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Characterization of Cellular and Viral Factors that Define the Stability of the Latent HIV-1 Reservoir

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CHARACTERIZATION OF CELLULAR AND VIRAL FACTORS THAT DEFINE THE STABILITY OF THE LATENT HIV-1 RESERVOIR.

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ABSTRACT

Human immunodeficiency virus (HIV) infection is a chronic viral illness that requires lifelong management due to the early formation of a latent viral reservoir within the patient's CD4+ T cells. These latent infection events lay dormant within infected cells and reactivate stochastically or during periods of T cell activation presenting a major hurdle to the development of an HIV cure. As our understanding of the factors that contribute to HIV latency has evolved, we have started to appreciate latency as more than a chromatin regulation phenotype, due to the vast number of cellular factors that have been described to impact the establishment of latent infection. Additionally, the heterogeneity of described molecular changes in latently infected cells has further complicated analysis of HIV latency. Therefore, it is important to develop an understanding of the interactions of host cell and virus that ultimately lead to the establishment of a latent infection event. To this end, we characterized the host cell phenotype during latency, and the impact of viral proteins on the establishment of latent infections. Using this approach, we found that responsiveness of latent HIV infections to activating stimuli was directly linked to transcriptomic and proteomic changes within the infected cell, and that modulation of these changes could alter responsiveness of latent

HIV proviruses to T cell activating stimuli. Additionally, we found that HIV Nef was able to modulate the establishment of latent infection, in a lineage dependent fashion, with HIV-1 Nef promoting active infection and HIV-2 Nef promoting latency establishment. Together these findings support the concept that HIV latency is established and maintained through alterations to the host cell that may occur during the initial infection event, although further work must be done to link these mechanisms. Therefore, it will be critical for future HIV cure strategies to account for alterations to the host cell phenotype during attempts to reverse HIV latency or enhance viral silencing. This additional step, modulation of the host cell phenotype, will be necessary to address the HIV latent reservoir and presents an exciting new direction in HIV cure research.

DEDICATION

My Family

Cris, Bonnie and Benjamin Carlin

For their support and encouragement

&

Dr. Olaf Kutsch, Dr. Alexandra Duverger, and Frederic Wagner

Whose support made this possible

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INTRODUCTION

1.1 HIV Virology

Human Immunodeficiency Virus (HIV) is a positive strand RNA virus from the *Retroviridae* family of the *lentivirus* genus (1). Two of the major pathogens of this family, HIV-1 and HIV-2, emerged from distinct zoonotic transfer events (1) and were identified in the 1980s by F. Barré-Sinoussi and L. Montagnier or F. Calvel and L. Montagnier, respectively (2, 3). Despite being closely related, these two pathogens have significantly different disease outcomes. HIV-1 has become a global epidemic contributing to the development of acquired immunodeficiency syndrome (AIDS) in millions of individuals across the globe (4), while HIV-2 has remained mostly limited to West African nations with a slower spread on the global scale (5) and a significantly slower disease progression to AIDS (6-8). Both viruses are roughly 10kB, with HIV-2 being slightly larger (Figure 1). These viruses primarily infect CD4+ T cells through interactions between the viral envelop protein (Env; gp41/gp120) and the cellular CD4 protein, using either the chemokine receptor CCR5 or CXCR4 as co-receptors (9). They can also infect other cell types with varying levels of productivity, including macrophages (10) and dendritic cells in the case of HIV-2 (11). If left untreated, HIV will deplete CD4+ helper T cell populations within the infected host leading to massive immunosuppression thereby increasing the risk of opportunistic infections or cancer (AIDS) and greatly reducing life expectancy. Currently, HIV is treated using

antiretroviral therapy (ART), a combination of inhibitors that target different viral proteins that are essential for viral replication, thus suppressing active viral infection (12),However, once an individual is infected with HIV-1 or HIV-2, a latent viral reservoir is established within T cells (13-21) or macrophages (22, 23) that persists for the lifetime of the patient (13, 20, 24). This latent reservoir prevents viral clearance by the host immune system and allows for viral rebound when therapeutic pressure (ART) is removed for even a short amount of time (15, 22, 25-27). Therapeutic elimination of this viral reservoir is an essential requirement for a cure for HIV-1 and HIV-2 infection. In spite of over a decade of studies into HIV latency, a therapeutically relevant latency reversal agent (LRA) has not been identified. Thus, a better understanding of the establishment and maintenance of HIV latency, is critical to the development of pharmacological HIV cure strategies.

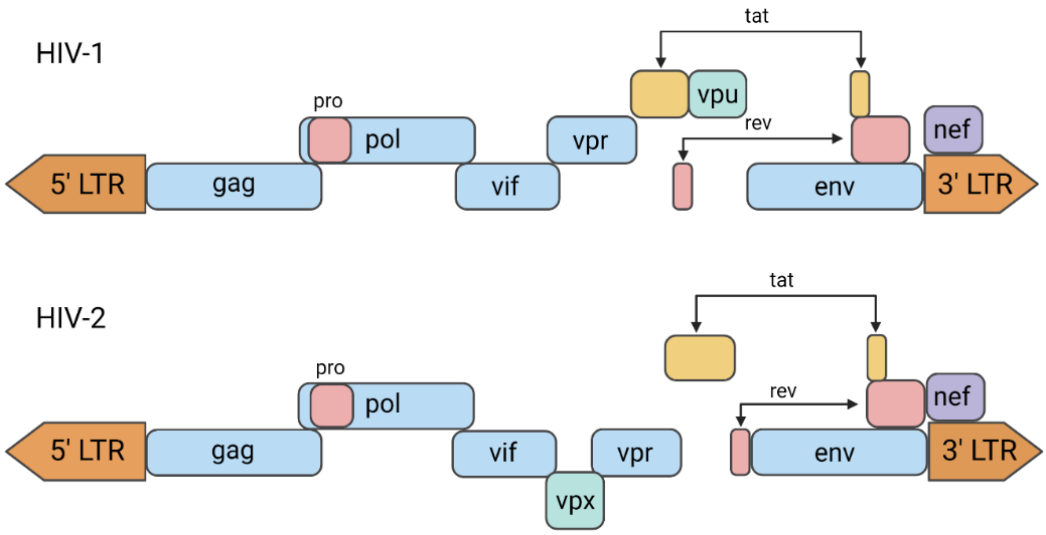

Figure 1. Genetic organization of HIV family genomes.

1.2 HIV Life Cycle

The HIV life cycle begins with the attachment of the virus to the target cell through nonspecific interactions between the envelope protein (Env) of HIV and charged moieties such as heparan sulfates or specific interactions between Env and integrin α4β7 (28, 29). After attachment, binding of the Env to the primary host cell receptor CD4 and one of the co-receptors, either CXCR4 or CCR5 (Figure 2.1), mediates fusion of the viral membrane to the host membrane allowing the viral core to be released into the cytoplasm (Figure 2.2) (30). Here the capsid proteins facilitate nuclear import of the viral core through interactions with host proteins, and once localized in the nucleus, positions the viral core near the future site of integration (31). At this point, the viral genome reverse transcribes the RNA genome into DNA and uncoats from the viral core (Figure 2.3) (32). The freshly synthesized DNA genome is then integrated into the host genome, hereafter the integrated viral genome is known as a provirus (Figure 2.4). Integration typically occurs in open chromatin regions and within actively expressed genes, but it has been described in a variety of other contexts, including closed regions of chromatin and rarely within intergenic regions. Once integrated, the viral life cycle has three possible transcriptional outcomes: permanent transcriptional silencing, silent but inducible integration also known as latent infection or HIV latency, and transcriptionally productive integration also known as active infection (13, 14, 20). Latent and transcriptionally silent events will be discussed in a later section. Once integrated, HIV is transcriptionally regulated by a lock and key system based on interactions between the viral protein Tat and a secondary RNA stemloop structure in the nascent RNA produced from the long terminal repeat (LTR) known as the TAR element (33, 34). This RNA stem-loop prevents elongation by

RNA polymerase II (Pol II) and is counteracted to some degree by the binding of transcription factors that allow Pol II to elongate the nascent viral transcript through the entire viral genome. While very inefficient, this allows Pol II to complete the viral transcript, leading to the production of viral proteins. As the viral protein Transactivator of Transcription (Tat) interacts with the TAR stem-loop in the nascent viral RNA, Tat recruits both CDK9 and CCNT1 (PTEFB complex) to induce phosphorylation of serine 6 on the C terminal domain of RNA Pol II (34, 35). This greatly increases the efficiency of elongation through the recruitment of additional elongation enhancing factors and the phosphorylation of SUPT5H and NELF, removing their suppressive effect on elongation (Figure 2.5) (34, 35). The full length genomic viral RNA is either shuttled out of the nucleus for packaging in progeny virions or spliced to allow for production of viral proteins. After translation, viral proteins assemble at the inside of the cell membrane where they recruit mature mRNA genomes into immature virions (Figure 2.7) (36). By usurping the activity of the host ESCRT (endosomal sorting complex required for transport) machinery, the still immature viral particles are released by the host cells through a process called budding (Figure 2.8). In this process, the virus acquires its external envelope from the host cell membrane, which bulges outwards and encloses the virion until it is released into the extracellular space. After budding, the viral protease begins cleavage of the Gag and Gag-Pro-Pol polyproteins at several sites to produce mature matrix (MA), capsid (CA), nucleocapsid (NC), and other accessory proteins. These processed proteins are then arranged into the mature infectious virion with the hallmark conical core (Figure 2.9) (36).

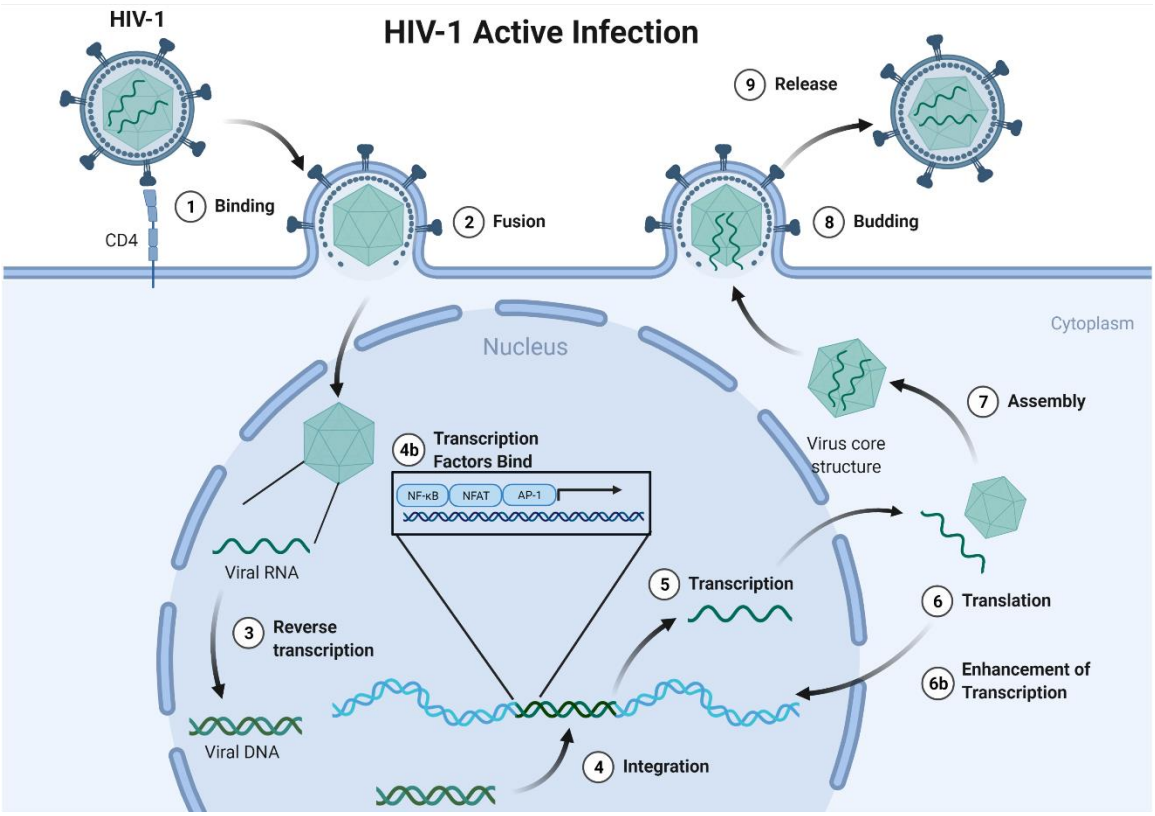

Figure 2. Life cycle of HIV infection.

1.3 HIV Inhibitors

Currently HIV treatment consists of suppression of active viral infection through the inhibition of multiple steps in the viral life cycle. These inhibitors are collectively referred to as Antiretroviral therapy (ART). ART consists of a cocktail of inhibitors targeting at least two steps of the viral life cycle to prevent the generation of resistance through mutation. Certain forms of ART are also currently used as a pre-exposure prophylaxis (PrEP) to protect individuals in high-risk groups from contracting HIV. The currently available inhibitors fall into one of the following categories.

Entry Inhibitors – (Entry inhibitors) block interactions necessary for viral binding and/or fusion. Broadly, they can disrupt interactions between the viral Env protein and CD4 or the coreceptors CCR5 and CXCR4 (37, 38), or they can act to prevent fusion of viral and cellular membranes. Approved medications include Enfuvirtide and Maraviroc.

Reverse Transcriptase Inhibitors (RTis) – RT inhibitors fall into two categories, (i) nucleoside reverse transcriptase inhibitors (NRTIs) and (ii) non-nucleoside reverse transcriptase inhibitors (NNRTIs). NRTI inhibitors mimic nucleosides but fail to allow for 3' processing causing a breakdown of reverse transcription (39, 40). NNRTIs bind to and inhibit the function of the reverse transcriptase through interactions with distal sites on the enzyme (40). Approved medications include Lamivudine, Tenofovir DF, and Emtricitabine.

Integrase Inhibitors – Also referred to as integrase strand transfer inhibitors (ISTIs), these block integration of the DNA provirus by preventing DNA strand transfer (41, 42). Approved medications include Raltegravir, and Dolutegravir.

Protease Inhibitors (PIs) – PIs block the activity of the viral protease (pol), preventing proper HIV protein processing and maturation of the virion (40, 43). Approved medications include Lopinavir, Nelfinavir and, Ritonavir.

Utilizing these inhibitors in combination has allowed for both viral suppression in HIV+ individuals and prevention of viral infection in PrEP adherent individuals. However, these treatments currently must be taken daily and require strict adherence although longer lasting therapeutics are in development. Even short therapy interruptions of 4-6 weeks allow for viral rebound in HIV+ individuals originating from the latent HIV

reservoir (25, 27). Thus, latent HIV infection events are the final barrier to the development of an HIV cure.

1.4 HIV Latency

Following integration into the host cell genome, HIV usually assumes a transcriptionally active state, but in rare instances, HIV can enter a state of transcriptional dormancy, in which no viral RNA is produced. This state is termed HIV latency. As the virus produces no viral proteins in this state, latently infected cells are undetected by ongoing immune surveillance, which is how the virus is thought to persist for years. In its latent state, the integrated virus can clonally expand through homeostatic host cell proliferation, generating a stable clonal population of latently infected cells (44, 45). Latent HIV infection events can reactivate as the resting host T cells become activated and cellular transcription factors such as NF-κB, NFAT, and AP-1 family members (discussed in section 1.2) among others, become available to drive HIV LTR transcriptional initiation (46). Once activated, these latent infection events can produce functional virus that can seed additional infection events, which necessitates the lifelong use of antiretroviral drugs (20). As the immune system's ability to clear latently HIV infected cells is insufficient, pharmacological intervention will be required. Given that all previous interventions have failed to produce even a relevant decline of the size of the latent viral reservoir, understanding the mechanism underlying the establishment and maintenance of HIV latency will be essential to develop better interventions.

It was originally thought that HIV latency occurred due to integration into restrictive chromatin environments, areas of tightly wound chromosomal DNA or heterochromatin that would block binding of transcription factors to the HIV LTR, thereby preventing

initiation of viral transcription (46). This finding was supported by initial characterizations of integration sites in latently infected immortalized cell lines (J-Lat cells) (46). Since, it has been shown that HIV preferentially integrates into euchromatin, regions of relaxed chromosomal DNA. As such, most integration events are found in introns of actively expressed genes. The same integration site preference is also found for latent HIV-1 integration events. While integration into heterochromatin occasionally occurs, it is rare and not a primary driver of latency establishment (47). The discovery that HIV preferentially integrates into actively expressed genes led to the development of two new hypotheses: the first hypothesis postulated that transcriptional interference between host cell transcription and viral transcription forced the virus into a latent state, and the second postulated that DNA methylation of the HIV promoter suppressed viral transcription. Transcriptional interference can occur when HIV is integrated into actively transcribed host genes as RNA Pol II reads through the host gene and collides with an RNA Pol II that is actively transcribing the HIV genome, thus preventing productive viral transcription. This interference can occur in either orientation of the proviral genome relative to the host gene, but it is more suppressive when the HIV genome is oriented in the opposite direction of the host gene due to the binding of transcription factors to the 5' LTR (46, 48). It was shown that while this process can compete with low levels of HIV transcription, tight binding of NF-κB during cellular activation can block read through of RNA Pol II from the host gene, allowing productive HIV transcription. The second mechanism, DNA methylation at the HIV promoter, has long been reported to contribute to the establishment and maintenance of transcriptional silencing and therefore HIV latency (49). These findings have been supported by reports that pharmacological

interventions with DNA methylation inhibitors such as 5-aza-2'-deoxycytidine (5-aza-CdR) can induce reactivation of latent HIV and synergize with other latency reversal agents (LRAs). Given the fundamental importance of DNA methylation for certain transcriptional regulation effects, this could provide a logical mechanistic explanation of HIV latency control. While these mechanisms certainly could contribute to the establishment of the HIV latent reservoir, there have been descriptions of HIV latency in the absence of these factors, such as the discovery of latent HIV events in intergenic regions, or the description of latent or silenced viruses without methylation events on the viral LTR (50). These findings support the idea that HIV latency has a multifaceted phenotype with several contributing factors.

While early theories on the biomolecular control of HIV latency were based mainly on genome level regulation, the field has come to appreciate that it is possible to maintain latency in the absence events that control viral promoter activity, but rather through alterations to the host cell proteome. This concept initially stemmed from the observation that latent HIV infection events occurred primarily in resting CD4+ central memory (T_{CM}) and transitional memory (T_{TM}) T cells (13, 51, 52). It was thought that this subset of T cells was predisposed to establishing latent infections due to reduced expression of important HIV transcription factors such as NF-κB, NFAT, and AP-1, which allowed negative elongation factors (NELFs) to prevent efficient elongation of the viral genome. This idea was supported by work from the Siliciano group that showed cells transitioning into a resting state are predisposed to establishing latent infections when compared to cells that are activated or cells in a resting state (51). While this established differentiation state is an important component of latency establishment, latent HIV-1

infections have been found in almost every cell state in the T cell lineage, including naïve cells (53, 54). These findings that HIV latent infections can occur in diverse cellular environments suggests that these different cellular contexts play a role in latency establishment. Additionally, it has been recently appreciated that the strength and type of T cell activation (Toll-Like Receptor, TCR signaling, or TNF-a) contributes both to the initial transcriptional outcome of infection and the stability of established latent infections (55). This is likely explained by differential regulation of the activation and modification of transcription factors by these signaling pathways, indicating that the host cell environment during the initial infection event is critical to the downstream transcriptional outcome of the integrated provirus.

To further add to the complicated nature of latent infections, it has been shown that the infection event itself can influence the host cell phenotype to alter establishment and stability of latent HIV infections. HIV has been shown to greatly alter the host cell environment during infection through the activity of viral proteins such as transactivator of transcription (Tat), viral protein R (Vpr), and negative regulatory factor (Nef). As these proteins are packaged within the viral particle, they may already affect infected cells prior to *de novo* protein production and induce biomolecular changes that could alter latency establishment in previously undescribed ways. There is no current research on how these viral proteins may function at the site of integration when the viral core uncoats (31, 32). Of those, the most likely viral proteins to influence latency establishment are Tat, Vpr, and Nef (described below).

HIV Tat- Due to Tat functioning as a transcriptional activator, attenuation of Tat function causes a larger portion of infection events to become latent. However, while viruses with

impaired Tat functionality are technically latent infection events, they would largely be considered defective proviral integration events and, upon reactivation, would not be able to drive efficient viral replication (56). Tat has also been reported to interact with over 180 nuclear proteins. Among these are several acetyltransferases and cellular kinases that alter the nuclear landscape during infection (57). Tat is important for acetylation of NF-κB and the degradation of Iκb-α supporting active HIV transcription (57, 58). Extracellular Tat has also been shown to induce secretion of important immunoregulatory cytokines such as interleukin-2 (IL-2), interleukin-6 (IL-6), and transforming growth factor-ß (TGF-ß), thereby having the ability to alter the infected cell and neighboring cells (57, 59-61).

HIV Vpr- HIV Vpr has been shown to halt cell cycle progression at the G2/M checkpoint by targeted depletion of the cellular protein CCDC137 (coiled-coil domain-containing protein 137) (62). This activity has been shown to enhance HIV gene expression through the activation of multiple transcription factors during infection, further driving productive HIV transcription (63). The activity of Vpr has been reported to disrupt the stability of latent infections through alterations to HDAC activity during infection (64, 65).

HIV Nef- Nef is another multifunctional viral HIV protein, with a large variety of characterized host protein interactions. As such, Nef has been shown to drastically alter host cells during infection. At the level of transcriptional regulation, Nef is known to drive activation or modulate regulation of critical TFs that regulate HIV transcription including NFAT, AP-1, and NF-kB. Nef has been shown to drive NF-κB activation through promoting degradation of its inhibitor IκB (66, 67) and is capable of activating NFAT through Ca^{2+}/calcineurin mediated signaling in the absence of TCR activation.

While Nef activates NFAT and NF-κB, it has been shown to both activate AP-1 (67, 68) and inhibit the DNA binding of AP-1 (69). In addition to the modulation of TFs, Nef can influence the activation potential of cells through disruption of cell signaling by inhibition or mis-localization of cellular kinases (70-73) or by altering the surface expression levels of several important cellular proteins including CD3, CD4, MHC-I, MHC-II, and CD28 (57, 70, 73, 74).

The findings that alteration to the host cell proteome impacts the establishment of latent HIV infections, coupled with the earlier descriptions of the genetic components of HIV latency serve to demonstrate the complex and intertwined nature of HIV latency. Due to the incomplete understanding of the multifaceted nature of HIV latency control, the field has failed to successfully develop a therapeutic intervention to treat latently infected cells despite decades of attempts.

1.5 Latency as a Barrier to HIV Cure

With the ability of ART to control active infection, life expectancy and quality of life has significantly increased for HIV+ individuals. However, as mentioned in the previous section, successful treatment requires strict adherence, and interruptions can prove extremely dangerous to the patient. Latent infection events are currently outside the therapeutic reach of ART and are the primary driver of viral rebound during treatment interruption (26, 27). However, if a therapeutic would be developed that can deplete this latent population, our ability to control active infection makes eradication of HIV a possibility. Therefore, latent infection events are currently thought to be the final hurdle to an HIV cure of either the “functional” (patient no longer requires treatment) or “sterilizing” (no virus present within the patient) type. Currently there are only a few

documented HIV cure cases, the most famous of them being Timothy Ray Brown, also known as the "Berlin patient". Brown was given a hematopoietic stem cell transfer from a CCR5-Δ32 donor for treatment of acute myeloid leukemia (AML) and then removed from antiretroviral therapy until his passing in 2020. After his transplant, there were no detectable traces of HIV in his body, although it is contested if he should be classified as a sterilizing or a functional cure. This case supports the idea that by diminishing or depleting the latent reservoir, it would be possible to truly cure HIV+ individuals allowing them to stop ongoing treatment. This idea has developed into a field of study termed HIV cure or HIV cure strategies and broadly falls into three major disciplines, which will be detailed in the sections below.

1.6 HIV Cure Strategies

As our understanding of HIV latency has progressed, so too have the strategies designed to address the latent reservoir. Currently there are three major approaches to HIV cure strategies: the modulation of the host immune response, the "Shock and Kill" strategy, and the "Block and Lock" strategy. In the following sections, these approaches will be discussed.

