# INFECTION OF HEMATOPOIETIC PROGENITOR CELLS BY HIV-1

**by**

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## DEDICATION

*To my family and friends*

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## ABSTRACT

**Infection of Hematopoietic Progenitor Cells by HIV-1**

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HIV causes a chronic infection characterized by depletion of CD4+ T lymphocytes and, at late stages, severe bone marrow abnormalities. Despite the development of drugs that inhibit viral spread, HIV infection is difficult to eradicate because of uncharacterized cellular reservoirs that are resistant to antiretroviral therapy and the immune response. One possible mechanism that could account for viral persistence and hematopoietic dysfunction is the direct infection of hematopoietic progenitor cells (HPCs) by HIV. For this reason, we sought to determine if HPCs can be infected by HIV, and to understand the role of HPC infection in disease pathogenesis.

Surprisingly, we found that a subset of HPCs became actively infected following *in vitro* exposure to HIV. Actively infected HPCs up-regulated apoptosis markers and were rapidly lost from culture. Moreover, HIV infection was detected in primitive, multipotent HPCs. In some HPCs, HIV became latent and stably persisted in cell culture

until viral gene expression was activated by differentiation factors. A novel reporter HIV that directly detects latently infected cells *in vitro* confirmed the presence of distinct populations of active and latently infected HPCs.

In light of these findings, we initiated studies to evaluate the infection of HPCs in HIV-infected individuals. We found that some subjects had actively infected HPCs that were detectable by flow cytometry immediately following isolation. Conversely, other subjects did not have detectable active HPC infection after isolation, but virus could be activated from the cells by treatment with differentiation factors.

Finally, we wished to determine if different HIV isolates differed in their ability to infect HPCs. We found that virus isolates that utilize CXCR4 as an entry co-receptor were capable of infecting primitive HPCs, whereas viral isolates that utilize CCR5 were not. This finding is especially interesting considering that the emergence of CXCR4-utilizing isolates corresponds with clinical disease progression. Studies in mouse model systems are currently underway to determine if CXCR4-utilizing HIV is capable of infecting hematopoietic stem cells.

These findings have important implications for understanding HIV bone marrow pathology, and provide mechanisms that may help explain viral persistence.

# CHAPTER I

# INTRODUCTION

## HIV: A GLOBAL PERSPECTIVE

Despite increasing global efforts to combat the HIV, the magnitude of the pandemic continues to be staggering. It is estimated that 38.6 million people in the world were HIV positive at the end of 2005, and over 5 percent of people aged 14-24 are living with HIV worldwide. In sub-Saharan Africa, the epicenter of the AIDS pandemic, HIV prevalence has leveled off at extremely high levels, due partially to anti-AIDS efforts but also due to increases in AIDS-related deaths. Some of the most severely affected nations include South Africa (18.8% HIV positive), Botswana (24.1%) and Swaziland (33.4%). Despite the stabilization of HIV prevalence, the actual number of people with HIV continues to rise, largely due to population growth. Equally disconcerting is the emergence of new HIV epidemics far from Africa, particularly in eastern Europe and Asia (Figure 1-1).

Despite the dismal statistics, AIDS prevention and treatment efforts are proving they can be successful. Several nations including Kenya, Zimbabwe, Haiti, and the Caribbean have seen declines in HIV prevalence corresponding with increased condom

use and safer sex practices. Additionally, global HIV funding and the availability of antiretroviral drugs have increased dramatically, owing largely to the establishment of global and national AIDS programs. Despite these improvements, the need for HIV education, condoms, and antiretrovirals strongly outweighs availability in most nations.

To successfully bring the HIV pandemic under control, continued and improved action will be needed on numerous fronts. Some of the most basic needs are social, including improvements in human rights, combating poverty and decreasing the negative stigma and discrimination associated with HIV. It is also of great importance to provide underprivileged nations with the means to prevent and treat HIV by improving education, condom and needle availability, and the availability of antiretroviral drugs. Finally, it is crucial that nations which have the resources to do so continue to improve the understanding of HIV disease and to develop better tools for HIV treatment and prevention [1].

## HIV Biology

### Viral structure

HIV is a retrovirus belonging to the family *Lentiviridae*. Structurally, the virus consists of a protein nucleocapsid surrounded by an envelope derived from host cell plasma membrane. The capsid contains 2 similar copies of the plus-strand RNA genome,

which is approximately 9 kilobases in size. The genomic organization of HIV-1 can be found in Figure 1-2.

**Viral entry**

Two viral proteins, gp120 and gp41, are encoded by the HIV envelope gene (*env*), and facilitate entry of the virus into host cells. The first step of viral entry occurs when HIV gp120 binds to CD4 on the target cell surface. CD4 expression alone is not sufficient for membrane fusion to occur; the virus must subsequently bind a coreceptor on the target cell surface. Binding to the coreceptor molecule triggers a conformational change in the HIV gp120/gp41 complex, which exposes gp41, triggering fusion of the viral and host cell membranes (reviewed in Ray 2006 [2]). The first and best characterized coreceptors are chemokine receptors CXCR4 and CCR5 [3-7]. All HIV isolates characterized to date are capable of using one or both of these receptors, and the coreceptor preference of a particular isolate is partially responsible for the cell types it can infect (discussed in detail below). HIV coreceptor usage is not limited to CXCR4 and CCR5, however, and other chemokine receptors have been shown to have coreceptor function, including CCR2B, CCR3, CCR8, CCR9 and CX3CR1 (reviewed in [8]). However, the importance of these coreceptors *in vivo* is questionable [9].

**Reverse transcription**

After fusion, the viral nucleocapsid is released into the cytoplasm. Uncoating of the nucleocapsid releases the RNA genome of the virus, and reverse transcription by the virion-packaged reverse transcriptase enzyme commences. During this process, viral

RNA is converted into a terminally redundant cDNA, which is the substrate for HIV integrase. The complexity of the reverse transcription process is necessitated by the fact that the viral genomic RNA is a transcript derived from an integrated provirus, and thus lacks the 5' U3 and 3' U5 regions of the long terminal repeats (LTRs) at the viral RNA termini, both of which must be regenerated during reverse transcription. HIV reverse transcription begins using a host-derived tRNA primer, which binds the HIV PBS and primes reverse transcription of the 5' viral RNA terminus. As reverse transcription proceeds, RNaseH degrades the RNA template. cDNA synthesis halts when the 5' end of the viral RNA is reached, and this product is termed strong stop cDNA (SS-cDNA). This cDNA then dissociates from the 5' region of the viral RNA and anneals to the 3' region, using homology between the R regions of the RNA termini. The SS-cDNA primes reverse transcription of the envelope, polymerase and gag portions of the vRNA. A complete 5' LTR has been regenerated at this point, but the 3' LTR is absent. Second strand synthesis then begins, generating a DNA strand complementary to the first strand cDNA. This second strand dissociates after reverse transcribing the PBS, and anneals to the truncated 3' end of the first strand. At this point, DNA synthesis can proceed in both directions, generating a complete cDNA with identical LTRs. The PBS-*gag* junction is the very last section to be synthesized, and thus is only present in completed reverse transcripts and integrated [10].

**Integration**

After reverse transcription has completed, a pre-integration complex containing viral cDNA, viral integrase and host proteins migrates to the nucleus. The viral integrase

then coordinates components of the host DNA repair machinery and the host LEDGF/p75 protein to facilitate integration of linear vDNA into the host genome [11]. Interestingly, the provirus selectively integrates into gene-rich, transcriptionally active regions of the host genome [12]. Although linear reverse transcripts are the substrate for integration, circularized forms of vDNA called 2LTR circles are also generated. 2LTR circles are believed to be a "dead-end" product, but they are notable because they are only formed within the nucleus, and can thus be used as a marker for successful nuclear import of the preintegration complex [13].

**Viral gene expression**

Upon integration into the host genome, HIV genes are transcribed by host cell machinery. The virus contains its own promoter/enhancer region, located within the LTR. The basal promoter, enhancer and a modulatory region are found within the U3 portion of the LTR, and the transactivation response element (TAR) is located within the R region of the LTR. HIV lacks a viral RNA polymerase and transcription initiation factors, and thus relies upon those found in the host cell. The HIV LTR binds many host-derived positive transcriptional regulators, the best-studied of which are AP-1, SP-1, NF-κB, and NF-AT (Figure 1-2). However, detailed studies of the LTR have revealed dozens of host transcription factors that may regulate the LTR promoter, both positively and negatively (reviewed in [14]). Interestingly, host transcription factor binding is insufficient for high level viral gene expression, which requires the viral protein Tat. In the absence of Tat, transcription initiates but elongation is very weak, and most RNAs generated are truncated. Many studies of Tat have revealed that it has a unique role as a

transcription elongation factor. Tat binds the TAR region of the HIV LTR, where is recruits CycT and Cdk9. Cdk9 in turn phosphorylates the RNA polymerase II C-terminal domain, allowing for efficient elongation (reviewed in [15]).

Efficient HIV transcription generates a single, full length transcript. To maximize genomic space, HIV uses alternative splicing to generate numerous mRNAs, which are fully processed and competent for nuclear export and translation. However, full-length unspliced RNA is the source of message encoding the *gag* and *pol* genes, and serves as the source of viral genomes for packaging into new virions. Since unspliced RNAs are inefficiently exported from the nucleus, HIV has adapted a specific transport system. The viral protein Rev binds the rev response element (RRE), which is a *cis*-acting domain found within the *env* region of the full-length viral RNA. Rev then recruits the cellular proteins Exportin-1 and Ran-GTP, which facilitate nuclear export of the ribonucleoprotein complex (reviewed in [15]).

**Viral assembly and release**

HIV Gag protein is the primary coordinator of viral assembly. In fact, the Gag p55 precursor alone is capable of inducing the production and release of virus-like particles (VLPs), even in the absence of envelope or other HIV proteins. Assembly begins when Gag p55 multimerizes. Multimeric Gag p55 is specifically targeted to the plasma membrane in infected cells. As they move to the plasma membrane, Gag complexes bind to numerous other molecules needed to generate infectious particles, carrying them to sites of viral assembly. Important Gag cargo include newly synthesized viral genomes and the viral proteins Env, Vif, Vpr, and the Pol precursor, all of which

must be packaged in newly forming virions. Additionally, Gag is responsible for selectively packaging certain host proteins. CyPA, for instance, is packaged into virions and is needed for virion infectivity. The aggregation of Gag complexes at the plasma membrane induces a curvature in the membrane, visible as an electron dense "bud" by electron microscopy. The process of pinching off viral buds is incompletely understood, but it is believed that Gag recruits cellular endosomal sorting proteins (e.g. Tsg101) to facilitate the process [16].

The released viral particle is immature and non-infectious; it is relatively unorganized and consists of radially-organized Gag complexes surrounded by envelope protein-enriched plasma membrane. After release, the viral protease (part of the Pol polyprotein) is activated, and cleaves Gag into 3 separate proteins: the nucleocapsid protein (p7, NC) binds and condenses the viral RNA, the capsid protein (CA, p24) forms a cone-shaped shell around the viral RNA, and matrix protein (MA, p17) lines the inner leaflet of the viral membrane. Only after this maturation process does the virion acquire infectivity (reviewed in [16]).

**HIV accessory genes**

HIV encodes several accessory genes which function in promoting the viral life cycle and modulating host biology. These genes, *nef, vif, vpr, and vpu*, encode small proteins that are remarkably multifunctional, allowing the virus to control many aspects of host cell biology with minimal genomic size.

The HIV *vpr* gene encodes a basic 96 amino acid protein. Vpr is specifically incorporated into HIV virions via its interaction with p55 Gag, and 200-300 Vpr

molecules are found per virion. Vpr has been shown to increase the fidelity of reverse transcriptase, possibly by recruiting the cellular uracil DNA glycosylase UNG2. Lentiviruses, unlike oncoretroviruses, can infect non dividing cells, necessitating a means to transport the preintegration complex (PIC) through an intact nuclear envelope. Although the details are poorly understood, Vpr clearly plays a role in the nuclear transport of the PIC. Vpr is also capable of regulating the cell cycle, causing a G2 arrest in infected T cells. This is thought to enhance viral gene expression, since the HIV LTR is most active during G2. Finally, Vpr has the ability to induce apoptosis in infected cells and in uninfected bystander cells. The details and clinical significance of this activity are topics of active investigation (reviewed in [17]).

The HIV *vpu* gene encodes a 9kDa protein important for the release of infectious HIV particles. Firstly, Vpu recruits members of the ubiquitination machinery, causing degradation of newly-synthesized CD4 molecules. This, in cooperation with Nef-mediated CD4 endocytosis, depletes the plasma membrane of CD4, preventing the superinfection of infected cells and preventing virions from fusing with one another following release (reviewed in [18]). It was recently discovered that Vpu also counteracts a cellular antiviral protein called Tetherin (Bst2). In the absence of Vpu, Tetherin prevents the release of viral particles, leading to the accumulation of virions on the cell surface [19].

The HIV *vif* gene encodes a 192aa protein that is expressed in infected cells and is incorporated into newly forming virions by its association with p55 Gag. It has been known for some time that Vif is needed for HIV to infect T cells and macrophages, and that some cell lines can be infected with Vif-deleted HIV whereas others cannot.

Recently it was discovered that Vif binds a cellular RNA-editing enzyme, APOBEC3G, targeting it for ubiquitin-mediated proteosomal degradation. In the absence of Vif, APOBEC3G is incorporated into virions, rendering the particle non-infectious. In the absence of Vif, APOBEC inhibits reverse transcription, deaminates viral deoxycytosine residues, and leads to accelerated degradation of the viral genome. Thus, Vif represents a crucial countermeasure to a unique host innate defense mechanism (reviewed in [16, 18].

The HIV *nef* gene encodes a 27kDa protein that modulates numerous host cell functions by acting as a cellular adaptor protein. As mentioned above, Nef modulates the surface expression of several proteins, the best-characterized of which are MHC Class I and CD4. HIV Nef targets MHC-I molecules early in the secretory pathway by recruiting the cellular trafficking adaptor AP-1, ultimately redirecting class I molecules for degradation rather than cell surface expression [20], [21]. In contrast, CD4 reaches the cell surface in infected cells but is rapidly removed from the cell surface by Nef, by mechanisms incompletely understood. These activities benefit the virus by protecting infected cells from CTL lysis and preventing superinfection of infected cells. Nef has also been shown to regulate cellular activation. Nef promotes activation of T cells by binding cellular PAK-2, generating a cellular environment more conducive to HIV replication (reviewed in [18]). T cell activation by Nef may be one of the critical pathogenicity factors that distinguishes HIV from less pathogenic primate lentiviruses, which actually inhibit T cell activation [22]. Nef also activates the CD40L signaling pathway in macrophages, stimulating them to release chemokines that in turn attract HIV-susceptible CD4+ T cells to the infected macrophage [23]. Finally, Nef has been

implicated in modulation of the host cell actin cytoskeleton and in cellular cholesterol trafficking (reviewed in [18]).

## CLINICAL ASPECTS OF HIV INFECTION

### Natural course of disease

In the first days to weeks after exposure, viral replication rises rapidly as the virus replicates in macrophages and CD4+ T cells. Typically, a decrease in circulating CD4+ T cell counts occurs during this period of poorly controlled replication. Within several weeks, the host's immune system responds with effective cellular and humoral responses. Neutralizing Anti-HIV antibodies and HIV-specific cytotoxic T lymphocytes (CTLs) effectively reduce the serum HIV load, and CD4 T cell counts frequently recover to normal levels. Unlike the vast majority of viral infections, HIV is not cleared from the host. Rather, the virus and immune system engage in an ongoing adaptive competition, which results in low-level viral replication and gradual depletion of CD4+ T cells. During this asymptomatic phase of disease, the level of persistent viremia varies between patients as well as within individuals over time. After a period of time ranging from months to decades, the CD4+ T cell count falls to levels that result in generalized immune system dysfunction. When T cell counts fall below 400/μL, patients may begin to develop constitutional symptoms and early opportunistic infections. Below 200/μL, severe opportunistic infections occur, and most patients succumb to opportunistic infection or neoplasms within 2-3 years of symptom onset.

**HAART therapy**

The advent of highly active antiretroviral therapy (HAART) has allowed for dramatic extension of the asymptomatic phase of disease in many patients. This clinical approach employs combinations of anti-HIV drugs to suppress viral replication, primarily reverse transcriptase inhibitors and protease inhibitors. By targeting several steps of viral replication simultaneously, the emergence of resistant mutants can be minimized. Patients can be maintained on such regimens for many years, and viral levels are typically extremely low or undetectable. However even with effective therapy, most patients eventually progress to disease. Additionally, many patients develop severe side effects after prolonged periods of HAART therapy [24]. New classes of antiretroviral drugs have recently become available for clinical use, including entry inhibitors that target CCR5 and integrase inhibitors. These newer drugs may help combat the emergence of treatment-resistant viral mutants, and may also carry less of the side effects associated with older antiretrovirals [25].

**HIV coreceptor usage and disease progression.**

HIV coreceptor usage varies during the course of disease and has important clinical correlates. Despite expression of both CCR5 and CXCR4 on many HIV target cells, the initial transmission of HIV occurs by viruses that utilize the CCR5 coreceptor (R5-tropic HIV) [26, 27]. Multiple mechanisms likely underlie the selective transmission of R5-tropic HIV, and together are referred to as the "gatekeeper effect." For instance, the antigen presenting cells that are thought to first encounter HIV at mucosal sites are more susceptible to R5-tropic HIV infection. Thus, the initial infection event may

preferentially occur via R5-tropic HIV. After the initial infection occurs, the majority of viral replication occurs within the mucosal associated lymphoid tissues (MALT) of the intestines. The lymphocytes found in the MALT express more CCR5 than CXCR4, and thus MALT HIV replication may be another contributing mechanism to the gatekeeper effect (reviewed in [28]).

As disease progresses, many patients develop CXCR4-utilizing (X4-tropic) HIV isolates and HIV isolates capable of using both coreceptors (R5X4-tropic). The mechanisms that account for the emergence of X4 and R5X4-tropic HIV are unclear, but likely involve a complex mix of virologic, immunologic and environmental selective pressures. Interestingly, only 2-3 amino acid substitutions are needed to convert R5-tropic envelopes to X4-tropism, and yet X4-tropic HIV emerges only late in disease (reviewed in [29]). It was initially estimated that 50% of patients developed X4-tropic HIV, but more recent genetic approaches have revealed that this may be an underestimate, and one study detected X4-tropic HIV in all subjects studied, emerging most frequently during the intermediate stages of disease [30].

The emergence of X4- and R5X4-tropic HIV has been shown to correlate with disease progression and is associated with a poor clinical prognosis [31, 32]. One study found that in rare instances when R5X4-tropic HIV initiates infection, the infected individuals display rapid CD4 depletion and clinical progression to AIDS [33]. This suggests that the X4 coreceptor utilizing HIVs may contribute to disease progression. The mechanisms that underlie the disease progression that is associated with the presence of X4-tropic HIV remain a major mystery of HIV pathogenesis.

## CELLULAR TARGETS OF HIV INFECTION

### Viral coreceptor tropism

As mentioned above, HIV can utilize CXCR4 or CCR5 as a coreceptor for entry [3, 5]. Therefore, it is not surprising that cellular coreceptor expression is a major determinant of HIV susceptibility in various cell types. Another level of complexity is that HIV envelopes vary in their ability to utilize the coreceptors. Some viral isolates may use CCR5 but not CXCR4, others may use CXCR4 but not CCR5, and others may utilize both. HIV isolates are often categorized by their coreceptor usage (tropism) and are termed R5-tropic, X4-tropic or X4R5-tropic (dual tropic). Because of the differences in tropism, certain HIV isolates may be able to infect a certain cell type, whereas others may not.

### CD4+ T cells

The first identified and best characterized targets of HIV are activated CD4+ T cells. Activated T cells express high levels of CD4, CXCR4 and CCR5, making them targets for X4, R5, and dual tropic (R5X4) viruses. X4 viruses are sometimes referred to as "T cell tropic," and R5 viruses are frequently referred to as "macrophage tropic", but this nomenclature is misleading, as primary CD4+ T cells express both coreceptors and can be infected by either viral envelope. Infection of activated T cells with HIV is rapidly cytotoxic. Shortly after infection, apoptosis is induced in the infected cell, leading to cell death [34, 35]. It has been estimated that the lifespan of an infected T cell

blast is approximately 2.2 days, demonstrating the rapid cytotoxicity of HIV in T cells [36]. Additionally, a bystander apoptosis effect takes place, wherein infected T cells induce apoptosis in uninfected T cells [37]. This effect may by facilitated by soluble viral envelope protein or by the secretion of pro-apoptotic cytokines by infected cells [38, 39].

The interactions of HIV and resting CD4+ T cells are considerably more complex. Resting T cells express CD4 and both chemokine receptors, however exposure to HIV does not result in productive infection. Early studies demonstrated that HIV enters resting T cells and viral DNA is synthesized, but integrated HIV DNA is absent [40]. Subsequent studies demonstrated that reverse transcription occurs at a sluggish rate in resting T cells [41], and that nuclear import of the pre-integration complex is inefficient [42]. A possible mechanism underlying these findings is the cellular restriction factor APOBEC3G. Resting T cells contain a highly active low-molecular-mass (LMM) APOBEC3G complex, which is converted into a less active high-molecular-mass (HMM) complex during cell activation. Indeed, depleting APOBEC3G from resting T cells renders them much more susceptible to productive HIV infection. Surprisingly, the reverse transcripts that do form in the presence of APOBEC3G lack G→A hypermutation, suggesting that the restriction activity of the enzyme occurs by a mechanism other that RNA editing [43]. Despite these blocks to infection, activation of HIV-exposed resting CD4+ T cells can induce productive infection by driving cells into the cell cycle stage G1b, which enhances reverse transcription and allows efficient integration to occur [44].

