTGTAGATCTTAGCCACTT
D	SLIC_EF1mCh_F	ACGAGCTGTACAAGTAAAGCGACCCTCGAGTACTAGGATCCATTAGG
	SLIC_EF1mCh_R	TAAGATCTACAGCTCATGAGAGCCATGGCTTCCCGCCGGCGGT
E	US RNA-gag-F	AAAAGAGACCATCAATGAGGAAGC
	US RNA-gag-R	TGGTGCAAT(c)AGGCCCTGC
	US RNA-gag-Probe	CAGAATGGGATAGAT(g)TGCATCCAGTGCA
F	MS RNA-total-F	ACAGTCAGACTCATCAAGC(t)TTCTC
	MS RNA-total-R	CTGTCGGGTCCCCTCG
	MS RNA-total-Probe	TTCTCTATCAAAGCAACCCA(t)CCTCCCA
G	SS RNA-env-F	CGGCGACTGGAAGAAGCGGA
	SS RNA-env-R	TACTATG(a)GACCACACAACTATTGC
	SS RNA-env-Probe	CGACGAAGAGCTCATCAGAACAGTCAGACTC

**Supplementary table 2.2. Primer table**
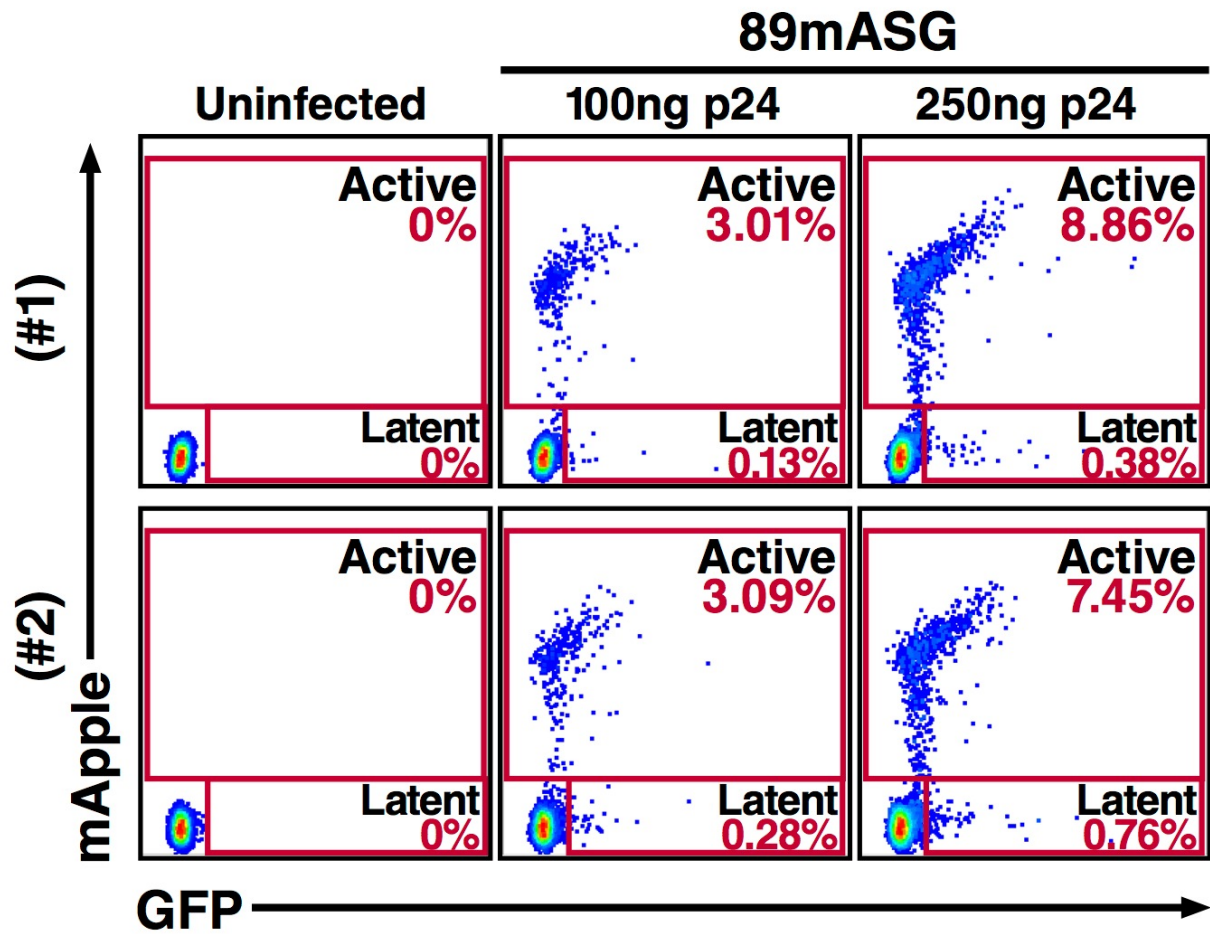
Supplementary Figure 2.1. Primary T cell infection with 89mASG virus. FACS

analysis of activated CD4$^+$ T cells from two human donors (#1, #2) infected with

89mASG virus at the indicated amounts.