1.6a Modulation of the host immune response

While not traditionally thought of as cure strategies, the field has long attempted to modulate or weaponize the host immune response to HIV to increase the protective ability of the resident immune cells. Rapidly after discovery of HIV and consistently since, there have been attempts to generate a protective vaccine to HIV; however, these have failed to produce effective immunity (75-77). This is due in large part to the

unprecedented ability of HIV to mutate, a result of the low fidelity of the HIV reverse transcriptase, which makes generation of neutralizing and protective antibodies difficult, as viral genotypes rapidly evolve even within one infected patient (78, 79). Despite this, there have been several broadly neutralizing antibodies (bNAbs) isolated and characterized that target critical regions of the viral envelop protein allowing them to prevent viral escape (80). These antibodies, such as 3BNC117 and VRC01, have been tested in passive transfer clinical trials but fail to provide lasting protection due to the short life span of antibodies in serum (81).

1.6b Shock and Kill

The most prominent HIV cure strategy is the "Shock and Kill" or "Kick and Kill" strategy, which is based on the idea of reactivating latent HIV infection events through some form of therapeutic intervention and allowing the immune system, the cytotoxic effect of the virus or additional therapeutic interventions to kill the now actively infected host cell. This would provide a sterilizing cure through depletion of the latent reservoir. "Shock" strategies have evolved with our understanding of HIV latency but so far have failed to produce any clinically efficient therapeutics. Initially, when latency was thought of as a chromatin restriction phenomenon, early latency reversal agents (LRAs) were focused on inducing chromosomal remodeling that would allow for more open chromatin near the HIV genome and the downstream recruitment of transcriptional machinery to the viral promoter. These LRAs were compounds, such as histone deacetylase inhibitors and cell differentiation agents, that would induce chromatin remodeling through interactions with chromatin regulatory factors (82, 83). During this time, DNA methylation inhibitors such as 5-aza-CdR were also employed to reduce DNA methylation at the HIV promoter.

These strategies were met with limited success *in vitro* and, failed to diminish the latent reservoir *in vivo* (84, 85). As the field began to appreciate the protein level regulation involved in HIV latency, LRAs began to target cell signaling pathways to induce HIV reactivation in more natural ways. These LRAs have been developed to target T cell activation pathways using strategies such as CD3 antibody stimulation and a variety of TLR (Toll-Like Receptor) and PKC (Protein Kinase C) stimulations, but again these have seen limited success (86-90). The biggest challenge for this approach is the need for a strong HIV reactivating stimulus which must be balanced with the off-target activation to limit detrimental side effects, a feat that has not been accomplished yet (86, 91, 92).

With this limitation in mind, the most recent strategies have focused on non-canonical forms of T cell activation, as these approaches could theoretically have lower risk of off-target effects. Promisingly, two recent approaches have seen success in animal models. The first therapeutic AZD5582, a SMAC (second mitochondria-derived activator of caspase) mimetic induces both non-canonical activation of NF-κB and pro-apoptotic genes to selectively activate and kill latently infected cells and has seen success in both humanized mice and SHIV infected macaques (93). The second therapeutic, compound called N-803, is a strong inducer of IL-15, which when combined with a depletion of CD8+ T cells (known to suppress viral transcription in latently infected cells), induced viral reactivation in the same animal models of HIV latency (94). However, while these attempts triggered measurable levels of HIV production, they failed to change the reservoir size. This is possibly due to absence of a dedicated “Kill” step in the treatment protocols. Recently, it has been shown that latently infected T cells can survive both viral cytopathic effects and cytotoxic T cell activity, indicating that the kill phase will likely

require a secondary therapeutic intervention even in the presence of ART treatment (95). Development of a dedicated "Kill" intervention has lagged behind the development of the "Shock" but some promising candidates have been explored. These interventions have focused on inhibitors of anti-apoptotic proteins such as the Bcl family, or inhibitors of inhibitor of apoptosis proteins (IAPs), such as the baculoviral IAP repeat (BIR) domain proteins, reviewed in (95). While recent developments in the "Shock and Kill" strategy have been promising, it is important to note that none of the interventions have measurably reduced the size of the latent reservoir.

1.6c Block and Lock

Conceptually the opposite of the "Shock and Kill" strategy, the "Block and Lock" strategy focuses on permanent transcriptional silencing of HIV. This functional cure approach has been proposed through two prevailing concepts, (i) direct disruption of HIV transcription or (ii) alteration of cellular responsiveness. The direct mechanisms for "Block and Lock" strategies focus on the disruption of the Tat/TAR interactions required for efficient HIV transcription. There are currently two major types of interventions studied for direct Tat disruption, either protein- or compound-based. The protein-based interventions take advantage of the established knowledge that non-functional Tat mutations are dominant over functional Tat (96, 97). As such, the introduction of null mutants (Nullbasic) or the introduction of nonfunctional fusion proteins (HT1) would suppress HIV transcription. Promisingly, both proteins were able to suppress HIV reactivation in cell line models of HIV latency (97-99). However, only Nullbasic has been studied in animal models, where exposure of humanized mice to Nullbasic before infection reduced viral transcription ~2800 fold in CD4+ cells from organs, and no viral

RNA was detected in the blood (97, 100). The most advanced compound-based interventions for Tat either function by stimulating proteasomal degradation of Tat (Triptolide) (97, 101), or through inhibition of Tat-dependent transcription (didehydro-cortistatin A(dCA)) (97). Triptolide is a compound derived from a Chinese herb that increases proteasomal degradation of Tat within the nucleus. The mechanism of enhanced degradation is currently under investigation (101). dCA is an analog to Cortistatin A (CA) derived from the marine sponge *Corticium simplex*. Unlike CA, dCA was shown to bind to the basic region of HIV Tat and specifically inhibit recruitment of RNA Pol II to the HIV LTR (102, 103). This inhibition led to the establishment of a strongly repressive chromatin environment at nucleosome 1 (downstream of the HIV LTR) and limited recruitment of PBAF (an activating chromatin remodeling protein) (104). Addition of dCA to an ART regimen in humanized mice both reduced viral mRNA in tissues and inhibited viral rebound after therapeutic interruption (105).

Since, several alternative host cell-focused approaches of the "Block and Lock" concept have been put forward. These strategies focus on suppression of HIV reactivation through either enhancement of a suppressive chromatin environment or on altering the reactivation threshold of latently infected cells.

At the level of transcriptional regulation, two approaches currently stand out. The first is RNA-induced epigenetic silencing, where siRNAs are used to recruit RISC (RNA Induced Silencing Complex) to the HIV LTR, which induces chromatin remodeling through modulation of histone acetylation and methylation near TF binding sites in the HIV genome (97, 106). The second relies on small molecule modulators of BRD4 (bromodomain-containing protein 4) activity. Modulation of BRD4 protein activity leads

to competition with Tat for P-TEFb, inhibiting productive transcription and causing chromatin remodeling at the HIV LTR (97, 107). Additionally, there are several recent approaches that focus on altering host cell responsiveness to both prevent HIV reactivation and limit spread to nearby T cells through anti-inflammatory effects of these compounds. As HIV is linked to the activity of a variety of cell signaling pathways through the activity of HIV regulatory TFs, these interventions have taken many forms such as mTor inhibitors (108), JAK-STAT inhibitors (109), kinase inhibitors (110), and HSP90 inhibitors (111). These approaches have shown some promise, at the level of humanized mouse studies (HSP 90 inhibitors (111)), or have already moved into clinical trials (Jak-STAT inhibitors (NCT02475655)). However, as many of these inhibitors target critical components of cellular signaling or immune responsiveness, these inhibitors will undoubtedly have serious off-target effects. Therefore, applications of these therapeutics as HIV block agents will have to be thoroughly studied and optimized prior to clinical translation.

This points to the largest problem of developing a cure strategy. Unlike cancer treatments, where side effects need to be balanced only to the impending death of the patient, the phenomenal success of ART at controlling active viral replication means that any intervention to deplete or address the latent reservoir must not place the ART treated patient at any risk. Therefore, these cure strategies that are originally designed as anti-cancer treatments or used in organ transplantation need to improve their ability to target and deplete the reservoir, but they will also need to control and reduce off-target effects.

1.7 Understanding the Establishment of HIV Latency

The studies described above represent progress on both our understanding of HIV infection and our ability to combat active infection, but the field has still failed to develop either a sterilizing or functional cure. While there are many current attempts at latency reversal or therapeutic interventions, most have failed to significantly deplete the latent reservoir due to the complicated and multifaceted nature of HIV latency. In particular, there is a huge knowledge gap regarding the interactions between HIV and the host cell that lead to the establishment and maintenance of latent infection. For example, our understanding of the virology of HIV infection needs to be revisited after the recent discovery that the viral core is translocated to the nucleus before reverse transcription and viral uncoating (31, 32). This opens the possibility that both host and viral proteins within the viral core may have nuclear activities in addition to their reported roles in the cytoplasm. This expanded understanding is not only limited to viral functions, but also our insights on the host cells of latent HIV infection events has evolved. A prime example is the recent characterization of the transcriptomic heterogeneity present within the latent reservoir that has recently been described using single cell RNA-seq (scRNA-seq) (112, 113).

The understanding that latency can be established and maintained in vastly different cellular contexts complicates our attempts to develop a therapeutic intervention for latently infected cells. Additionally, this finding also seemingly explains the failure of previous LRAs to reach a large portion of the latent reservoir, as the design of those interventions was based on an incomplete understanding of the heterogeneous latent HIV reservoir. Thus, understanding the establishment of latent HIV in a variety of cellular

contexts and the contributions of viral proteins to the establishment of latent infection is critical to generating a more complete understanding of HIV latency, a foundation which will allow for the development of future cure strategies.

The work presented hereafter serves to enhance our understanding of how virus-host cell interactions (i) lead to the establishment of latent HIV infection events (ii) and contribute to the stability of latent infections. We studied two examples of virus-host interactions and their impact on HIV latency. First, we provide direct evidence for the presence of TCR/CD3 activation inert latent infection events within the latent reservoir. Using transcriptomic and proteomic systems biology analysis, we have here detailed the biomolecular phenotype of the host cells of these reactivation inert latent infection events. We further describe a functional role of HIV Nef during the establishment of latent infections. Collectively, this work begins to close some of the current knowledge gap regarding host cell/virus interactions that control latent HIV infection and suggests that therapeutic restoration of host cell signaling will need to be an integral part of future cure strategies.

EXTENSIVE PROTEOMIC AND TRANSCRIPTOMIC CHANGES QUENCH THE TCR/CD3 ACTIVATION SIGNAL OF LATENTLY HIV-1 INFECTED T CELLS

by

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ABSTRACT

The biomolecular mechanisms controlling latent HIV-1 infection, despite their importance for the development of a cure for HIV-1 infection, are only partially understood. For example, *ex vivo* studies have recently shown that T cell activation only triggered HIV-1 reactivation in a fraction of the latently infected CD4+ T cell reservoir, but the molecular biology of this phenomenon is unclear. We demonstrate that HIV-1 infection of primary T cells and T cell lines indeed generates a substantial amount of T cell receptor (TCR)/CD3 activation-inert latently infected T cells. RNA-level analysis identified extensive transcriptomic differences between uninfected, TCR/CD3 activation-responsive and -inert T cells, but did not reveal a gene expression signature that could functionally explain TCR/CD3 signaling inertness. Network analysis suggested a largely stochastic nature of these gene expression changes (transcriptomic noise), raising the possibility that widespread gene dysregulation could provide a reactivation threshold by impairing overall signal transduction efficacy. Indeed, compounds that are known to induce genetic noise, such as HDAC inhibitors impeded the ability of TCR/CD3 activation to trigger HIV-1 reactivation. Unlike for transcriptomic data, pathway enrichment analysis based on phospho-proteomic data directly identified an altered TCR signaling motif. Network analysis of this data set identified drug targets that would promote TCR/CD3-mediated HIV-1 reactivation in the fraction of otherwise TCR/CD3-reactivation inert latently HIV-1 infected T cells, regardless of whether the latency

models were based on T cell lines or primary T cells. The data emphasize that latent HIV-1 infection is largely the result of extensive, stable biomolecular changes to the signaling network of the host T cells harboring latent HIV-1 infection events. In extension, the data imply that therapeutic restoration of host cell responsiveness prior to the use of any activating stimulus will likely have to be an element of future HIV-1 cure therapies.

AUTHOR SUMMARY

A curative therapy for HIV-1 infection will at least require the eradication of a small pool of CD4+ helper T cells in which the virus can persist in an inactive, latent state, even after years of successful antiretroviral therapy. It has been assumed that activation of these viral reservoir T cells will also reactivate the latent virus, which is a prerequisite for the destruction of these cells. Remarkably, this is not always the case and following application of even the most potent stimuli that activate normal T cells through their T cell receptor, a large portion of the latent virus pool remains in a dormant state. Herein we demonstrate that a large part of latent HIV-1 infection events reside in T cells that have been rendered activation inert. We provide a systemwide, biomolecular description of the changes that render latently HIV-1 infected T cells activation inert and using this description, devise pharmacologic interference strategies that render initially activation inert T cells responsive to stimulation. This in turn allows for efficient triggering of HIV-

1 reactivation in a large part of the otherwise unresponsive latently HIV-1 infected T cell reservoir.

INTRODUCTION

Antiretroviral therapy (ART) efficiently suppresses HIV-1 replication below detection levels of diagnostic assays, but ART does not eliminate latent viral reservoirs, enabling HIV-1 to persist for the lifetime of a patient and rebound whenever ART is interrupted [1]. The most comprehensive evidence for viral persistence has been presented for latent HIV-1 infection events residing in long-lived, resting CD4 memory T cells [2–4]. Intuitively, this would explain the stability of the latent HIV-1 reservoir, as T cell memory can persist for the life-time of an individual. However, while immunological memory can persist for a life-time, individual memory T cells are relatively short lived. The half-life of individual $CD4^+$ central memory T cells (T_{CM} cells) that are thought to serve as host cells of latent HIV-1 infection events ranges between 20 and ~100 days and is generally shorter in HIV patients than in healthy individuals, with most studies suggesting a half-life $\tau_{1/2}$ <50 days [5–8]. With an assumed half-life of $\tau_{1/2}$ = 50 days and an initial reservoir consisting of $1x10^6$ latently HIV-1 infected $CD4^+$ T_{CM} cells, it should take less than three years after the onset of ART for the last latently infected T_{CM} cell to disappear. This is obviously not the case and evidence has been provided that preferential or homeostatic proliferation of latently HIV-1 infected T cells in the absence of reactivation can contribute to the stability of the latent reservoir [9,10]. While

homeostatic T cell proliferation must contribute to the stability of the latent HIV-1 reservoir, it is only one contributor to lifelong immunological memory. The second component, repeated exposure to cognate antigen and subsequent re-expansion of the specific memory T cells are also crucial components of lifelong memory maintenance. How the latent HIV-1 infection pool in the memory $CD4^+$ T cell population remains stable despite the expected and likely required exposure of latently HIV-1 infected T cells to their cognate antigen, and the resulting potent biological activation of the host T cells, has not been detailed. Over time, encounters with cognate antigen should cause a continuous contraction of the latent HIV-1 reservoir [11]. The absence of a measurable reservoir decay despite the expected continuous encounter of cognate-antigen and subsequent T cell activation could be explained by the idea that memory T cells hosting latent HIV-1 infection events have been altered to exhibit an activation-inert phenotype. TCR/CD3 activation-inertness would explain the inability of early therapeutic interventions (e.g. IL-2 or anti-CD3 mAb OKT3) to trigger a meaningful decrease in the size of the latent HIV-1 reservoir [12–14]. An activation-inert host cell phenotype would also explain findings that a significant part of the latent HIV-1 reservoir is resistant to *ex vivo* T cell activation, mostly based on impaired host cell signaling pathways, without any requirement for a repressive chromatin environment at the viral LTR, which is often not present, as latent HIV-1 is usually integrated into actively expressed genes [15].

A detailed description of the biomolecular biology of latently HIV-1 infected T cells that are TCR/CD3 activation inert is rather complicated. Their presence can only be indirectly detected by activating T cells from the same donor or cell population-based model of HIV-1 latency with anti-CD3 antibody and a subsequent or parallel activation with a more potent stimulus such as PMA/ionomycin, followed by quantification of the level of the resulting reactivated virus production. Should PMA/ionomycin stimulation result in higher levels of measurable HIV-1 production, the differential between these two activation methods would indicate the presence of a TCR/CD3 activation inert latent reservoir. However, per definition, TCR/CD3-activation inert latently HIV-1 infected T cells cannot be directly identified in any bulk T cell population, neither in *ex vivo* cell material from patients, nor in *in vitro* generated populations of primary T cells or T cell lines holding latently HIV-1 infected cells. Thus, direct biomolecular studies describing the detailed biomolecular baseline phenotype of TCR/CD3 activation-inert host cells of latent HIV-1 infection events can only be done in clonal T cell lines in which the inert phenotype has been established prior to analysis.

In this study we formally demonstrate that TCR/CD3-inertness occurs in population-based *in vitro* models of latent HIV-1 infection that utilize primary T cells and is reproduced in populations of HIV-1 infected long-term T cell line cultures. Following the identification of clonal latently infected T cell lines that were TCR/CD3-(re)activation responsive or inert, we were able to describe the molecular basis of this phenomenon in

these models using a systems biology approach that described TCR/CD3-inertness at the transcriptome and proteome level. We further demonstrate that network analysis of these data can be used to identify drug targets that can be addressed to restore TCR/CD3 responsiveness. Strikingly, genome-wide RNA-level analysis failed to identify the underlying biomolecular phenotype, which was efficiently detected by proteomic analysis. In either case, the data suggest that stable changes to the biomolecular host cell environment are key to TCR/CD3 reactivation inertness and we discuss the implications of these findings for the development of therapeutic approaches to potentially eradicate the latent HIV-1 reservoir.

RESULTS

HIV-1 infection generates a TCR/CD3 activation inert population of latently infected T cells

Previous reports had provided evidence that a large part of the latent HIV-1 reservoir would be recalcitrant to T cell activation, including stimulation of the TCR/CD3 pathway [15,16]. We initially sought to confirm this phenomenon in a well-established primary T cell model of HIV-1 latency [16–22]. Briefly, activated CD4+ T cells from healthy donors were infected with a full length, replication competent HIV-GFP reporter virus and RT inhibitors were added beginning 72hr post infection. At four weeks post infection, the cell cultures were sorted to remove all actively infected and therefore GFP-

positive T cells to lower the signal background. The sorted CD4+T cells were then left unstimulated, stimulated with anti-CD3/CD28 mAbs or stimulated with PMA/ionomycin. The latter is widely considered the most potent experimental means to trigger activation and proliferation in primary T cells while completely bypassing the TCR/CD3/CD28 pathway. In line with the idea that a fraction of the host T cells of latent HIV-1 infection events had been rendered inert to TCR/CD3 activation, PMA/ionomycin stimulation of CD4+ T cell HIV-1 infected cultures consistently triggered higher levels of HIV-1 reactivation levels than antibody mediated CD3/CD28 stimulation (Fig 1A).

While these results formally demonstrate that HIV-1 infection establishes both, a TCR/CD3 activation-responsive and a TCR/CD3 activation-inert latent viral reservoir in primary T cells, the model would not lend itself to biomolecular analysis of the observed reactivation inertness phenomenon. To directly describe the biomolecular baseline phenotype of TCR/CD3 activation-inert latently HIV-1 infected T cells it would be necessary to isolate TCR/CD3-responsive and TCR/CD3-inert T cells from these cultures without applying any manipulation to the cells. In the absence of any accepted marker that identifies latently HIV-1 infected primary T cells, or markers for TCR/CD3 reactivation-responsive or TCR/CD3-inert latently HIV-1 infected T cells, direct identification of TCR/CD3 activation-inert T cells within a population is mechanistically impossible. The only possible strategy to accomplish this goal is thus the generation of latently HIV-1 infected T cell clones (based on PMA stimulation), followed by a second

round of characterization for TCR/CD3 responsiveness (anti-CD3/CD28 mAb stimulation). This approach requires that HIV-1 infection of T cell lines also generates a separation into TCR/CD3-responsive and TCR/CD3-inert latently HIV-1 infected T cell populations as observed for primary T cells.

Jurkat T cells were early on used to decipher the fundamental molecular biology of TCR/CD3 signaling [23–26] and are now a commonly used CD4+ T cell line to study HIV-1 latency [27–29], making them an ideal system to investigate TCR/CD3 reactivation response in the context of HIV-1 infection. Similar to previous efforts, we infected Jurkat T cells with a replication competent full-length HIV-GFP reporter virus and followed the establishment of latent HIV-1 infection at the population level [18,19,28–31]. After 6 weeks of culture in the presence of RT inhibitors, active background infection was reduced to <2% (control) and addition of PMA triggered reactivation in ~8% of the T cell population over this background (Fig 1B). Antibody mediated stimulation of CD3/CD28 only triggered HIV-1 reactivation in an additional ~3% of the cells over background, suggesting the presence of TCR/CD3-inert latently

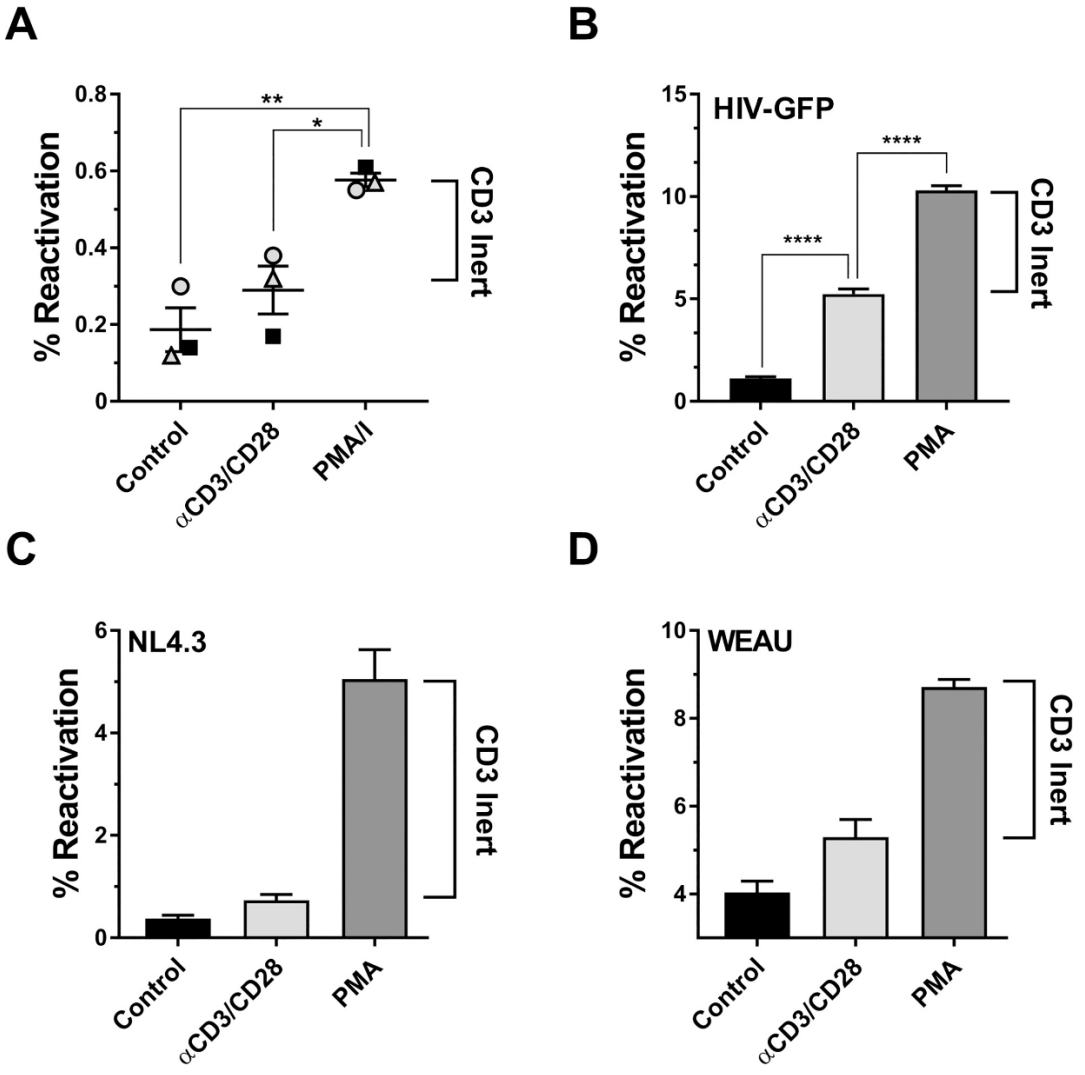


Fig 1. HIV-1 infection induced TCR/CD3 activation-inertness is conserved between primary T cells and T cell lines.