While the aforementioned mechanisms render resting CD4+ T cells resistant to productive HIV infection, the block appears not to be absolute. It has been shown that HIV integration does take place within unstimulated T cells at a low frequency, and activation of these cells results in productive infection [45]. The physiological significance of this occurrence is reinforced by the finding of HIV infection in naïve ($CD45RA^{+}CD4^{+}$) T cells in HIV-infected individuals and in ex-vivo infected lymphoid tissue [46, 47].

**Antigen presenting cells**

HIV infection of macrophages and dendritic cells (DC) has been an area of intense study since the discovery of the virus. As the interactions of these cells with HIV are elucidated, it has become clear that these cells play a vital role in HIV pathogenesis. Areas of particular importance include the role of APC infection in perturbing normal immune function and in the potential for these cells as HIV reservoirs (reviewed in [48]). Tissue macrophages expressing CD4 are susceptible to productive HIV infection [49, 50]. Although it was initially thought that only CCR5-tropic HIV isolates could infect macrophages, it has more recently been determined that CXCR4-tropic viruses can also infect macrophages [51]. While T cells are rapidly destroyed by active HIV infection, macrophages can persist for as long as 40 days after infection, all the while releasing infectious virus [49, 52]. This makes macrophages uniquely suited as a short-term reservoir for HIV. HIV also appears to manipulate macrophages to enhance infection of T cells within the host. In one such mechanism, HIV Nef activates signal transduction pathways in infected macrophages, leading to the release of paracrine factors that

stimulate nearby T cells. This mechanism is thought to increase the available pool of HIV-susceptible T cells [23]. It has also been proposed that macrophages can take up infectious virions and maintain them in intracellular compartments. The virions can later be released during the formation of a macrophage-T cell synapse, leading to efficient trans-infection of T cells [53]. Further studies, however, have questioned whether HIV virions are in fact maintained in intracellular compartments [54].

Dendritic cells also have clear importance in HIV pathogenesis, but the role of these cells is more controversial. Several groups reported infection of DC by HIV, but studies have also argued that DC are not susceptible to infection [55][56]. A unique characteristic of DC is the ability to transfer HIV to T cells during the formation of immune synapse. The DC protein DC-SIGN interacts with HIV envelope, and the virion is maintained in a protective state until an activated T cells binds the DC, at which point the virion is transferred to the T cell [57]. The original studies demonstrated the ability of DC to transfer virus in *trans*, i.e. without infection of the DC, however it has also been suggested that infected DC may transfer HIV to other cells using this mechanism [58]. These observations have led to the model that DC may be the first cell type infected after host-to-host sexual transmission, but this model remains unproven [54].

**Neuronal cells**

The effects of HIV on the central nervous system (CNS) are evidenced by the incidence of HIV-related encephalitis, neuropathy and dementia. It has been shown that HIV infection occurs in CNS macrophages as well as in microglial cells, however infection of neurons appears to be a rare or absent event. Current models suggest that

HIV is initially introduced into the CNS when infected macrophages or lymphocytes cross the blood-brain barrier during the initial high viremic phase of infection. Once HIV is introduced into the immune-privileged CNS environment, viral replication is thought to occur at a low but persistent rate. Additionally, it appears that factors secondary to direct infection of CNS cells also contribute to HIV-related neurological disorders (reviewed in [59]).

**Hematopoietic progenitor cells**

The infection of hematopoietic progenitor cells (HPC) by HIV is a highly controversial topic. Several studies have reported the *in vitro* and *in vivo* infection of HPC by HIV, whereas others have reported that HPC are highly resistant to infection. Several studies revealed subsets of HPC that express CD4 in addition to CCR5 or CXCR4 (Figure 1-3 and [60-64]), suggesting that HIV infection of HPC could occur using the same viral entry method as is observed in T cells. Interestingly, it was found that CXCR4 expression was found on immature phenotype HPC [65] as well as in immature lymphoid progenitors [66]. Likewise, CD4 expression is frequently found on populations of immature phenotype HPC [67]. An initial study suggested that HPC could be infected with HIV, however detection of HIV in cultures required an extended period of *ex vivo* culture (>40 days) [60]. This indirect method of detection made it difficult to exclude the possibility that contaminating non-progenitor cells could be the source of HIV in the cultures. Two additional studies using similar long-term culture approaches also reported the replication of HIV in HPC cultures [68, 69]. Another study reported that HIV could infect HPC with clonogenic capacity. In this study, HIV RNA was

detected in hematopoietic colonies derived from infected HPC cultures. Viral RNA was detected in erythroid and myeloid colonies, but not in multilineage colonies derived from the most primitive HPC. In addition to the aforementioned studies of *in vitro* HIV infection of HPC, one study used PCR and virus isolation approaches to demonstrate the presence of HIV in HPC isolates from some HIV-infected individuals [70].

On the other hand, a number of studies reported that HPC are highly resistant to HIV infection. Two studies reported that HPC are intrinsically resistant to HIV infection [62, 71], several studies of HPC derived from HIV infected individuals reported that HPC from infected people are not infected, [72, 73] and subsequent studies proposed mechanisms to explain the resistance of HPC to HIV infection. Several reports confirmed the expression of HIV receptors on HPC, but proposed that the secretion of receptor-blocking chemokines prevents HIV entry [61, 64, 74]. Recently it was also reported that expression of the cell cycle regulator p21 may protect HPC from infection by HIV [75].

In the aftermath of the studies discussed above, the general consensus among HIV biologists is that HPC cannot be infected by HIV (reviewed in [76]). However, due to the vastly conflicting reports on this topic, the idea of HIV infection of HPC remains an inadequately addressed question. There are also significant shortcomings in the studies of HPC infection that have been performed. For instance, nearly all of the aforementioned studies used indirect methods to detect HIV infection, such as long-term culture assays and PCR. These techniques can produce false-positive results due to contamination from non-HPC cell types. Conversely, false-negative conclusions can be reached by these approaches if the assays lack sufficient sensitivity to detect rare

infection events. Furthermore, if viral infection led to cell death in HPC as it does in T cells, long-term culture assays would fail to detect infected cells. Another potential source of conflicting results is the extreme variability between HIV isolates. For instance, X4-tropic and R5-tropic HIVs could vary in their ability to infect HPC, or could infect distinct subsets of HPC. In light of recent advances in the understanding of HIV latency, it is clear that even rare infection of HPC could have significant clinical ramifications. This has raised a renewed interest in the interactions between HPC and HIV, and high-quality studies are needed to understand this controversial topic [77].

## HIV PERSISTENCE: VIRAL LATENCY AND CELLULAR RESERVOIRS

### Immune system evasion

Since the discovery of HIV as the etiologic agent causing AIDS, intense research efforts have sought to understand why HIV is not cleared by the immune system. One contributing factor is the virus' remarkable ability to evade detection and destruction by the immune system. While a detailed description of HIV immune evasion is beyond the scope of this chapter, a few mechanisms will be described here for the purpose of general background. Firstly, HIV has a remarkably high mutation rate due to the high error rate of the viral reverse transcriptase enzyme. This generates constant antigenic variation among viral progeny, which allows evasion of antibodies and CTLs. The few regions of viral genes that cannot tolerate mutation are structurally inaccessible or are poorly antigenic. Secondly, HIV encodes gene products that specifically target aspects of the immune response. HIV Nef, for instance, allows HIV-infected cells to escape lysis by

CTLs [78] by preventing antigen-presenting MHC I molecules from reaching the cell surface. Finally, HIV targets the immune system by directly infecting immune system cells, including CD4+ T cells, macrophages and dendritic cells. In fact, it has been shown that HIV preferentially targets HIV-specific CD4+ T cells [79].

**The latent T cell reservoir**

In addition to evading the immune system, HIV persistence is also facilitated by the virus' ability to become latent in certain cell types, the best characterized of which are resting T cells. It was first discovered that extrachromosomal HIV DNA exists in the resting T cells of HIV patients, and that stimulating these cells causes some cells to become productively infected [80]. This reservoir is termed the pre-integration reservoir, since it consists of viral cDNAs that have not integrated into the host chromosomes. This latent reservoir is quite labile; it is believed to only persist within a given cell on the order of days, and in patients it disappears after effective HAART therapy [41],[81].

It was subsequently discovered that resting T cells also carry integrated HIV DNA in patients [82]. Quantification of this reservoir revealed a very low-frequency but consistently present post-integration reservoir of resting T cells [83]. In this study, the frequency of resting T cells bearing integrated HIV DNA ranged from 16 to 410 per $10^6$ cells. HIV DNA integrated within the host genome will persist for the life of the cell, and will be maintained as if it were host DNA. Due to the stability of integrated HIV DNA and the long life of memory T cells, it is reasonable to surmise that the post-integration resting T cell reservoir would be very stable over time. Indeed, long term follow-up studies have revealed that this reservoir decays extremely slowly, with a half-life

averaging 44 months in patients on HAART [84]. An important clinical consequence of this stable latent reservoir is that it prevents eradication of HIV by both the immune system and HAART. All available anti-retrovirals target actively replicating HIV and have no effect on integrated HIV DNA or the cells harboring it. Thus, even if replication is completely abolished, latent HIV DNA remains poised to reinitiate active infection as soon as therapy is halted. Several studies have confirmed this model, showing that the resting T cell reservoir persists even in patients on HAART with undetectable viral replication [85, 86].

The mechanisms of HIV latency are unclear, and most likely result from one of several mechanisms. One possibility is that HIV integrates into heterochromatin, which inhibits expression of viral genes. A second possibility is transcriptional interference, a phenomenon that occurs when transcription from an upstream promoter reads through a downstream promoter, blocking transcriptional activation from the downstream promoter. This could occur if the HIV genome integrated downstream from an active cellular promoter. A third possible mechanism is explained by host transcription factor availability. If HIV integrates into a cell that does not express the necessary transcription factors, then the viral genome will remain dormant. A fourth mechanism is the availability of HIV Tat protein. As discussed above, Tat is required to form the transcription complex that activates the HIV LTR. If insufficient Tat protein is present following integration, it is possible that transcriptional activation of the LTR would fail. Finally, it has recently been suggested that cellular micro-RNAs exist that specifically suppress HIV gene expression in resting T cells to maintain latency [87, 88].

### Other cell types that may function as viral reservoirs

In addition to quiescent T cells, other cell types have been implicated as HIV reservoirs. Macrophages, for instance, have been implicated as viral reservoirs. These cells have not been shown to undergo latent infection *per se*, however they survive for long periods of time following infection, and can release infectious virus for a considerable period of time [52]. Similarly, dendritic cells have been proposed to function as a cellular reservoir for HIV. These cells could function as a reservoir via direct infection or by transfer of viral particles to T cells during the formation of immune synapses [57, 58, 89]. It is also possible that infected cells in the central nervous system could function as reservoirs that are uniquely protected from the immune response (reviewed in [59]). The significance of these and other cell types in HIV persistence remains unknown and is an important area for future study [48].

## HEMATOPOIESIS

### The hematopoietic cascade

The process of hematopoiesis is responsible for replenishing all of the cells of the blood and immune system. Hematopoiesis is organized in a hierarchical developmental cascade consisting of hematopoietic progenitor cells (HPC), which begin as hematopoietic stem cells and gradually differentiate into mature effector cells. The process originates with long-term reconstituting hematopoietic stem cells (LT-HSCs), which are capable of giving rise to all cell types of the hematopoietic system. The

hallmark feature of LT-HSC is the ability to undergo self renewal, a cell division process that gives rise to progeny that maintain the multipotency of the parental cell. Thus, LT-HSCs persist for the life of the organism. HSC are relatively quiescent, however by definition they must undergo regular, regulated cell divisions. In fact, it has been shown in the mouse that 8% of the LT-HSC pool undergoes cell division each day [90].

The next class of cells in the hematopoietic hierarchy are short-term reconstituting cells (ST-HSC) and multipotent progenitors (MPP). These cells retain the multipotency of LT-HSC, but have absent or highly limited self-renewal capacity. Next, MPP are thought to undergo additional cell division, giving rise to common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). Each of these cell types retains some multipotency, but is limited to differentiation into lymphoid or myeloid cell types. From this point, additional cell divisions give rise to progenitors that have lost multipotency, and are thus restricted to differentiation into a singular cellular lineage. In humans, this process correlates with the expression of CD38 and markers specific for hematopoietic cell lineages (e.g. CD3 expression on T lymphocyte committed progenitors). As cells further develop, they begin to acquire the characteristics of the mature effector cell and lose expression of CD34 (reviewed in [91]).

**Cellular markers of HPC subsets**

Different classes of hematopoietic cells can be identified in the bone marrow based on the expression of cell surface antigens. Unique immunophenotypes have been identified for several classes of HPC, allowing for identification and purification of cells

with specific differentiation capacities. A summary of the hematopoietic cascade and important surface markers can be found in Figure 1-4.

*Markers for total human HPC.* CD34 expression is the broadest and most commonly employed marker for human hematopoietic progenitors. The $CD34^+$ phenotype is found on nearly all progenitor cells, ranging from HSC at the origin of the hematopoietic cascade all the way to more differentiated progenitors at the bottom of the hierarchy.

A newer marker for HPC is CD133, also called Prominin-1. CD133 expression marks a more primitive population of HPC than does CD34, and the $CD133^+$ compartment is enriched for HSC [92, 93]. CD133 is also a useful marker for identifying immature HPC in cultivated cells. It has been found that other markers for immature HPC may lose reliability after extended culture periods, especially CD38 [94]. In contrast, cultivated $CD133^+$ cells undergo asymmetric cell divisions give rise to more differentiated HPC that are $CD133^-$/low and more primitive HPC that continue to express CD133 [95].

One area of controversy in basic HPC markers stems from several reports that HSC may reside in the $CD34^-$ fraction. These studies identified $Lin^-CD34^-CD133^+$ cells that were highly immature and capable of re-engraftment in NOD-SCID mice [96-98]. The role of $CD34^-$ HPCs in human hematopoiesis remains unclear, but they likely represent a small minority of HSCs.

*Murine HSC markers.* In the mouse, hematopoietic stem cells can be isolated to near purity based on their immunophenotype. One commonly used phenotype to enrich HSC in the mouse is $Lin^-Sca\text{-}1^+c\text{-}kit^+Thy\text{-}1^{low}$, where Lin represents a panel of antibodies

against markers of specific hematopoietic lineages [90, 99, 100]. Another immunophenotype, $CD150^{+}CD48^{-}CD41^{-}$, also identifies murine HSC with great specificity [101, 102].

*Human HSC markers.* In Humans, identification of HSC by immunophenotype is far less efficient. It is thought that most human HSC reside in the $Lin^{-}CD34^{+}CD38^{-}CD90^{+}$ population, but this phenotype falls far short of isolating HSC to purity [103-105]. Identification of HSC in humans is further complicated by the experimental limitations, as identification of HSC requires xenotransplantation approaches using immunocompromised mice. While these techniques have been very useful in understanding human hematopoiesis, there are limitations related to the poor homing efficiency of human cells in the murine context as well as residual immune activity within the murine recipient.

*Markers for human multipotent progenitors.* It is difficult to distinguish multipotent progenitors (MPP) from HSC in humans based on immunophenotype. MPP are found in the $Lin^{-}CD34^{+}CD38^{-}$ fraction along with HSC [106].

*Markers for the common lymphoid and myeloid progenitors in humans.* Markers have recently been established that allow for the identification and purification of immature HPC that are restricted to lymphoid or myeloid development. In human bone marrow, the common lymphoid progenitor (CLP) is identified by a $Lin^{-}CD34^{+}CD38^{-}CD10^{+}$ immunophenotype The CLP in human umbilical cord blood is slightly different, bearing a $Lin^{-}CD34^{+}CD38^{-}CD7^{+}$ phenotype [107]. The common myeloid progenitor (CMP) in humans has a $Lin^{-}CD34^{+}CD38^{-}$IL-3R$\alpha^{low}$$CD45RA^{-}$ phenotype, both in bone marrow and in umbilical cord blood [108]. This same study also identified granulocyte-

macrophage restricted progenitors ($Lin^{-}CD34^{+}CD38^{-}IL\text{-}3R\alpha^{low}CD45RA^{+}$) as well as megakaryocyte-erythroid committed progenitors ($Lin^{-}CD34^{+}CD38^{-}IL\text{-}3R\alpha^{-}CD45RA^{-}$), allowing for identification of a number of oligopotent progenitors.

**Hematopoietic abnormalities during HIV infection**

Numerous hematologic disorders occur with high frequency during the course of HIV disease. The most common disorder seen is anemia, which presents in the majority of HIV patients at some point during the course of the disease and correlates with poor prognosis regardless of CD4 counts. The mechanism of anemia is most likely multifactorial. It is likely that the secretion of pro-inflammatory cytokines has a general inhibitory effect on hematopoiesis. Additionally, superinfection with *Mycobacterium Avium*, cytomegalovirus and parvovirus B19 can cause or worsen anemia. Antiretroviral and antimicrobial drugs, especially zidovudine, amphotericin B and ganciclovir are all capable of causing anemia and are frequently used in HIV management (reviewed in [109, 110]).

Thrombocytopenia is another common hematologic disorder seen in HIV-infected individuals. While thrombocytopenia can be caused by infectious, immune-mediated and iatrogenic mechanisms as in anemia, a major difference is that thrombocytopenia often occurs early in HIV disease, before other symptoms of AIDS manifest. Initiation of antiretroviral treatment frequently reverses thrombocytopenia in these cases, suggesting a direct effect of viral infection [109, 111]. Less commonly, neutropenia and thrombosis are observed as sequellae of HIV infection [110].

The defects that occur in the hematopoietic process seem to originate early in the hematopoietic cascade. A study of progenitor cell subsets in HIV-infected patients demonstrated that the frequency of primitive phenotype ($CD34^{+}CD38^{-}$) HPC was lower in HIV-positive individuals as compared to healthy control subjects, suggesting that hematological abnormalities may originate from defects early in the hematopoietic progenitor cell pool [112]. Additionally it has been reported that exposure to HIV reduces the ability of primitive HPC to undergo multilineage hematopoiesis in vitro [113] and in a murine model of human hematopoiesis [114]. Another study used a macaque animal model to study hematopoietic function during simian immunodeficiency virus (SIV) and HIV infection. The authors found that substantial hematopoietic defects occur shortly following infection, and that effective antiretroviral therapy failed to restore normal hematopoiesis [115]. This suggests that HIV infection can cause an early hematopoietic defect that likely originates at the most primitive HPC level.

The cause of HIV-associated hematopoietic abnormalities are unclear, and most likely result from numerous factors. One possibility is the secretion of chemokines in excess during HIV disease. TNF-α, interferon-α/β, MIP-1α, and RANTES are known to be secreted in excess in HIV infection, and may have negative effects on hematopoiesis (reviewed in [116]). Additionally, the interaction of the viral envelope protein gp120 may induce signaling cascades that impair the function of HPC [111, 117-119]. It is also possible that soluble viral proteins can affect HPC without directly infecting the cells. It has been reported that soluble HIV Nef protein can impair hematopoietic function by activation of STAT5 signaling [120]. Another intriguing possibility is that HIV directly infects HPC, leading to dysregulation of hematopoiesis over time. This possibility has

not been explored adequately, however it is interesting to note that effective antiretroviral therapy often improves hematopoietic function [121, 122]. Further studies are clearly needed to understand how HIV infection leads to dysregulation of the hematopoietic system.

## VIROLOGICAL METHODS

The following section will describe important virological methods employed in this study.

**Production of replication competent HIV.** Because HIV naturally has a DNA intermediate step in the viral lifecycle, virus can easily be produced by transfection of proviral DNA in a producer cell line. To accomplish this, a full-length HIV provirus is cloned into a carrier plasmid, which is then transfected into HEK-293T cells. Viral RNA is then transcribed from the plasmid, and viral gene products are produced just as if the virus genome had integrated into the host genome during natural infection. Virus is shed from the transfected cells, and infectious virus is collected from the cell culture supernatant (Figure 1-5A).

**Production of envelope-deleted HIV and viral pseudotyping.** In many experimental settings, it is desirable to produce HIV which can infect target cells, but not undergo further rounds of viral replication. Such single-round infections minimize the pleiotropic effects that occur due to viral mutations during viral replication, and also allow the researcher to ensure that cells were only exposed to infectious virus for brief, defined period of time. To accomplish single round infections, proviral DNA constructs

are created that have deletions in the viral *env* gene To create infectious virions, the *env*-deleted provirus is transfected into producer cells along with a second plasmid that expresses the HIV envelope. Thus the virus produced will have functional Env protein, but will be unable to produce infectious virions after infecting target cells. This approach, termed virus pseudotyping, allows for the production of viruses with the same genetic backbone but bearing different envelopes, which is useful in studying the effects of different viral envelopes (Figure 1-5B).

**Production of self-inactivating HIV.** In several experiments in this work, we wished to create infectious HIV that would only express a reporter gene following infection, and not the viral proteins. To do this, we employed self-inactivating mutations in the viral LTR, an approach initially developed to create HIV-based gene therapy vectors [124]. In self-inactivating viral constructs, a deletion is made in the U3 region of the 3' LTR of the proviral construct, which eliminates the crucial transcription factor binding sites (Figure 1-2). Since the 3'LTR serves as the template for both LTRs during reverse transcription, after infection the integrated virus has the U3 deletion in the 5' and 3' LTRs, and no viral gene products are produced (Figure 1-5C).