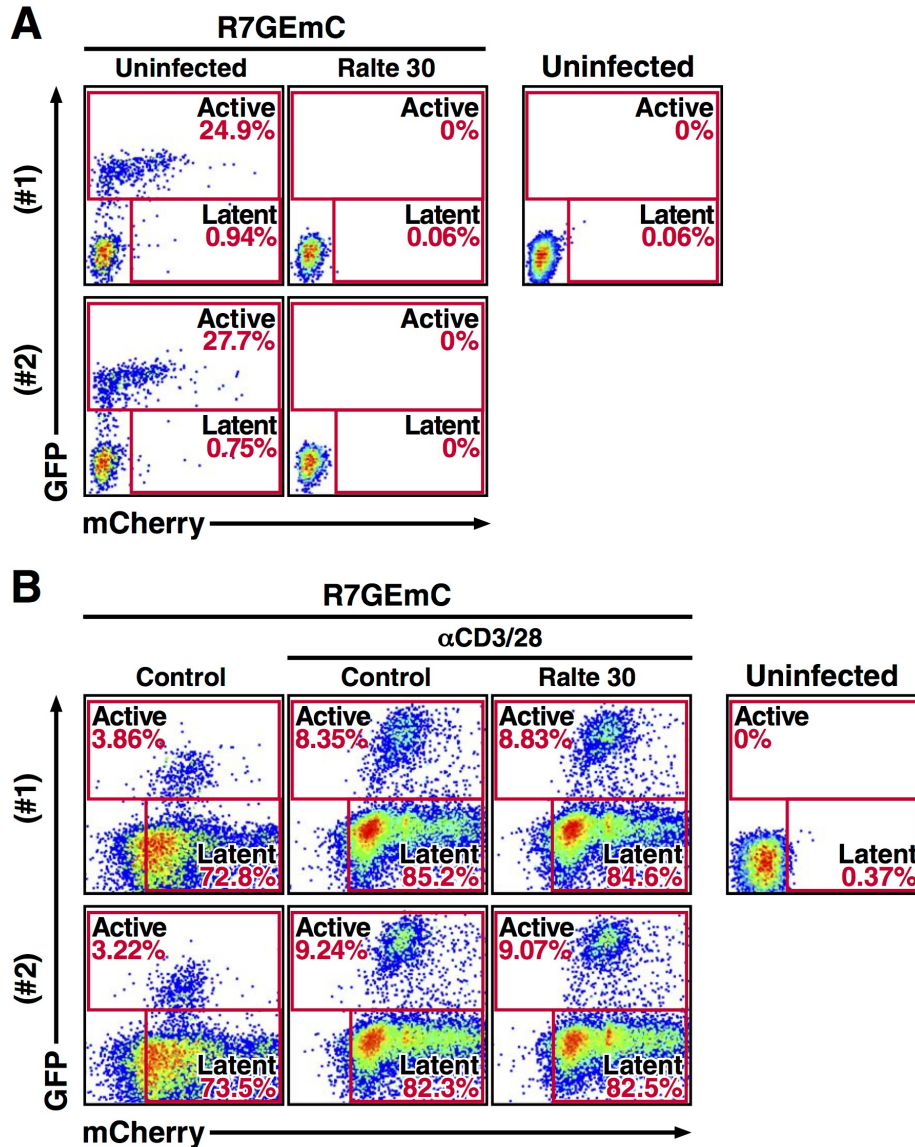
Supplementary Figure 2.2. Primary T cell infection and reactivation with integrase inhibitor. (a) FACS analysis of activated CD4$^+$ T cells from two human donors (#1, #2) infected with R7GEMC alone or with 30 μM Raltegravir (RALTE 30). (b) Analysis of sorted CD4$^+$ T cell populations from donors 1 and 2, after 48 h with anti-CD3/CD28-coated Dynabeads (αCD3/28), alone, with 30 μM Raltegravir, or untreated (CTR).