(**A**) *In vitro* latently HIV-1 infected primary CD4+ T cells were generated as described under Methods using PBMCs from three different donors infected with a full-length GFP-reporter virus. The T cell infection cultures were then left unstimulated (control), stimulated with anti-CD3/CD28 mAb coated beads or stimulated with PMA/ionomycin. Using GFP expression as a surrogate marker, levels of active/reactivated HIV-1 expression were determined 72 hours post stimulation using flow cytometric analysis for GFP expression. The difference between the GFP+ frequencies following PMA/ionomycin stimulation and CD3/CD28 mAb stimulation for each donor represents

the TCR/CD3 activation-inert reservoir. (**B**) Jurkat T cells were infected with a HIV-1 NL4-3 based GFP reporter virus and cultured for a total of 60 days in the presence of RT inhibitors until a stable viral reservoir had been established as previously described [29,30]. At this time, samples of the infection cultures were either left unstimulated, treated with anti-CD3/CD28 mAb coated beads or stimulated with PMA. Baseline HIV-1 expression levels in the untreated cells and HIV-1 reactivation in the stimulated cultures were determined using flow cytometric analysis for GFP expression. The difference between the GFP+ frequencies following PMA stimulation and CD3/CD28 mAb stimulation for each donor represents the TCR/CD3 activation-inert reservoir. Similar experiments were performed using a reporter T cell line that expresses GFP when actively HIV-1 infected (J-R5D4 cells). J-R5D4 T cells were infected with (**C**) HIV-1 NL4-3 or (**D**) HIV-1 WEAU, a primary HIV-1 patient isolate. Following six weeks of culture in the presence of RT inhibitors reactivation experiments were performed as in (B) and reactivation levels were determined by flow cytometric analysis for GFP expression. Data plotted as mean ± standard deviation of three independent experiments.

HIV-1 infected T cells consisting of 5% of the total cell population or ~60% of the overall latently HIV-1 infected subpopulation. This phenomenon could be reproduced in a GFP-reporter T cell population (J-R5D4 cells [30]) that were infected with either the recombinant clone HIV-1 NL43 (Fig 1C) or with HIV-1 WEAU, a primary R5-tropic patient isolate (Fig 1D) [30,32]. In these cells, Tat protein derived following reactivation of the integrated latent full-length virus is required to *in trans* drive the expression of an integrated LTR-gag-pol-GFP-LTR reporter vector. Here, PMA induced 10-fold or 3-fold more reactivation than CD3/CD28 stimulation, indicating that the phenomenon of HIV-1 infection induced TCR/CD3 reactivation that we observed in primary T cells is effectively reproduced in T cell lines. This finding provided us with the ability to generate relevant T cell clones that represent inert and responsive phenotypes.

Generation of TCR/CD3 activation-responsive and -inert latently HIV-1 infected T cell clones

We used the infection culture shown in Fig 1D to generate latently HIV-1 infected T cell clones that would represent TCR/CD3-responsive (JWEAU-A10) and TCR/CD3-inert (JWEAU-C6) T cells. In these T cell clones, PMA triggered an efficient HIV-1 reactivation response (Fig 2A), but even saturating amounts of CD3/CD28 mAbs that triggered reactivation in ~60% of JWEAU-A10 T cells, would not trigger HIV-1 reactivation in JWEAU-C6 cells. (Figs 2B and S1). TCR/CD3-reactivation inertness in JWEAU-C6 T cells was not a function of the utilized antibody clone (OKT3, UCHT1, HIT3A) (Fig 2C) and flow cytometric analysis demonstrated that TCR/CD3 inertness was also not the result of reduced levels of CD3 or CD28 expression on JWEAU-C6 (Fig 2D).

TNF-α, which also triggered NF-κB activation, albeit through a different upstream pathway, would induce similar levels of HIV-1 reactivation in JWEAU-A10 and JWEAU-C6 T cells (~65%; Fig 2E). Bacterial flagellin, another NF-κB agonist that signals through toll-like receptor 5 (TLR5), triggered reduced levels of HIV-1 reactivation in JWEWAU-A10 T cells (~45%) and was further compromised in JWEAU-C6 T cells (<30%) (Fig 2F). A diminished flagellin-induced reactivation response was also observed at the population level, where two-thirds of the latent population remained unresponsive to TLR5 activation (Fig 2G). Differences in the host cell signaling networks

that promote latent HIV-1 infection and restrict reactivation are thus not limited to the TCR/CD3 signaling pathway and extend to other activating receptors/pathways that are currently investigated as targets of latency reversing agents (LRA) [33–37].

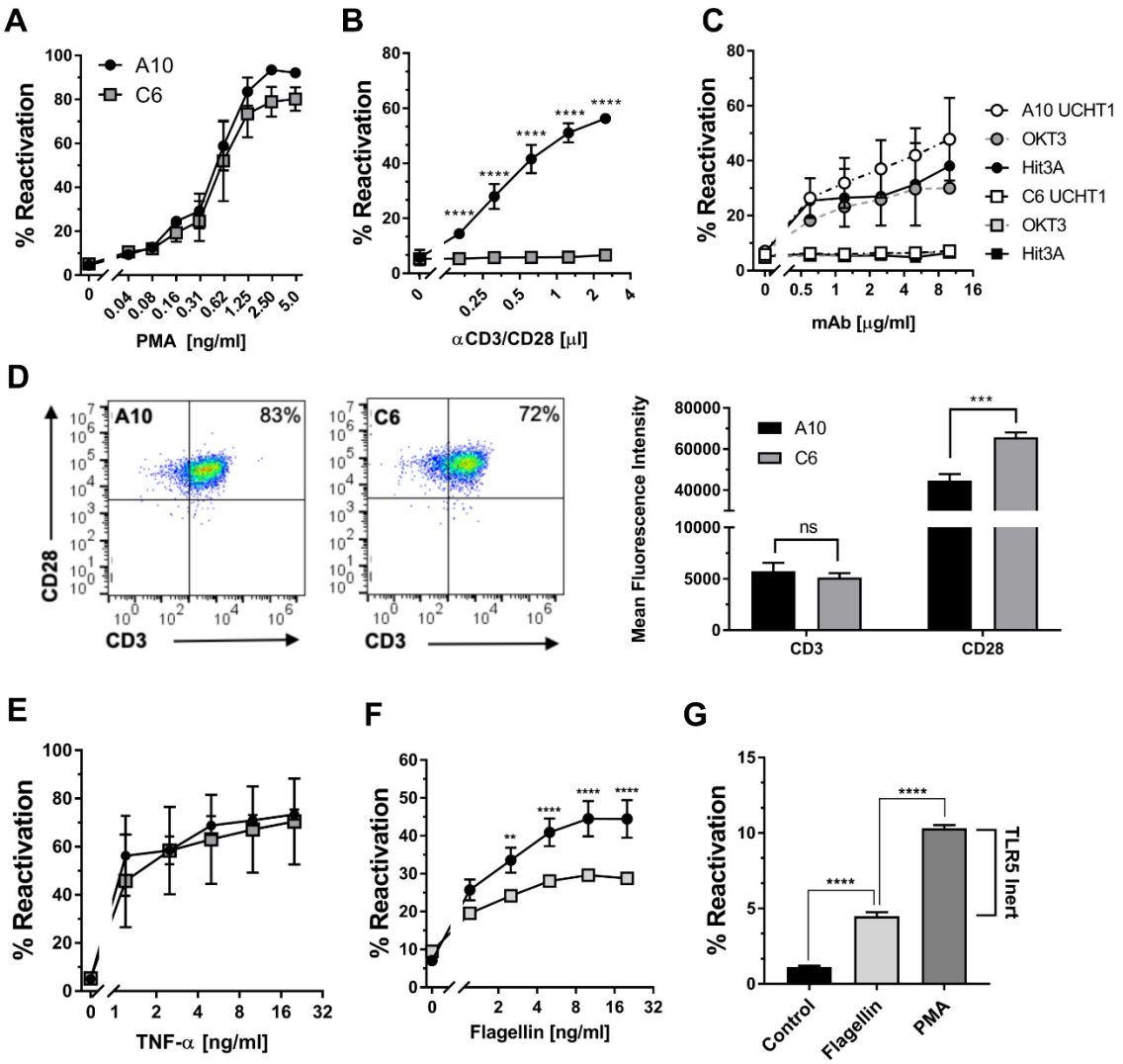


Fig 2. Generation of TCR/CD3-inert and -responsive latently HIV-1 infected T cell clones.

(**A**) HIV-1 reactivation levels in JWEAU-A10 and JWEAU-C6 T cells following stimulation with increasing concentrations of the PKC/NF-κB activating phorbolester PMA as determined by flow cytometric analysis for GFP expression. (**B**) HIV-1 reactivation levels in JWEAU-A10 and JWEAU-C6 T cells following stimulation with increasing concentrations of α-CD3/CD28 mAb as determined by flow cytometric analysis for GFP expression. (**C**) HIV-1 reactivation levels in JWEAU-A10 and JWEAU-C6 T cells following stimulation with increasing concentrations of different α-CD3 mAbs (OKT3, UCHT1, HIT3A) as determined by flow cytometric analysis for GFP expression.

(**D**) Expression levels of CD3 and CD28 proteins on JWEAU-A10 and JWEAU-C6 T cells as determined by flow cytometric analysis. (**E**) HIV-1 reactivation levels in JWEAU-A10 and JWEAU-C6 T cells following stimulation with increasing concentrations of TNF-α. (**F**) HIV-1 reactivation levels in JWEAU-A10 and JWEAU-C6 T cells following stimulation with increasing concentrations of the TLR5 agonist flagellin. (**G**) HIV-1 reactivation levels in a long-term HIV-1 infected T cell population holding a 10% latently infected subpopulation following stimulation with optimal concentrations of the TLR5 agonist flagellin or PMA. Where indicated, data represent the mean ± standard deviation of at least three independent experiments.

Analysis of the T cell activation markers CD38 and CD69 at baseline and following stimulation revealed additional insights into the fundamental differences between the responsive JWEAU-A10 and the inert JWEAU-C6. CD38, a type II glycoprotein that marks T cells with an activated phenotype [38] was highly expressed already at baseline on Jurkat T cells and the responsive JWEAU-A10 T cells, but not on the activation-inert JWEAU-C6 T cells (Fig 3A). Stimulation with PMA (Fig 3A) or anti-CD3/CD28 mAbs (Fig 3B) only triggered minimal or no induction of CD38 expression on JWEAU-C6 T cells. CD69, an immediate early T cell activation marker at baseline was absent on all three cell types, but upon PMA activation was efficiently upregulated and expressed on >80% of all cells, with no discernable difference between the three cell lines (Fig 3C) [39,40]. CD3/CD28 mAb stimulation triggered CD69 upregulation in ~50% of Jurkat and ~40% of JWEAU-A10 T cells, but completely failed to trigger CD69 expression in JWEAU-C6 T cells (Fig 3D). As CD69 expression, similar to HIV-1 or CD38 expression, is driven by NF-κB family transcription factors [41], these data show that CD3/CD28 reactivation inertness is not specific to viral transcriptional control, but reflective of a

general cellular CD3/CD28 activation inertness in JWEAU-C6 T cells. The data further indicate that the activation inert JWEAU-C6 are characterized by an already lower baseline activation state compared to their activation responsive counterparts JWEAU-A10.

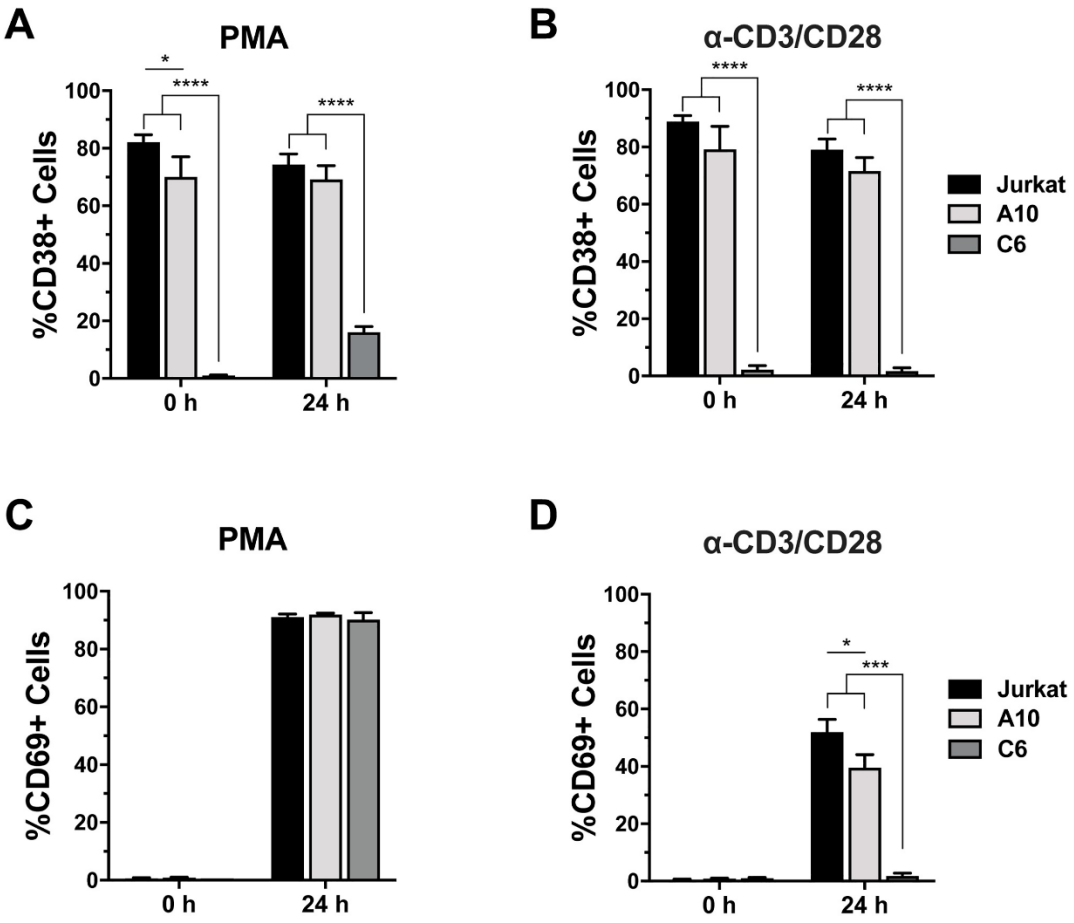


Fig 3. Regulation of T cell activation markers on TCR/CD3-inert and -responsive latently HIV-1 infected T cell clones.
(**A**) CD38 expression on uninfected Jurkat, JWEAU-A10 and JWEAU-C6 T cells was determined at baseline and 24 hours post PMA stimulation using flow cytometric analysis. (**B**) CD38 expression on uninfected Jurkat, JWEAU-A10 and JWEAU-C6 T cells was determined at baseline and 24 hours post α-CD3/CD28 mAb stimulation using

flow cytometric analysis. (**C**) CD69 expression on uninfected Jurkat, JWEAU-A10 and JWEAU-C6 T cells was determined at baseline and 24 hours post PMA stimulation using flow cytometric analysis. (**D**) CD69 expression on uninfected Jurkat, JWEAU-A10 and JWEAU-C6 T cells was determined at baseline and 24 hours post α-CD3/CD28 mAb stimulation using flow cytometric analysis. Data represent the mean ± standard deviation of at three independent experiments.

CD3 stimulation fails to generate a NF-κB signal in inert latently HIV-1 infected T cells

Given that different NF-κB agonists, such as PMA, TNF-α or flagellin efficiently or at least partially triggered HIV-1 reactivation in JWEAU-C6 T cells, it stands to reason that TCR/CD3 reactivation inertness in these cells is not the result of a generally dysfunctional canonical NF-κB pathway, but a phenomenon that is specific for the TCR/CD3 signaling pathway. To detail the kinetic NF-κB response in the two latently HIV-1 infected T cell lines relative to uninfected Jurkat T cells, we performed TransAM assays measuring NF-κB p65 phosphorylation 15, 30, 45, and 60 minutes post PMA or anti-CD3/CD28 mAb stimulation (Fig 4). Interestingly, despite the ability to induce efficient HIV-1 reactivation, the kinetic NF-κB signal induced by PMA in either latently HIV-1 infected T cell line already differed from the signal in uninfected T cells. Whereas PMA stimulation produced the expected sinus-wave shaped signal with increasing amplitude in the uninfected control cells over the monitored 60 minutes time period [42], PMA stimulation resulted in a single high-amplitude peak in both latently HIV-1 infected T cells, that within the 60 min observation period returned to baseline in JWEAU-C6 T cells, but not in JWEAU-A10 T cells (Fig 4A). The CD69 expression data shown in Fig 3C are derived from this experiment, demonstrating that stimulation was equally

successful for al cell types. CD3/CD28 stimulation triggered identically shaped kinetic NF-κB activation response curves in Jurkat and JWEAU-A10 T cells, which for Jurkat T cells differed from the PMA-induced activation pattern (Fig 4B). Both cell types produced a single peak signal in the 60 min observation period, with Jurkat T cells possibly showing a slightly more extended signal response. In the inert JWEAU-C6 T cells the ability of CD3/CD28 stimulation to induce a NF-κB signal was completely abrogated, conclusively explaining the inability of TCR/CD3 activation to trigger HIV-1 reactivation.

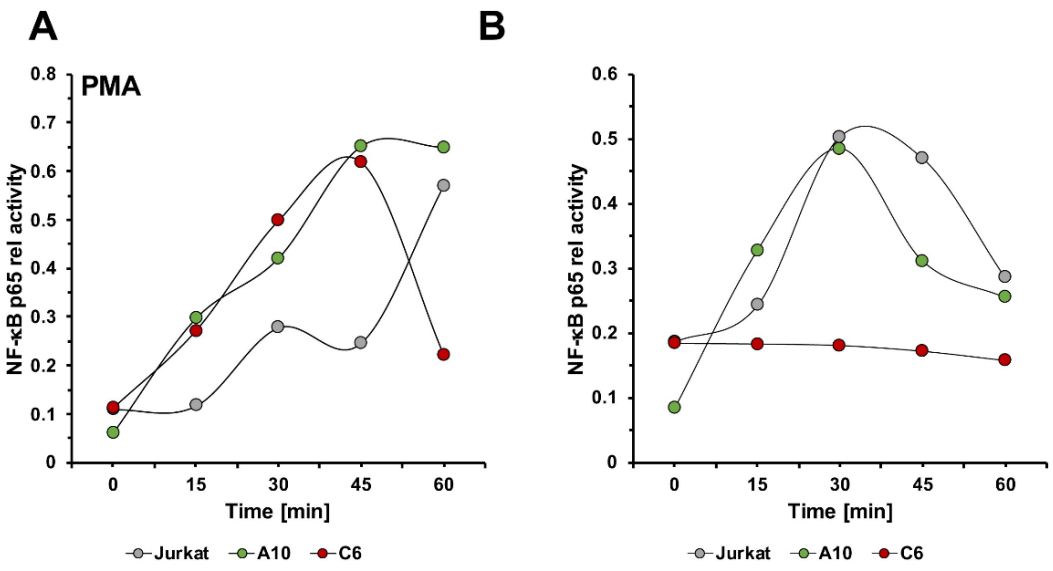


Fig 4. NF-κB activation kinetics in TCR/CD3-inert and -responsive latently HIV-1 infected T cell clones.
Uninfected Jurkat, JWEAU-A10 and JWEAU-C6 T cells were stimulated with either (**A**) PMA or (**B**) α-CD3/CD28 mAbs and sampled at the indicated timepoints. Phospho-p65 NF-kB signals were quantified with a TransAM ELISA and plotted over a time axis.

Gene expression patterns associated with TCR/CD3 activation-inert latently HIV-1 infected T cells

To identify the biomolecular basis of TCR/CD3 activation inertness, we first generated RNA-seq data describing the gene expression signatures of uninfected T cells, the TCR/CD3-inert JWEAU-C6 T cells and the TCR/CD3-responsive JWEAU-A10 T cells. A total of 7,151 genes (of 20,830 genes with any reads) were differentially expressed (likelihood ratio test, adjusted *P*-value < 0.01) across the three cell types. Pathway analysis of the complete mRNA-level data set did not produce any information that would suggest impairment of the TCR signaling or any related pathway, which was surprising given that this was the predetermined phenotype. In fact, pathway enrichment analysis produced a series of completely unrelated motifs the highest ranked being cardiac development (3.74E-03) and leukocyte chemotaxis (3.77E-03). Also, despite the high number of input genes, all motifs were suggested with very low confidence (S1 Table).

We reasoned that hierarchical clustering of the gene expression data followed by cluster-based pathway enrichment analysis could improve the likelihood of discovering motifs that control latency and TCR/CD3 reactivation inertness (Fig 5). For two of the six clusters, regulation of genes was largely shared between the two latently HIV-1 infected T cell lines, but different from the parental T cells (cluster #2 (960 genes) and cluster #6 (2353 genes)). We would expect these clusters to contain motifs that are associated with a

general latency phenotype. Gene Ontology (GO) enrichment analysis of cluster #2 genes (upregulated in latently HIV-1 infected T cells) suggested changes to lipid metabolism, regulation of cellular component size, and actin filament-based processes [43]. Cluster #6 genes were reported as critical to RNA metabolism, adaptive immune system function, the antiviral response, regulation of the mitotic cell cycle, and, interestingly, T cell activation (Fig 5) (for more details see S2 Fig). Many of these motifs have been associated with HIV-1 latency, but the data would suggest that TCR signaling pathway impairment is a general feature of latently HIV-1 infected T cells and that the magnitude of changes to this pathway is variable. TCR/CD3 reactivation inertness would then only be the most extreme phenotype on a spectrum of possible inertness phenotypes, which may be supported by the data that even optimal anti-CD3/CD28 mAb stimulation remains inferior to PMA activation and only triggers up to 70% reactivation in JWEAU-A10 T cells.