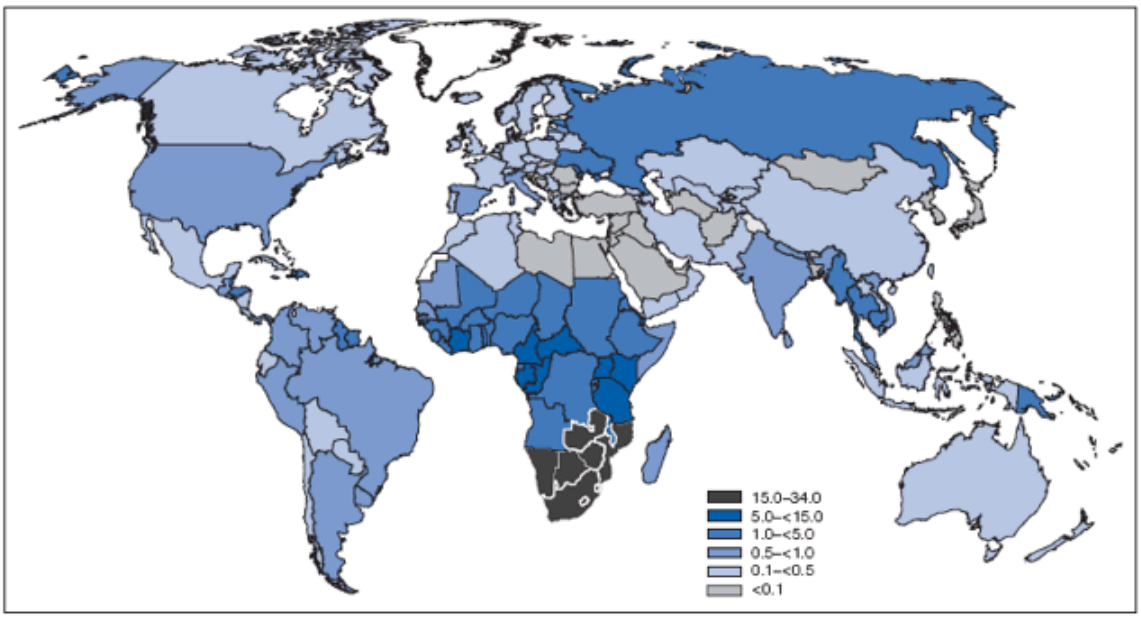



**Figure 1-1. Current state of the HIV pandemic [1].** Shown is the percent of the population estimated to be infected with HIV. Source: Joint United Nations Program on HIV/AIDS (UNAIDS), 2006 report on the global AIDS epidemic, Geneva, Switzerland.

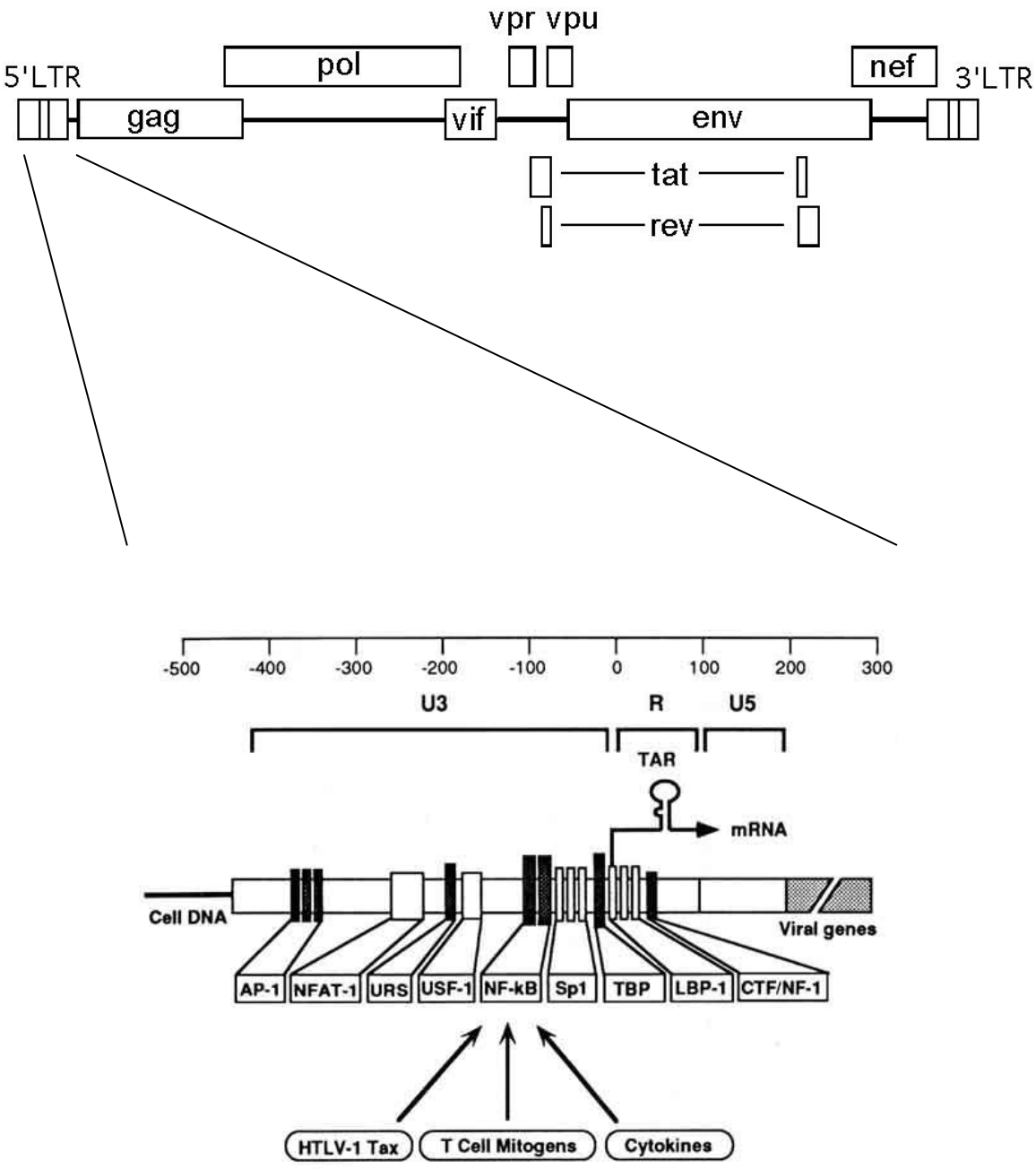



**Figure 1-2. Organization of the HIV genome.** Complete viral genome is shown in top graphic. A detailed schematic of the viral LTR with transcription factor binding sites is shown in the bottom graphic. Adapted from [125].

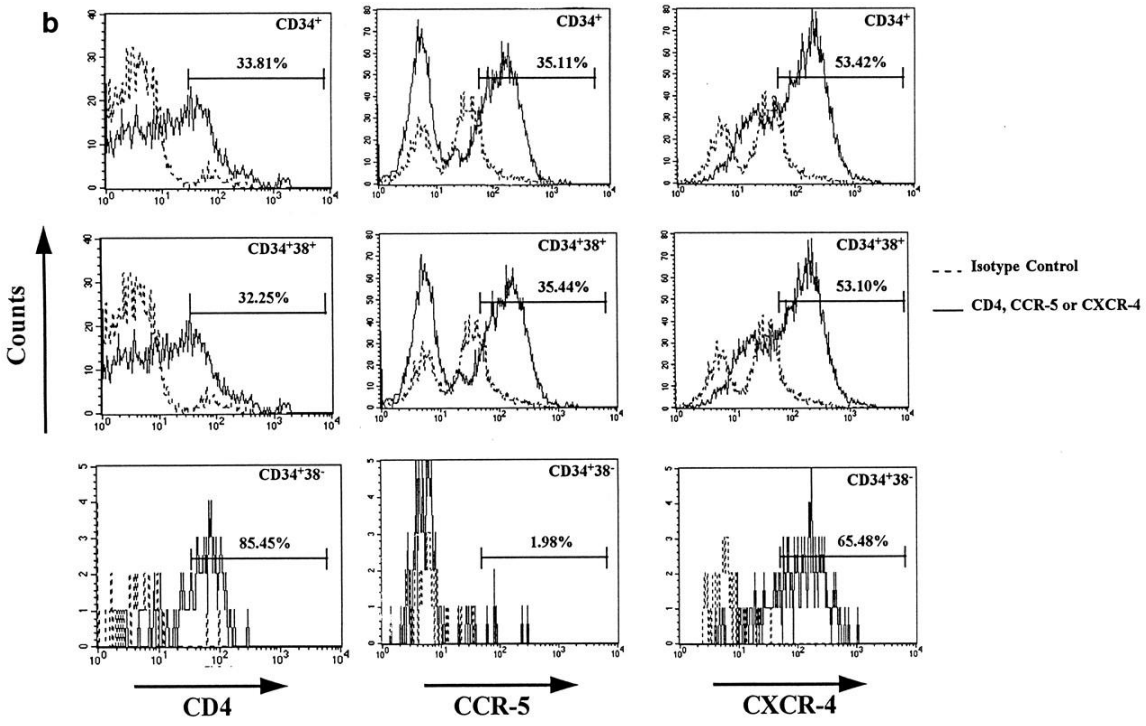



**Figure 1-3. Expression of CD4, CCR5 and CXCR4 on HPC.** Bone marrow mononuclear cells were stained with antibodies against CD34, CD38, CD4, CCR5 and CXCR4 (solid lines), or with matched isotype controls (dotted lines). Expression of CD4, CCR5 or CXCR4 is shown within total $CD34^+$ cells (top row), $CD34^+CD38^+$ cells (middle row) or $CD34^+CD38^-$ cells (bottom row). Adapted from [62].

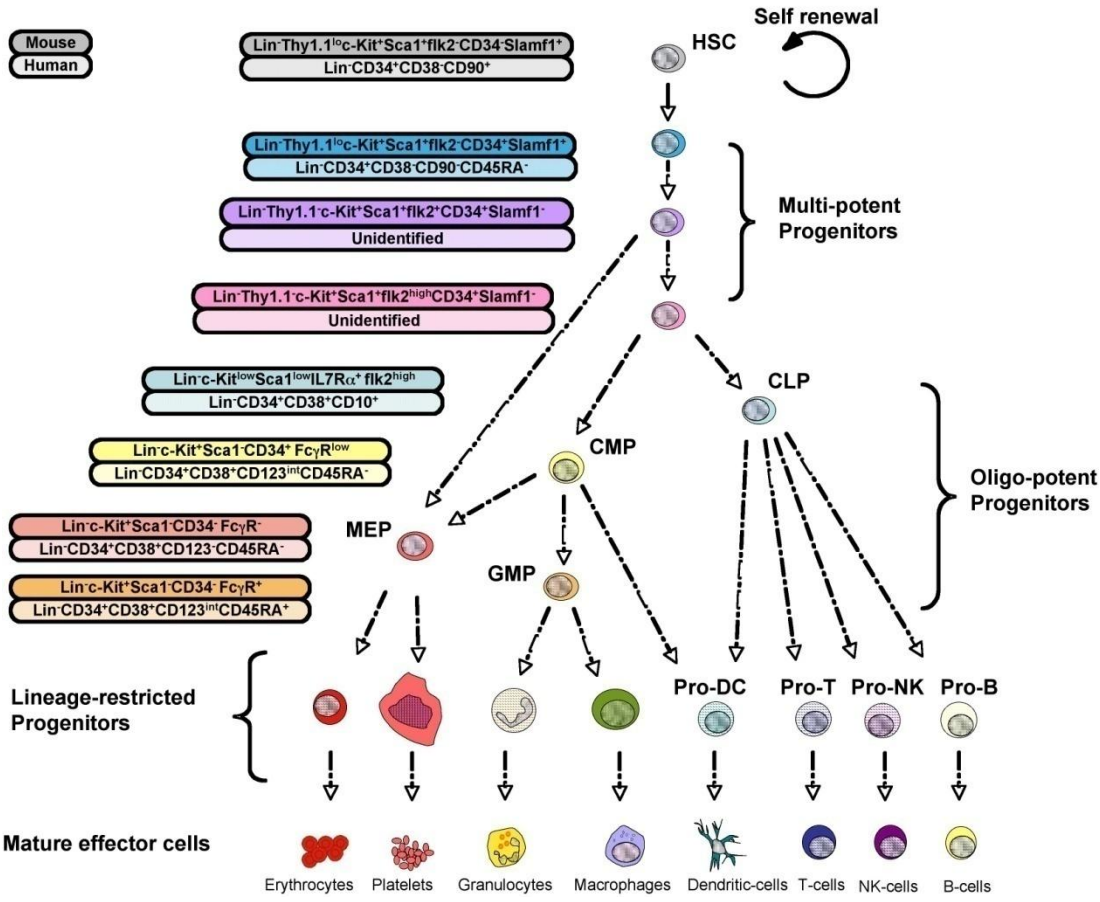
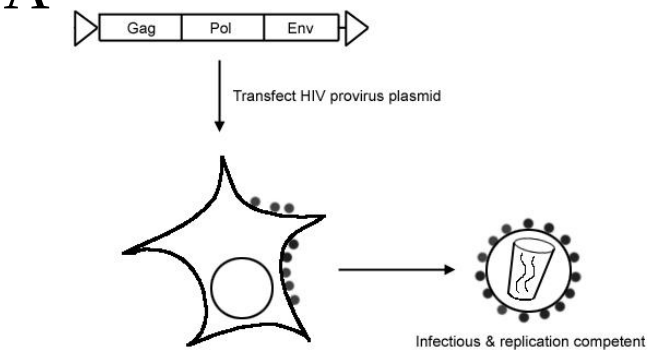


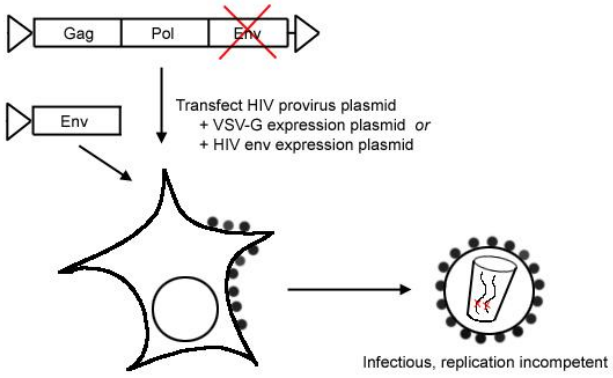

**Figure 1-4. The hematopoietic cascade in human and mouse.** Adapted from [123].

**Figure 1-5. Virological methods.** A, Production of replication competent HIV. B, Production of pseudotyped HIV for single-round infections. C, Self-inactivating mutations.

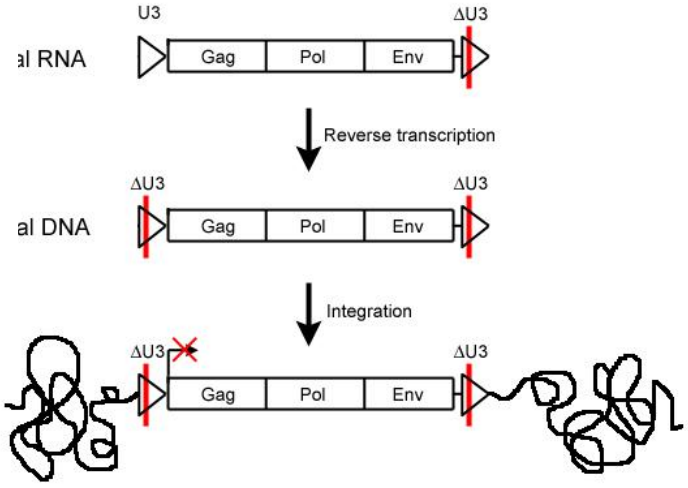
A


Gag
Pol
Env
Transfect HIV provirus plasmid
Infectious & replication competent

B


Gag
Pol
Env
Env
Transfect HIV provirus plasmid
+ VSV-G expression plasmid or
+ HIV env expression plasmid
Infectious, replication incompetent

C


U3
ΔU3
al RNA
Gag
Pol
Env
Reverse transcription
ΔU3
ΔU3
al DNA
Gag
Pol
Env
Integration
ΔU3
ΔU3
Gag
Pol
Env

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# CHAPTER II

## HIV-1 INFECTS PRIMARY MULTIPOTENT PROGENITOR CELLS CAUSING CELL DEATH AND ESTABLISHING LATENT CELLULAR RESERVOIRS

## ABSTRACT

HIV causes a chronic infection characterized by depletion of CD4+ T lymphocytes and, at late stages, severe bone marrow abnormalities. Despite the development of drugs that inhibit viral spread, HIV has been difficult to treat because of uncharacterized reservoirs of infected cells that are resistant to highly active antiretroviral therapy and the immune response. Here we used CD34 cells from infected people as well as in vitro studies of wild type HIV to demonstrate infection and killing of $CD34^+$ hematopoietic progenitor cells (HPCs). Moreover, we found that even primitive, multi-potent HPCs became infected. However, in some HPCs, HIV was latent and stably persisted in cell culture until viral gene expression was activated by differentiation factors. A novel reporter HIV that directly detects latently infected cells *in vitro* confirmed the presence of distinct populations of active and latently infected HPCs. These findings have important implications for understanding HIV bone marrow pathology and the mechanisms by which HIV causes a persistent infection.

## INTRODUCTION

HIV causes a persistent infection, despite the host immune response and effective treatment with highly active antiretroviral therapy (HAART). Viral persistence is due in part to latent HIV reservoirs that do not express viral proteins, but that can be induced to active infection by a variety of stimuli. Latently infected cells are resistant to the host immune response and to antiviral therapy, and thus facilitate viral persistence [1-3]. One important cellular reservoir is comprised of resting $CD4^+$ T cells [1], but studies of viral genetics have revealed the presence of additional viral reservoirs that remain uncharacterized [4]. The presence and slow decay kinetics of these viral reservoirs means that eradication of HIV infection will require reservoir-targeting therapeutics, and thus better understanding of viral reservoirs is crucial [5].

A second clinically important feature of HIV infection is the gradual collapse of the hematopoietic system. One simple manifestation of this hematopoietic dysfunction is the gradual decline in the ability of the hematopoietic compartment to regenerate T cells lost due to viral infection, despite the fact that only a tiny fraction of activated T cells are infected at any given time [2]. Perturbation of the hematopoietic system is also

manifested in the frequent occurrence of hematological abnormalities, including anemia, thrombocytopenia, and neutropenia (reviewed in [6]).

From the hematologic manifestations of HIV disease, it is clear that hematopoietic progenitor cells are affected by HIV infection. The term hematopoietic progenitor cell (HPC) refers to a heterogeneous collection of hematopoietic cells that are generally identifiable by the expression of CD34 in humans. The most primitive HPCs are hematopoietic stem cells (HSCs), which are capable of self-renewal and are multipotent. HSCs give rise to multipotent progenitor cells (MPPs), which retain a broad development capacity but cannot self renew. Both HSCs and MPPs have a $Lin^{-}CD34^{+}CD38^{-}$ surface phenotype, where "Lin" represents markers of specific hematopoietic lineages. Further development of HPCs gives rise to myeloid, lymphoid or erythroid-committed cells, which have a $Lin^{-}CD34^{+}CD38^{+}$ phenotype. As HPCs become further differentiated, they acquire markers of specific hematopoietic lineages, becoming $Lin^{+}CD34^{+}CD38^{+}$ (reviewed in [7]). Another useful marker for primitive HPCs is CD133, which identifies a more primitive subset of cells than CD34 and enriches for MPPs and HSCs [8, 9]. CD133 is also useful as a marker of primitive HPCs in culture, whereas other markers of these cells may lose significance under these conditions [10, 11].

An intriguing explanation for HIV persistence and HIV-associated hematopoietic failure is direct infection of HPCs by HIV. Interestingly, subpopulations of HPCs are known to express the HIV receptors (CD4 and CCR5 or CXCR4 [12-15]), and thus it is possible that if infected, these long-lived cells function as long-term cellular reservoirs

for the virus. Despite these observations, the most recent published data have suggested HPCs are not targets of HIV infection [12, 16-18]. The trouble in clearly determining whether HPCs are infected stems from the fact that they are difficult to maintain in culture. Additionally, commonly used approaches based on PCR and viral release cannot distinguish between low-frequency infection of HPCs and contamination of the sample by more well-established HIV targets. To more clearly determine whether HIV infects HPCs, we utilized flow cytometric techniques to directly assess the susceptibility of HPCs to active and latent HIV infection. In addition, we took advantage of recently developed culture techniques that allowed the maintenance and expansion of highly immature subsets *in vitro* [19]. We report here that a considerable proportion of HPCs become actively infected following exposure to HIV. HIV infected both lineage-committed progenitors, as well as the most primitive HPC subsets as assessed by flow cytometry and colony formation assays. Active infection of HPCs increased annexin V reactivity and resulted in loss of the infected population from the culture, helping to explain why detection of these cells has been difficult in past studies. We also report direct detection of latently infected HPCs and demonstrate that active infection was induced by treatment of HPCs with factors that induce cell differentiation. Together these findings demonstrate that HPCs are susceptible to both active, cytotoxic as well as latent, inducible HIV infection and thus reveal important mechanisms by which HPCs can contribute to disease pathogenesis.

## RESULTS

**HIV actively infects hematopoietic progenitor cells.** To initially assess susceptibility of HPCs to HIV infection, we purified total HPCs from bone marrow based on CD34 expression. Cells were sorted to high purity, typically 96-100% $CD34^+$ ( **Fig. 2-7a**) and treated with HIV 89.6ΔE/$env^{89.6}$ (**Fig. 2-1b**). After three days in culture, as expected, some of the $CD34^+$ HPCs had differentiated and become $CD34^-$; however our flow cytometric approach allowed us to specifically gate on cells expressing CD34, revealing that 6% were infected based on Gag expression (**Fig. 2-2a**, middle panel). Gag expression was blocked by AZT treatment, verifying that Gag expression was a result of *bona fide* infection. (**Fig. 2-2a**, lower panel).

To determine whether the capacity to infect HPCs was a feature of HIV in general we also examined infection by the molecular clones NL4-3, 89.6, 94UG, MJ4 and YU-2. NL4-3 utilizes the CXCR-4 co-receptor, 94UG, MJ4 and YU-2 utilize the CCR5 co-receptor, and 89.6 is "dual tropic" meaning that it can utilize both types of co-receptors to infect target cells. All five molecular clones were able infect $CD34^+$ HPCs ( **Fig. 2-8a**).