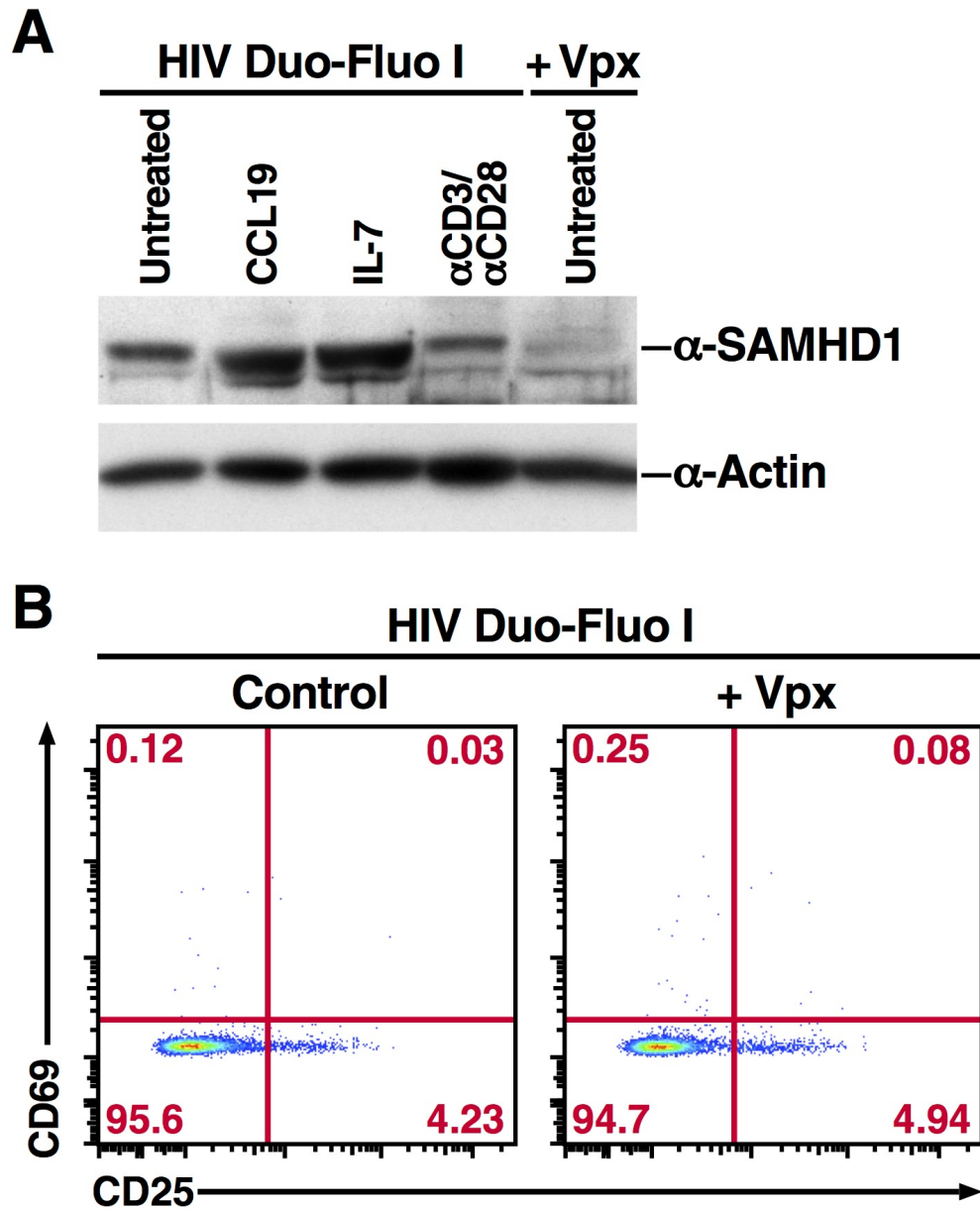
Supplementary Figure 3.1. Infection of primary CD4+ T cells with HIV Duo-Fluo I containing Vpx leads to SAMHD1 degradation, and has no effect on T cell activation. (A) Protein expression levels of SAMHD1 in resting primary CD4+ T cells infected with either HIV Duo-Fluo I alone or HIV Duo-Fluo I containing Vpx six days post

infection. (B) Expression of activation markers, CD69 and CD25, in untreated resting primary CD4+ T cells infected with either HIV Duo-Fluo I alone or HIV Duo-Fluo I containing Vpx 6 days post infection. Data shown is from a single donor, but is representative of three separate donors.