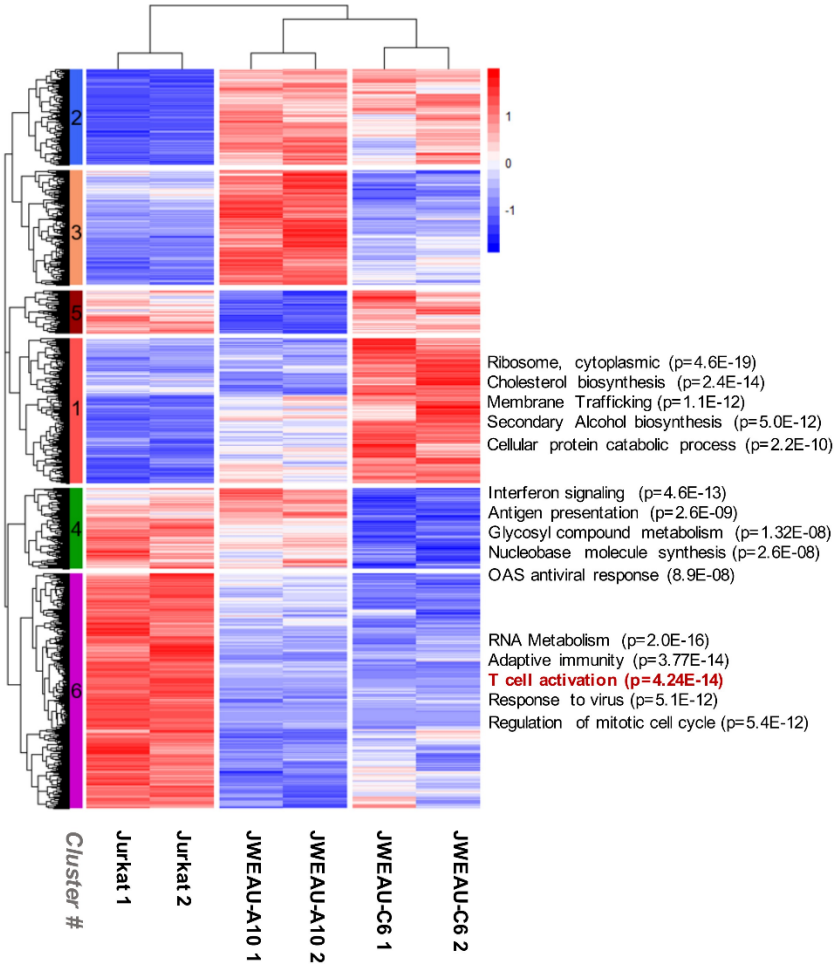


Fig 5. Transcriptomic analysis reveals extensive differences between TCR/CD3-inert and -responsive latently HIV-1 infected T cell clones.
RNA-seq analysis was used to describe the genome-wide RNA expression signature of uninfected Jurkat T cells, the TCR/CD3 responsive JWEAU-A10 and the TCR/CD3-inert JWEAU-C6 T cells. For each T cell line, RNA-seq data were obtained for low and high cell density conditions. 7,151 genes with differential expression (likelihood ratio test, adjusted *P*-value < 0.01) across the three cell types were subjected to hierarchical clustering. Subtrees representing genes that were lowly or highly expressed throughout all three cells were automatically omitted through the selection criteria. Results for motif enrichment analysis for clusters #1, #4 and #6 are presented.

In the remaining four clusters, gene regulation differed between the two latently HIV-1 infected T cells, but one or the other had gene regulation patterns similar to the parental T cells (cluster #1 (1449 genes), #3 (1155 genes), #4 (805 genes), #5 (429 genes). We had expected to extract information content regarding the predetermined TCR/CD3-activation inertness from these clusters, but surprisingly, none of the pathway analysis for these clusters suggested critical relevance to CD3-responsiveness (S2 Fig). A focused analysis of the genes involved T cell activation (GO term 0042110) confirmed that there was no significant difference between the two latently HIV-1 infected T cell clones, but both latently infected T cell lines differed in their gene expression signature from uninfected T cells (S3 Fig). Somewhat surprisingly, the RNA-level data only supported the conclusion that TCR/CD3 signaling impairment is a common feature of latently HIV-1 infected T cells, but could not explain the observed TCR/CD3 stimulation response differences.

Transcriptomic heterogeneity as the result of extensive stochastic changes to gene expression patterns

The extent of the observed transcriptomic shift between the latently HV-1 infected cells was certainly surprising. Of the total observed changes, 3,313 (46%) were shared between both cell lines, but 3,838 (54%) occurred only in one of the latently infected T cell clones. These findings are consistent with recent reports suggesting vast heterogeneity between individual latently HIV-1 infected T cells and emphasize that HIV-1 latency can be maintained in extensively different cellular environments [44,45].

Mechanistically the data raised the possibility that the extensive amounts of gene expression changes that are not shared between latently HIV-1 infected T cells. Instead, these changes may be stochastic in nature and create an unspecific reactivation threshold by broadly affecting the overall signal transduction ability of host cells of latent HIV-1 infection events.

To explore the idea that the changes to the transcriptome were mostly stochastic in nature, we first tested whether network analysis software would be able to efficiently link genes with altered expression into a functional network. Specifically, MetaCore network analysis software can build protein interaction networks based on reported molecular interactions collected in a manually curated database consisting of information derived from ~1 million peer reviewed publications. Based on the vast extent of curated interactions spanning from transcriptional regulation to post translational modifications, MetaCore software should be expected to efficiently recognize possible links between altered signals from our data set. A high degree of linkage in the network would indicate a functionally interlinked nature of the genes with altered expression, whereas low linkage would suggest stochastic gene regulation effects.

To avoid overloading the network software, we condensed the data set by performing more stringent pairwise comparison between the parental Jurkat T cells and each of the latently HIV-1 infected T cell clones. Significant signals were defined to have a padj < 0.01, a fold-change ≥2; and the read count for at least one the cell types had to be >250.

As expected, this process did not alter the proportion of shared (45%, 626 genes) and non-shared genes (55%) between the two latently infected T cell lines, relative to the initial analysis (Fig 5).

A direct interaction algorithm, which builds a protein-protein interaction network exclusively used the genes that were shared between the two latently HIV-1 infected T cell lines, but differed from the uninfected Jurkat T cells as network nodes, only integrated 53% of the seed nodes. Especially, as these were the altered genes that were shared between the two latently infected T cell lines and that would have to be responsible for a common latency control motif (as opposed to non-shared genes that would immediately seem more randomly expressed) this low linkage rate would suggest a very low degree of controlled gene regulation events in the data set (S4 Fig). 50% of the linked genes (293 genes) in the network were connected with only a single link meaning that their regulation were endpoint events and did not contribute to any network functionality (S4 Fig). Only 8 genes had more 20 or more links with other genes. On average each node of the network had only three interactions. Both the failure to efficiently link all altered signals into the network and the low level of interactions per node suggest that a large portion of the observed gene expression changes are not functionally related and represent stochastic gene regulation events, possibly triggered by cellular mechanisms/pathways induced by the actual infection event. We will henceforth refer to this extensive group of functionally unconnected, stochastic gene regulation

events as transcriptomic noise. Largely stochastic gene regulation effects also would conclusively explain why motif enrichment analysis of the transcriptomic data set produced only T cell unrelated motifs with very low statistical confidence (S1 Table).

Validating a role of transcriptomic noise as reactivation threshold for TCR/CD3 signaling

Outside the field of HIV-1 research, histone deacetylase (HDAC) inhibitors, BET protein inhibitors or cell differentiating agents are well known to trigger genome-wide changes to gene expression patterns, through a variety of specific and off-target effects, and as such create transcriptomic noise [46–56]. In the context of HIV-1 latency research Dah *et al*. have demonstrated that HDAC inhibitors would trigger or facilitate HIV-1 reactivation by inducing an increase in genetic noise [57] and the varying potential of different histone deacetylase inhibitors to trigger HIV-1 reactivation has been linked to the induction of differential host cell responses [58]. To confirm that our experimental system would reproduce the findings by Dah et al., we initially tested the effect of the histone deacetylase inhibitor suberanilohydroxamic acid (SAHA; vorinostat) on PKC agonist mediated HIV-1 reactivation. As described by Dah et al. pretreatment with SAHA increased the ability of specifically lower concentrations of prostratin and bryostatin to trigger HIV-1 infection in JWEAU-A10 T cells (Figs 6A and 3B). Having confirmed the experimental findings by Dah *et al*., we reasoned that if transcriptomic noise contributes to a TCR/CD3 activation threshold in latently HIV-1 infected T cells, then treatment of

the TCR/CD3-responsive JWEAU-A10 T cells with HDAC inhibitors, BET inhibitors or cell differentiating agents, would increase the level of transcriptomic noise, but in contrast to what was observed for PKC agonists, would reduce the ability of TCR/CD3 activation to trigger HIV-1 reactivation.

Following a 24 hours pretreatment period to allow each inhibitor to exert its full effect, non-toxic concentrations of the histone deacetylase inhibitor SAHA had no direct effect on HIV-1 reactivation in either, JWEAU-A10 and JWEAU-C6 T cells (Fig 6C). As predicted, SAHA decreased the TCR/CD3 stimulation-induced HIV-1 reactivation response of JWEAU-A10 T cells, while not restoring a TCR/CD3 response in JWEAU-C6 T cells. The cell differentiating compound hexamethylene bisacetamide (HMBA), which has been reported to trigger HIV-1 reactivation in some experimental systems [59,60], did not directly trigger HIV-1 reactivation (Fig 6D). However, in line with its ability to induce transcriptomic noise HMBA suppressed the TCR/CD3 stimulation-induced HIV-1 reactivation response of JWEAU-A10 T cells. HMBA had no effect on latent HIV-1 infection in JWEAU-C6 T cells, neither by itself nor following TCR/CD3 stimulation. Lastly, we tested the effect of JQ1, a Brd4 inhibitor that has been reported to not only affect global cellular gene expression, but to trigger low level HIV-1 reactivation [61–63]. JQ1 triggered modest levels of HIV-1 reactivation by itself in JWEAU-A10 T cells, but completely suppressed the ability of TCR/CD3 stimulation to trigger any additional HIV-1 reactivation (Fig 6E). As each of these compounds is a documented

LRA, their inhibitory effect on TCR/CD3 mediated HIV-1 reactivation of necessity cannot come from interactions of the compounds with functionalities on the HIV-1 LTR, but must be caused by the reported ability of these compounds to trigger transcriptomic noise that affects the CD3 signaling efficacy. As such, the findings indirectly validate a role for transcriptomic noise as a factor affecting TCR/CD3 activation mediated HIV-1 reactivation.

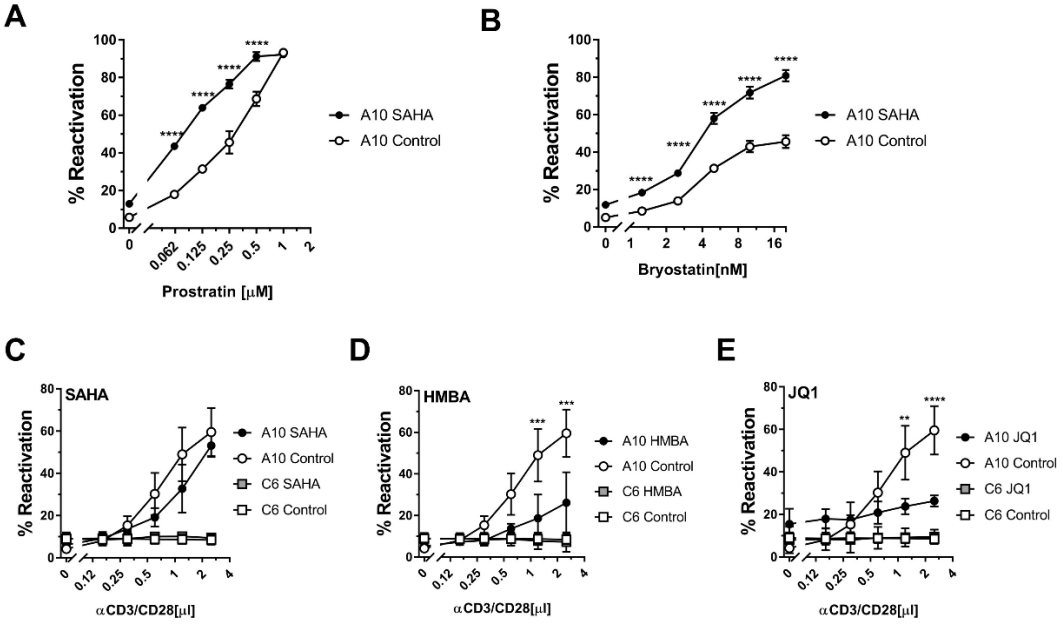


Fig 6. Induction of genetic noise suppresses TCR/CD3 induced HIV-1 reactivation. To determine if transcriptomic noise contributes to the stability of latent HIV-1 infection we pretreated the TCR/CD3 responsive JWEAU-A10 and the TCR/CD3 inert JWEAU-C6 T cells with compounds known to induce transcriptomic noise by promoting unspecific transcriptional elongation and then stimulated the cells with either PMA or α-CD3/CD28 mAbs. To confirm that JWEAU-A10 T cells reproduce previous findings by Dah et al. the cells were treated for overnight with SAHA and then stimulated with (**A**) increasing concentrations of prostratin or (**B**) bryostatin. Cells were treated overnight with (**C**) the HDAC inhibitor SAHA (300 nM), (**D**) the cell differentiating agent HMBA (2mM), or (**E**) the BET inhibitor JQ1 (10μM) and then treated with increasing

concentrations of α-CD3/CD28 mAbs. HIV-1 reactivation levels were determined by flow cytometry for GFP expression 24 hours post CD3/CD28 activation. Data represent the mean ± standard deviation of three independent experiments.

Kinomic description of CD3-activation inert latently HIV-1 infected T cells

A possible explanation for the inability of the RNA-level analysis to directly identify the underlying functional impairment (TCR/CD3-inertness) that stabilizes latent HIV-1 infection is that major cellular regulation effects often occur at the level of post-transcriptional or even post-translational modifications (e.g. protein phosphorylation). Changes to the proteome at the level of protein expression and phosphorylation would be the most direct indicators of functional modifications of the host cell signaling network that cause TCR/CD3-inertness and contribute to the stability of latent HIV-1 infection. To identify protein regulation effects that differ between TCR/CD3-responsive and TCR/CD3-inert T cells we used antibody arrays (Kinexus KAM-900). Each antibody array was spotted with 265 pan-specific antibodies and 613 phosphosite specific antibodies. Between JWEAU-A10 and JWEAU-C6 T cells the array identified a total of 123 significant differences. 74% of the signals were phospho-site specific and 26% were pan-specific, which is proportional to the bias of the antibody array for phospho-specific antibodies. Relative to the parental Jurkat T cells the majority of these differences were found in the TCR/CD3-inert JWEWAU-C6 T cells, where a total of 58 signals were found upregulated and 29 downregulated relative to Jurkat T cells, as opposed to 10

upregulated and 33 downregulated signals in JWEAU-A10 T cells. A detailed breakdown regarding regulation of protein expression and tyrosine- versus serine/threonine phosphorylation signals relative to uninfected control cells displays the obvious dysregulation of the phospho-kinome, particularly in the inert JWEAU-C6 cells (Fig 7).

Other than for the transcriptomic data, the MetaCore direct interaction algorithm generated an efficient network from these differentially regulated signals. 86% of the signals were linked into a direct protein-protein interaction network, which had an average degree of 14 interactions per node, a sign that likely more than one pathway within the overall network was affected, and no single master switch controlled the recorded changes (Fig 8A). The three highest linked nodes in the network were p53 (55 interactions), STAT3 (47 interactions), and c-Src (46 interactions) (Fig 8B). Also, consistent with the functional phenotype, pathway enrichment analysis identified *Immune Response/TCR signaling* ($p = 6.78E-14$) as a highly ranked altered motif in addition to other motifs that could be reasonably relevant for HIV-1 latency control (G1-S growth factor regulation, 3.716E-16; regulation of initiation, 2.196E-15; NOTCH signaling, 1.874E-16) (Fig 8C).

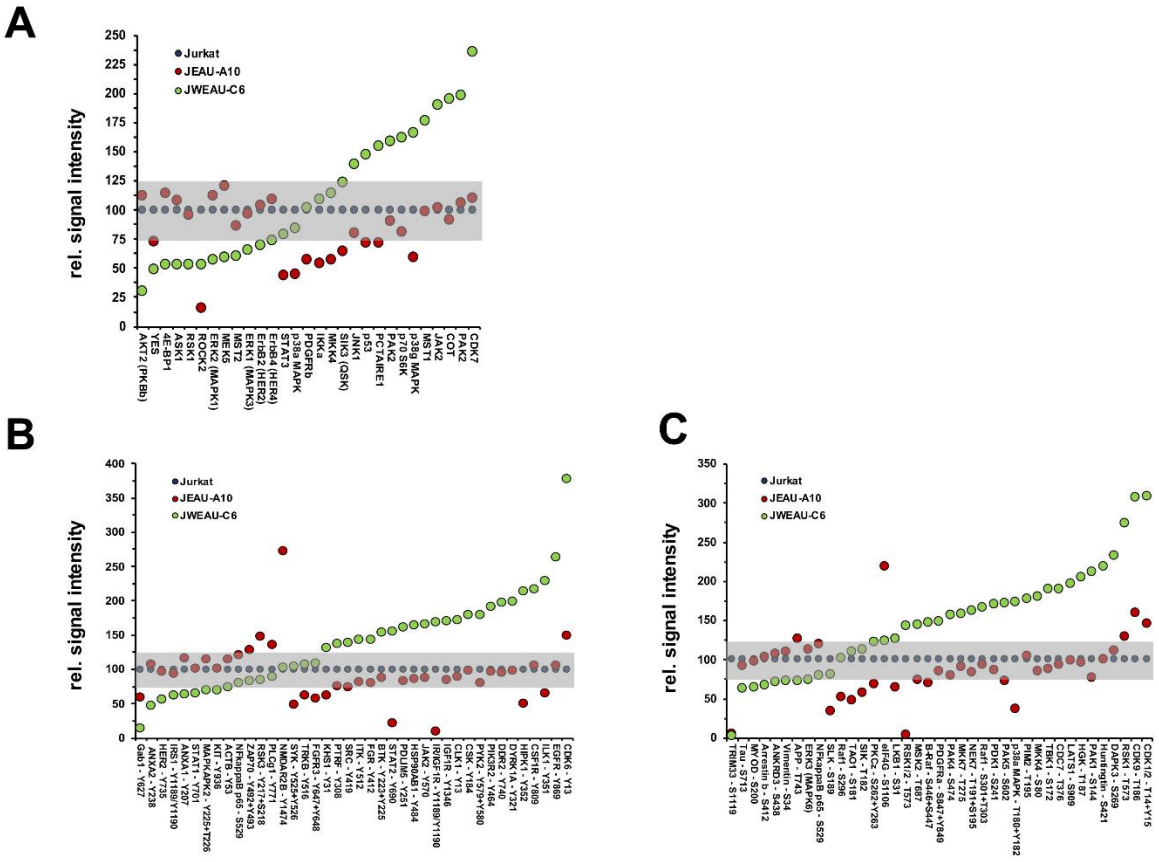
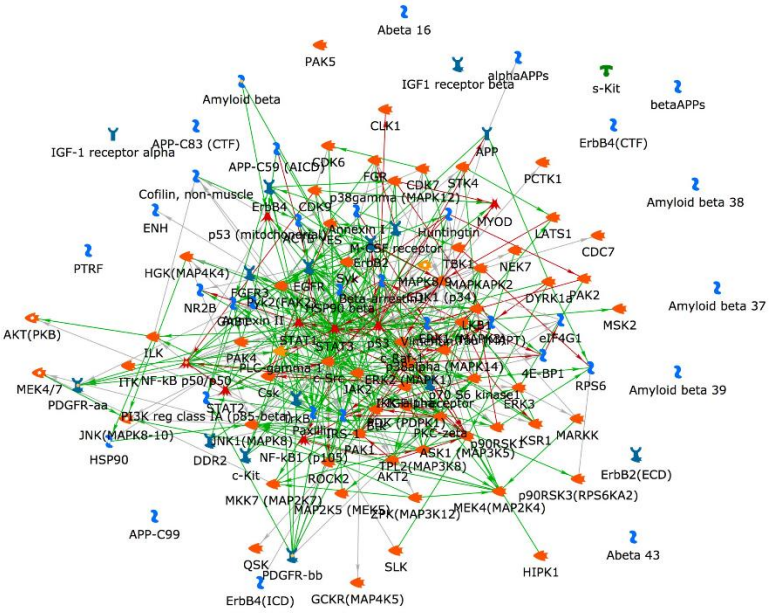


Fig 7. Kinomic analysis of TCR/CD3-inert and -responsive latently HIV-1 infected T cell clones.

Cell lysates from JWEAU-A10 (responsive) and JWEAU-C6 T cells (inert) were loaded on antibody array chips (Kinexus KAM-900) to determine differences in protein expression and protein phosphorylation. Each of the two identical arrays on the chip is spotted with 265 pan-specific antibodies and 613 phosphosite specific antibodies. (**A**) Protein expression signals that differed in JWEAU-A10 or JWEAU-C6 T cells from Jurkat T cells were plotted as relative signal percentage normalized to expression in uninfected Jurkat T cells. Signals were ordered based on their relative expression in the activation-inert JWEAU-C6 T cells. Signals in the gray zone (max. 25% deviation from controls) would be considered not altered. (**B**) Tyrosine phosphorylation signals that differed in JWEAU-A10 or JWEAU-C6 T cells from Jurkat T cells were plotted as relative signal percentage normalized to expression in uninfected Jurkat T cells. Signals were ordered based on their relative signal intensity in the activation-inert JWEAU-C6 T cells. (**B**) Serine-threonine phosphorylation signals that differed in JWEAU-A10 or JWEAU-C6 T cells from Jurkat T cells were plotted as relative signal percentage

normalized to expression in uninfected Jurkat T cells. Signals were ordered based on their relative signal intensity in the activation-inert JWEAU-C6 T cells.

A



B

Rank	Name	Number of Interactions
1	p53	55
2	STAT3	47
3	Src	46
4	RelA (p65)	39
5	EGFR	34
6	ERK2 (MAPK1)	32
7	CDK1 (p34)	29
8	STAT1	28
9	Pyk2(FAK2)	26
10	JAK2	24
11	IRS-1	22
12	c-Raf-1	20
13	PKC-zeta	20
14	ERK1 (MAPK3)	19
15	p38alpha (MAPK14)	19
16	PLC-gamma 1	19
17	Paxillin	18
18	Vimentin	18
19	ErbB2	17
20	IGF-1 receptor	17

C

Rank	Networks	p-value
1	Hemopoiesis, Erythropoietin pathway	3.101E-17
2	NOTCH signaling	1.874E-16
3	G1-S Growth factor regulation	3.716E-16
4	Regulation of initiation	2.196E-15
5	ERBB-family signaling	2.879E-14
6	G1-S Interleukin regulation	3.979E-14
7	TCR signaling	6.781E-14
8	Positive regulation cell proliferation	6.678E-13
9	Regulation of epithelial-to-mesenchymal transition	8.713E-13
10	Anti-Apoptosis (MAPK and JAK/STAT)	1.333E-12

Fig 8. Network analysis of kinomic data describing the biomolecular basis of reactivation inertness in T cells.

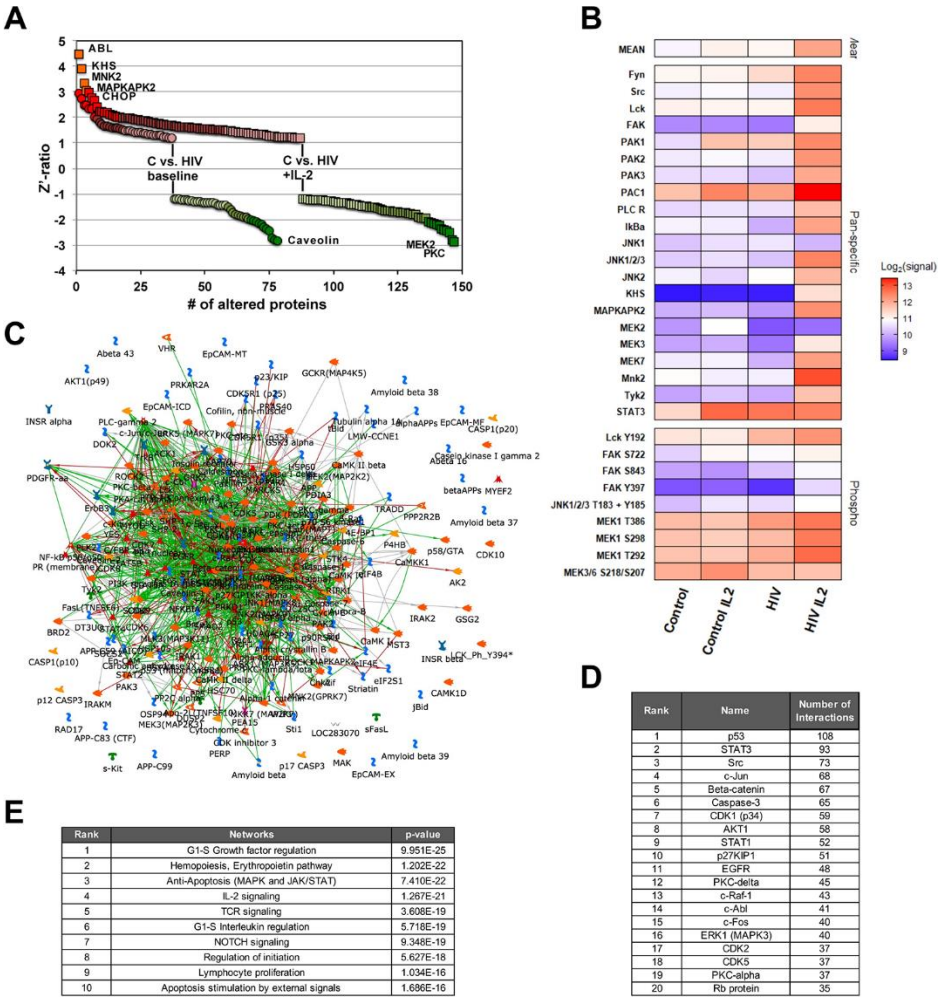
(**A**) The altered kinomic signals identified between activation responsive JWEAU-A10 and activation-inert JWEAU-C6 T cells were used to generate a protein-protein interaction network using the direct interaction algorithm of MetaCore software. The network visualizes the high level of interactions with few seed nodes (proteins with altered signals) not being connected. (**B**) List of the 20 highest connected altered proteins and their numbers of interactions with other proteins in the network. (**C**) List of the highest ranked functional motifs associated with the network.