As another approach to verify infection of $CD34^+$ HPCs, we analyzed cell surface levels of major histocompatibility class I molecules (MHC-I), because active infection leads to downmodulation of surface MHC-I by HIV Nef in order to evade the immune response [20, 21]. For this experiment, $CD34^+$ HPCs were purified from umbilical cord blood (UCB) and infected with 89.6ΔE/$env^{89.6}$. We found that $CD34^+$ $Gag^+$ HPCs

downmodulated MHC-I compared to the $Gag^-$ cells in the culture (**Fig. 2-2b**). This confirms that the HIV Gag detected in HPCs results from active infection, rather then endocytosed viral particles or non-specific adhesion of virions to the cells.

Since $CD34^+$ HPCs are a heterogeneous population of primitive and differentiated cells, we sought to determine if active HIV infection was restricted to lineage-committed HPCs or if more primitive progenitors were infected. We infected bone marrow (BM)-derived $CD34^+$ HPCs with 89.6 (**Fig. 2-1a**) and co-stained the cells for Gag, CD34 and Lin. Infection of $Lin^+$ cells was very robust (40%), however a significant frequency of the more primitive $Lin^-$ HPCs were also infected (4.4%), demonstrating that HIV infection was not limited to highly differentiated cells (**Fig. 2-2c**). Interestingly, infection appeared to be cytotoxic, as a significant loss of $Lin^+$ cells was seen in the infected culture after only three days.

To more directly assess whether HIV kills the infected HPCs, as occurs in infected T lymphocytes [22], we infected umbilical cord blood (UCB)-derived $CD34^+$ HPCs with wild type HIV 89.6 and monitored the frequency of $Gag^+$ cells over time. As shown in **Fig. 2-2d**, we found that $Gag^+$ cells were lost rapidly in culture, consistent with the virus being toxic to the cells. Similar results were obtained with four other wild type HIV molecular clones (Supplementary Figure 2b). Additionally, we found that infected cells displayed increased annexin V binding, a measure of phospatidylserine exposure to the cell surface during apoptosis (**Figure 2-2e).** Taken together, these findings demonstrate that HPCs are susceptible to active infection by HIV, and that cell death

results from the infection. These data provide support to the notion that profound bone marrow defects noted in HIV-infected people are due in part to direct infection and killing of HPCs by HIV.

**Multipotent HPCs are susceptible to HIV infection**

The induction of cell death following infection presented a significant obstacle to understanding the identity and developmental capacity of HIV-susceptible HPCs, because standard assays to identify progenitors require long term culture. To overcome this obstacle, we employed a highly deleted HIV genome, which does not express any HIV genes (HIV-7SF-GFP, **Fig. 2-1c**). When co-expressed with an HIV envelope-expressing plasmid and a packaging-defective $HIV^{NL4-3}$ genome, virus with the same cell tropism as wild-type HIV is produced, but the viral genome only expresses GFP from the internal SFFV promoter following integration. This virus allowed us to "tag" susceptible cells so that their developmental capacity could be understood, without the cell death that resulted from natural infection.

To assess which progenitors are susceptible to HIV infection, we infected HPCs with HIV-7SF-GFP pseudotyped with 89.6*env* (HIV-7SF-GFP/$env^{89.6}$). In this case we used $CD133^+$ HPCs from UCB, since this population is highly enriched for immature progenitors [23, 24]. Cells were sorted to >90% purity ( **Fig. 2-7b**). Following infection, we used flow cytometry to assess infection in different HPC populations. We found a significant proportion of infected ($GFP^+$) cells within the $CD34^+$ HPC population, ranging from 1-6% (**Fig. 2-3a**). Within the primative $CD34^+CD38^-CD133^+$ fraction we

detected $GFP^+$ HPCs at frequencies similar to that in the total $CD34^+$ fraction [**Fig. 2-3a and 2-3b**, 2.4% of total $CD34^+$ versus 3.8% of the most immature subset ($CD34^+CD38^-CD133^+$)], indicating that very primitive HPCs are susceptible to infection. Identical experiments conducted with $CD133^+$ HPCs purified from BM instead of UCB produced similar results (**Supplementary Fig. 2-9**).

To confirm that HIV can infect primitive HPCs, we assessed the ability of infected HPCs to form hematopoietic colonies in semisolid medium. $CD133^+$ HPCs from UCB infected with HIV-7SF-GFP/$env^{89.6}$ were sorted by FACS and identical numbers of $GFP^+$ and $GFP^-$ cells were plated in complete methylcellulose medium. Colonies were then assessed for morphology and GFP expression. We detected $GFP^+$ colonies of erythroid (CFU-E), myeloid (CFU-M and CFU-GM) and multilineage (CFU-GEMM) origin, demonstrating that HIV can infect highly primitive, multipotent HPCs (**Fig. 2-3c**). Quantitation revealed similar numbers of total colonies in uninfected and infected cells (**Fig. 2-3d**), although the infected cells had a somewhat greater frequency of CFU-GM and fewer CFU-E when compared to the uninfected cell fraction and we noted a modest reduction in CFU-GEMM.

To ensure that the pHIV-7SF-GFP virus did not inaccurately portray HIV susceptibility due to smaller genome size, we performed similar experiments using a full-length HIV reporter that has a LTR mutation preventing expression of viral genes (89.6-SIΔE-SF-GFP, **Fig. 2-1d**). Similar results were obtained using this virus (**Fig. 2-3e and**

**f**). In sum, these findings demonstrate that primitive, multipotent HPCs can be infected by HIV.

**Induction of latent HIV from infected HPCs**

To determine whether HPCs might harbor latent HIV genomes in an analogous manner to resting memory T cells, we infected highly purified BM-derived HPCs (>98% $CD34^{+}$) with replication defective HIV ($HXB\text{-}ePLAPenv^{VSV\text{-}G}$), which expresses the marker gene placental alkaline phosphatase (PLAP) (**Fig. 2-1e**). Infected cells treated with DMSO solvent control showed a modest infection rate (5.6%), however cells that were induced to differentiate with PMA immediately after infection expressed PLAP at a 12-fold higher rate (**Fig. 2-4a**), suggesting that latent HIV exists in these cultures and that it can be activated by cellular maturation.

To determine whether the effect of PMA was pre- or post-integration, we assessed the relative abundance of integrated HIV in HPCs using nested real-time PCR [25]. As shown in **Figure 2-4b**, we found similar levels of integrated genomes plus or minus PMA, demonstrating that activation of HIV expression was a post-integration event.

The high infection rate we obtained using $HXB\text{-}ePLAPenv^{VSV\text{-}G}$ allowed us to examine the biology of the HIV genome under conditions in which a high fraction of the cells were infected. However, the expanded range of targets infected with VSV-G envelope may have included cells not normally susceptible to HIV infection. Thus, we also performed similar experiments using $HXB\text{-}ePLAP/env^{89.6}$. As expected, the

infection rate was lower than what had been observed with the VSV-G envelope (1.5% in the DMSO control), but viral expression was induced several fold by PMA stimulation (5%) (**Fig. 2-4c**). Together, these data indicate that HPCs can harbor integrated HIV genomes in a latent but inducible form.

We next asked whether a more physiological HIV and cytokine cocktail would induce HIV release from infected HPCs. For these experiments, BM-derived $CD34^+$ HPCs infected with wild type HIV-89.6 (**Fig. 2-1a**) were cultured either in STF medium [stem cell factor (SCF), thrombopoietin (TPO) and Flt3 ligand (Flt3-L)] to maintain HPCs or with GM-CSF and TNF$\alpha$ to induce myeloid differentiation [26]. We conducted the experiment with highly purified (>98% $CD34^+$) HPCs as well as CD34-depleted bone marrow mononuclear cells (BM-MNC) to control for viral release from contaminant cells. Using reverse transcriptase activity to quantify viral particle production, we found only small amounts of HIV release from STF-treated cells. In contrast, GM-CSF/TNF$\alpha$-treated HPCs showed a rapid and sustained release of HIV into the culture supernatant (**Fig. 2-4d**). Control cultures depleted for $CD34^+$ cells never showed RT activity over background. Flow cytometric analysis of the cells, staining for HIV Gag, confirmed the absence of actively infected cells in the STF-treated cells and a high frequency of actively infected cells in GM-CSF/TNF$\alpha$-treated cultures (**Fig. 2-4e**). FACS analysis of the differentiated cells revealed many infected $Lin^-HLA\text{-}DR^+$ cells, which is consistent with a dendritic cell phenotype. These findings demonstrate that cellular maturation down the myeloid lineage induces robust HIV expression from infected HPCs.

To determine whether latent HIV in HPCs was stable over time, we infected HPCs with wild type 89.6 and cultured the cells in STF medium, monitoring the cells for Gag expression over time. Once the culture was uniformly Gag-negative (7 days), the culture was split and half was stimulated with GM-CSF/TNFα and the other half was cultured in STF. The culture that was stimulated with GM-CSF/TNFα displayed an immediate resurgence in HIV production, triggering a robust spreading infection. The culture treated with STF resulted in only a small degree of infection, most likely resulting from spontaneous cellular maturation activating HIV (**Fig. 2-4f** and 2-**4g**). These results confirm that latent HIV infection occurs in HPCs, and can be induced by cellular maturation.

**HIV establishes latency in T cells and HPCs.** The approaches used above and in other HIV latency studies required indirect methods like PCR or retrospective identification of latently infected cells by cellular stimulation (reviewed in [27]). To directly detect latent infection *in situ* without inducing changes in the infected cells, we developed a novel latency reporter virus. This molecular clone, 89.6-ΔE-SF-GFP (**Fig. 2-1f**), contains a reporter cassette consisting of the spleen focus forming virus (SFFV) promoter driving GFP expression independent of the HIV LTR, allowing us to "tag" infected cells that are not expressing viral genes. To determine if 89.6-ΔE-SF-GFP/env$^{89.6}$ can detect HIV latency, we infected T cell lines and primary T cells and analyzed the expression of GFP and HIV Gag. As shown in **Fig. 2-5a**, a large proportion of infected CEM T cells were Gag$^{+}$, indicating active viral infection. Many of the Gag$^{+}$ cells did not express GFP, most likely due to transcriptional interference silencing the SFFV promoter [28]. In contrast,

other cells expressed GFP but not Gag, suggesting that they were latently infected. To verify latency versus active infection in the various cell populations by another method, we assessed CD4 downmodulation in infected cells, which occurs only if HIV Nef, Vpu or Env are expressed [29]. As expected, we found that CD4 was robustly downmodulated in $Gag^+$ cells. In contrast, we observed no downmodulation in the $GFP^+Gag^-$ cells, confirming that infection in $GFP^+Gag^-$ cells is latent. In contrast, when cells were infected with a virus that expresses GFP from the HIV LTR (89.6-ΔE-IRES-GFP, **Fig. 2-1g**), all GFP-expressing cells downmodulated CD4, even those lacking Gag expression (**Fig. 2-5a**). We performed similar experiments in CD8-depleted, PHA/IL-2 stimulated peripheral blood mononuclear cells (PBMCs). Following infection of stimulated PBMCs with 89.6ΔE-SF-GFP $env^{89.6}$, we were able to detect actively infected ($Gag^+$) cells as well as latently infected ($GFP^+Gag^-$) cells within the $CD3^+HLA\text{-}DR^+$ activated T cell population. Again we found that the actively infected cells downmodulated CD4 robustly, whereas the latently infected cells displayed normal CD4 levels (**Fig. 2-5b**). Thus, this methodology allowed us for the first time to detect populations of latently infected cells within cell populations that are also actively infected. We then used 89.6-ΔE-SF-GFP $env^{89.6}$ infected Jurkat cells to determine if latently infected cells could be induced to actively express viral genes by treatment with PMA and ionomycin. As shown in **Figure 2-5c**, PMA and ionomycin treatment led to a reduction in $GFP^+Gag^-$ cells and a concurrent increase in $Gag^+$ cells, consistent with our expectations that the $GFP^+Gag^-$ cells represented latently infected T cells that could be induced to active infection.

In UCB-derived $CD34^+$ HPCs infected with 89.6-ΔE-SF-GFP $env^{89.6}$ we found $Gag^+$ cells as well as cells that expressed GFP only, indicating that both active and latent infection occurs in infected HPCs (**Fig. 2-5d**). To assess the stability of latently infected HPCs, we passaged infected HPCs in culture under non-maturing conditions and monitored actively and latently infected cells over time. The actively infected Gag+ cells were lost from culture rapidly, whereas the latently infected cells persisted (**Fig. 2-5e**). Analysis of the latently infected HPCs revealed that most were $CD34^+$ Lin-, or CD34+CD38- (**Fig. 2-5f**). Together these results demonstrate that latent infection indeed occurs in primitive HPCs.

**Freshly isolated CD34+ cells from HIV-infected people are infected with endogenous virus.** To establish the physiologic relevance of our results, we obtained fresh bone marrow aspirates from HIV-infected people with viral loads greater than 50,000 copies per mL and normal white blood cell counts. The yield of CD34+ cells from these donors was drastically reduced in five of the six analyzed and had an average of 8,000 (+/-6000) CD34+ cells per mL of bone marrow. The sixth donor had CD34+ counts that fell slightly above the normal range [70,000 (+/- 21,000) CD34+ cells per mL of bone marrow (n=6)]. Despite the low yield of cells, we were able to directly detect Gag+CD34+ cells in the freshly isolated, uncultured samples from 50% (3/6) of people tested (**Fig. 2-6**, upper panels are representative). Additionally, cells from two donors were expanded in STIF media and then cultured in GMCSF and TNFα for two weeks. These conditions led to cellular differentiation and to an increase in the percentage of Gag positive cells in both donors (Figure 6, lower panels are

representative). In contrast, bone marrow mononuclear cells specifically depleted of CD34+ cells were not viable under these culture conditions, as described above for our in vitro data shown in Figure 4d. Thus, these data provide compelling confirmatory evidence that HIV infects CD34+ progenitor cells in vivo and that productive infection can be enhanced by culturing CD34+ bone marrow cells in cytokines that induce differentiation.

# DISCUSSION

HIV latency represents a critical obstacle in the eradication of the virus by the immune system or antiretroviral therapy. In order to fully understand HIV pathogenesis and ultimately develop novel therapeutic approaches aimed at eradicating the virus, it is critical to understand the reservoirs of viral latency and infection. Here, we demonstrate that HIV is capable of infecting hematopoietic progenitor cells in vivo and in vitro to cause an active cytotoxic infection as well as a latent infection, which can be induced by cellular maturation.

We found that infection of HPCs with HIV that expressed normal viral gene products was capable of inducing cell death, as seen by the loss of infected cells and by the presence of phosphatidylserine on the cell surface, detected by increased annexin V binding. Infection and death of HPCs could have the effect of depleting crucial progenitor cells, which could contribute to the gradual inability of the hematopoietic system to replenish lost $CD4^+$ T cells over time, as well as to the numerous cytopenias that develop during HIV disease. Other models such as those suggesting that hematopoietic dysfunction results from heightened systemic inflammation or from the effects of soluble HIV proteins in the systemic circulation [30-37] are not necessarily mutually exclusive with the model implicated here; as the effects of HIV on the hematopoietic system are clearly complex and are likely the result of multiple mechanisms.

Other groups have reported that HPCs are resistant to HIV infection, and indeed the majority of these cells appear not to undergo infection. Importantly, we demonstrate here that a subset of HPCs reproducibly become infection following exposure to HIV. The finding that HIV kills infected HPCs may help explain why other groups have had difficulty demonstrating infection of HPCs. Our results indicate that long-term culture, such as that required for colony formation, typically results in loss of the infected cells due to the toxicity of the viral infection. To overcome these obstacles, we focused on experimental approaches that allowed direct detection of wild type, endogenous HIV infection on a single cell basis and we also used inactivated HIV constructs to minimize the effects of HIV-induced cell death. Using transcriptionally inactive HIV constructs, we demonstrated that HIV infected immature phenotype HPCs, including subsets ($CD133^+$, $CD34^+CD38^-$, $lin^-$) enriched for stem cells capable of completely repopulating the hematopoeitic lineages in SCID mice [9, 24]. This observation was confirmed by colony formation assays, which demonstrated that immature lineage-committed progenitors as well as multipotent progenitors were infected by HIV. The infection of immature progenitors has clear ramifications for HIV disease, as these immature cells are longer lived and thus could carry latent HIV for long periods of time. Additionally, if active infection occurred in immature HPCs, the downstream effects in the hematopoietic system would be far greater than if infection only occurred in more differentiated cells.

We also report here that latent infection occurs in HPCs in vitro and in vivo. Both chemical stimulation with PMA and stimulation with GM-CSF and TNF$\alpha$ were able to induce latent HIV, as assessed by flow cytometric detection of active infection as well as

viral release by reverse transcriptase assay. Interestingly, we found that infected dendritic cells arose following stimulation of infected HPCs. These cells are known to be uniquely capable of transmitting HIV to T cells [38], and thus even infrequent infection of HPCs could be readily amplified in this manner.

To definitively demonstrate that latent infection can occur in HPCs, we utilized an HIV with an internal promoter-reporter cassette to facilitate the detection of latently infected cells. To our knowledge, this is the first approach that allows direct detection of latently infected cells *in situ*. This technique allows for the first time the detection of latent genomes in cell systems that also support active infection. Unexpectedly, we found that latent infection was detected in transformed T cell lines as well as in primary activated T cells. This was surprising because current models propose that latency in T cells only occurs when an actively infected cell reverts to a memory T cell state following infection. We confirmed that $Gag^{-}GFP^{+}$ cells were indeed latently infected by absence of CD4 downmodulation, which would take place if HIV *nef* or *vpu* genes were expressed. Additionally we found that PMA and ionomycin stimulation was capable of inducing viral gene expression in a considerable proportion of these cells.

Using 89.6ΔE-SF-GFP $env^{89.6}$, we were also able to demonstrate latent infection in HPCs. When the infected cells were passaged in culture, the actively infected cells were lost rapidly, as expected from previous experiments. The latently infected cells, however, persisted at constant levels in the cultures, despite significant expansion of the total culture. This suggests that latently infected cells divide in culture at a rate similar to

uninfected cells. The division and expansion of latently infected HPCs makes them quite different from latently infected resting T cells, which are more quiescent. This could provide a unique mechanism for expansion of latently infected cells, enhancing the ability of these cells to function as a reservoir.

It is interesting that both active and latent infection can be detected in HPCs and in T cell blasts, and it will be important to determine the mechanisms that trigger active versus latent infection. In the heterogeneous HPC pool, it is possible that some cells may have the correct transcription factor milieu to activate the HIV LTR promoter whereas others do not. Alternatively, chance mechanisms could trigger latency, such as integration of the HIV genome into transcriptionally inactive chromatin regions.

We have also assessed the phenotype of latently infected HPCs. These cells are primarily found in immature cell fractions ($CD34^+Lin^-$ and $CD34^+CD38^-$), suggesting that immature HPCs can undergo latent infection, and verifying that the latently infected cells we detected were not contaminating non-HPC cells. This has important implications for the ability of HPCs to function as a viral reservoir, as immature cells have a longer lifespan within the marrow. An important question that remains is whether *bona fide* stem cells are susceptible to HIV infection. Since these cells have the ability to undergo self-renewal and thus persist indefinitely, they could potentially function as a reservoir for the life of the host. Further studies will be needed to determine whether specific HPC subsets are more likely to undergo latent versus active infection, and whether certain viral isolates or viral envelopes could vary in their tendency to establish latent infection.

In sum, these studies have demonstrated that HPCs are indeed targets for both active and latent HIV infection in vivo and in vitro. Because viral reservoirs remain the primary obstacle in eradicating HIV infection from the host, these findings have important implications in the understanding of HIV pathogenesis and the development of novel therapeutic strategies.

# METHODS

**Antibodies and reagents**. The antibody for CD4 was purified from ascites made from the OKT4 hybridoma and FITC conjugated according to the manufacturer's instructions (Fluorotag, Sigma-Aldrich). Additional antibodies were: anti-CD34-FITC (BD Biosciences), anti-CD34-APC (Caltag), anti-CD38-PE/Cy7 (eBioscience), anti-CD133-PE (Miltenyi), anti-CCR5-PE and anti-CXCR4-PE (eBioscience), anti-Gag-FITC and anti-Gag-PE (clone KC57, Coulter), Lin1-FITC (BD Biosciences). Lineage cocktail was prepared by combining anti-CD3, anti-CD14, anti-CD19, anti-CD20, anti-CD56 and anti-glycophorin A antibodies in equal amounts (BD Biosciences). The cocktail was then biotinylated according to manufacturer's instructions (EZ Link Sulfo-NHS-Biotin, Pierce Biotechnology) or conjugated to PE-Cy5.5 according to manufacturer's instructions (Lightning Link PE/Cy5.5, Innova Biosciences). CC110 cytokine cocktail (10µg/mL rhSCF, 10µg/mL rhTPO and 10µg/mL rhFlt3-L; Stemcell Technologies) was used alone or supplemented with recombinant human IGFBP-2 (R&D systems). StemSpan medium was purchased from StemCell Technologies. StemlineII medium was purchased from Sigma-Aldrich and supplemented with penicillin/streptomycin/L-glutamine and Plasmocin (Invitrogen). PMA, PHA and ionomycin were purchased from Sigma-Aldrich. rhGM-CSF and rhIL-2 were purchased from R&D systems. rhTNF-$\alpha$ was purchased from Biolegend. Complete methylcellulose medium with cytokines (Methocult 4034) was purchased from StemCell Technologies.