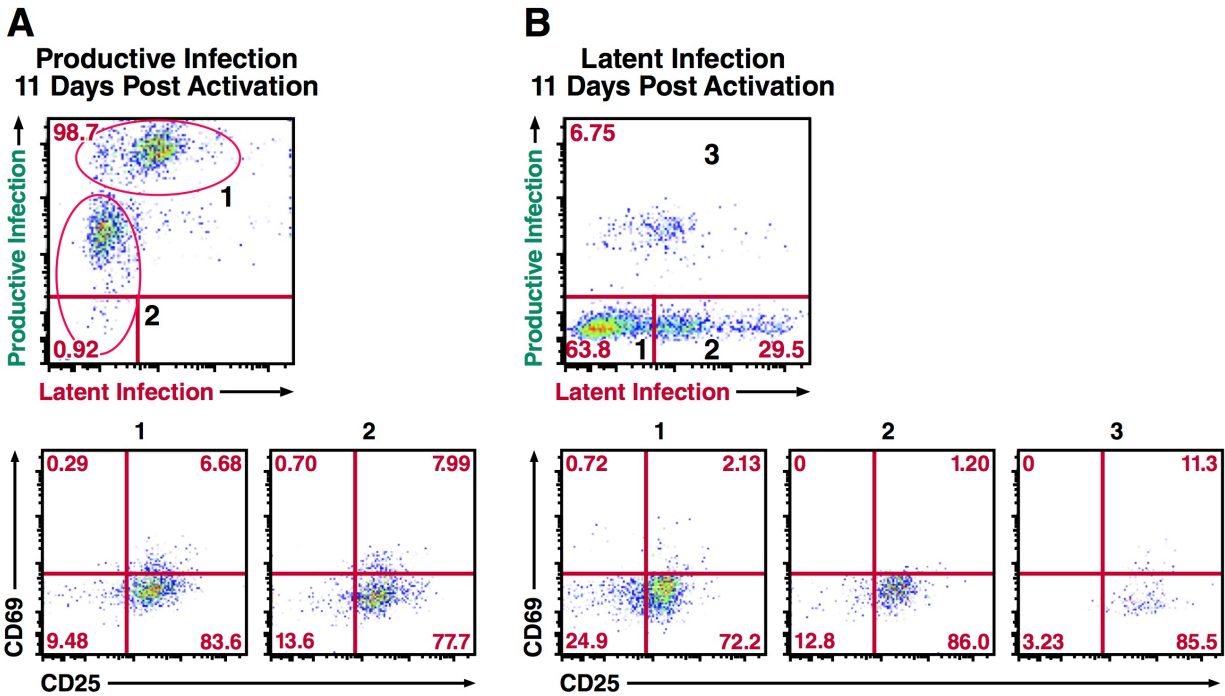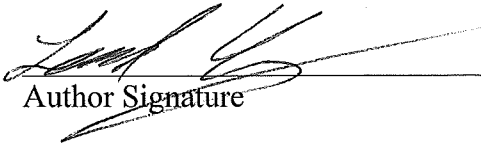

Supplementary Figure 3.2. Productively infected and latently infected primary CD4+ T cells that lose expression of their fluorescent marker are are more likely to exhibit a resting phenotype. (A) Expression of activation markers, CD69 and CD25, within GFP-high (1) and GFP-low (2) cell populations from productively infected primary CD4+ T cells 11 days post activation (Figure 3.3C). (B) Expression of activation markers, CD69 and CD25, within GFP/mCherry double negative (1), mCherry single positive (2) and GFP/mCherry double positive (3) cells from latently infected primary CD4+ T cells 11 days post activation (Figure 3.3C).

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