Being cognizant of the fact that these data were generated in T cell lines, we chose to generate a second phospho-kinome data set for a primary T cell system that could ultimately guide drug target prioritization among the identified central nodes of the protein-protein interaction network. Obviously, as a result of the scarcity of latently HIV-1 infected T cells *in vivo,* the inability to identify these rare cells in the absence of previous activation, and the complete impossibility to identify TCR/CD3-inert primary T cells, we needed to generate phospho-kinome data using a relevant surrogate T cell population that also provides the required amount of cells (~5x10e6 cells per array). Given the substantial body of literature describing a broadly impaired ability of T cells from HIV/ART patients to respond to stimulation, also after the initiation of ART [64–72], we isolated CD4+ T cells from HIV/ART patients (n = 8) that were on ART for less than 2 years and from healthy controls (n = 11). We chose two experimental conditions, (i) unstimulated and (ii) overnight exposure to IL-2, a simple experimental proxy condition for T cells that would reside in an immunologically more active environment such as lymph nodes. Consistent with the idea that T cells from HIV/ART patients have

an altered biomolecular phenotype, under unstimulated conditions, kinome array experiments identified a total of 78 differences (37 up-/41 down-regulated) between T cells from healthy controls and HIV/ART patients, and 147 differences (87 up/60 down) following overnight exposure to IL-2 (Fig 9A). A focused analysis of the data for significantly altered proteins reported to be involved in TCR/CD3 signaling further highlighted the impairment of this pathway in T cells from HIV/ART patients, in particular following IL-2 exposure (Fig 9B). MetaCore driven network analysis integrated 83% of the altered signals into a direct interaction network (Fig 9C). 54 nodes had more than 20 interactions in the network, suggesting an overall high degree of linkage between the nodes and a relatively flat signaling hierarchy. Within this network the central proteins (hubs) were p53 (108 interactions), STAT3 (93 interactions), Src (73 interactions), c-Jun (68 interactions), and ß-catenin (67 interactions), all factors that play key roles in TCR/CD3 signaling (Fig 9D) [73–80]. Pathway enrichment analysis ranked G1-S growth factor regulation (p = 9.91E-25), IL-2 signaling (p = 1.26E-21) and TCR signaling (p = 3.60E-19) in the top five altered network motifs (Fig 9E), showing clear similarities to the changes we observed in latently HIV-1 infected T cells.

These data again validate the ability of kinome analysis in conjunction with MetaCore network analysis software to accurately detect and describe experimental conditions (IL-2 signaling) and phenotypes (TCR signaling) and should provide an efficient tool to guide target identification by providing the ability to distinguish between HIV-1 infection

induced changes that are shared by primary and immortalized T cells, and changes that are found in latently HIV-1 infected T cell lines, but tat are not present in primary T cells.



Rank	Name	Number of Interactions
1	p53	108
2	STAT3	93
3	Src	73
4	c-Jun	68
5	Beta-catenin	67
6	Caspase-3	65
7	CDK1 (p34)	59
8	AKT1	58
9	STAT1	52
10	p27KIP1	51
11	EGFR	48
12	PKC-delta	45
13	c-Raf-1	43
14	c-Abl	41
15	c-Fos	40
16	ERK1 (MAPK3)	40
17	CDK2	37
18	CDK5	37
19	PKC-alpha	37
20	Rb protein	35

Rank	Networks	p-value
1	G1-S Growth factor regulation	9.951E-25
2	Hemopoiesis, Erythropoietin pathway	1.202E-22
3	Anti-Apoptosis (MAPK and JAK/STAT)	7.410E-22
4	IL-2 signaling	1.267E-21
5	TCR signaling	3.608E-19
6	G1-S Interleukin regulation	5.718E-19
7	NOTCH signaling	9.348E-19
8	Regulation of initiation	5.627E-18
9	Lymphocyte proliferation	1.034E-16
10	Apoptosis stimulation by external signals	1.686E-16

Fig 9. T cells from HIV-1 patients on ART exhibit an altered kinomic phenotype. (**A**) Kinome array analysis was performed using T cells from 9 healthy individuals and 8 HIV/ART patients. Samples were produced either at baseline or following 24h of IL-2 incubation. The samples for each experimental conditions were pooled to minimize individual variations and loaded onto Kinexus antibody arrays. Signals that were altered in samples from HIV/ART patients relative to healthy controls at baseline or following IL-2 stimulation were plotted. (**B**) Focused analysis of kinase and transcription factor

signals from the kinome array involved in the TCR/CD3 signaling pathway represented as a heat map (expression or phosphorylation) for each of the four experimental conditions. (**C**) The altered kinomic signals identified between T cells from healthy individuals and T cells from HIV/ART patients were used to generate a protein-protein interaction network using the direct interaction algorithm of MetaCore software. The network visualizes the high level of interactions with few proteins with altered signals not being connected. (**D**) List of the 20 highest connected altered proteins and their numbers of interactions with other proteins in the network. (**E**) List of the 10 highest ranked functional motifs associated with the network.

Probing the role of individual network nodes in CD3 inertness and HIV-1 latency control

Under the assumption that individual, highly linked network nodes act as molecular switches that control latent HIV-1 infection, or are key to TCR/CD3-inertness, we chose three targets that were identified through network analysis (Figs 8 and 9). Targets needed to be (i) central nodes in the network describing the kinomic differences between TCR/CD3-responsive and -inert latently HIV-1 infected T cell clones, (ii) needed to be also central nodes in the network describing changes in the T cell populations of HIV/ART patients and (iii) clinically relevant inhibitors against the targets needed to be available. Based on these criteria, we chose to target (i) Src (dasatinib), (ii) Raf (sorafenib) and (iii) STAT3 (S31-201).

As the generated protein-protein interaction networks are not directional, it is not possible to predict whether e.g. an inhibitor against a central node will inhibit or activate down-stream events, in this case trigger or inhibit HIV-1 reactivation. The networks provide no information on the connection between a particular central kinase/protein, its actual role

in the altered network and how this affects latency stability. Network analysis strictly increases the probability of successful target identification. We would not expect that interference with these key network nodes would directly trigger HIV-1 reactivation, but anticipated that pharmacological targeting of the selected network nodes would either boost or abrogate TCR/CD3 activation-induced HIV-1 reactivation. Ideally, interference would restore the ability of TCR/CD3 activation to trigger HIV-1 reactivation in the otherwise inert JWEAU-C6 T cells.

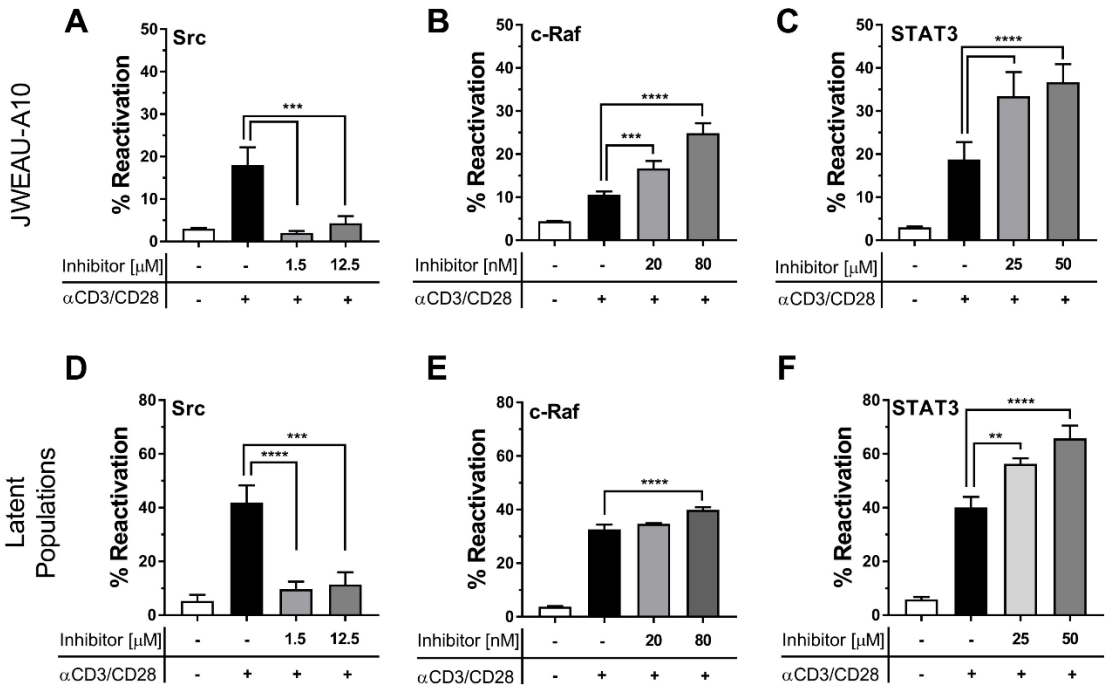


Fig 10. Pharmacological targeting of network hubs alters TCR/CD3 responsiveness of latently HIV-1 infected T cells.

To validate that network analysis correctly predicted and prioritized drug targets that affect the ability of TCR/CD3 stimulation to trigger HIV-1 reactivation we tested the effect of inhibitors against Src, Raf and STAT3 on the ability of a-CD3/CD28 mAbs to trigger HIV-1 reactivation. JWEAU-A10 T cells (A-C) or populations enriched for latently HIV-1 infected T cells (D-F) were incubated overnight with different concentrations of the Src inhibitor dasatinib (**A, D**), the c-Raf inhibitor sorafenib (**B, E**), or the STAT3 inhibitor S31-201 (**C, F**) before stimulation with a sub-optimal concentration of α-CD3/CD28 mAbs. Data represent the mean ± standard deviation of three independent experiments.

In a first step, we pretreated JWEAU-A10 T cells or long-term HIV-1 infected T cell populations that we enriched for latently HIV-1 infected T cells (see Material and Methods section) with increasing concentrations of the three drugs, and, after 24 hours, stimulated with a suboptimal concentration of anti-CD3/CD28 mAbs. Suboptimal CD3/CD28 stimulation would facilitate the discovery of potential additive/synergistic effects of the drugs on TCR/CD3 signaling. Inhibition of Src by sub-cytotoxic concentrations of dasatinib effectively abrogated TCR/CD3 triggered HIV-1 reactivation, in both JWEAU-A10 T cells and the latently HIV-1 infected T cell population (Fig 10A and 10D) [81–84]. While this finding is likely therapeutically irrelevant, it demonstrates the ability of network software to correctly identify and prioritize functionally important protein targets. The Raf inhibitor sorafenib boosted the ability of suboptimal CD3/CD28 mAb combinations to trigger HIV-1 reactivation in JWEAU-A10 T cells in the nM-range (Fig 10B), but its effect was minimal in the latently HIV-1 infected T cell population (Fig 10E). Finally, the STAT3 inhibitor S31-201 showed robust enhancement of CD3/CD28

triggered HIV-1 reactivation in JWEAU-A10 T cells (Fig 10C), and also in the latently HIV-1 infected T cell populations (Fig 10F).

We next tested whether Raf or STAT3 inhibition would permit optimal CD3/CD28 stimulation to trigger HIV-1 reactivation in the otherwise TCR/CD3 activation-inert subpopulation that is only responsive to PMA treatment. Consistent with its limited effectiveness in combination with suboptimal CD3/CD28 activation, sorafenib treatment only slightly increased the percentage of HIV-1 expressing T cells following optimal CD3/CD28 activation, but the majority of the TCR/CD3 activation-inert T cell fraction that was revealed by PMA stimulation was not addressed (Fig 11A). The STAT3 inhibitor S31-201 exhibited a stronger reactivation-boosting effect. Pretreatment with S31-201 followed by CD3/CD28 stimulation produced HIV-1 reactivation levels that were similar to the reactivation levels produced by PMA (Fig 11B), indicating that STAT3 inhibition would restore the ability of a large fraction of the initially CD3-inert T cells to generate an effective TCR/CD3 pathway signal. Of note, neither sorafenib nor S31-201 had any effect on the ability of CD3/CD28 mAb combinations to trigger HIV-1 reactivation in the inert JWEAU-C6 T cells, suggesting that extreme CD3-inertness may not be reversible (S5 Fig).

Finally, we tested whether these findings would be transferable to HIV-1 latency in primary T cells, and whether sorafenib or S31-201 would also promote reactivation of latent HIV-1 infection in an *in vitro* model of latency in primary T cell. This possibility

was suggested by the similarity of signaling network changes identified in primary T cells from HIV/ART patients (Fig 8B) and latently HIV-1 infected T cell lines (Fig 9C). To test this possibility, we again generated latently HIV-1 infected primary T cell cultures using the same *in vitro* infection model utilized in Fig 1A. Similar to what was observed in latently HIV-1 infected T cell populations, neither sorafenib nor S31-201 by themselves had any direct HIV-1 reactivating effect, but both drugs exhibited the ability to boost CD3/CD28 mAb-induced HIV-1 reactivation in a donor-dependent manner. Throughout the experiments (n = 5), sorafenib provided a stronger reactivation boosting effect than S31-201, confirming that the network analysis derived target predictions could be transferred into a primary T cell-based model of latent HIV-1 infection. However, donor variation was apparent and while some infection cultures responded as predicted (Fig 11D) the drugs seem to have no boosting effect at all in other infection cultures (Fig 11E). It stands to reason that heterogeneity caused by the immunological history of primary T cells likely adds to the complexity of HIV-1 latency control in primary T cells that thus is largely, but certainly not fully reflected in latently HIV-1 infected T cell lines.

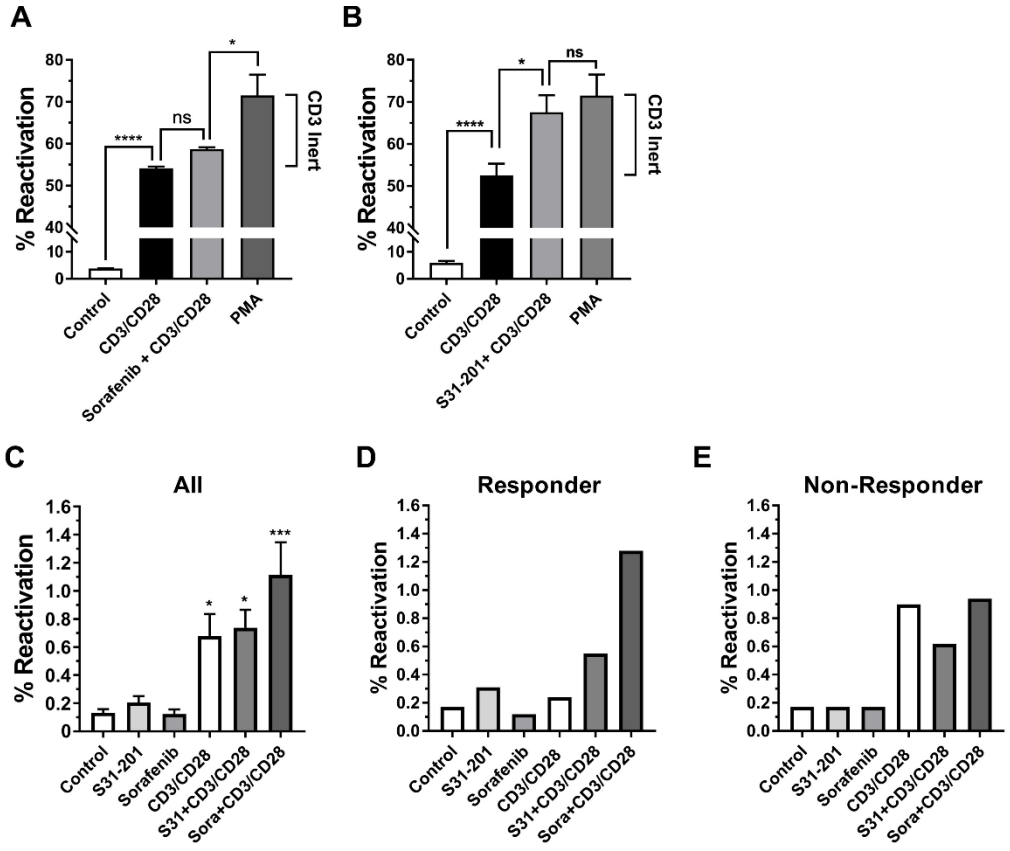


Fig 11. Restoring CD3 responsiveness in latently HIV-1 infected T cells.
To test whether pharmacological interventions that were prioritized by network analysis would specifically restore the ability of the CD3 signaling pathway to trigger latent HIV-1 infection in CD3-inert T cells, we stimulated latently HIV-1 infected T cell populations with α-CD3/CD28 mAbs without any pretreatment or following overnight pretreatment with optimal concentrations of (**A**) the c-Raf inhibitor sorafenib or (**B**) the STAT3 inhibitor S31-201 (50 μM). PMA stimulation was used as a positive control to determine the maximum achievable reactivation level in the T cell population. The difference between the reactivation levels accomplishable by α-CD3/CD28 mAb activation and PMA stimulation constitutes the TCR/CD3-inert T cell reservoir (CD3 inert). Any increase of HIV-1 reactivation levels above the ability of α-CD3/CD28 mAbs to trigger HIV-1 reactivation means that the therapeutic intervention allows antibody stimulation to target previously activation inert latently HIV-1 infected T cells. (**C**) Using a full-length HIV-GFP reporter virus latently HIV-1 infected T cell populations were established in primary CD4+ T cell populations obtained from five different donors. The T cells were left unstimulated (control) or treated overnight with optimal concentrations of the STAT3

inhibitor S31-201 or the Raf inhibitor sorafenib. Each of these cultures was then split and left either untreated or stimulated with α-CD3/CD28 mAbs. HIV-1 reactivation was determined by flow cytometry 72 hours post α-CD3/CD28 mAbs addition using GFP expression as a surrogate marker of active HIV-1 expression. Stars indicate conditions that significantly differed from the control condition (see Statistical Analysis in methods).

DISCUSSION

At the time it is assumed that latent HIV-1 infection events are established as activated T cells become HIV-1 infected before transitioning to a long-lived resting memory phenotype [1,85–89]. The resting status of memory T cells is thought to deplete HIV-1 of essential transcription factors and thereby restrict its ability to express its genes, forcing the virus into a latent state [90–92]. Based on this concept, T cell activation in general, or specifically through antibody-mediated stimulation of the TCR/CD3 complex, the experimental equivalent of cognate antigen recognition, should trigger HIV-1 reactivation. It has by now been conclusively demonstrated that this is correct only for a part of the latent HIV-1 reservoir, whereas a large portion of the latent reservoir remains recalcitrant to TCR/CD3 activation [15,16]. We thus hypothesized that a part of the HIV-1 reservoir is established in T cells that are either TCR/CD3 activation-resistant prior to infection or are rendered TCR/CD3 activation-inert by the cellular response to the actual infection event.

In this study we provide formal experimental evidence that TCR/CD3 activation-inertness (i) can indeed be generated by HIV-1 infection (Figs 1 and 2), (ii) must a

fundamental mechanism that is conserved between primary T cells and T cell lines (Fig 2) and (iii) affects a substantial portion of the T cells serving as host cells for the latent HIV-1 reservoir (Figs 2 and 7). The existence of such an activation-inert reservoir T cell population would conclusively explain why cognate antigen recognition does not deplete the latent HIV-1 reservoir over time. We further demonstrate that host cells of latent HIV-1 infection events are characterized by extensive stable changes to their signaling networks (Figs 8 and 9). (iv) Our data suggest that high-level transcriptomic noise may have the potential to amplify TCR/CD3 activation-inertness (Fig 5), but (v) TCR/CD3 inertness was clearly driven by selective changes to the TCR/CD3 signaling pathway, which could be targeted and at least partially reversed by inhibitors (Figs 6 and 7).

The observed presence of stochastic gene expression changes or transcriptomic noise explains the findings by others that described a high degree of heterogeneity between individual latently HIV-1 infected T cells [44,45]. Our research extends on these studies in primary T cells and demonstrates that this diversity is not necessarily the result of a pre-existing heterogeneity caused by different immunological histories or differentiation status of T cells that later on become latently HIV-1 infected. While such T cell diversity most likely adds to the observed heterogeneity in primary T cells (Fig 11), we show that heterogeneity is generated by a fundamental mechanism that must be part of the cellular response to the actual infection event. Heterogeneity between latently HIV-1 infected T cells was observed at the gene and protein expression level (Figs 5 and 7). Given that

gene expression analysis is now the basis of most studies, it was somewhat surprising that RNA-seq analysis would indicate extensive changes to the gene expression landscape, but network analysis software could not efficiently integrate these data into interaction networks or for that matter assign system-relevant motifs to the data (S1 Table). Even hierarchical clustering followed by subtree cluster analysis would not directly identify potential molecular switches that were to affect TCR/CD3 mediated HIV-1 reactivation, but rather suggested that TCR/CD3 activation impairment could be a general feature of latently HIV-1 infected T cells. This was disconcerting as the phenotype, TCR/CD3 activation-inertness, was already predetermined by functional assays (Figs 1–4). In contrast, proteomic analysis immediately identified the underlying molecular phenotype (Fig 8). The apparent failure of RNA-level analysis to identify a defined, preexisting phenotype exposes the apparent limitations of RNA-focused analysis methods. While the failure of RNA-seq analysis to detect a specified phenotype is not necessarily surprising as it is common knowledge that gene regulation effects often are not translated into protein level effects, there are no true alternatives available at the time. Proteomic analysis, no matter whether done using antibody arrays or mass spectrometry, while powerful, requires relatively large amounts of cell material. Other than transcriptomic analysis which already offers acceptable coverage of genome-wide gene expression at the single cell level, proteomic analysis is likely trailing this success by more than a decade. The inability of current proteomic analysis to perform proteome-wide analysis at the single cell level or at least with small amounts of cell material presents a major

bottleneck to an immediate translation of our research approach to the study of latently infected T cells from patients or even relevant *in vitro* models of HIV-1 latency in primary T cells. Either way, the data clearly pinpoint a major shortcoming of the current RNA-level focused research efforts.

At the same time, the formal demonstration that heterogeneity at the gene expression and protein expression/phosphorylation level extends to functional diversity has implications for therapeutic strategies that seek to trigger HIV-1 reactivation in patients. The data imply that single drug interventions will be insufficient to accomplish complete viral reactivation and subsequent elimination. TCR/CD3 inertness was not the only functional consequence of the molecular changes that can occur in host cells of latent HIV-1 infected T cells. While we do not detail this phenomenon, we found that TLR pathway signaling can also be impaired (Fig 2F), which would have implications for efforts to identify HIV-1 reactivating agents that address the TLR/Myd88 pathway [33–37]. Differential reactivation responsiveness to varying stimuli, was actually paralleled by differential regulation of NF-κB dependent cellular genes, seen for the T cell activation markers CD38 and CD69 (Fig 3), and explained by the finding that while PMA would trigger an altered, but effective kinetic NF-κB signal in activation-responsive and -inert T cells, α-CD3/CD28 mAb stimulation would completely fail to trigger a NF-κB signal in the reactivation-inert JWEAU-C6 T cells, conclusively demonstrating that reactivation-

inertness is indistinguishable from activation-inertness and is strictly a cellular restriction phenomenon.