**Plasmid constructs.** p89.6, pNL4-3, p94UG-114.1, pYU-2 and pMJ4 were obtained from the NIH AIDS Reference and Reagent program. pCMV-HIV-1 and pCMV-G were kind gifts from Shin-Tai Chen [39]. pHIV-7/SF-GFP was a kind gift from Jiing-Kuan Yee [40].

p89.6ΔE was generated by cutting p89.6 with BsaBI and StuI, blunting and re-ligating the ends. The resulting construct has a termination codon after the first 656 nucleotides of *env*, and thus expresses a highly truncated Env protein. The 89.6 *env* expression vector pCDNA-89env was created by digesting p89.6 with HindIII and EcoRV and ligating the resulting 3180bp fragment into HindIII/EcoRV-cut pcDNA3.1(+).

To construct p89.6-ΔE-SF-GFP we first assembled a cassette comprised of *nef* $^{89.6}$ followed by the SFFV promoter and EGFP. The SFFV promoter was isolated from pHIV-7/SF-GFP as follows: DNA was digested with BamHI, blunted by Klenow treatement, and digested with KpnI. This fragment was ligated into MluI(blunt)/KpnI cut pCDNA3.1(+), resulting in pSFDNA, which has the SFFV promoter in place of the original CMV promoter. Next, EGFP was isolated as follows: pEGFP-N2-LAMP1 (provided by Norma Andrews) was digested with BamHI, blunted by Klenow treatment, and digested with XbaI. This fragment was then ligated into EcoRV/XbaI cut pSFDNA, generating pSFDNA-EGFP. Next, *nef*$^{89.6}$ was amplified from p89.6 using oligos AACAATTGTTTAAACATGGGAGGCAAGTGG and TTCAATTGTCAGTTCTTGAAGTACTCCGGATGC. The forward oligo adds MfeI

and PmeI sites before the initiation codon, and the reverse oligo adds a MfeI site following the termination codon. This PCR product was digested with MfeI and ligated into MfeI cut pSFDNA-EGFP, generating pNef-SFFV-EGFP.

The next step in generating p89.6-ΔE-SF-GFP was to generate 89.6ΔNE, an *env/nef*-deleted p89.6 backbone capable of accepting the Nef-SFFV-EGFP cassette. To make this construct, p89.6 was used as a template for PCR amplification using oligonucleotides 5'CACCATTATCGTTTCAGACCCT3' and 5'TCTCGAGTTTAAACTTATAGCAAAGCCCTTTCCA3'. The 5' oligo spans the XhoI site found in 89.6 *env*. The 3' oligo recognizes the 3' end of *env* and includes 3' XhoI and PmeI sites. p89.6 was digested with XhoI, excising the 3' portion of *env* and the first 112bp of *nef*. The PCR product described above was then cut with XhoI and ligated into p89.6, thus reconstituting *env, tat* and *rev*, but leaving *nef* deleted and introducing a novel PmeI site immediately after *env*. Next, a large deletion was created in *env* as described for p89.6ΔE. The final step in generating p89.6-Δ E-SF-GFP was to cut pNef-SFFV-EGFP with PmeI, excising the cassette containing Nef-SFFV-EGFP and ligating into PmeI-cut p89.6dNE.

To a create a self-inactivating version of p89.6-ΔE-GFP, the parental HIV construct was subjected to PCR-mediated deletion of the 3' U3 region of the viral LTR, generating a SIN deletion analogous to those employed in Lentiviral vectors [41]. Two separate PCR reactions amplified the regions flanking the desired deletion using oligos bearing complementary linkers, allowing for subsequent annealing of the two products. The first

reaction amplified from the XbaI site in the Nef-pSFFV-GFP cassette to the beginning of the desired deletion (400bp upstream of the LTR R region), using oligos GCATGGACGAGCTGTACAAG and CAGGCAAAAAGCAGCGACCCACAGATCAAGG. The second reaction amplified from the end of the desired deletion (20bp upstream of the LTR R region) to the novel BglI site found in the plasmid *ori*, using oligos CCTTGATCTGTGGGTCGCTGCTTTTTGCCTG and GCAACAACGTTGCGCAAA. The resulting 2 PCR products were combined and PCR amplified with the two external oligos, generating a product that corresponds to the region of p89.6-ΔE-GFP from the XbaI site to the BglI site of but lacks 380bp of the U3 region. This product was cut with XbaI and BglI and ligated into similarly cut p89.6-ΔE-SF-GFP. The resulting U3 deletion eliminates all consensus AP-1, NF-AT, NF-κB SP-1 and TATA motifs, but maintains the polyA site found in the R region.

**Cell culture**. Fresh whole bone marrow aspirates were obtained commercially (AllCells Ltd.). Umbilical cord blood was obtained following scheduled cesarean section procedures, in accordance with an IRB-approved protocol. Bone marrow mononuclear cells (BM-MNC) and cord blood mononuclear cells (CB-MNC) were prepared by density separation using Ficoll-Paque (GE healthcare) according to the manufacturer's instructions. CB-MNC were often cryporeserved by suspending cells $50x10^6$ cells/mL in 10%DMSO/FBS and freezing at a controlled rate of -1ºC/min. BM-MNC were always used fresh. Prior to cell separation, MNC were adherence depleted by incubating in StemlineII for at least 2 hours in tissue culture coated flasks.

To prepare enriched $CD34^+$ cells, adherence depleted mononuclear cells were subjected to immunomagnetic cell sorting using commercially available kits (EasySep CD34 positive selection kit, StemCell Technologies and CD34 MACS positive selection, Miltenyi) according to the manufacturer's instructions. Fluorophore-labled antibodies were included during immunomagnetic labeling to monitor sort purity. $CD133^+$ cells were isolated similarly, using CD133 positive selection MACS (Miltenyi). Following isolation, $CD34^+$ cells were maintained in StemSpan medium supplemented with recombinant cytokine cocktails as indicated in the text. To maintain and expand total $CD34^+$ HPCs, media was supplemented with STF cytokine cocktail (100ng/mL SCF, 100ng/mL Flt3-L and 100ng/mL TPO). Cells were cultured for no longer than 24h prior to infection. To expand immature progenitor cells for colony formation assays, media was supplemented with STIF cytokine cocktail (50ng/mL stem cell factor, 50ng/mL Flt3-L, 50ng/mL TPO and 100ng/mL IGFBP-2) as described elsewhere [19]. Cells were propagated for up to 4 days in this medium prior to infection.

Methylcellulose colony forming assays were conducted by according to the manufacturer's recommendation (Methocult H4034, StemCell Technologies). Briefly, infected or control cells were diluted in IMDM+2%FBS and mixed with complete methylcellulose medium. The mixture was then plated on non-coated 6-well plates (500-1500 cells per well) and incubated for 14 days. Colonies were scored based on morphology using an inverted brightfield microscope at 40x or 100x magnification.

CFU-GEMM morphology was verified at high power (200x). GFP expression was analyzed on an inverted epifluorescent microscope at indicated magnifications.

For chemical stimulation purified $CD34^+$ HPCs, cells were infected as described above, washed twice with medium and incubated with 10ng/mL PMA in DMEM supplemented with 10% FBS as described elsewhere [42, 43]. For cytokine stimulation of HPCs, cells were infected as described above, washed five times with medium and cultured with 100ng/mL rhGM-CSF, 2.5ng/mL TNF$\alpha$, and 20ng/mL SCF in StemlineII.

Jurkat and CEM-SS cells were cultured in RPMI1640 medium supplemented with 10% FBS, 10mM HEPES, 0.292mg/mL L-glutamine, 100U/mL penicillin and 100U/mL streptomycin. For Jurkat cells were stimulated by treatment with 100ng/mL PMA and 500ng/mL ionomycin for 24h. HEK 293T cells were cultured in DMEM supplemented with 10% FBS, 0.292mg/mL L-glutamine, 100U/mL penicillin and 100U/mL streptomycin.

**HIV preparation**. Infectious supernatants were prepared by transfection of proviral plasmids into 293T cells using polyethylenimine [43]. For unmodified clinical isolates, $5x10^6$ 293T cells were transfected with 8-12µg plasmid DNA. Media was replaced 24 hours post transfection and supernatant was collected 48 hours post-transfection. Supernatants were 0.45µM filtered and stored at -80ºc. For *env*-deleted and internal promoter viruses, 10µg proviral plasmid was transfected along with 2µg *env* expression plasmid as indicated in the text. Supernatants were collected as described above and

virions were concentrated using high-molecular weight polyethylene glycol (PEG) precipitation as described elsewhere [44]. Briefly, filtered supernatants were combined with an equal volume of 20% PEG/0.9% NaCl (20kDa average molecular weight, Fluka) and cooled overnight at 4ºc. Virions were then pelleted by centrifugation at 17800xg for 20 minutes. Pellets were resuspended in $1/5^{th}$ to $1/10^{th}$ the original volume of StemSpan medium and stored at -80oc. Virus infectivity was determined by infection of CEM-SS or CEM-R5 cells under identical conditions. MOI were calculated by applying the percent of infected CEM-SS cells to the formula MOI = -Ln(1-p) where p is the proportion of cells infected.

**HIV infections**. HIV infections were conducted using a standard spin infection technique for primary cells. Briefly, cells were resuspended in infectious supernatants and spun at room temperature for 2 hours at 2500rpm. After infection cells were cultured in media supplemented with cytokines as described in the text.

**Flow cytometry**. For cell analysis, cells were stained in FACS buffer (2%FBS, 1% human serum, 2mM HEPES, 0.025% $NaN_3$/PBS) for 20 minutes on ice. Cells were then washed and fixed in 2% paraformaldehyde/PBS for 20 minutes at room temperature prior to analysis. For experiments analyzing Gag expression, cells were extracted after fixation by incubating for 5 minutes in 0.1% Triton-X100/PBS at room temperature. Cells were then washed and incubated with anti-Gag antibody in FACS buffer for 30 minutes on ice. Cells were analyzed on a FACScan or FACSCanto flow cytometer.

For cell purification, cells were sorted on a FACSVantage SE in Normal-R mode with a sorted drop envelope of 1.0. Sorted cells were collected in media, counted and plated in complete methylcellulose medium as described above.

**qPCR integration assay.** Integration assay was performed as described elsewhere [25]. We modified the forward oligo in the second step PCR reaction ($2^{nd}$-LTR-F) to be homologous to a wider range of HIV molecular clones ($2^{nd}$-LTR-F-univ, GTGTIGAAAATCTCTAGCAGTGGC). Reactions were run in triplicate on a Bio-Rad iCycler. A serial dilution of cellular DNA with a known number integrated HIV DNA copies was run in parallel to verify the amplification efficiency.

**Reverse transcriptase assay**. Cells were cultured in 96-well flat-bottom plates. Every 2-4 days half of the culture volume was collected and replaced with fresh medium. Supernatants were frozen at -80ºc until analysis. Supernatants were then mixed with RT reaction buffer (50mM Tris pH8.0, 1mM EGTA, 75mM KCl, 10mM DTT, 0.05% NP-40, 100µg/mL polyA RNA, 2.5µg/mL oligo-$dT^{18}$, 11mM $MgCl_2$) and incubated for 2 hours at 37ºC. Each reaction was run in triplicate. Reactions were spotted on DEAE membrane and washed extensively with 2xSSC. Membranes were then exposed to storage phosphor and analyzed on a Typhoon Trio imager (GE Healthcare). Signal was quantified on GelQuant software. Mean signal intensities for triplicate reactions were calculated and error is represented as one standard deviation of the mean.

**Isolation of CD34+ cells from HIV-positive people**. HIV+ people with viral loads greater than 50,000 copies per milliliter and normal white blood cell counts were recruited from the University of Michigan Infectious Disease Clinic. The human subjects protocol was approved by the Institutional Review Board and General Clinical Research Center and, as outlined in the protocol all subjects signed informed consent documents. CD4 counts of the subjects ranged from 109 to 840 (mean =408). Viral loads ranged from 260,000 to 61,000 (mean =147,000).. Using sterile procedure, a Jamshidi needle was used to aspirate one mL of marrow aspirate from the posterior iliac crest. The sample was evaluated for spicules to ensure adequate quality, and then ten mL of marrow aspirate was obtained in preservative free heparin. The subjects experienced no adverse events from the procedure. CD34+ cells were isolated as described above, although the low yield from HIV+ patients resulted in decreased purity in some cases. A portion of these cells were immediately stained for anti-p24 Gag directly conjugated to alexafluor or an isotyped-matched control antibody that was also directly conjugated to alexafluor as described above. The remainder of the cells, as well as the column flow-through (CD34- cells) were cultured in media containing GM-CSF plus TNFα for 14 days. The cells were then harvested and stained for CD34 and p24 Gag as described above. Under these conditions, the CD34- control cells died, and thus only cells derived from CD34+ were further evaluated.

## ACKNOWLEDGEMENTS

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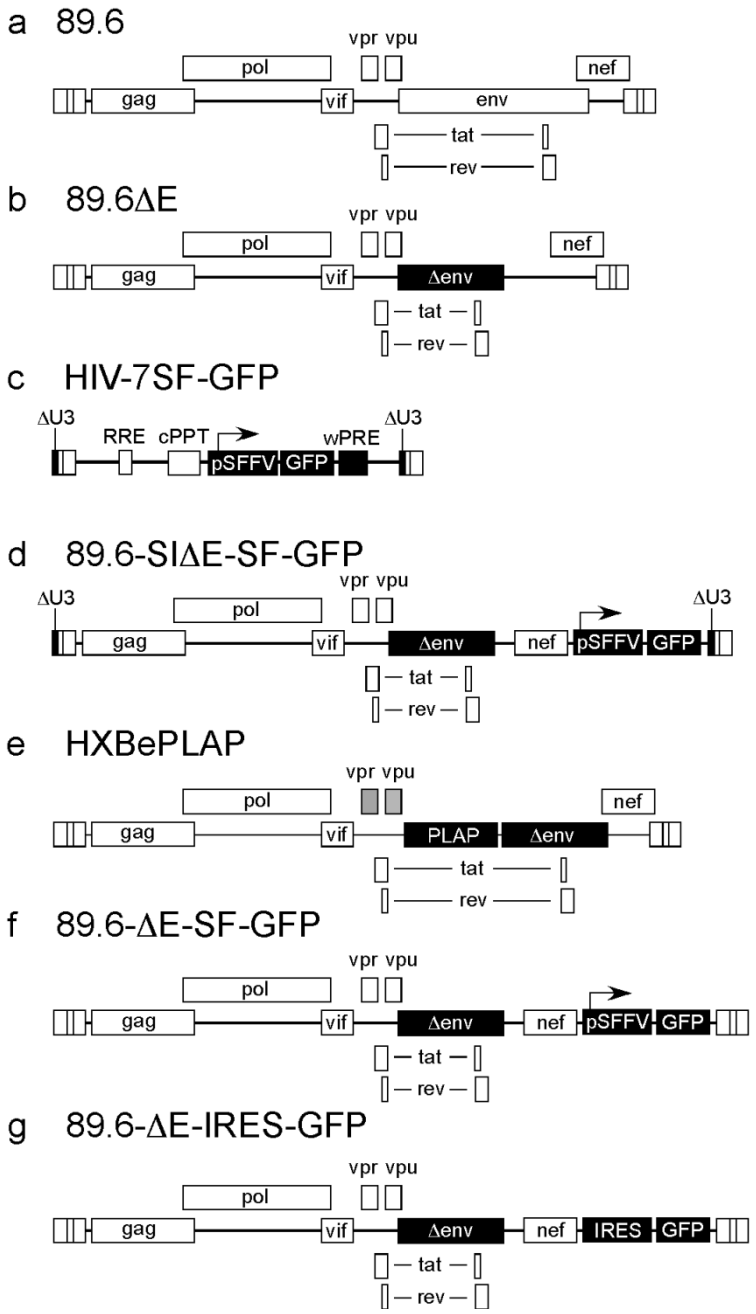
**Figure 2-1.** HIV genomes. a, b, e, f and g are derived from the molecular clone p89.6. c and e have been described elsewhere and are derived from HXB and NL4-3 [29, 39]. Expressed viral genes are shown in white, deletions and additions to the viral genome are shown in black, and non-functional viral genes are shaded in gray. Viral constructs lacking *env* were co-transfected with a separate plasmid expressing the indicated *env* open reading frame. The resultant pseudotyped virus is capable of a single round of infection, but cannot spread.


a 89.6
vpr vpu
pol
nef
gag
vif
env
tat
rev
b 89.6ΔE
vpr vpu
pol
nef
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Δenv
tat
rev
c HIV-7SF-GFP
ΔU3
RRE
cPPT
wPRE
ΔU3
pSFFV
GFP
d 89.6-SIΔE-SF-GFP
vpr vpu
ΔU3
pol
ΔU3
gag
vif
Δenv
nef
pSFFV
GFP
tat
rev
e HXBePLAP
vpr vpu
pol
nef
gag
vif
PLAP
Δenv
tat
rev
f 89.6-ΔE-SF-GFP
vpr vpu
pol
gag
vif
Δenv
nef
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tat
rev
g 89.6-ΔE-IRES-GFP
vpr vpu
pol
gag
vif
Δenv
nef
IRES
GFP
tat
rev

**Figure 2-2.** HIV actively infects HPCs, causing cell death. **a,** Flow cytometric analysis of HPCs infected by HIV 89.6. BM-derived HPCs (>95% $CD34^+$) were infected with a variant of 89.6 in which the envelope gene had a large deletion (89.6ΔE, **Fig. 1b**). Infectivity was restored by pseudotyping with 89.6 *env* expressed on a separate plasmid ($89.6\Delta E/env^{89.6}$). Where indicated, cells were cultured in the presence of 20mM AZT. At 3 days post-infection cells were analyzed for CD34 and intracellular Gag expression. $CD34^+$ cells were gated (left panels) and $Gag^+$ cells were quantified within the $CD34^+$ population in the right panels. Numbers indicate the percent of $CD34^+$ cells expressing Gag. Gray histograms represent isotype control staining. **b**, MHC class I protein is downmodulated in infected HPCs. UCB-derived HPCs (>95% $CD34^+$) were infected with $89.6\Delta E/env^{89.6}$ and analyzed for CD34, HLA-A,B,C (w6/32) and intracellular Gag expression 48 hours post-infection. HLA-A,B,C and Gag were analyzed within the $CD34^+$ population as in a. **c,** HIV infects $Lin^+$ and $Lin^-$ HPCs. BM-derived HPCs (>95% $CD34^+$) were infected with 89.6 and analyzed for CD34, Lin and intracellular Gag expression 3 days post-infection. Cells were first analyzed for CD34 and Lin expression. Gag expression was then analyzed within the $CD34^+Lin^-$ population (gray gates, middle panels) and the $CD34^+Lin^+$ population (black gates, right panels). Numbers indicate the percent of gated cells expressing Gag. Gray events represent isotype control staining. **d,** Actively infected HPCs are rapidly lost in culture. UCB-derived HPCs (>95% $CD34^+$) were infected with 89.6 and the proportion of the cells expressing Gag was monitored over time by flow cytometry. Numbers represent the percent of the cells in culture expressing Gag. **e,** HIV-infected HPCs undergo apoptosis. UCB-derived HPCs (>95% $CD34^+$) were infected with $89.6\Delta E/env^{89.6}$ and stained with anti-CD34, annexin V, and anti-Gag 48h post-infection. $CD34^+Gag^-$ (gray gate and histograms) and $CD34^+Gag^+$ cells (black gate and histogram) were analyzed for annexin V reactivity, shown in the overlayed histograms in the right panel.