This would have theoretical implication for the design of future HIV-1 cure strategies. Given that our data detail the dysfunctional nature of latently HIV-1 infected T cells, and the seemingly random nature of observed changes, it needs to be assumed that the further upstream a NF-κB activating pathway is targeted, the less likely it is that an efficient, HIV-1 reactivating NF-κB signal is being triggered. The results thus support the idea that optimal HIV-1 reactivation agents should induce NF-κB activation by targeting proteins as immediate upstream of NF-κB as possible, which reduces the likelihood that the observed stochastic changes to the host cells affect drug efficacy. Pathways that activate NF-κB, but are essential to the long-term survival of the infected T cell could be most attractive, as changes to these essential pathways would result in rapid cell death after infection. This may make the CD3 or TLR pathways suboptimal targets for LRAs, unless possibly used in a combination therapy. By the same token, the extensive heterogeneity would favor the development of drugs like SMAC mimetics such as AZD5582 that also activate the non-canonical NF-κB pathway, but more importantly, they also target an important survival pathway for cells. SMAC mimetics imitate the activity of a protein call Second Mitochondrial-derived Activator of Caspases, a pro-apoptotic mitochondrial protein that is an endogenous inhibitor of a family of cellular proteins called the Inhibitor of Apoptosis Proteins (IAPs), which in turn are important regulators of cell death and

survival. The importance of such a pathway for long-term survival of the cells should make it less likely that this pathway is impaired in host cells of latent HIV-1 infection events. Functional conservation of this pathway would increase the likelihood of SMAC mimetics to broadly trigger HIV-1 reactivation within the population of latently HIV-1 infected T cells, despite the heterogeneity and pre-existing inertness in other pathways. SMAC mimetics, including AZD5582 have already been demonstrated to trigger HIV or SIV reactivation [93–95] and their potency may be increased in conjunction with drugs that restore the full signaling capacity of latently HIV-1 infected T cells.

Conceptually, the provision of experimental evidence that many host cells of latent HIV-1 infection events are extensively altered in their signaling networks and gene expression patterns and in extension, are altered in many of their functionalities, raises the possibility to affect the stability of latent HIV-1 infection events in these cells through an alternative approach. Therapeutic interventions that would restore host cell functionality may enable TCR/CD3 activation-inert latently HIV-1 infected T cells to properly respond to cognate antigen recognition, and the ensuing cellular increase in NF-κB activation would be sufficient to trigger HIV1 reactivation. Such a scenario would focus on the development of cellular reprograming strategies and remove the requirement for a potent, systemwide, stimulatory therapeutic intervention, which would here be provided by a natural process, specific antigen recognition.

Material and methods

Ethics statement

Human Sample disclosure and Information. Healthy, HIV-seronegative adults and HIV-seropositive subjects were recruited from the 1917 Clinic cohort at the University of Alabama at Birmingham to donate peripheral blood. All HIV-1 seropositive subjects were on ART and with undetectable viral loads (VL <50 copies/mL) for a median of 10 months (6.5–17.3 months). In accordance with the specific protocol for this project that was approved by the UAB Committee on Human Research (IRB-160715008), all subjects provided written informed consent for all biologic specimens and clinical data used in this study.

Cell culture and reagents

All T cell lines were maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat inactivated fetal bovine serum. Fetal bovine serum (FBS) was obtained from HyClone (Logan, Utah) and was tested on a panel of latently infected cells to assure that the utilized FBS batch did not spontaneously trigger HIV-1 reactivation [28,96]. The phorbol esters, Phorbol 12-myristate 13-acetate (PMA), prostratin, along with HMBA, and Flagellin were purchased from Sigma. Recombinant human TNF-α a well established trigger of HIV-1 reactivation was purchased from Gibco. The PKC activator Bryostatin and Sodium Butyrate (NaBu) were purchased from EMD Millipore. Vorinostat (SAHA) was purchased from Selleck

chemicals. Specific Inhibitors or recombinant proteins such as the STAT3 inhibitor S31-201 (NSC 74859), the Src inhibitor dasatinib, or the c-Raf inhibitor sorafenib were all purchased from Fisher Scientific. Dasatinib is approved for the treatment of chronic myelogenous leukemia and acute lymphoblastic leukemia. Sorafenib is approved for the treatment of certain kidney and liver cancers and certain forms of Acute Myeloid Leukemia. The selective STAT3 inhibitor S31-201 had been successfully used in several mouse cancer models [97,98]. Anti-CD3/CD28 antibody-coated beads (ImmunoCult) were purchased from Stemcell technologies (Vancouver, CA).

Model of HIV-1 latency in primary T cells

CD4+ T-cells were isolated from PBMCs from healthy donors using a negative CD4+ T cell isolation kit (Stemcell), and were then rested overnight in RPMI (10% FBS, 1% Pen/Strep, 1% L-Glutamine, and IL-2 (10 IU/ml). The T cells were stimulated with Immunocult (Stemcell) at a concentration of ~2 beads/cell and incubated at 37° for 3 days. On day three post stimulation the T cells were placed in fresh RPMI medium at a density of 5 million cells/well in a 6 well plate and infected with a HIV-GFP reporter virus (pBR43IeG-NA7nef) [99]. Infection using full-length replication competent viruses generally results in lower level reservoir formation than what can be accomplished by utilization attenuated HIV vectors that are often used by others [90,100], but fails to reproduce the impact of viral accessory genes on HIV-1 latency establishment [101]. After infection cells were pelleted by centrifugation and placed in fresh RPMI medium.

After 48 hours GFP levels were determined as surrogate marker of HIV-1 infection using flow cytometry to confirm successful infection. To prevent further viral replication reverse transcriptase inhibitors 3TC(lamivudine) and EFV(efavirenz) (NIH AIDS Reagent Program) were added and the cells were cultured for 30 days. Medium was replenished twice a week. After 30 days the T cell cultures were sorted using a BD FACSAria cell sorter to remove all remaining GFP+, and therefore actively infected T cells to reduce the signal background. Cells were then plated at a density of $5x10^5$ to $1x10^6$/ml into the described experimental conditions. 3 days post reactivation the cell cultures were analyzed for the frequency of GFP+ T cells using a BD LSRII flow cytometer acquiring at least 100,000 events per experimental condition.

Generation of latently HIV-1 infected T cell populations and T cell clones

The latently HIV-1 infected T cell populations were generated by infecting a Jurkat T cell-based reporter cell population (JR5D4 cells) [30] with the primary HIV-1 patient isolate WEAU [32] or the classic laboratory strain HIV-1 NL43. Starting two days post infection (3TC) was continuously added to prevent viral replication and the establishment of pre-integration latency. On day 4 post infection we selected cultures with infection levels around 40% were selected and maintained until day ~60 when active infection levels had mostly declined to levels between 1–5%. In the HIV-1 WEAU infected T cell population, addition of PMA revealed the presence of a reactivatable HIV-1 reservoir that varied in size between 4–10%. The HIV-1 WEAU infected bulk cell populations were

plated in a limiting dilution and tested for CD3 inertness and PMA responsiveness, to generate the differentially responsive cell clones JWEAU-A10 (responsive) and JWEAU-C6 (inert). To generate T cell populations that were enriched for latently HIV-1 infected T cells, we exploited the reported ability of host cells of latent HIV-1 infection events to repeatedly shut down the virus into a latent state. HIV-1 reactivation was triggered using PMA and on day 2 post activation cell sorting was performed for GFP+ T cells. Over a period of 3–4 weeks, active HIV-1 infection in these cells was again shut down into a latent state, resulting in an enriched population in which ~80% of the cells would hold reactivatable, latent HIV-1 infection events.

TransAM assays to resolve kinetic NF-κB response

T cells were stimulated with either PMA or anti-CD3/CD28 mAb combinations and cell material was harvested at different time points post stimulation (10x10e6 cells per time point). Nuclear extracts were generated and NF-κB p65 activity in the nuclear extracts of cells was determined using TransAM assays (Active Motif). All experiments were performed according to the manufacturer's instructions. TransAM assays measure the ability of activated NF-κB to bind to an NF-κB consensus sequence in solution, with a 5- to 10-fold-higher sensitivity than gel shift assays. At 0 and 24hrs post stimulation one million cells were harvested for activation marker staining, and compared to isotype controls.

Kinexus antibody microarray-based analysis

Kinome analysis to study protein expression and phosphorylation levels was done using Kinexus microarray analysis. 50 μg of cell lysate protein (~$5x10^6$ cells) from each sample or experimental condition were covalently labeled with a proprietary fluorescent dye according to the manufacturer's instructions (Kinexus, Canada). After the completion of the labeling reaction, any free dye was removed by gel filtration. After blocking non-specific binding sites on the array, an incubation chamber was mounted onto the microarray to permit the loading of two side by side samples on the same chip. Following sample incubation, unbound proteins were washed away. KAM-850 arrays detect 189 protein kinases, 31 protein phosphatases and 142 regulatory subunits of these enzymes and other cell signaling proteins. This array provided information on the phosphorylation state of 128 unique sites in protein kinases, 4 sites in protein phosphatases and 155 sites in other cell signaling proteins. KAM-900 chips are spotted in duplicates with over 870 antibodies. 265 pan-specific antibodies and 613 phosphosite specific antibodies. Each array produced a pair of 16-bit images, which are captured with a Perkin-Elmer ScanArray Reader laser array scanner (Waltham, MA). Signal quantification was performed with ImaGene 8.0 from BioDiscovery (El Segundo, CA) with predetermined settings for spot segmentation and background correction. The background-corrected raw intensity data were logarithmically transformed with base 2. Since Z normalization in general displays greater stability as a result of examining where each signal falls in the

overall distribution of values within a given sample, as opposed to adjusting all of the signals in a sample by a single common value, Z scores are calculated by subtracting the overall average intensity of all spots within a sample from the raw intensity for each spot, and dividing it by the standard deviations (SD) of all of the measured intensities within each sample [102]. Z' ratios were further calculated by taking the difference between the averages of the observed protein Z scores and dividing by the SD of all of the differences for that particular comparison. Calculated Z' ratios have the advantage that they can be used in multiple comparisons without further reference to the individual conditional standard deviations by which they were derived.

RNA-seq analysis

Total RNA was extracted using the Qiagen RNeasy mini kit. Genewiz (Plainfield, NJ) prepared cDNA libraries and performed sequencing. For RNA-seq data processing and analysis, raw paired reads were first adapter-trimmed from fastq files using TrimGalore! (http://www.bioinformatics.babraham. ac.uk/projects/trim_galore/). Reads were aligned to Hg19 using STAR [103], and count matrices were generated using HTSeq-count [104]. DESeq2 was used to normalize read counts (CPM) and analyze differential expression [105]. For the analysis of global transcriptional regulation, genes were considered differentially expressed if they had an adjusted *P*-value <0.01 by the likelihood ratio test. For pairwise comparisons, genes were considered differentially expressed if they had at least a 1.5-fold change, adjusted *P*-value < 0.01, and at least one signal > 250 CPM, as

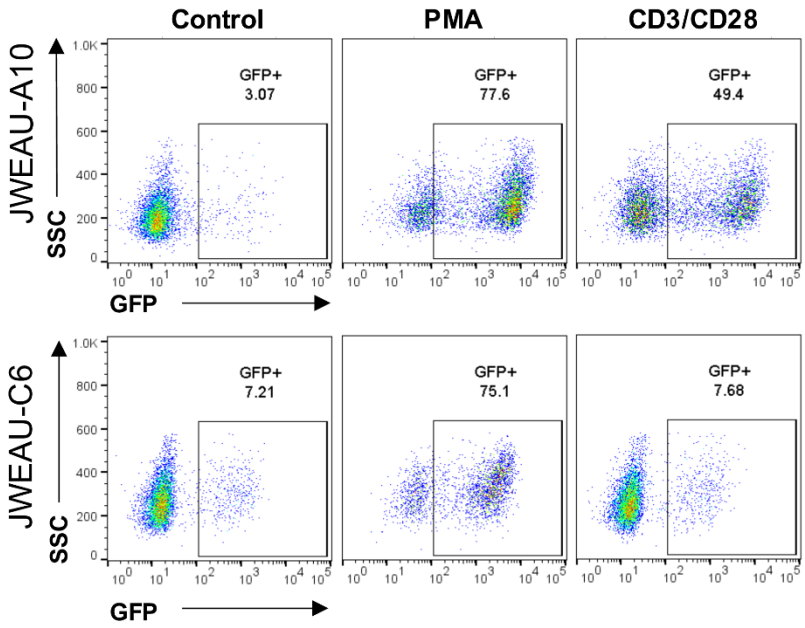
qPCR validation repeatedly failed to confirm lower signals. Pheatmap (*) was used to row-normalize, cluster (via hclust, using the complete linkage method), and visualize global transcriptional changes. Gene Ontology analysis was conducted using Metascape [43].

Network analysis

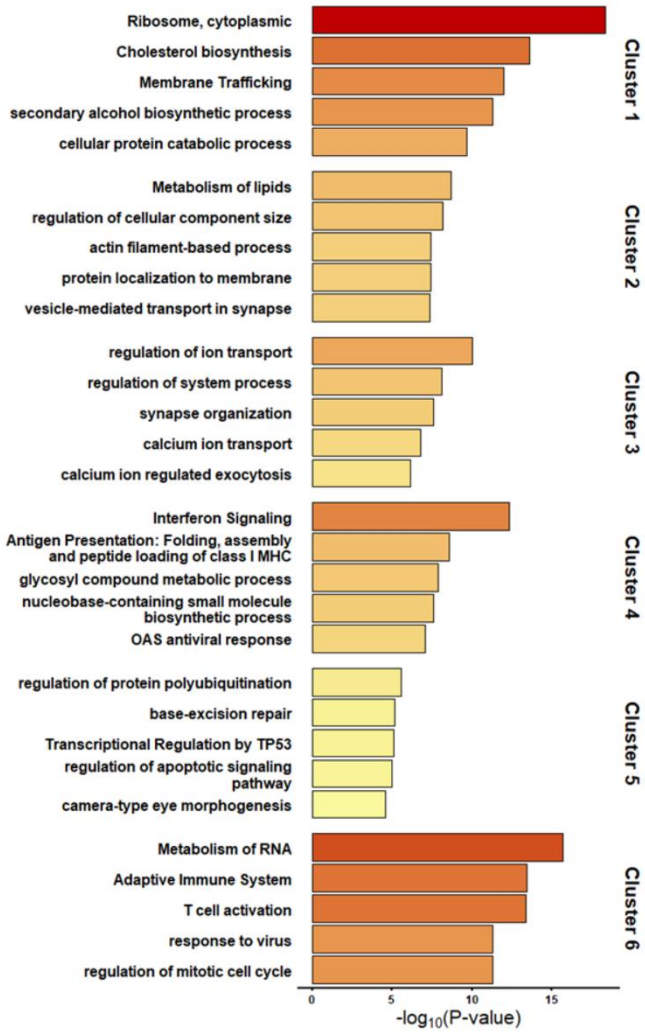
MetaCore software (Clarivate Analytics) was used to generate shortest pathway interaction networks that are presented. Pathway specific GO filters or tissue specific filters were used to prioritize nodes and edges. MetaCore was further used to identify pathway associations of the identified input dataset, using network enrichment analysis.

Statistical analysis

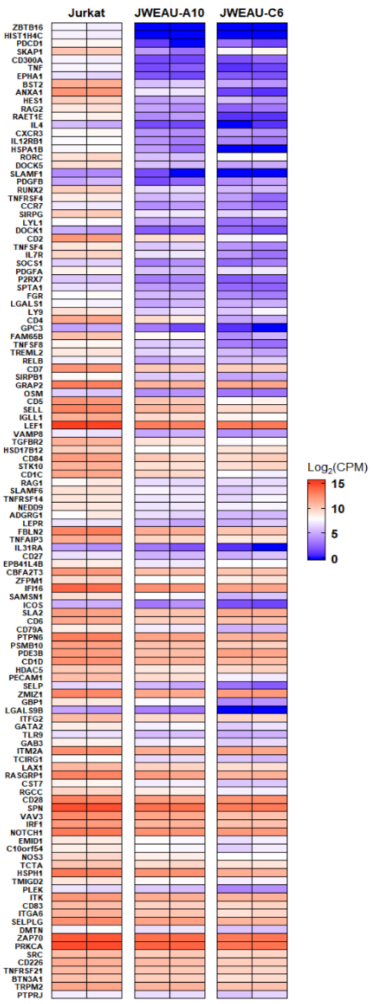
Statistical significance of differences between multiple experimental conditions was determined by ANOVA with multiple comparisons (Tukey's correction) (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$) or students T-test for comparison between two conditions.



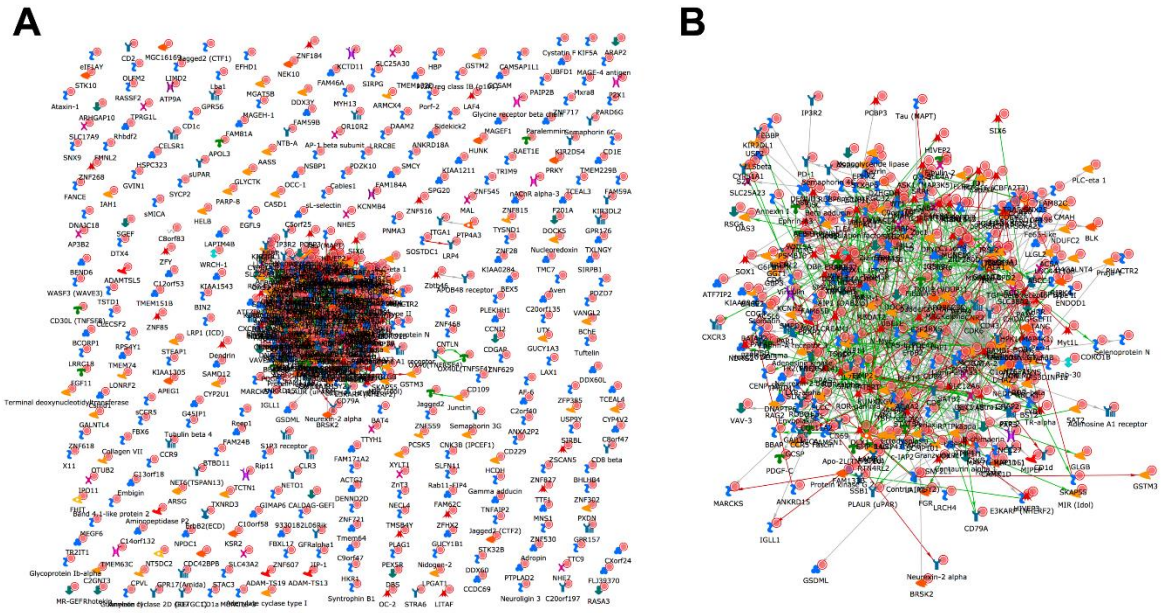
S1 Fig. Representative flow cytometric dot plot analysis of GFP expression as a surrogate marker of HIV-1 expression in the TCR/CD3-responsive JWEAU-A10 T cells and the TCR/CD3-inert JWEAU-C6 T cells at baseline (control) and following activation by α-CD3/CD28 mAb or PMA.



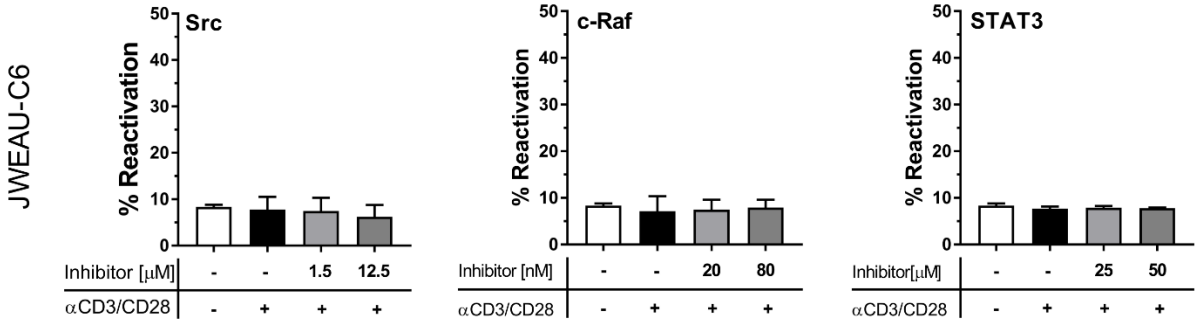
S2 Fig. Motif enrichment analysis of the genes in each cluster of the RNA-seq analysis data shown in Fig 5 was performed using MetaScape [43].
The top 5-ranked motifs for each motif are listed and the p-values for each motif are depicted as histograms.



S3 Fig. Focused analysis of the genes involved in T cell activation (GO term 0042110) from the RNA-seq data set presented in Fig 5 visualizes the presence of extensive difference between uninfected and latently HIV-1 infected T cells, while demonstrating the absence of relevant differences between the latently HIV-1 infected T cell clones in this pathway motif.



S4 Fig. Protein-protein interaction network for the RNA-seq data describing the shared transcriptomic features between activation-inert and activation-responsive latently HIV-1 infected T cells that differ from uninfected T cells.
(**A**) The depiction visualizes the lack of connectivity of a large portion of seed nodes (proteins) around a central interaction network. (**B**) Visualization of the core network hiding the unconnected genes.



S5 Fig. Targeting network hubs fails to restore reactivation responsiveness to TCR/CD3 stimulation in CD3-activation inert latently HIV-1 infected T cells.
To determine if Src, Raf and STAT3 inhibition would also affect or restore the TCR/CD3 responsiveness of JWEAU-C6 T cells, dasatinib (Src), sorafenib (Raf) or S31-201 (STAT3) were titrated on JWEAU-C6 T cells, which were then stimulated with αCD3/CD28 mAbs. HIV-1 reactivation was determined after 24h by flow cytometric analysis using GFP expression as a surrogate marker of active HIV-1 infection. Data represent the mean ± standard deviation of three independent experiments.

Supplemental Table 1: Network enrichment analysis of genes that are differentially expressed in JWEAU-A10 and JWEAU-C6 T cells.

Rank	Motif	p-value
1	Cardiac development - β-catenin, Notch, VEGF	3.74E-03
2	Leucocyte chemotaxis	3.77E-03
3	Cardiac development - FGF, ErbB signaling	3.83E-03
4	Cell adhesion: Platelet-endothelium-leucocyte interactions	9.31E-03
5	Cytoskeleton - Actin filaments	9.99E-03
6	Cardiac development - BMP, TGF-β signaling	3.27E-02
7	Development - Hedgehog signaling	3.47E-02
8	Development - Regulation of angiogenesis	3.75E-02
9	Immune response - Antigen presentation	4.91E-02
10	Proliferation - Lymphocyte proliferation	6.50E-02
11	Immune response - T helper cell differentiation	6.75E-02
12	Development - Neurogenesis synaptogenesis	7.63E-02
13	Regulation of epithelial-to-mesenchymal transition	8.89E-02
14	Development - Blood vessel morphogenesis	9.42E-02
15	Proteolysis - Connective tissue degradation	9.45E-02
16	Cytoskeleton - Macropinocytosis and its regulation	9.65E-02
17	Development - Neurogenesis in general	1.01E-01
18	Anti-Apoptosis mediated by external signals via PI3K/AKT	1.05E-01
19	Cell adhesion - Platelet aggregation	1.06E-01
20	Blood coagulation	1.23E-01

S1 Table. Network enrichment analysis of genes that based on RNA-seq analysis data are differentially expressed in the activation -responsive JWEAU-A10 T cells than in the activation-inert JWEAU-C6 T cells.

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NEF PROTEINS DIFFERENTIALLY GOVERN HIV LATENCY ESTABLISHMENT

by

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ABSTRACT

The ability of HIV-1 to persist in a latent state necessitates lifelong treatment of patients. Attempts to deplete the latent viral reservoir have been unsuccessful, which is largely the result of our limited biomolecular understanding of how latent infection events are established and maintained. We here investigated whether different lentiviruses would have a different capacity to establish latent infections. Consistent with reports that HIV-2 infection in patients has much lower viral RNA/viral DNA ratio than HIV-1 infection, we show that HIV-2 is more likely to remain in a transcriptionally silent infection state. Large parts of this phenomenon could be attribute to the Nef proteins of these viruses. In both T cell lines and primary T cells, replication competent HIV-1 vectors that expressed HIV-2 Nef would establish significantly higher levels of latent infection than the same HIV-1 backbone vector carrying a HIV-1 Nef gene. T cells that overexpressed HIV-1 Nef at the time of HIV infection were less likely to establish latent infection events than control T cells, while overexpression of HIV-2 Nef promoted latency establishment, suggesting that HIV-1 Nef drives active infection, while HIV-2 Nef actively promotes latency establishment. Given that Nef represents the only difference in these HIV-1 vectors, and that Nef is known to interact with numerous cellular factors, these data add further support to the idea that latency is primarily a host cell response phenomenon and therapeutic strategies aimed at curing HIV-1 infection must focus on the host cells of latent HIV-1 infection events.

INTRODUCTION

HIV-1 latency, the ability of HIV-1 to persist in a transcriptionally dormant state, is one, if not the ultimate challenge to be addressed by a curative therapy. HIV-1 latency has been comprehensively studied in CD4+ T cells, as it was initially thought that central memory T cells were the primary host cells of this reservoir. However, latent HIV-1 infection has recently been shown in other T cell types, including T follicular helper cells, Th17 cells and naïve T cells (1-7). At the biomolecular level, HIV-1 latency was initially explained by the presence of a restrictive chromatin environment (8-13). Subsequent findings that latent HIV-1 is predominantly integrated into actively expressed genes, coupled with the failure of clinical trials based on histone deacetylase inhibitors, served as clear demonstrations that latency was maintained in the absence of chromatin restrictions, leading to the development of other theories on latency establishment. Recently, increasing evidence has been presented that host cells of latent HIV-1 infection events can be altered to be activation inert. A milestone study was published by Ho et al. (14), in which the Siliciano team demonstrated that large parts of the viral reservoir in patient-derived T cells were actually resistant to activation, despite the absence of a restrictive histone code/chromatin environment at the viral promoter. In extension, we have demonstrated that in vitro HIV-1 infection alters T cell lines and primary T cells leading to activation inertness to TCR/CD3 mediated reactivation in a significant portion of the latent reservoir (15). It seems that host cell activation inertness would present a major obstacle to a shock

and kill strategy designed to eradicate the latent HIV-1 reservoir through reactivation of latent infection events. Therefore, a better understanding of the host cell response to HIV-1 infection that leads to latency establishment and/or the establishment an inert host cell phenotype and would be an important step towards addressing HIV-1 latency.

To this end, we first explored whether HIVs from the two lineages would have different latency establishment profiles. This possibility was suggested by early studies comparing HIV-1 and HIV-2 infections which found that HIV-2 produced a much lower viral RNA/viral DNA ratio than HIV-1. In other words, integrated HIV-2 infection events (vDNA) would produce significantly fewer viral particles (vRNA) that could be detected in the serum than what was seen in HIV-1 patients (16-18), despite similar frequencies of viral integration events (19). This could suggest that HIV-2 is more likely to produce latent infection events than HIV-1. Herein we show that, at least in T cell line-based model of latency, HIV-2 indeed produced increased levels of transcriptionally silent infection events when compared to HIV-1.

While HIV-1 and HIV-2 share many principal properties, there are many differences between these viruses, including the structure of the TAR element, LTR-TAR interaction, and several protein functions, that could account for these differential latency establishment profiles. Given that we have demonstrated that the activation status of the host cell at the time of infection is crucial for the ability of the virus to establish latent infection events (20), and an abundance of literature that describes differential of abilities of Nef proteins from HIV and SIV lineages to promote T cell activation, we decided to

explore a direct role for HIV-1 or HIV-2 Nef in latency establishment. Initially, HIV-1 Nef was thought to be a transcriptional repressor of HIV-1 LTR, though this was not confirmed (21). Since, Nef has been assigned several functions through interactions with the host cell proteome (reviewed in: (22)). Nef proteins from the HIV lineages, and related SIVs, have been shown to have different capacities to modulate TCR/CD3 availability on the cell surface altering the activation potential of infected T-cells (23). While Nef proteins are known to interact with Src proteins, a kinase family involved in T cell activation, it has been shown that HIV-2 Nef proteins target different Src family members than HIV-1 Nef. This phenomenon was later traced to a triple amino acid substitution in the SH3 domains, impacting signal transduction following activation (24). Nef interactions with NFAT and meditation of NFAT inhibition following TCR stimulation differ between HIV-1 subtypes (25). Even after stimulation, Nef has been found to enhance Tat-mediated viral transcription through a hnRNP-K-nucleated signaling complex (26) suggesting a post transcriptional role for Nef in latency regulation. By changing the cellular activation status or the availability of transcription factors essential for HIV expression, Nef could certainly affect latency establishment.

We here provide evidence that Nef functionalities are crucial to the establishment of HIV-1 latency, with HIV-1 Nef driving active infection, while HIV-2 Nef promoted HIV-1 latency establishment levels in T cell lines and primary T cells.

RESULTS

Differential latency establishment capacity of HIV-1 and HIV-2.

Previous reports suggested that in patients, relative to the detected viral DNA, HIV-1 produces higher levels of viral RNA than HIV-2 (16-18). This could suggest that HIV-2 may be more likely to produce transcriptionally quiescent or latent viral integration events. To test this idea, we infected a GFP-reporter T cell line (NOMI)(27), with either HIV-1 TYBE, HIV-1 SPL3, two clinical X4-tropic patient isolates, or with HIV-2 ROD, or HIV-2 7312A. On day 3, samples from each infection culture were either left unstimulated (control) or activated with PMA to trigger activation of transcriptionally inactive or latent integration events. As expected from previous studies (20, 28, 29), with increasing multiplicity of infection (MOI), active HIV-1 infection levels increased, and PMA stimulation revealed the presence of a matching reservoir of latent integration events (Figure 1A). Infection patterns for HIV-2 viruses were completely different. Despite increasing MOIs, active infection levels would not increase, and remained almost absent; however, the reservoir of latent integration events increased with increasing virus concentrations (Figure 1B). The latency establishment rate, which is calculated as the slope of a linear regression comparing active infection (X axis) and latent infection (Y axis), suggested that between 40-80% of the HIV-2 infection events integrated in a transcriptionally silent state, compared to only 2-5% of HIV-1 infections (Figure 1C). This

is consistent with the observation that HIV-2 is less likely to produce viral RNA following integration than HIV-1 (16-18).

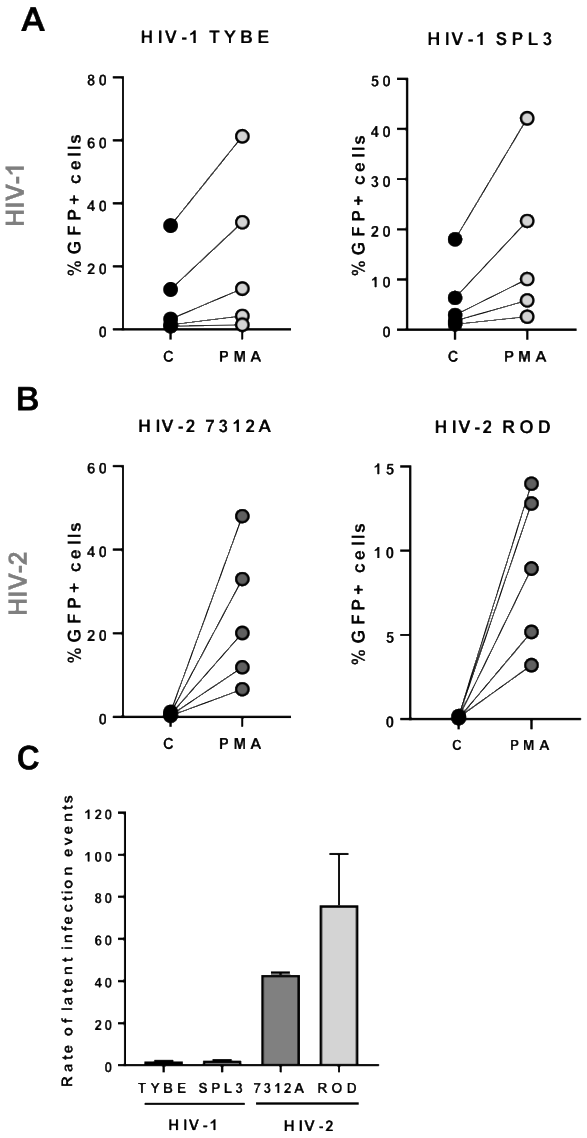

Figure 1: Differential latency establishment capacity of HIV-1 and HIV-2. NOMI GFP-reporter T cells were infected with increasing concentrations of either (**A**) HIV-1 TYBE, HIV-1 SPL3 or (**B**) with HIV-2 7312A, HIV-2 ROD. On day 3, samples from each infection culture were either left unstimulated (C) or activated with PMA to trigger activation of transcriptionally inactive or latent integration events. Samples were subjected to flow cytometric analysis to determine the percentage of GFP+, and therefore HIV-1 expressing T cells in each experimental condition. (C) The latency establishment rate, calculated as the size of the silent reservoir divided by the total infection over a range of infection levels.

We next sought to determine whether it would be possible to identify a specific viral factor responsible for the increased latency establishment levels seen in HIV-2 infections. We decided to focus on the possible role of Nef proteins, a decision that was primarily driven by our previous finding that host cell activation prior to infection prevents latency establishment (20) and by the multitude of reports that assign Nef proteins from HIV-1 and HIV-2 greatly different functionalities, including differential T cell activation effects (23). For this purpose, we tested the ability of a replication competent, full-length HIV-1 vector (pBR-NL43-IRES-eGFP-nef+) to establish latent infection when carrying a Nef gene derived from HIV-1 (Na7; nef1) or from HIV-2 (Ben; nef2) (23). For clarity, we will henceforth refer to these viruses as NL-Nef1 (pBR-NL43-IRES-eGFP-Na7) and NL-Nef2 (pBR-NL43-IRES-eGFP-Ben). To study whether a potential Nef effect on latency establishment would be a function of the viral lineage, we further used NL-Nef_{cpz} or NL-Nef_{smm} carrying SIV Nef genes that are phylogenetically related to HIV-1 and HIV-2, respectively (23)(Supplemental Figure 1).

In these experiments we infected Jurkat T cells with increasing concentrations of the HIV-1 vectors carrying different Nef genes. On day 2 post infection RT inhibitors were added to the infection cultures to prevent viral replication. On day 3 samples from each infection culture were stimulated with PMA and 24 hours later, control and stimulated samples were subjected to flow cytometric analysis to determine the relation between active infection and early latent reservoir establishment. At this time point, the size of the early latent reservoir established by NL-Nef2 (or NL-Nef_{smm}) (max. 40-50%), clearly

exceeded the reservoir size established by NL-Nef1 (or NL-Nef$_{cpz}$) (max at ~30%)(Figure 2A-D). Notably, for NL-Nef1 infections the early latent reservoir was depleted by increasing the MOI past an optimal concentration (Figure 2A; blue symbols). On day 14 post infection, the initial NL-Nef1 and NL-Nef$_{cpz}$ reservoirs had collapsed, leaving maximum reservoirs <10% while the maximum reservoirs for NL-Nef2 or NL-Nef$_{smm}$ were still ~40% or ~30%, respectively. For day 21, when the latent reservoir is stable in this experimental system, Figure 2E shows the massively greater propensity of HIV-2 lineage viruses to establish latent infection events. A more detailed comparison of spontaneously active infection levels of NL-Nef1 (Figure 2F) and NL-Nef2 (Figure 2G) on day 4 post infection, highlights that these differences could be explained by NL-Nef1 being more able to overcome a host cell intrinsic activation threshold. For both HIV-1 vectors, as MOIs increased, total infection levels (determined as % GFP+ T cells 24 hours following PMA treatment) converged to a maximum infection level around 60%. However, as NL-Nef1 MOIs increased, active infection levels converged towards this maximum saturation level, as this virus can establish increasing amount of active superinfection events (30), that for NL-Nef1 would conceal the initial latent infection event (Figure 2F). This was not the case for NL-Nef2, where even high MOIs that are expected to establish superinfection events are not capable of establishing active infections (Figure 2G). This more detailed analysis of NL-Nef1 and NL-Nef2 infection data shows that in this system the differences in latency establishment capacity of HIV-1 vectors carrying Nef proteins from different lentiviral lineages are related to differences in the ability to transition into a transcriptionally active

state and therefore likely due to the ability of the Nef proteins to alter the host cell activation state. However, these experiments cannot determine whether HIV-1 Nef is activating, HIV-2 Nef is inhibitory, or whether both scenarios are relevant.

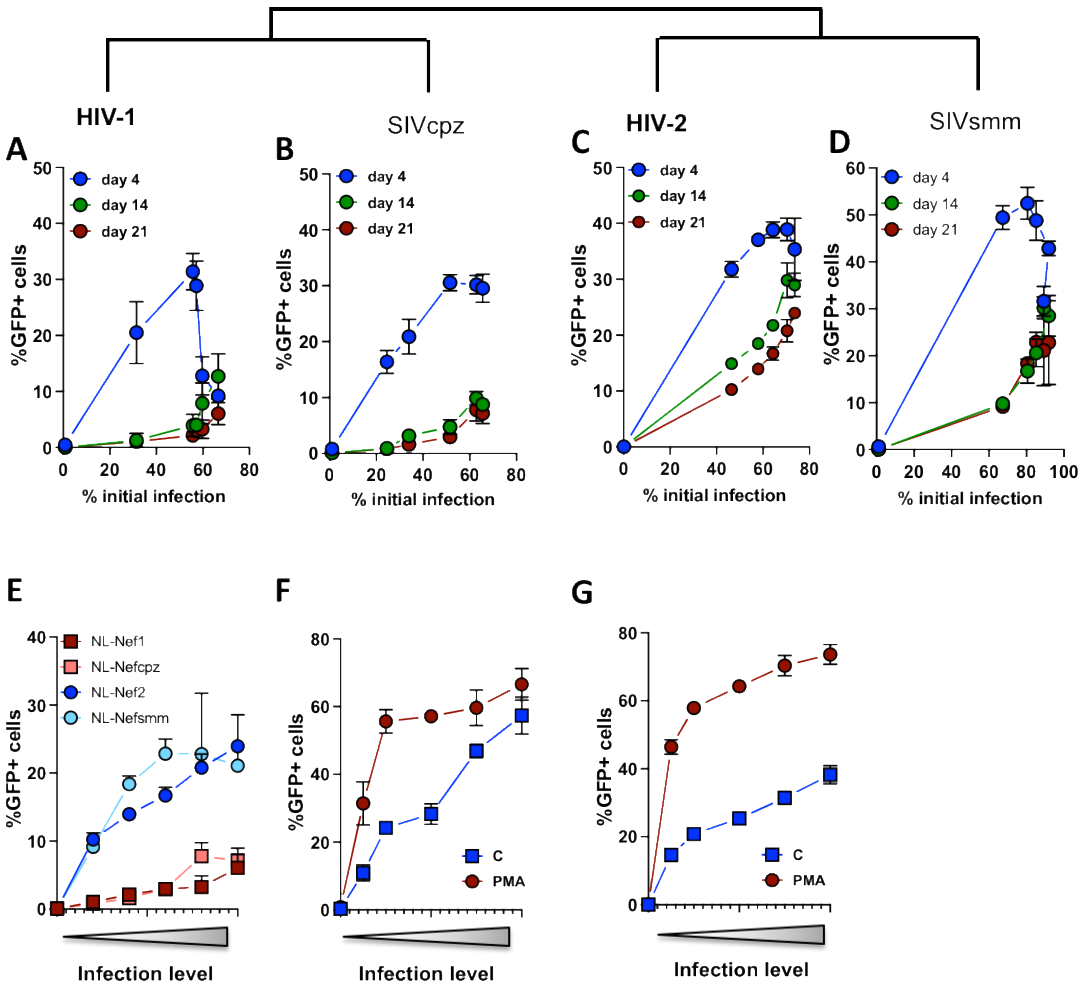

Figure 2: Differential effects of HIV-1 Nef and HIV-2 Nef on HIV latency establishment. Jurkat T cells were infected with increasing concentrations of the HIV-1 vectors carrying Nef genes from (**A**) HIV-1, (**B**) SIV_{cpz}, (**C**) HIV-2 and (**D**) SIV_{smm}. On day 3 post infection samples from each infection culture were stimulated with PMA and 24 hours later, control and stimulated samples were subjected to flow cytometric analysis to determine the relation between active infection and early latent reservoir establishment (blue symbols). This analysis was repeated on day 14 (green symbols) and on day 21 (red symbols). (**E**) On day 21 post infection, when the latent reservoir is stable in this experimental system, latent reservoir size is plotted over increasing initial active infection for HIV-2 lineage viruses (blue symbols) and HIV-1 lineage viruses (red symbols). On day 4 post infection a differential ability of (**F**) HIV-1 vectors carrying HIV-1 Nef and (**G**) the HIV-1 vector carrying HIV-2 Nef to establish active infections relative to the total infection level is observed.

HIV-1 Nef prevents and HIV-2 Nef promotes HIV latency establishment.

To determine the exact effect of HIV-1 and HIV-2 Nef gene products on latency establishment, we next transduced Jurkat T cells to stably express either HIV-1 Nef or HIV-2 Nef, using a MSCV-vector carrying mCherry as a reporter gene. Nef is known to downregulate CD4, so successfully transduced cells that produce functional Nef could be easily identified by their CD4 expression status (Figure 3A) and Nef expression was confirmed by RT-PCR (Figure 3A; inserts). Based on the expressed Nef allele, the T cell populations were termed J-Nef1 and J-Nef2, respectively.

J-Nef1 and J-Nef2 cells were next infected with increasing concentrations of either NL-Nef1 or NL-Nef2. Jurkat T cells that were transduced with an empty MSCV-mCherry vector were used as controls. To compare the capacity of J-Nef1 and J-Nef2 cells to establish latent HIV-1 infection independent of the actual infection levels, we calculated the rate of latency establishment for each individual infection experiment on day 4 post

infection. As seen in Figure 3B, relative to control cells, J-Nef1 cells produced lower levels of latent infection events when infected with NL-Nef1. This would be consistent with reports that suggest that HIV-1 Nef has activating effects on T cells. Interestingly, J-Nef2 cells produced higher levels of latent infection events, suggesting that HIV-2 Nef expression drives HIV-1 into a transcriptionally quiescent state. Similar analysis of the experiments using NL-Nef2 confirmed these findings (Figure 3C). Consistent with the results shown in Figure 2, infections with NL-Nef2 produced overall more latency. The presence of HIV-1 Nef in J-Nef1 cells also reduced the ability of NL-Nef2 to establish latency and, again, the presence of HIV-2 Nef prior to infection in J-Nef2 cells further boosted the already high capacity of latency establishment NL-Nef2. These data clearly demonstrate that Nef proteins are potent modulators of latency establishment.

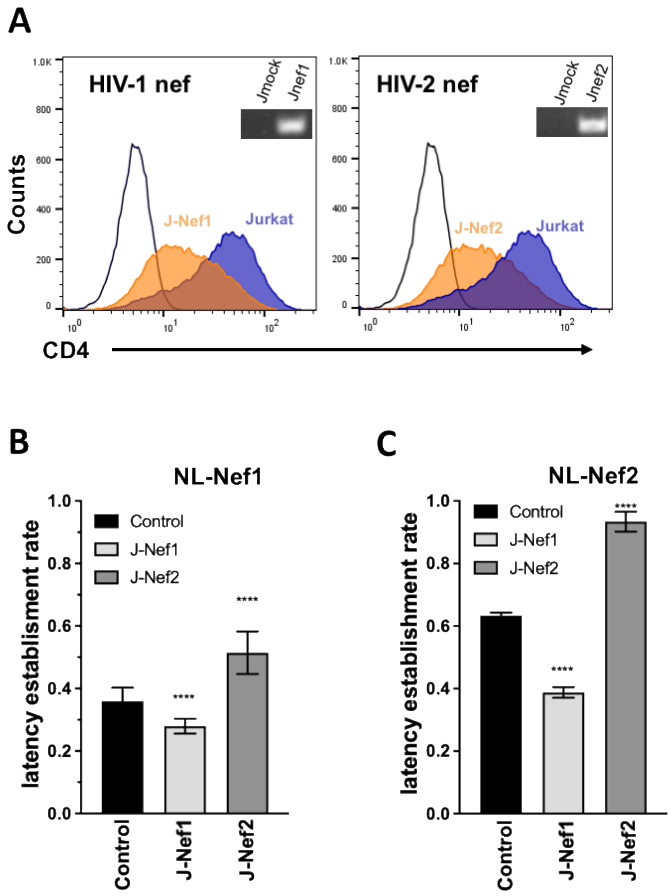

Figure 3: HIV-1 Nef prevents, and HIV-2 Nef promotes HIV latency establishment. (A) Jurkat T cells were retrovirally transduced to express HIV-1 Nef (J-Nef1 cells; left panel) or HIV-2 Nef (J-Nef2 cells; right panel). Transduction was confirmed by PCR for Nef mRNA (inserts) and functional Nef protein expression was demonstrated by down-regulation of CD4 expression as determined by flow cytometric analysis. J-Nef1 and J-Nef2 cells were next infected with increasing concentrations of either (B) NL-Nef1 or (C) NL-Nef2. Jurkat T cells that were transduced with an empty MSCV-mCherry vector were used as controls. To compare the capacity of J-Nef1 and J-Nef2 cells to establish latent HIV-1 infection independent of the actual infection levels, we calculated the rate of latency establishment for each individual infection experiment on day 4 post infection. Statistical significance determined by two-way ANOVA with multiple comparisons (***$p<0.001$, ****$p<0.0001$)

Nef proteins influence latency establishment in primary T cells.

In comparison to T cell lines, such as Jurkat T cells, in which latent HIV-1 infection events can be readily established (31, 32), primary T cells are relatively refractory to HIV-1 latency establishment. As such, we sought to determine whether the HIV-2 Nef effect on latency establishment would be sufficiently potent to also increase HIV-1 latency establishment in primary T cells. For this purpose, we used a model system established by the Siliciano group that is based on Bcl-2-transduced primary T cells (33). Briefly, we infected Bcl-2-transduced T cells with increasing amounts of virus (either NL-Nef1 or NL-Nef2), and on day 7 post infection, stimulated parts of the infection cultures with anti-CD3/CD28 mAb-coated beads (Immunocult). 24 hours later we determined latent infection events by flow cytometric analysis, defined as the increase in the percentage of GFP+ T cells in stimulated cultures over background infection in untreated cell cultures. As seen in Figure 4A, NL-Nef1 infections established relatively small latent reservoirs. Similar to what we observed for NL-Nef1 infections in Jurkat T cells, beyond an optimal infection level for maximum reservoir establishment, increases in viral inoculation doses actually decreased the size of the latent reservoir (Figure 2A). In contrast, reservoir size for the NL-Nef2 vector increased with higher infection levels, again recapitulating the observations in Jurkat T cells (Figure 2C). As seen in the experiments using Jurkat T cells, NL-Nef2 infection produced vastly higher levels of latent infection events in the primary T cell model. Most importantly unlike the NL-Nef1 infections, the absolute size of the latent NL-Nef2 reservoir continued to increase with the level of initial infection. The

latency establishment rate for NL-Nef2 was ~2.5-times higher than for NL-Nef1 (Figure 4B) confirming that Nef proteins play a major role in latency establishment even in a primary model. Given their superior ability to establish latent HIV-1 infection, NL-Nef2 viruses generated in the Kirchhoff laboratory could provide extremely useful tools to study the biomolecular mechanisms of HIV-1 latency in primary T cells.