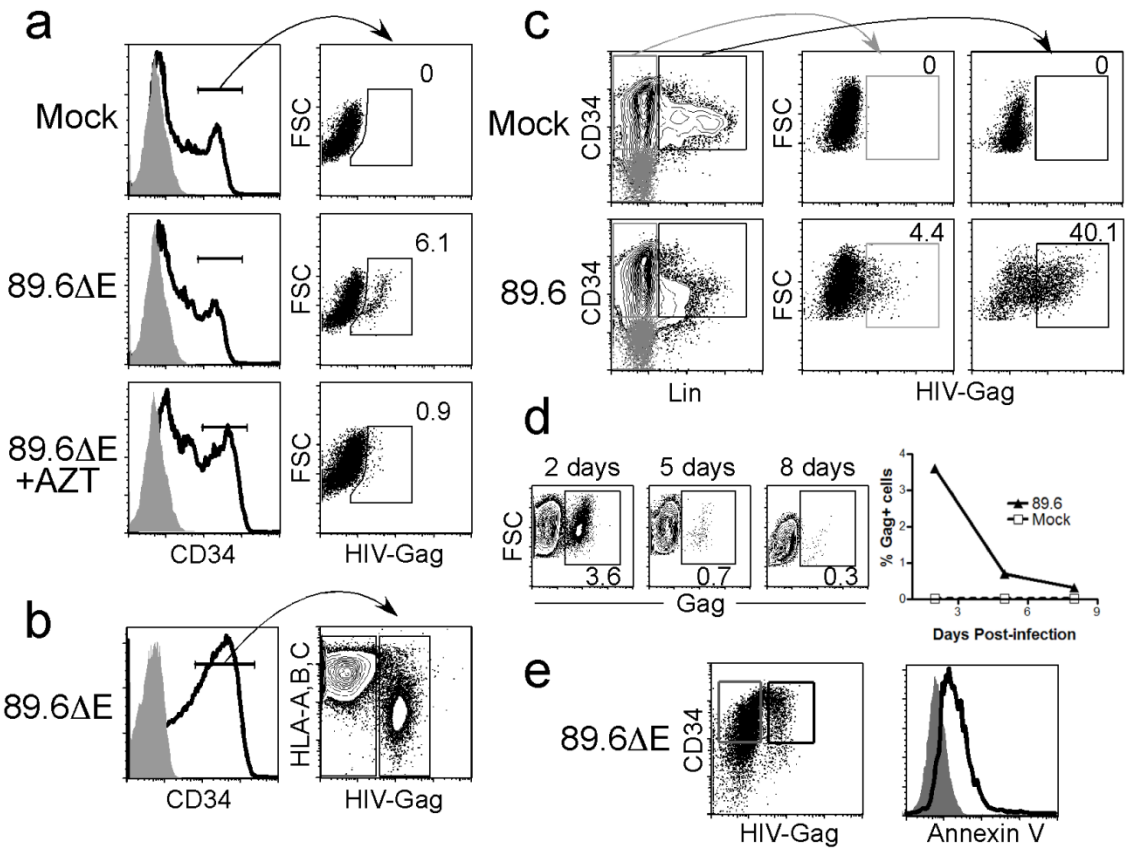

a
Mock
89.6ΔE
89.6ΔE +AZT
FSC
0
6.1
0.9
CD34
HIV-Gag
b
89.6ΔE
HLA-A,B,C
CD34
HIV-Gag
c
Mock
89.6
CD34
FSC
0
4.4
40.1
Lin
HIV-Gag
d
2 days
5 days
8 days
FSC
3.6
0.7
0.3
Gag
% Gag+ cells
89.6
Mock
Days Post-infection
e
89.6ΔE
CD34
HIV-Gag
Annexin V

**Figure 2-3.** HIV infects primitive and multipotent HPCs. **a**, HIV-7SF-GFP/env$^{89.6}$ infects CD34$^{+}$ HPCs. UCB-derived HPCs (>90% CD133$^{+}$) were infected with HIV-7SF-GFP/env$^{89.6}$ and analyzed for CD34 and GFP expression 3 days post-infection. CD34$^{+}$ cells were gated in the left panels and GFP+ cells were quantified in the right panels. Numbers represent the percent of CD34$^{+}$ cells expressing GFP. **b**, HIV-7SF-GFP/env$^{89.6}$ infects primitive phenotype HPCs. Cells shown in part **a** were analyzed for CD133, CD34, CD38 and GFP expression. Cells were first gated for CD133$^{+}$ phenotype, then this population was subgated for CD34$^{+}$CD38$^{-}$ phenotype and the resulting cells were analyzed for GFP expression. Numbers represent the percent of CD133$^{+}$CD34$^{+}$CD38$^{-}$ cells expressing GFP. Gray histograms and events represent isotype control staining. **c,** HIV-7SF-GFP/env$^{89.6}$ infects primitive and multipotent HPCs. UCB-derived HPCs (>90% CD133$^{+}$) were infected with HIV-7SF-GFP/env$^{89.6}$ and sorted by FACS for GFP$^{+}$ cells 3 days post-infection. Cells were then plated in complete methylcellulose medium for 14 days and colonies were counted and scored by phase contrast and epifluorescence microscopy. **d**, Quantification of colony formation potential in infected HPCs. UCB-derived HPCs were infected as in part **c** and were purified by FACS into GFP$^{+}$ and GFP$^{-}$ populations. Equal numbers of cells (6000 per condition) were then plated in complete methylcellulose medium for 14 days after which colonies were counted and scored as erythroid (CFU-E), granulocyte-macrophage (CFU-GM) or multilineage (CFU-GEMM). **e**. 89.6-SIΔE-GFP/env$^{89.6}$ infects immature HPCs. UCB-derived HPCs (>90% CD133$^{+}$) were infected with 89.6-SIΔE-GFP/env$^{89.6}$ and analyzed by colony formation assay as in **c**. **f**. Quantification of colony formation potential in 89.6-SIΔE-GFP/env$^{89.6}$ infected HPCs. UCB-derived HPCs were infected with 89.6-SIΔE-GFP/env$^{89.6}$ and analyzed by colony formation assay as in part **d**. Magnifications for parts **c** and **e** were CFU-E: 100x, CFU-M and CFU-GM: 40x, CFU-GEMM: 40x (left panel) and 100x (right panel).

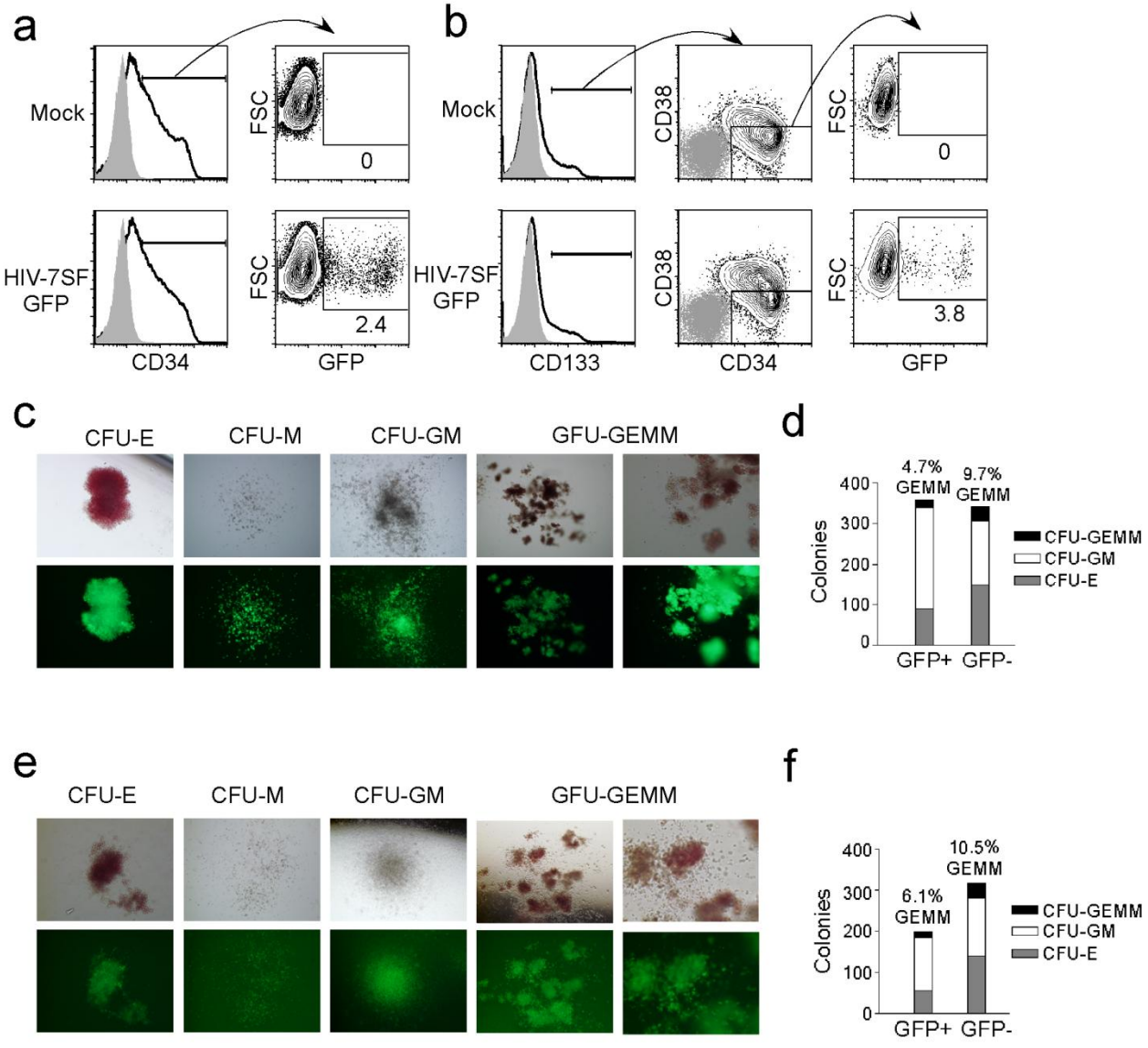

a
Mock
FSC
0
HIV-7SF GFP
2.4
CD34
GFP
b
Mock
CD38
FSC
0
HIV-7SF GFP
3.8
CD133
CD34
GFP
c
CFU-E
CFU-M
CFU-GM
GFU-GEMM
d
4.7% GEMM
9.7% GEMM
Colonies
400
300
200
100
0
GFP+
GFP-
CFU-GEMM
CFU-GM
CFU-E
e
CFU-E
CFU-M
CFU-GM
GFU-GEMM
f
10.5% GEMM
6.1% GEMM
Colonies
400
300
200
100
0
GFP+
GFP-
CFU-GEMM
CFU-GM
CFU-E

**Figure 2-4.** Induction of HIV from latency. **a,** PMA induces HIV expression in infected HPCs. Bone marrow-derived $CD34^+$ HPCs (>98% pure) were infected with HXB-ePLAP/$env^{VSVG}$ and stimulated with 10mM PMA or DMSO as solvent control. Cells were then analyzed for PLAP expression by flow cytometry. Numbers represent the percent of cells expressing PLAP. **b,** HIV integration is unaffected by PMA stimulation. Bone marrow derived $CD34^+$ cells were infected and cultured as in **a**, and total DNA was prepared from equal numbers of cells. Integrated DNA was quantified by nested qPCR as described elsewhere [25]. First round PCR reactions were performed with and without polymerase to account for amplification of non-integrated HIV genomes. Data are displayed as mean relative amount of integrated HIV DNA from three reactions with 1 standard deviation error bars. **c,** BM-derived $CD34^+$ HPCs (>98% pure) were infected with HXB-ePLAP/$env^{89.6}$ and stimulated with 10mM PMA or DMSO. The cells were then analyzed for intracellular Gag expression by flow cytometry. Numbers indicate the percent of cells expressing Gag. **d,** GM-CSF/TNFα stimulation induces HIV expression from infected HPCs. BM-derived HPCs were sorted into $CD34^+$ (>98% $CD34^+$) and $CD34^-$ (<1% $CD34^+$) fractions. $CD34^+$ and $CD34^-$ cells were then infected with HIV 89.6 and treated with GM-CSF (100ng/mL), TNFα (2.5ng/mL) and SCF (20ng/mL) or with STF cocktail (50ng/mL STF, 50ng/mL TPO, 50ng/mL Flt3-L). Supernatants were collected at the indicated time points and HIV release was measured by reverse transcriptase assay. Data are displayed as the mean plus and minus standard deviation , n=3 **e,** Infected dendritic cells and macrophages arise in GM-CSF/TNFα stimulated HPC cultures. BM-derived HPCs were infected and cultured as in c. After 14 days in culture, cells were analyzed for intracellular Gag, HLA-DR and Lin expression by flow cytometry. $Gag^+$ events were gated in the FSC vs. Gag plots (GM-CSF/TNFα plots, center panels) and overlayed on HLA-DR vs. Lin plots (right panels). Light gray events are $Gag^-$ cells whereas black events represent $Gag^+$ cells. **f,** Latently infected cells are stable in culture. BM-derived HPCs were infected with HIV 89.6 and cultured in STF cocktail. Cells were monitored for intracellular Gag expression at indicated time points. When $Gag^+$ cells were no longer detectable (day 7) the culture was split in two and half was cultured further in STF cocktail and half was treated GM-CSF/TNFα. Numbers indicate the percent of cells expressing Gag. Asterisks represent Gag reactivity that was less than or equal to mock infected control. **g,** Graphical representation of the experiment depicted in part **f**.

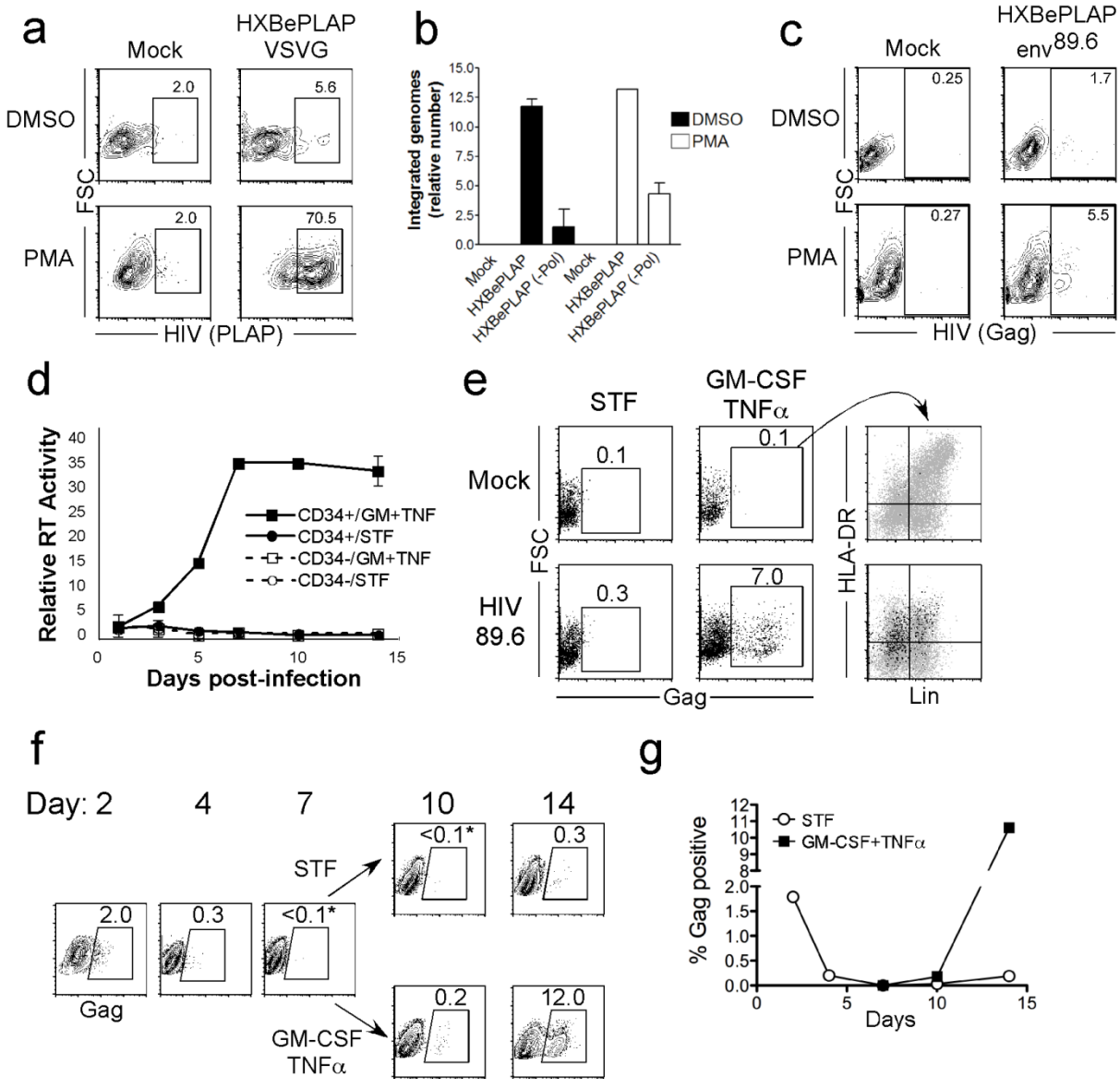
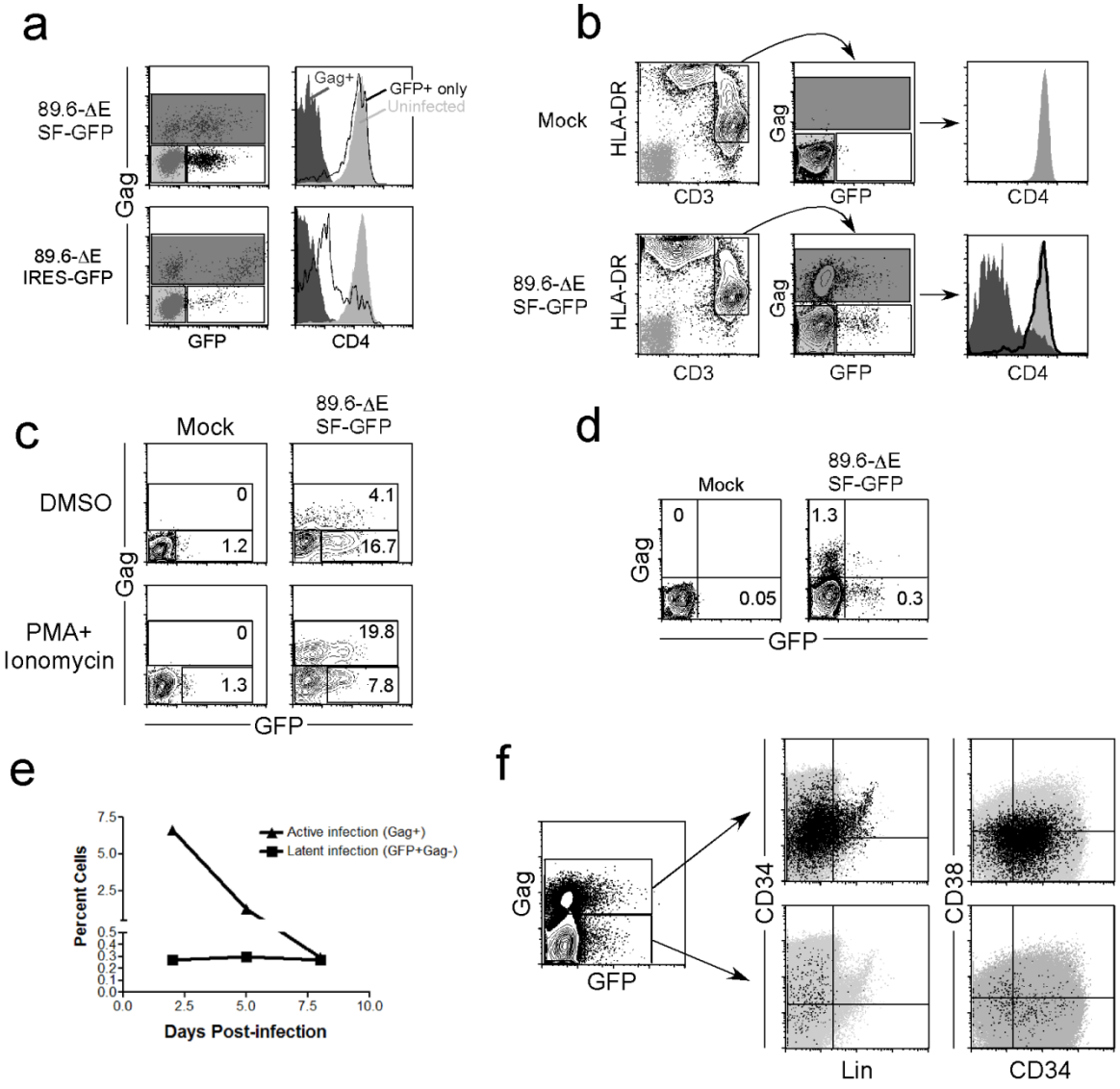

a
Mock
HXBePLAP VSVG
DMSO
PMA
FSC
2.0
5.6
2.0
70.5
HIV (PLAP)
b
Integrated genomes (relative number)
DMSO
PMA
Mock
HXBePLAP
HXBePLAP (-Pol)
c
Mock
HXBePLAP env89.6
0.25
1.7
0.27
5.5
HIV (Gag)
d
Relative RT Activity
CD34+/GM+TNF
CD34+/STF
-CD34-/GM+TNF
-CD34-/STF
Days post-infection
e
STF
GM-CSF TNFα
Mock
HIV 89.6
0.1
0.1
0.3
7.0
Gag
HLA-DR
Lin
f
Day: 2
4
7
10
14
2.0
0.3
<0.1*
STF
<0.1*
0.3
Gag
GM-CSF TNFα
0.2
12.0
g
% Gag positive
STF
GM-CSF+TNFα
Days

**Figure 2-5.** HIV latency probe detects both active and latent infection in T cells and HPCs. **a,** 89.6-ΔE-SF-GFP/env$^{89.6}$ "tags" latently infected cells. CEM-SS cells were infected with 89.6-ΔE-SF-GFP/env$^{89.6}$ or 89.6-ΔE-IRES-GFP/env$^{89.6}$. Seven days post-infection, cells were analyzed for intracellular Gag, GFP and CD4 surface expression. Actively infected cells (Gag$^{+}$, dark gray gates and histograms and GFP+ for 89.6-ΔE-IRES-GFP/env$^{89.6}$, white gates and histograms), latently infected cells (Gag$^{-}$GFP$^{+}$ for 89.6-ΔE-SF-GFP/env$^{89.6}$, white gates and histograms) and uninfected cells (Gag$^{-}$GFP$^{-}$, light gray gates and histograms) were gated in the left panels, and CD4 expression was analyzed within these populations in the right panels. **b,** 89.6-ΔE-SF-GFP env$^{89.6}$ "tags" latently infected primary T cells. CD8-depleted PBMC were stimulated with PHA and IL-2 for 48h before being infected with 89.6-ΔE-SF-GFP/env$^{89.6}$. 48h post-infection cells were analyzed for CD3, CD4, HLA-DR, GFP and intracellular Gag expression. Activated T cells were gated based on CD3$^{+}$HLA-DR$^{+}$ phenotype (left panels), and GFP and Gag expression was analyzed within this subset (right panels). CD4 expression was then analyzed within the actively infected population (Gag$^{+}$, dark gray gates and histograms) and the latently infected population (Gag$^{-}$GFP$^{+}$, white gates and histograms). Gray events in the left panels represent isotype control staining. **c,** PMA and ionomycin stimulation induces active infection from latently infected Jurkat cells. Jurkat cells were infected with 89.6-ΔE-SF-GFP/env$^{89.6}$ and cultured for 7 days without stimulation. Culture was then split in two and half was treated with PMA (100ng/mL) and ionomycin (500ng/mL) and the other half with DMSO solvent control. After 48h of stimulation, cells were analyzed for GFP and Gag expression by flow cytometry. Numbers represent percent of cells within the Gag$^{+}$ and Gag$^{-}$GFP$^{+}$ gates. **d**, HIV establishes latent infection in HPCs. UCB-derived CD34$^{+}$ HPCs were infected with 89.6-ΔE-SF-GFP/env$^{89.6}$. Three days post-infection cells were analyzed for GFP and intracellular Gag expression by flow cytometry. **e**, Latently infected HPCs are maintained in culture. UCB-derived CD34$^{+}$ HPCs were infected as above and cultured in STF. Cells were analyzed for GFP and intracellular gag expression at the indicated time points. The percent of actively infected (Gag$^{+}$) and latently infected (Gag$^{-}$GFP$^{+}$) cells is shown. **f**. Immature phenotype HPCs undergo latent infection. UCB-derived CD34$^{+}$ HPCs were infected as above and cultured for 3 days in STF medium. Cells were then analyzed for CD34, CD38, Lin, GFP and intracellular Gag expression. Actively infected (Gag$^{+}$) cells and latently infected (Gag$^{-}$GFP$^{+}$) cells were gated on the left plot and overlayed on CD34 vs. Lin plots (middle panels) or CD34 vs. CD38 plots (right panels).