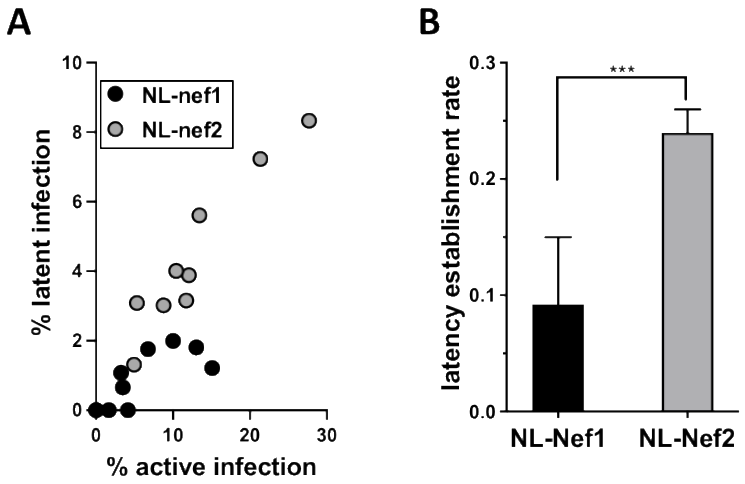

Figure 4: Nef proteins influence latency establishment in primary T cells. (**A**) Bcl-2-transduced primary T cells were infected with increasing amounts of virus (n=10; either NL-Nef1 or NL-Nef2), and on day 7 post infection stimulated parts of the infection cultures with anti-CD3/CD28 mAb-coated beads (Immunocult). 24 hours later we determined latent infection events by flow cytometric analysis, as increase in the percentage of GFP+ T cells in stimulated cultures over background infection in untreated cell cultures. (B) Latency establishment rate for NL-Nef1 or NL-Nef2 determined over the full range of infection levels. Statistical significance determined by two-way ANOVA with multiple comparisons (***p<0.001, ****p<0.0001)

DISCUSSION

Herein we demonstrate that the presence of Nef genes derived from different lentiviral lineages, in particular from HIV-1 and HIV-2, greatly affected the ability of HIV-1 vectors to establish latent HIV-1 infection events. Substitution of HIV-2 Nef for HIV-1 Nef in full-length, replication competent HIV-1 vectors greatly increased latency establishment efficacy in T cell lines (Figure 2) and primary T cells (Figure 4). The data further supported the conclusion that HIV-2 Nef actively promoted the establishment of latent HIV-1 infection, whereas HIV-1 Nef exerted an activity that would prevent latency establishment (Figure 3).

Amongst other functionalities of Nef proteins, such as their ability to trigger endocytotic cell surface removal of cellular proteins such as CD4, MHC I or CD3 (23, 34), it has been demonstrated that Nef can interact with host cell proteins involved in signal transduction pathways relevant to T cell activation (24, 25, 35, 36). Nef proteins from HIV-2 (and related SIV Nefs) were shown to would downregulate TCR/CD3 from the cell surface of infected T cells, effectively mitigating the ability of the primary T cell activation pathway to activate these cells while, HIV-1 Nef proteins and phylogenetically related SIV Nef proteins had lost this ability (23).

At the kinase level, HIV-2 Nef lacks the ability to bind to the SH3 domain of Hck, which is bound with high affinity by HIV-1 Nef (37, 38). In contrast, HIV-2 lineage Nefs bind with high affinity to the SH3 domains of Fyn or Src, kinases that HIV-1 Nef can only

weakly interact with (24). Mutational and structural studies have pinpointed relevant lineage dependent differences in Nefs that conclusively explain these findings (24, 39). As Nef is considered an early viral gene and Nef proteins are contained in the viral particle, it stands to reason that Nef proteins can modulate the activation state of the infected T cells from the moment of viral entry.

Given that we have demonstrated that HIV-1 latency establishment is heavily influenced by the activation status of the host cell at the time of infection (20), and that latency establishment in primary T cells seems to occur during transition to a resting, low-activation state (40), it is plausible that Nef proteins could alter latency establishment efficacy by modulating the activation state of the infected cell in the first hours following infection. This would certainly be supported by our experiments using stably Nef-transduced T cells that had altered latency establishment profiles (Figure 3). As Nef gene substitutions are the only manipulation in our experimental system, and in the absence of reports that would suggest that different Nef genes can cause an integration site bias, the data show that different lentiviruses have developed Nef protein-based mechanisms to either promote or prevent latent infections.

Future studies will aim at identifying means for how these findings can be used to destabilize an existing latent HIV-1 reservoir, but an immediate implication of our finding is that HIV-1 vectors carrying HIV-2 lineage Nef genes can be used to increase the efficacy of HIV-1 latency studies in primary T cells. The demonstrated ability of these hybrid HIV-1 vectors to establish oversized latent reservoirs provides an optimized model to study

HIV-1 latency in primary T cells (Figure 4) and should greatly facilitate biomolecular studies designed to develop efficient therapeutic intervention strategies to deplete the latent HIV-1 reservoir.

MATERIAL AND METHODS

Cell culture and reagents.

All T cell lines and populations were maintained in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat inactivated fetal bovine serum. Fetal bovine serum (FBS) was obtained from HyClone (Logan, Utah) and was tested on a panel of latently infected cells to assure that the utilized FBS batch did not spontaneously trigger HIV-1 reactivation (32, 41). Jurkat T cells were initially obtained from the ATCC (clone E6-1; ATCC TIB-152). The utilized NOMI reporter cells have been described earlier (27). Bcl-2 transduced primary T Cells were generated at previously described (33). Different from the original protocol, we utilized a pMSCV-puro based retroviral system to Bcl2 transduce primary T cells. Purity of the generated T cell population was ensured by staining for CD3+CD4+ T cells and CD3+CD4+ T cell enrichment at the time of experiments was >97%. The replication competent, full-length HIV-1 vector containing various Nef alleles (pBR-NL43-IRES-eGFP-Nef+) were generously provided by Dr. Frank Kirchhoff (University of Ulm, Germany) and can also be obtained through the NIH HIV Reagent Program. The CXCR4 tropic virus isolates

TYBE and SPL-3 were kindly provided by Dr. Collman (University of Pennsylvania), while HIV-2 ROD and HIV-2 7312A were made available to us by Dr. Beatrice Hahn (University of Pennsylvania). For all HIV-1 reactivation experiments GFP was used as a surrogate marker of HIV-1 expression (42, 43). GFP expression was routinely measured 24 or 48 hours post induction using flow cytometric analysis and data were presented as percent of GFP-positive cells. In all experiments where compounds or drugs were added to latently HIV-1 infected T cells, these compounds/drugs were not removed prior to the addition of a secondary stimulus. Flow cytometric analysis was performed on a GUAVA easyCyte^{tm} BGR HT (Luminex) or an LSRII (Becton&Dickinson). Data were analyzed using FlowJo software (Treestar, Ashland, OR). All antibodies were purchased from Becton Dickinson (Franklin Lakes, NJ). The phorbol ester Phorbol 12-myristate 13-acetate (PMA), was purchased from Sigma. ImmunoCult (anti-CD3/CD28 mAb coated beads) was obtained from STEMCELL Technologies.

HIV-1 latency establishment assays.

To determine latency establishment levels under defined experimental conditions, a series of individual cell cultures of Jurkat T cells, J-Nef T cells or primary Bcl-2 transduced T cells were infected over a range of multiplicities of infection (MOIs) using the indicated pBR-NL43-IRES-eGFP-Nef+ reporter viruses. HIV Inhibitors (3TC and EFV) were added at day 3 post infection to prevent multiple rounds of viral infection. To determine the size of the formed latent reservoir, a sample of each infection culture was taken and

stimulated overnight. Jurkat T cell experiments were stimulated with PMA overnight, primary T cells were stimulated with Immunocult due to high levels of cell toxicity cause by PMA in this model. After overnight incubation samples were measured for GFP expression using flow cytometry. In overexpression experiments (J-Nef1, J-Nef2) mCherry was used as a marker for Nef expression. Latent infection event percentages were determined by subtracting the active GFP background from the stimulated GFP values. Latency establishment rate is calculated using a linear regression of the percentage of latent infection over the active infection percentage.

Generation of stably Nef expressing Jurkat T cells.

Nef genes contained in pBR-NL43-IRES-eGFP-Na7 (HIV-1) and pBR-NL43-IRES-eGFP-Ben (HIV-2) were used as templates to generate the retroviral pMSCV-mCherry vectors used in the experiments, by cloning the PCR products into the Hpa1/EcoR1 (HIV-1 Na7 Nef) or Xho1/EcoR1 (HIV-2 Ben Nef) restriction sites of the vector. 5'Hpa1NA7nef-taccgttaacatgggtggcaagtggtcaaaa; 3'EcoR1NA7nef-attcgaattcctagcagtctttgtagtactccg; 5'Xho1BENnef-gtaactcgagatgggtgcgagtggatcca; 3'EcoR1BENnef-attcgaattcttattcactatatggtatccctcttgc). Retroviral vectors were generated as previously described (XX) and supernatants were used to transduce Jurkat T cells. Transduced Jurkat T cells were selected by mCherry expression and expression of functional Nef protein was confirmed by CD4 downregulation of the transduced mCherry+

Jurkat cells. Production of the correct Nef gene was confirmed by RT-PCR using primers that would not be cross-reactive. The Na7Fwd-5'- GTGGGGTTTCCAGTCAGACC / Na7Rev-5'-CATGCAGGCTCATAGGGTGT primer pair produced a 319bp amplicon for HIV-1 Na7 Nef and the BenFwd-5'- AAGCATTCGCGAGGACTACG / BenRev-5'-TTCCACAGCCACCCGAAGTA primer pair produced a 497bp amplicon for HIV-2 Ben Nef.

ACKNOWLEDGEMENTS

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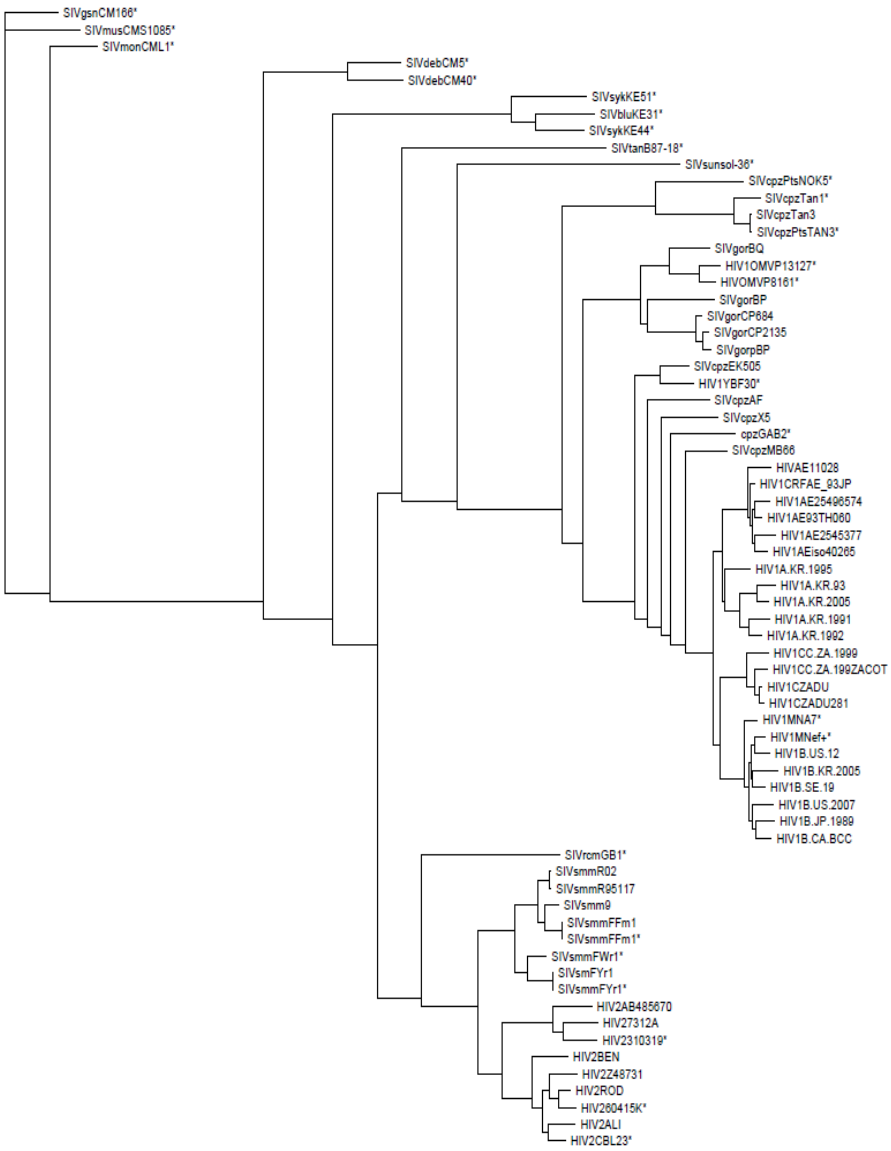

Supplemental Figure 1: Phylogenetic relationship of analyzed lentiviral Nef genes. Phylogenetic analysis of lentiviral nef gene sequences using a BioNJ starting tree with subtree pruning and regrafting with approximate Likelihood Ratio Test (aLRT) for branch support. Utilized nef genes are marked with red boxes.

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Conclusion of Findings

HIV-1 continues to be a worldwide epidemic, with roughly 37 million people living with HIV-1 infection and an additional 1 million or more new infections each year. Despite years of dedicated research and a vast array of attempted therapeutic approaches a functional cure for HIV has not been developed. Currently, ART allows for control of active viral replication but does not deplete the latent reservoir within the infected patient, requiring HIV+ individuals to adhere to treatment regimens throughout their lifetime. As described above (chapter 1.6 and 1.7), current approaches for depletion of the latent reservoir have failed to generate significant results in clinical trials, seemingly due to an incomplete understanding of the control mechanisms of HIV latency. In order to expand our understanding of HIV latency, we utilized a combination of studies into the molecular host cell phenotype during latent infection, and analysis of the role of viral proteins on latency establishment to reveal important biomolecular factors in the establishment and stability of latent HIV-1 infection.

In **Chapter 2,** we demonstrate the importance of the host cell phenotype on the stability of latent HIV infections. Our data show that a substantial portion of latent HIV-1 infection events have drastically altered cellular signaling networks rendering them unresponsive to TCR/CD3 stimulation, a finding that would explain the lack of reservoir depletion by cognate antigen exposure over the lifetime of an HIV+ patient. Our data show that reactivation inertness is not limited to the TCR/CD3 pathway but can also affect other signal transduction pathways that are currently targeted by therapeutic interventions. Due to the biomolecular heterogeneity found between host cells of latent

HIV infection events, activation responsiveness differs between individual reservoir cells, thereby limiting the effectiveness of “narrow spectrum” stimuli.

In addition to our host focused studies, in **Chapter 3** we demonstrate that the viral accessory protein Nef is involved in the establishment of HIV latency during the initial infection event in a lineage specific manner. Our data show that HIV-1 Nef drives active infection, while the presence of HIV-2 Nef favors the establishment of latent infection, demonstrating for the first time that HIV Nef impacts transcriptional outcomes early in infection. With the recent demonstration of viral uncoating in the nucleus, these findings also point to a critical role for proteins within the viral core on latency establishment

Collectively, these discoveries support a model where viral proteins alter the host cell during the initial infection event (Figure 3.1), altering the transcriptional fate of early infection (Figure 3.2) and the responsiveness of established latent events (Figure 3.3). Finally, this work suggests that expanding our understanding of the biomolecular mechanisms that control HIV latency well be critical to the future development of a successful HIV cure.

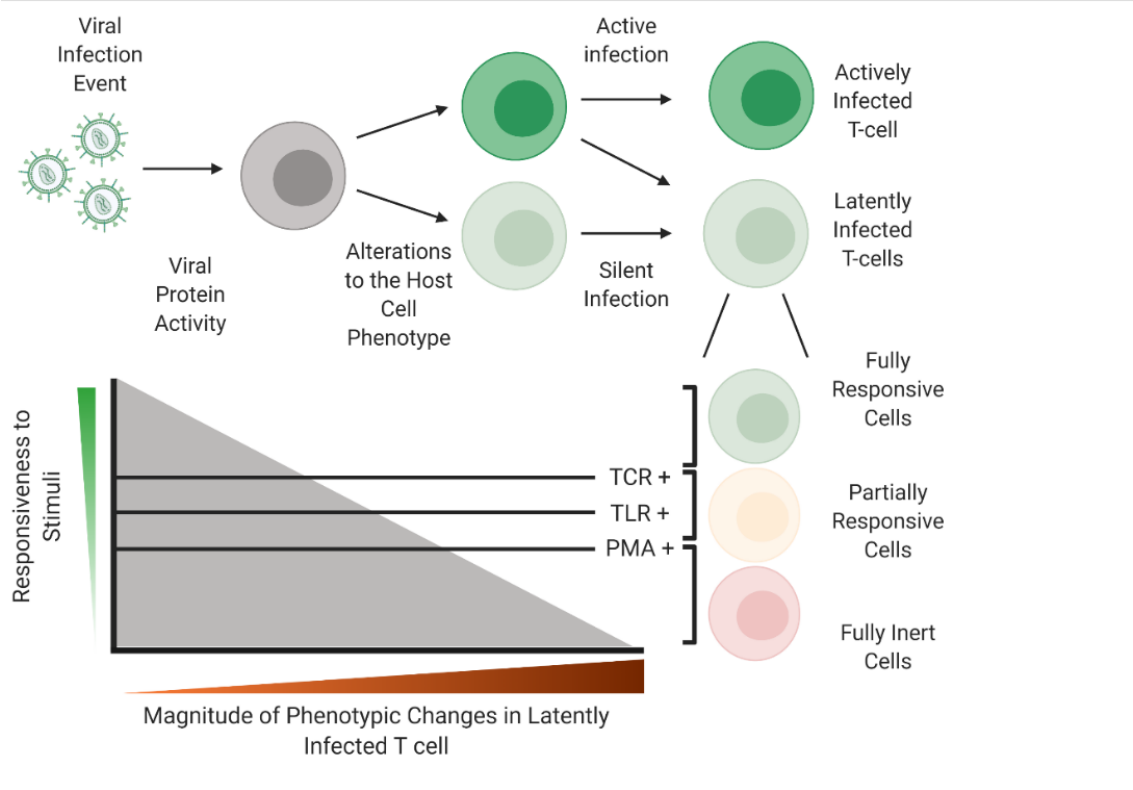

Figure 3. Consequences of viral infection.

Implications

While antiretroviral therapy fails to address the latent reservoir, it is a very effective therapeutic against HIV, drastically lowering viral load and preventing viral rebound in infected individuals. Therefore, any future cure strategies must balance off target effects and effectiveness in a way that is comparable to the impact of ART in HIV infected individuals. Current cure strategies that have shown the most promise such as the SMAC mimetics and TLR agonists have avoided major off target effects but have failed to diminish the latent reservoir. This likely due to both a lack of a dedicated kill step and the cellular inertness we demonstrated in chapter 2. Our findings show that cure strategies of the "Shock and Kill" conceptual framework will have to grapple with major differences

in cellular responsiveness between latently infected cells within each infected individual. As our work demonstrated these differences in responsiveness were due to alterations in the host cell, it stands to reason that other factors that are well characterized to impact viral infection such as cell differentiation state or activation state would also impact the responsiveness of downstream latent infections. When the entire latent reservoir is considered, we would expect differential responsiveness from cells infected in different contexts, an idea supported by the recent descriptions of heterogeneity with the responsive latent reservoir. Thus, "Shock and Kill" therapeutics will need to either address the host cell phenotype through an intervention aimed at the restoration of cellular responsiveness, or target pathways that do not establish inert reservoirs. However, the heterogeneity within the latent reservoir will complicate the design of an intervention aimed at restoring host cell responsiveness, as latently infected cells will each require unique interventions to restore cellular function. This makes any "Shock and Kill" approach that targets a pathway that can become inert in latently infected cells such as the TCR or TLR based interventions, therapeutically difficult and supports focusing on the discovery and therapeutic targeting of pathways that do not demonstrate inert latent infections. Until such a pathway is discovered, our findings support a "Block and Lock" approach as a more feasible cure strategy. As the "Block and Lock" approach is far more specific to the viral protein Tat and its function, it is far less dependent on the cellular environment. While not a sterilizing cure, prevention of viral rebound in support of ART may be enough for HIV infected individuals to maintain a healthy life, even through therapy interruption.

Additionally, the work presented in chapter 2 hints at loss of responsiveness as an additional mechanism of latent reservoir persistence. It's well established that the latent reservoir persists for the lifetime of a patient and that in long-term HIV infected individuals there is clonal expansion of latently infected cells, however how latently infected cells proliferate without immune intervention or HIV reactivation was unknown. Our work suggests that latently infected cells that are inert to forms of activation may bridge this gap, while we were not able to rescue completely inert cells, we were able to therapeutically enhance TCR induced reactivation in multiple systems. This suggests to us the inertness is far more common than previously thought and cellular environments can alter reactivation in a dynamic way such as in response to cytokine exposure. This would allow for cells to proliferate, without activating the latent HIV, bridging the gap between clonal expansion and latent reservoir persistence although further primary patient material studies must be done to validate this hypothesis. This therapeutic enhancement of TCR signaling also means these latently infected cells could still reactivate and produce virus under the right conditions, meaning they must be accounted for in future cure strategies as productive members of the latent reservoir.

In addition to the implications for current cure strategies, our work also presents new considerations for ART/PREP treatments and informs our understanding of the early stages of latency establishment. Our work in chapter 3 demonstrated that proteins within the viral core can directly influence the outcome of the initial infection event. These findings combined with the recent discovery that HIV uncoats within the nucleus near the site of viral integration, raises the possibility that the transcriptional outcome of infection can be modulated by altering the makeup of the viral core. By studying the nuclear

impact of proteins within the viral core on latency establishment, as we did with the Nef protein, it may be possible to identify other factors that greatly contribute to latency establishment and therapeutically prevent their incorporation into the viral core. This would help to reduce or prevent seeding of the latent reservoir during ART therapy interruption or early in HIV exposure, further increasing our ability to prevent latent reservoir formation.

In conclusion our work has expanded our understanding of how latent HIV-1 infections are established and reactivate after stimulation. While these findings raise concerns and challenges for future cure strategies, they have also opened new avenues for HIV latency research to progress and have provided both mechanisms and consequences for previous observations in the field.

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APPENDIX A

IRB APPROVAL

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APPROVAL LETTER
TO: Kutsch, Olaf
FROM: University of Alabama at Birmingham Institutional Review Board
Federalwide Assurance # FWA00005960
IORG Registration # IRB00000196 (IRB 01)
IORG Registration # IRB00000726 (IRB 02)
IORG Registration # IRB00012550 (IRB 03)
DATE: 18-May-2021
RE: IRB-140801006
Kinomic Analysis of Host Cell Factors Controlling Latent HIV-1 Infection
The IRB reviewed and approved the Continuing Review submitted on 12-May-2021 for the above referenced project. The review was conducted in accordance with UAB's Assurance of Compliance approved by the Department of Health and Human Services.
Type of Review: Expedited
Expedited Categories: 2, 7,
Determination: Approved
Approval Date: 13-May-2021
Approval Period: One Year
Expiration Date: 12-May-2022
The following apply to this project related to informed consent and/or assent:
Waiver of HIPAA
Waiver of Informed Consent
Documents Included in Review:
CONTINUING REVIEW EFORM
To access stamped consent/assent forms (full and expedited protocols only) and/or other approved documents:
1. Open your protocol in IRAP.
2. On the Submissions page, open the submission corresponding to this approval le□er.
NOTE:
The Determination for the submission will be "Approved."
3. In the list of documents, select and download the desired approved documents. The stamped consent/assent form(s) will be **APPROVAL LETTER**

TO: Kutsch, Olaf

FROM: University of Alabama at Birmingham Institutional Review Board

Federalwide Assurance # FWA00005960

IORG Registration # IRB00000196 (IRB 01)

IORG Registration # IRB00000726 (IRB 02)

IORG Registration # IRB00012550 (IRB 03)

DATE: 18-May-2021

RE: IRB-140801006

Kinomic Analysis of Host Cell Factors Controlling Latent HIV-1 Infection

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Type of Review: Expedited

Expedited Categories: 2, 7,

Determination: Approved

Approval Date: 13-May-2021

Approval Period: One Year

Expiration Date: 12-May-2022

The following apply to this project related to informed consent and/or assent:

Waiver of HIPAA

Waiver of Informed Consent