a
89.6-ΔE SF-GFP
89.6-ΔE IRES-GFP
Gag
GFP
CD4
Gag+
GFP+ only
Uninfected
b
Mock
89.6-ΔE SF-GFP
HLA-DR
CD3
Gag
GFP
CD4
c
Mock
89.6-ΔE SF-GFP
DMSO
PMA+ Ionomycin
0
1.2
4.1
16.7
19.8
1.3
7.8
d
0
0.05
1.3
0.3
e
Active infection (Gag+)
Latent infection (GFP+Gag-)
Percent Cells
Days Post-infection
f
CD34
CD38
Lin

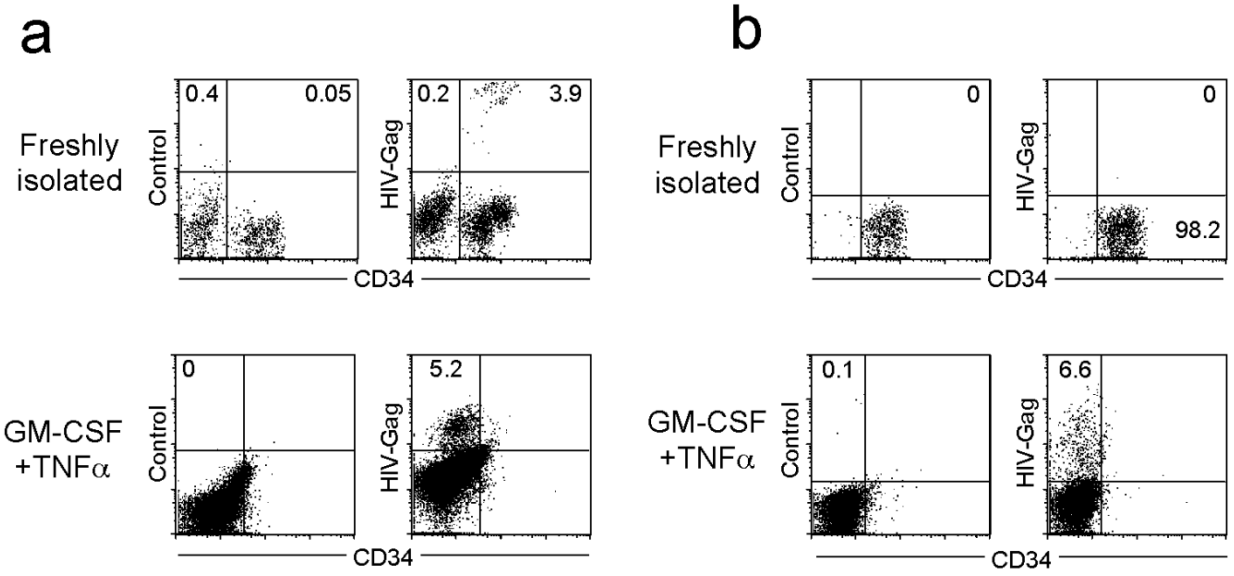



**Figure 2-6.** Infected HPCs are found in HIV-infected individuals. Bone marrow aspirates were collected from HIV-infected individuals with a viral load >50,000 copies/mL in accordance with an IRB-approved protocol. BM-MNC were adherence-depleted and CD34+ cells were enriched by immunomagnetic cell sorting. Immediately following isolation, cells were stained for CD34 and intracellular Gag expression (top panels) or an isotype-matched antibody (Control). Gag+ cells were detected in freshly isolated CD34+ cells in 50% of patients analyzed (n=6). For two of these donors, an aliquot of the CD34-enriched cells was pre-cultured for four days in STIF media to expand the number of CD34+ cells. The cells were then cultured for 2 weeks in GM-CSF/TNFα, after which the cells were analyzed for CD34 and Gag expression as above (lower panels). Under these conditions, CD34-depleted, control cells grown in parallel were not viable, and thus it was concluded that all living cells must have been derived from CD34+ cells.

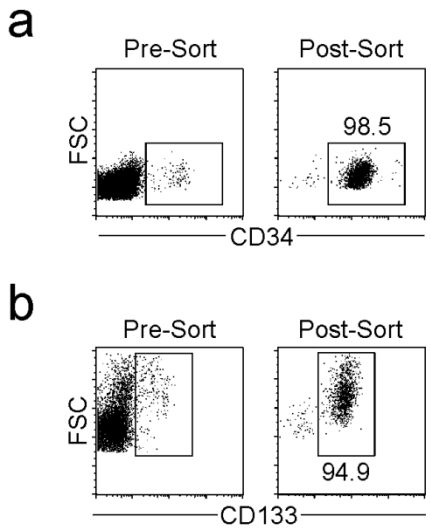



**Figure 2-7.** HPC purification. Bone marrow mononuclear cells were adherence depleted and sorted for CD34 (**a**) or CD133 (**b**) using commercially available immunomagnetic cell separation kits, as described in the results section. Antibodies against CD34 and CD133 were included during cell labeling to assess sort purity.

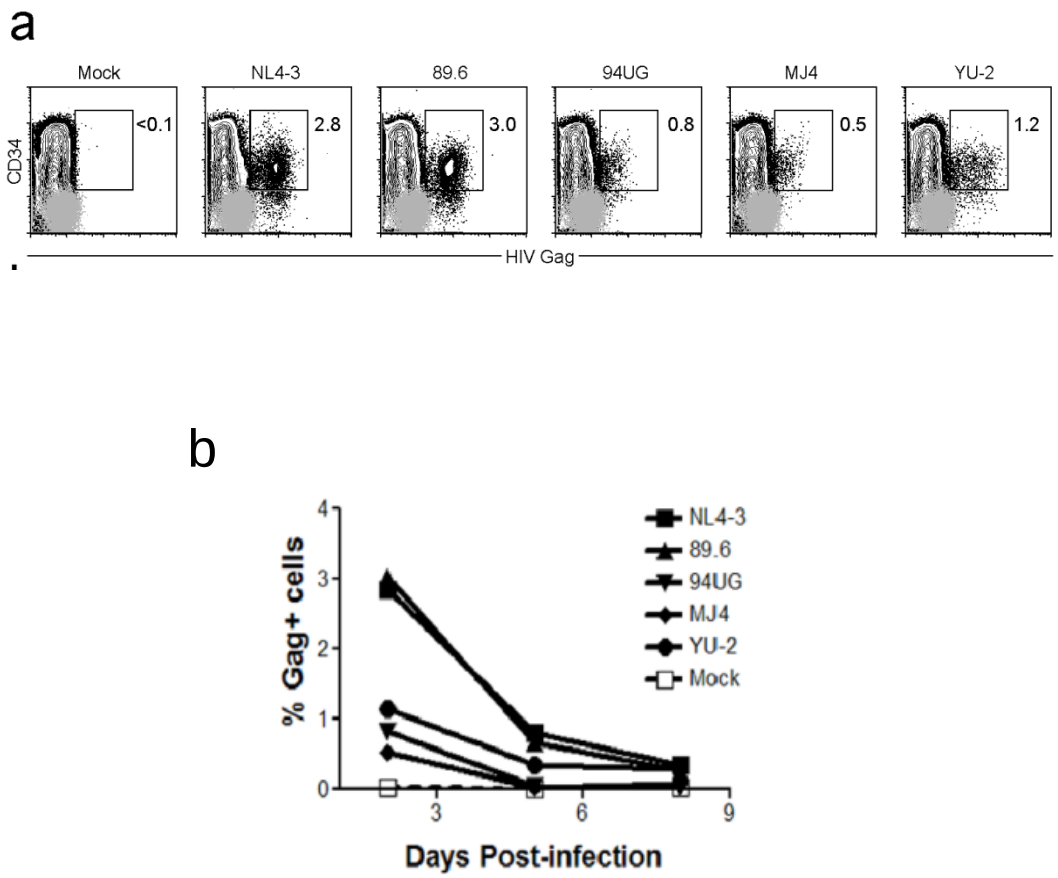



**Figure 2-8.** Infection of HPCs by HIV molecular clones. **a**, HPCs can be infected by a wide range of HIV molecular clones. Cord blood derived $CD34^+$ HPCs were infected with infectious supernatants derived from NL4-3, 89.6, 94UG, MJ4 or YU-2. 48 hours post-infection cells were analyzed for CD34 and intracellular Gag expression. Numbers represent the percent of $CD34^+Gag^+$ cells. The shaded population represents mock-treated CD34+ cells stained with an isotype-matched control antibody. **b,** Loss of HPCs due to active infection by several HIV molecular clones. Cells were infected as in **a** and cultured in STF medium. Gag expression was analyzed periodically by flow cytometry. Data are shown as percent $Gag^+$ cells.

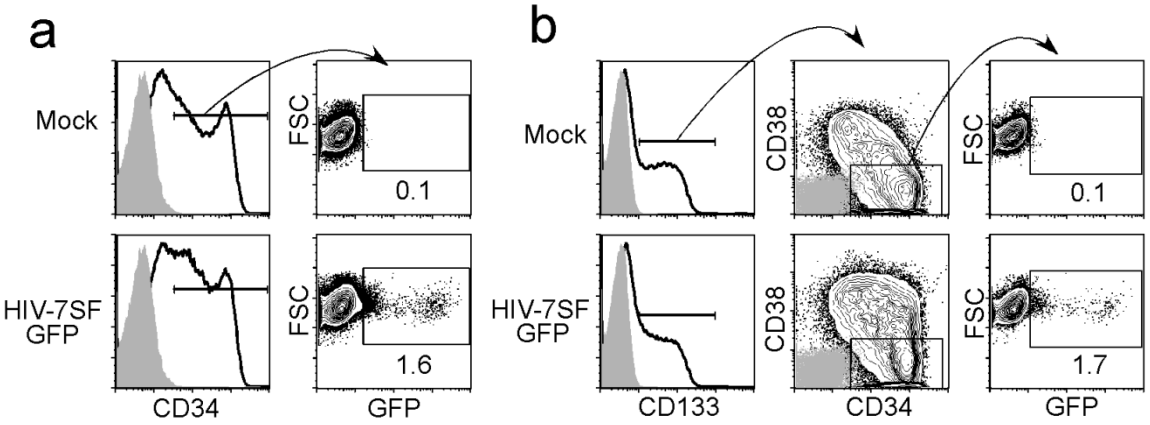



**Figure 2-9.** HIV infects immature phenotype bone-marrow derived HPCs. **a**, HIV-7SF-GFP/env$^{89.6}$ infects CD34$^{+}$ HPCs. Bone marrow-derived HPCs (>90% CD133$^{+}$) were infected with HIV-7SF-GFP/env$^{89.6}$ and analyzed for CD34 and GFP expression 3 days post-infection. CD34$^{+}$ cells were gated in the left panels and GFP$^{+}$ cells were quantified in the right panels. Numbers represent the percent of CD34$^{+}$ cells expressing GFP. **b**, HIV-7SF-GFP/env$^{89.6}$ infects immature phenotype HPCs. Cells shown in part **a** were analyzed for CD133, CD34, CD38 and GFP expression. Cells were first gated for CD133+ phenotype, then this population was subgated for CD34$^{+}$CD38$^{-}$ phenotype and the resulting cells were analyzed for GFP expression. Numbers represent the percent of CD133$^{+}$CD34$^{+}$CD38$^{-}$ cells expressing GFP. Gray histograms and events represent isotype control staining.

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## CHAPTER III

# CXCR4-utilizing HIV envelopes facilitate the infection of primitive hematopoietic progenitor cells


## ABSTRACT

HIV establishes a persistent infection in human hosts, characterized by gradual collapse of the immune system as well as hematopoietic dysfunction. As described in the previous chapter, HIV can establish both active and latent infection in hematopoietic progenitor cells (HPCs), which may provide an explanation for viral persistence as well as hematopoietic dysfunction. Here, we show that viral envelopes capable of using CXCR4 as a coreceptor for viral entry infect the most primitive HPCs, whereas viral envelopes that use CCR5 exclusively fail to infect primitive HPCs. By assaying a panel of HIV envelopes with known coreceptor usage profiles, we found that CXCR4-tropic envelopes infected primitive HPCs as assessed by immunophenotype and by colony formation capacity. Additionally, the ability of dual-tropic (CXCR4 and CCR5-utilizing) HIV envelopes to infect primitive HPCs was blocked by the CXCR4-antagonist AMD3100. We also employed the NOD-SCID xenotransplantation system to determine if hematopoietic stem cells (HSCs) could be infected by CXCR4-triopic HIV envelopes. These studies are currently underway, but preliminary results have shown engraftment of HIV-infected cells in several animals, suggesting infection of HSCs may indeed occur.

These results have significant implications for the virus' ability to persist within the host despite effective antiretroviral therapy, and may provide an explanation for the poor prognosis associated with the emergence of CXCR4-tropic HIV isolates.

## INTRODUCTION

The natural course of HIV disease is characterized by progressive destruction of the host immune system, manifested by decline in CD4+ T cell counts over several years. Depletion of CD4+ T cells invariably causes an immunocompromised state in the host, leading to the onset of AIDS, and ultimately death from opportunistic infections. Despite extensive study, the exact mechanisms that trigger the progression to AIDS remain unclear.

HIV entry into permissive cells is facilitated by interactions with the HIV envelope protein gp120, CD4 and a chemokine coreceptor. The chemokine coreceptors predominantly used by HIV are CCR5 and CXCR4 [1-4]. Other chemokine receptors have been implicated as HIV coreceptors [5-12], although the *in vivo* significance of these receptors is questionable [13, 14]. The initial transmission of HIV is facilitated exclusively by CCR5-utilizing (R5-tropic) HIV regardless of the route of transmission [15, 16], and these R5-tropic isolates predominate during the early course of disease. The reasons for the selection of R5-tropic HIV in early disease are likely numerous, and collectively constitute the "gatekeeper effect," which restricts the transmission of X4-tropic HIV isolates and favors the transmission of R5-tropic HIV (reviewed in [17]). As disease progresses, CXCR4-utilizing (X4-tropic) and dual coreceptor-utilizing (R5X4-

tropic) isolates emerge in many patients. Initially, it was thought that X4-tropic isolates emerged in about 50% of infected individuals [18], however methodological factors may underestimate the prevalence of X4 virus emergence. One study used newer genetic approaches to detect X4-tropic HIV isolates and found that X4 HIV emerged in all patients studied [19]. The conversion of R5-tropic HIV envelopes to an X4-tropic phenotype requires a small number of mutations in the envelope V3 region, and yet evolution of X4-tropic HIV occurs only in the later stages of disease. The factors that trigger the evolution of X4-tropic isolates are unclear, but likely involve a combination of virological, immunological and environmental selective pressures (reviewed in [20]).

It has been well documented that the emergence of X4-tropic HIV in infected individuals correlates with disease progression [21, 22]. Individuals with X4-tropic HIV typically display reduced CD4 T cell counts and have a poor clinical prognosis [23, 24]. This phenomenon is particularly profound in the rare instances when infection is initiated by X4-tropic HIV, a scenario that results in rapid decline of CD4 T cell counts and disease progression [25]. The reasons for the association between coreceptor switch and disease progression are unknown.

One possible explanation for viral persistence and the hematopoietic abnormalities associated with HIV infection is the infection of hematopoietic progenitor cells (HPCs). It is known that a subset of HPC express CD4 and CCR5 or CXCR4 [26, 27]. Early studies of HPC infection by HIV yielded conflicting results, with many studies concluding that HPCs are inherently resistant to HIV infection. In Chapter II, we demonstrated that subsets of HPCs are in fact susceptible HIV infection, including highly primitive multipotent progenitors. HPCs can be infected by X4, R5 and R5X4 tropic

HIV isolates, as expected considering that subsets of HPCs exist that express these receptors. Interestingly, it has been reported that primitive phenotype HPCs and common lymphoid progenitors express CXCR4 [28, 29]. This raises the intriguing possibility that different HPC subsets could be differentially susceptible to HIV infection by R5-tropic and X4-tropic HIV. To assess this question, we employed flow cytometry, colony formation assays and murine xenotransplantation approaches to study the infection of HPCs by R5, X4 and R5X4 –tropic HIV enveloped virus. We report here that X4-tropic envelopes have the capacity to infect primitive multipotent HPCs, whereas R5-tropic envelopes infect more differentiated HPCs, which are primarily myeloid-committed. These findings provide substantial insight into HIV pathogenesis, providing a possible mechanism for the poor prognosis associated with the emergence of X4-tropic HIV.

## RESULTS

**X4-tropic envelopes infect primitive-phenotype HPCs**. To assess the susceptibility of HPCs to infection by R5, X4 and R5X4-tropic envelopes to HIV infection, we used the HIV-7SF-GFP system, as described in Chapter 2 (Figure 2-1). HIV-7SF-GFP is a minimal HIV construct, which encodes only EGFP under the control of the SFFV promoter. We co-transfected this construct along with a packaging-null/envelope-null HIV genome and an HIV envelope-expressing plasmid. When these constructs are co-expressed in producer cells, infectious viral particles are produced that bear the envelope of interest and only contain the reporter cassette. These viral particles infect cells the same as wild-type HIV but only express GFP following integration. This approach

allows us to "tag" susceptible cells while avoiding the cytopathic effect associated with active HIV infection. We prepared HIV-7SF-GFP virus pseudotyped with VSV-G envelope or with HIV envelopes of known coreceptor usage profiles (**table 3-1**) and used these viruses to infect cord-blood derived HPCs. After infection, we assessed GFP expression and cell surface phenotype by flow cytometry. HIV-7SF-GFP pseudotyped with the receptor-independent envelope VSV-G robustly infected $CD34^+$ and $CD34^-$ cells as well as $CD133^+$ and $CD133^-$ cells, demonstrating that no post-entry block to viral infection exists in any of these cell populations. Remarkably, we found that the X4-tropic envelope HXB and the R5X4-tropic envelope 89.6 infected $CD34^{Hi}$ as well as $CD133^+$ HPCs, indicating infection of more primitive HPCs. In contrast, the R5 tropic envelope YU-2 failed to infect CD133+ cells, and most infection was seen in $CD34^{Lo}$ and $CD34^-$ cells, indicating infection of more differentiated HPCs **(fig. 3-1a and b**).

Having demonstrated that X4-tropic HIV envelopes facilitate the infection of primitive-phenotype HPCs using the HIV-7SF-GFP reporter virus, we next wished to determine if intact HIV molecular clones with differing envelope tropism also infect different HPC subsets. We infected cord blood-derived HPCs with the HIV molecular clones NL4-3, 89.6, YU-2 and 94UG. The cells were then stained for CD34, hematopoietic lineage markers (Lin), and intracellular HIV Gag. Flow cytometry was then used to analyze the surface phenotype of the infected cells. When cells were infected with NL4-3 (X4-tropic) or 89.6 (R5X4-tropic), the infected cells were largely of a $CD34^+Lin^-$ phenotype. Conversely, when cells were infected with YU-2 or 94UG (R5-tropic), the infected cells were largely of the differentiated $Lin^+$ phenotype (**fig. 3-2a**).

This shows that X4-tropic HIV molecular clones infect non-committed HPCs more frequently then R5-tropic HIV.

To ensure that these findings were not unique to cord-blood derived HPCs, we performed similar experiments using bone marrow-derived HPCs. We infected bone marrow-derived HPC with the molecular clones NL4-3, 89.6 and YU-2. Cells were then analyzed for CD34, CD133 and HIV gag expression. We found that NL4-3 infected cells of the $CD34^{+}CD133^{+}$ phenotype, whereas 89.6 and YU-2 infected primarily $CD34^{+}CD133^{-}$ cells (**fig. 3-2b**). These findings indicate that X4-tropic HIV molecular clones infect more primitive HPCs, whereas R5-tropic molecular clones primarily infect more differentiated HPCs, similar to the results seen using HIV envelope pseudotyped HIV-7SF-GFP.

**X4-tropic HIV envelopes facilitate infection of multipotent HPC.** To determine whether X4-tropic and R5 tropic envelopes are capable of infecting multipotent HPCs, we infected cord-blood derived HPCs with HIV-7SF-GFP pseudotyped with Hxb envelope (X4-tropic) or YU-2 envelope (R5-tropic). Three days after infection, $GFP^{+}$ cells were purified (>95% pure, **fig. 3-3a**), and identical numbers of $GFP^{+}$ cells were plated in complete methylcellulose medium. After two weeks, colonies were analyzed for morphology and GFP expression. We found numerous colonies of erythroid (CFU-E), myeloid (CFU-GM), and multilineage (CFU-GEMM) morphology when cells had been infected with HXB enveloped virus. In contrast, only small colonies arose from HPCs infected with YU-2 enveloped virus (**fig. 3-3b**). When colonies were quantified, it was clear that the X4-tropic HXB envelope infected numerous primitive and multipotent

HPCs, whereas the R5-tropic YU-2 envelope rarely infected primitive HPCs (**fig. 3-3c**). It was possible that the differential ability of HXB and YU2 envelopes to infect primitive HPC was coincidental, so we analyzed another pair of envelopes, NL4-3 (X4-tropic) and BaL (R5-tropic). Cord blood derived HPCs were infected with HIV-7SF-GFP pseudotyped with NL4-3 or BaL envelope and analyzed by colony formation assay as described above. Again, we found that the X4-tropic envelope (NL4-3) was able to infect erythroid, myeloid and multipotent HPCs, whereas the R5-tropic envelope (BaL) was not (**fig. 3-3d**). These findings demonstrate that X4-tropic envelopes have the capacity to infect primitive HPCs, whereas R5-tropic envelopes primarily infect more differentiated HPCs.

We hypothesized that infection of primitive HPC with X4-tropic envelopes was occurring by the canonical mechanism, wherein HIV envelope triggers membrane fusion after binding CD4 and CXCR4 on the target cell. However, numerous reports have documented HIV infection by CD4-independent mechanisms, usually involving the use of CXCR4 alone to facilitate entry [30-34]. To explore this possibility, we treated cord-blood derived HPCs and CEM T cells with the CD4-blocking antibody L3T4 before infecting the cells with HIV-7SF-GFP pseudotyped with the 89.6 envelope. As expected, pre-treatment with CD4-blocking antibody reduced the infection of CEM T cells significantly (~10-fold, **fig. 3-4a**, top panels). Pretreatment of HPCs with CD4-blocking antibody blocked infection even more robustly, causing a near-complete block in infection (~17-fold reduction in infection, **fig. 3-4a**, bottom panels). This demonstrated that infection of HPCs is strictly dependent on CD4. We then assessed the immunophenotype of infected HPCs with and without CD4 blockade. CD4 antibody

treatment blocked infection of primitive HPCs ($CD34^{High}/CD133^{+}$) as well as more differentiated cells, suggesting that infection of all HPCs is CD4-dependent (**fig. 3-4b**).

Although infection of primitive HPCs only occurred using X4-tropic HIV envelopes, this does not necessarily mean that infection is occurring using CXCR4 as a coreceptor. In fact, many other cell surface proteins have been reported to function as HIV entry co-receptors (reviewed in [35]). Additionally, it is possible that the envelopes we studied above could have differing capacities to infect primitive HPCs due to other factors besides CXCR4 usage. We hypothesized that if infection of primitive HPCs truly requires CXCR4, then blockade of CXCR4 would reduce the infection of primitive HPCs by dual-tropic HIV envelopes. To accomplish this, we used a potent small molecule CXCR4 antagonist, AMD3100 [36]. To validate this approach, we used CEM-SS T cells, which express only CXCR4, and CEM-R5 T cells, which express both CXCR4 and CCR5. Cells were treated with AMD3100 and infected with HIV-7SF-GFP pseusotyped with the dual-tropic envelope 92HT. AMD3100 blocked infection of CEM-SS cells almost completely, demonstrating effective blockade of CXCR4. Conversely, AMD3100 only partially blocked infection of CEM-R5 cells, demonstrating usage of CCR5 by 92HT envelope in these cells (**fig. 3-4c**). We then treated cord-blood derived HPCs with AMD3100 and infected the cells with HIV-7SF-GFP/89.6env. FACS analysis revealed that AMD3100 significantly but incompletely inhibited infection of HPCs (~5-fold, data not shown). We sorted $GFP^{+}$ cells from AMD3100-treated and control-treated cultures and plated the cells in methylcellulose medium. Control treated cells formed numerous erythroid, myeloid and multi-lineage colonies, whereas few colonies formed form

AMD3100-treated cells (**fig. 3-4d**). This demonstrates that CXCR4 usage is in fact necessary for the infection of primitive HPCs by HIV.

**X4-tropic HIV envelopes infect hematopoietic progenitor cells with NOD-SCID engraftment potential**. Having demonstrated that X4-tropic HIV envelopes can infect multipotent HPCs, we next asked whether hematopoietic stem cells were also susceptible to infection. Because we observed no infection of multipotent HPCs with R5-tropic envelopes, we limited our studies to X4-tropic envelope. To assay for infected HSCs, we performed murine xenotranplantation experiments as outlined in **Figure 3-5a**. Cord blood-derived HPCs were infected with HIV-7SF-GFP pseudotyped with HXB envelope. Three days after infection, GFP+ cells were enriched to 50-70% purity (**fig. 3-5b**). Cells were partially purified in order to provide an internal positive control for engraftment. Cells were then intrafemorally injected into sublethally irradiated NOD-SCID IL-2R$\gamma^{-/-}$ mice.

8 weeks after transplantation, peripheral blood was collected from the mice and analyzed for human and murine CD45, CD3, CD19, CD33 and GFP expression by flow cytometry. The flow cytometric approach used to identify human cells in transplanted mice is shown in **Figure 3-5c**. Although these experiments are in progress and are still in early stages, several animals have shown low level engraftment of human cells with $GFP^+$ cells in the peripheral blood. One animal (1-1-N, **fig. 3-6c**) showed human cell engraftment with $GFP^+$ B cells in the peripheral blood. The frequency of human cells in the blood of this animal peaked at 8 weeks post-transplant and has gradually declined

(data not shown). Two other animals (2-2-2R and 3-3-N, **fig. 3-6d and e**) showed $GFP^+$ B and myeloid cells in the peripheral circulation which has been sustained to date. Several other animals failed to engraft any human cells (**fig. 3-6a**). To date, only one animal transplanted with $GFP^+$ cells has shown engraftment of GFP-negative cells only (**fig. 3-6b**). While these results are preliminary in nature, they indicate that primitive lymphoid progenitors can be infected by X4-tropic HIV, and suggest that multipotent HSCs may also be susceptible to infection.

## DISCUSSION

In the previous chapter, we demonstrated that HIV can infect primitive HPCs, establishing either active or latent infection. Here we demonstrate that the infection of primitive HPCs is facilitated by CXCR4-utilizing HIV envelopes. We found that CXCR4-utilizing envelopes infected primitive phenotype HPCs as well as HPCs with multi-potent colony forming capacity. In contrast, HIV envelopes that only utilize CCR5 as a coreceptor failed to infect primitive phenotype HPCs, and HPCs infected with R5-tropic HIV envelopes failed to form colonies in semi-solid medium. Importantly, the infection of HPCs was dependent on CD4, as CD4-blocking antibodies prevented infection of all HPCs. Furthermore, blockade of CXCR4 inhibited the infection of primitive HPCs by dual-tropic HIV envelope, showing that CXCR4 usage facilitates infection of primitive hematopoietic cells. Finally, we show that X4-tropic envelopes infect HPCs with NOD-SCID engrafting capacity, demonstrating that HIV may be able to

infect hematopoietic stem cells. These findings provide significant insights into the HIV pathogenesis, and may help explain the clinical decline associated with the emergence of CXCR4-tropic HIV isolates.

Using a panel of HIV-expression constructs, we found that X4-tropic HIV envelopes have a unique capacity to infect HPC with a primitive surface phenotype, including CD34-high and $CD133^+$ cells. The ability to infect $CD133^+$ cells was particularly profound, as R5-tropic envelopes completely failed to infect this population. Interestingly, the dual tropic HIV envelope from the molecular clone 89.6 seemed to have an intermediate phenotype, infecting many HPCs with a differentiated phenotype, but also infecting primitive phenotype cells. Importantly, a similar pattern was observed when cells were infected with intact HIV molecular clones, verifying that our findings are not an artifact of the HIV-7SF-GFP pseudovirus system. For most of the experiments performed in this work we employed umbilical cord blood-derived HPCs, because large numbers of these cells can be obtained from donors without an additional invasive procedure, and because these cells are highly enriched for primitive HPCs [37]. However, we found similar results when experiments were performed using adult bone marrow-derived HPCs, suggesting that findings from cord blood-derived cells are generalizable to adult HPC.

Because the value of surface phenotype may become compromised when HPCs are cultured *ex vivo*, it was important to validate our immunophenotype observations with an assay that assesses cell physiology. For this reason, we analyzed several HIV envelopes using methylcellulose colony formation assays. We found that X4-tropic envelopes infected cells with colony formation capacity, whereas R5-tropic envelopes

infected cells with very little clonogenic potential. The colonies that formed from X4-tropic HIV infected HPCs included large mixed myeloid colonies as well as multi-lineage colonies, showing that these envelopes infect very primitive HPCs. The few colonies that arose from R5-tropic envelope infection were small and abnormal in appearance, suggesting that these envelopes fail to infect primitive HPCs. Thus far, we have analyzed 2 X4-tropic envelopes, one dual-tropic envelope, and 2 R5-tropic envelopes using this technique. In these experiments, all of the envelopes that are capable of using CXCR4 have been able to infect primitive HPCs. However, because HIV isolates are so genetically diverse, there is a possibility that this observation could be unique to a few isolated envelopes. While this seems unlikely, we are currently evaluating a larger panel of HIV envelopes to determine if our observations can be generalized to many HIV envelopes. It is also important to note that a number of the envelopes we used are direct isolates, suggesting that our findings are not an artifact of lab-adapted HIV strains.

One possible explanation for our results is that CCR5 is expressed on primitive HPCs, but that ligation of the receptor by HIV envelope triggers signaling pathways that perturb cell physiology, such that colony formation does not occur. Indeed, a few reports have suggested that HIV envelope may be toxic to HPCs [38, 39]. We feel that this is unlikely to explain our results for several reasons. Firstly, unlike CXCR4, the expression of CCR5on primitive HPCs has not been documented. In fact, we have been completely unable to detect CCR5 expression on primitive HPC subsets (data not shown). Also, we have shown that the dual tropic HIV envelope 89.6 is capable of infecting primitive HPC despite being able to utilize either major coreceptor. This suggests that it is the presence of X4-binding capacity, not the absence of R5-binding capacity, which facilitates

infection of primitive HPCs. Nevertheless, this possibility remains and is difficult to exclude completely, since pharmacologic interference with CCR5-mediated signal transduction could have unpredictable affects on other aspects of cell physiology, such as affecting cell differentiation or survival.

It was also important to determine if HIV infection of HPCs was occurring using canonical receptors. In T cells, HIV entry uses CD4 in combination with CXCR4 or CCR5. However, several HIV-1 and HIV-2 isolates have been identified that can enter cells using only CXCR4, without the need for CD4 [30-34, 40]. Likewise, several other cell surface molecules besides CXCR4 and CCR5 have been reported to function as coreceptors for HIV entry [35]. One such example of non-canonical receptor usage occurs in central nervous system astrocytes, which may undergo CD4-independent infection using mannose receptor as an entry factor [32]. To determine what receptors facilitate infection of HPCs, we treated the cells with CD4-blocking antibodies or with the CXCR4-antagonist AM3100 prior to infection with a dual-tropic HIV envelope. We found that CD4 blockade completely blocked infection of HPC. Additionally, CXCR4 blockade partially reduced infection of HPC, and dramatically reduced the infection of primitive HPC. These findings demonstrate that infection of primitive HPC occurs using canonical receptors, specifically CXCR4. This is relevant to in vivo infection, because it suggests that most commonly-arising viral envelopes would be capable of infecting HPC.

We have previously shown that multipotent HPCs can be infected by HIV, but we wished to determine whether HSCs could also be infected. This possibility has significant implications for viral persistence: since HSCs are capable of self-renewal, infected HPCs could persist indefinitely, forming a long-term viral reservoir. The

implications for hematopoietic pathogenesis could be equally profound if active infection occurred in HSCs. This process could trigger cell death in HSCs, causing serious detriment to the entire hematopoietic cascade. Our preliminary findings suggest that infection of HSCs could be possible. One animal showed GFP+ Human B cells in the periphery, which peaked at 8 weeks post-transplant and slowly declined thereafter. This engraftment kinetic is most likely consistent with engraftment of an infected primitive lymphoid progenitor. This finding is important, as the methylcellulose assay approach we employed earlier cannot detect the infection of lymphoid progenitors. Two other animals developed $GFP^+$ human B cells and myeloid cells in the periphery. This engraftment profile is consistent with the engraftment of an infected HSC. However, the hallmark of HSC engraftment is long-term release of multiple lineage blood cells into the periphery, and so continued monitoring of these animals over time is required before concrete conclusions can be made. Interestingly, in six out of seven animals which showed detectable human cell engraftment there was a mix of $GFP^+$ and $GFP^-$ cells. Only one animal transplanted with infected cells showed engraftment of $GFP^-$ cells without $GFP^+$ cells. This shows that infected cells may be equally capable of engrafting as their uninfected counterparts, meaning that highly primitive HPCs are not inherently resistant to HIV infection.

In sum, in this chapter we have shown that primitive HPCs can be infected by HIV, and that this infection is primarily accomplished by CXCR4-utilizing HIV envelopes. Additionally, we have intriguing preliminary evidence suggesting that HSCs may also be susceptible to infection by X4-tropic HIV. These results provide substantial insight into mechanisms of HIV pathogenesis.

## MATERIALS AND METHODS

**Antibodies and reagents.** Antibodies for flow cytometry were as follows: anti-CD34-FITC (BD biosciences), anti-CD34-APC (Caltag), anti-CD38-FITC (eBioscience), anti-CD38-PE/Cy7 (eBioscience), anti-CD133-PE (Miltenyi Biotech), anti-CD133-Biotin (eBioscience), anti-Gag-FITC (Coulter), anti-Gag-PE (Coulter), anti-CD45-APC/Cy7 (BD Biosciences), anti-CD33-PE (BD Biosciences), anti-CD3-PE/Cy5 (BD Biosciences), anti-CD19-APC (BD Biosciences). Lineage cocktail was created by combining equal amounts of antibodies against CD3, CD14, CD19, CD20, CD56 and Glycophorin A (BD Biosciences) and conjugating the antibodies to biotin using Sulfo-NHS-Biotin according to the manufacturer's instructions (Thermo Scientific). Biotinylated antibodies were detected with Streptavidin-PE/Cy5.5 or Streptavidin-APC/Cy7 (eBiosciences). For receptor blocking experiments, the functional grade antibodies used were anti-CD4 (clone L3T4, eBiosciences), and anti-CXCR4 (clone 12G5, eBiosciences).

Stem cell factor (SCF), Thrombopoietin (TPO) and Flt3-L were purchased from Stem Cell Technologies. IGFBP-2 was purchased from R&D systems. StemSpan serum-free medium was purchased from StemCell Technologies. Complete methylcellulose medium (Methocult H4034) was purchased from StemCell Technologies. AMD3100

was obtained from the NIH AIDS Research Reference and Reagent program. Neomycin sulfate and polymyxin B were purchased from sigma.

**Plasmid constructs.** HIV molecular clone plasmids pNL4-3, p89.6, p94UG114.1.6, pYU2, and pMJ4 were obtained from the NIH AIDS Research and Reference Reagent Program. pHIV-7SF-GFP was a kind gift from Jiing-Kuan Yee [41]. pCMV-HIV-1 and pCMV-G were kind gifts from Shin-Tai Chen [42]. Envelope expression plasmids pHXB 2-env, pCAGGS-SF162-gp120, pCRII-92HT593.1 and pcDNA3.1-BaL.01 were obtained from the NIH AIDS Research and Reference Reagent Program. The YU2 envelope expression plasmid was a kind gift from Joseph Sodroski.

The envelope expression plasmid pcDNA-89.6env was created by digesting p89.6 with HindIII and EcoRV. The resulting 3180 nucleotide fragment was then ligated to HindIII/EcoRV digested pcDNA3.1(+). The envelope expression plasmid pcDNA-94UGenv was created by digesting p94UG114.1.6 with XbaI and SmaI. The resulting 3021 nucleotide fragment was then ligated to pcDNA3.1(+) which had been digested with ApaI, blunted by Klenow treatment and digested with XbaI. The envelope expression plasmid pEBB-NLenv was created by digesting pNL4-3 with SalI and NotI and blunting the resulting fragment by Klenow treatment. The resulting fragment was then ligated to pEBB that had been digested with NotI and blunted by Klenow treatment.

**Cell culture**. Umbilical cord blood (UCB) was obtained following scheduled caesarian section procedures in accordance with an IRB-reviewed protocol. Whole bone marrow (BM) was obtained commercially (AllCells ltd.). UCB and BM were processed by Ficoll

separation to enrich mononuclear cells (MNC). UCB-MNC were frequently cryopreserved by resuspending cells in 10% DMSO in FBS and freezing at a controlled rate -1°/min overnight. Cells were then transferred to liquid nitrogen storage. BM-MNC were always used fresh. Fresh or thawed MNC were suspended in StemSpan medium (StemCell Technologies) and incubated for 2 hours at 37°c to deplete adherent cells. $CD133^+$ cells were then purified using magnetic cell sorting (CD133 positive selection MACS, Miltenyi Biotech). $CD133^+$ HPC were then cultured in STIF medium (StemSpan medium supplemented with 50ng/mL SCF, 50ng/mL TPO, 100ng/mL IGFBP-2, and 50ng/mL Flt3-L) as described elsewhere [43].

Methylcellulose colony forming assays were conducted by according to the manufacturer's recommendation (Methocult H4034, StemCell Technologies). Briefly, infected or control cells were diluted in IMDM+2%FBS and mixed with complete methylcellulose medium. The mixture was then plated on non-coated 6-well plates (500-1500 cells per well) and incubated for 14 days. Colonies were scored based on morphology using an inverted brightfield microscope at 40x or 100x magnification. CFU-GEMM morphology was verified at high power (200x). GFP expression was analyzed on an inverted epifluorescent microscope at indicated magnifications.

293T cells were maintained in DMEM supplemented with 10% FBS, 0.292mg/mL L-glutamine, 100U/mL penicillin, 100U/mL streptomycin and Plasmocin (Invivogen). CEM-SS and CEM-R5 cells were maintained in RPMI1640 supplemented with 10% FBS, 0.292mg/mL L-glutamine, 100U/mL penicillin, and 100U/mL streptomycin.

**HIV preparation.** Infectious supernatants were prepared by transfection of proviral plasmids into 293T cells using polyethylenimine. For unmodified clinical isolates, $5x10^6$ 293T cells were transfected with 8-12µg plasmid DNA. Media was replaced 24 hours post transfection and supernatant was collected 48 hours post-transfection. Supernatants were 0.45µM filtered and stored at -80ºc. For HIV-7SF-GFP, 3µg pHIV-7SF-GFP was co-transfected with 3µg pCMV-HIV-1 and 3-5µg *env* expression plasmid as indicated in the text. Supernatants were collected as described above and virions were concentrated using high-molecular weight polyethylene glycol (PEG) precipitation as described elsewhere [44]. Briefly, filtered supernatants were combined with an equal volume of 20% PEG/0.9% NaCl (20kDa average molecular weight, Fluka) and cooled overnight at 4ºc. Virions were then pelleted by centrifugation at 17800xg for 20 minutes. Pellets were resuspended in $1/5^{th}$ to $1/10^{th}$ the original volume of StemSpan medium and stored at -80oc. Virus infectivity was determined by infection of CEM-SS or CEM-R5 cells under identical conditions. MOI were calculated by applying the percent of infected CEM-SS cells to the formula MOI = -Ln(1-p) where p is the proportion of cells infected.

**HIV infections**. HIV infections were conducted using a standard spin infection technique for primary cells. Briefly, cells were resuspended in infectious supernatants and spun at room temperature for 2 hours at 2500rpm. For single-round infections, virus was used at MOIs of 0.7-1.2 (50-70% infectivity in CEM cells). After infection cells were cultured in media supplemented with cytokines as described in the text. For CD4 blocking experiments, cells were incubated prior to infection for 1 hour at 37ºc with 20µg/mL of antibody against CD4 (clone L3T4) or with an irrelevant control antibody. For CXCR4-

blocking experiments, AMD3100 or DMSO solvent control was added directly to viral supernatants prior to infection to a final concentration of 20μg/mL.

**Flow cytometry**. For cell analysis, cells were stained in FACS buffer (2% FBS, 1% human serum, 2mM HEPES, 0.025% $NaN_3$/PBS) for 20 minutes on ice. Cells were then washed and fixed in 2% paraformaldehyde/PBS for 20 minutes at room temperature prior to analysis. For experiments analyzing Gag expression, cells were extracted after fixation by incubating for 5 minutes in 0.1% Triton-X100/PBS at room temperature. Cells were then washed and incubated with anti-Gag antibody in FACS buffer for 30 minutes on ice. Cells were analyzed on a FACScan or FACSCanto flow cytometer.

Cell sorting was performed using a FACSVantage SE cytometer. For high-purity sorting, cells were sorted in Normal-R mode with a 1.0 sorted drop envelope. For cell enrichment, cells were sorted in Enrich mode with a 1.0 sorted drop envelope. Sort purities were checked immediately after sorting.

**Mice**. Nonobese diabetic severe combined immunodeficiency mice (NOD/SCID; NOD.CB17-Prkdcscid/J) were purchased from Jackson Laboratory. Mice were maintained at the University of Michigan by the Unit for Laboratory Animal Medicine. All experiments were conducted in accordance to research protocols approved by the University Committee on the Use and Care of Animals.

**NOD-SCID transplantation**. FACS-enriched cells were allowed to recover overnight in STIF medium prior to transplantation. 12 hours prior to transplant, mice received a

sublethal radiation dose (340cGy). Cells were resuspended in 25µL PBS and loaded into a 50µL Hamilton syringe fitted with a 27Ga needle. The animal's knee was then maximally flexed, the needle passed through the distal articular surface of the femur into the marrow space and the cells injected. Mice were given antibiotic water (1.1g/L neomycin and 0.121g/L polymyxin B) following the proceedure. Engraftment was analyzed by collecting peripheral blood from transplanted animals at 4 and 8-weeks post-transplant and analyzing for human CD45, GFP, CD3, CD19 and CD33 by flow cytometry.

**Table 3-1. HIV envelopes and coreceptor usage.**

| Envelope | Coreceptor usage |
|---|---|
| HXB 2 | X4 |
| NL4-3 | X4 |
| JC2 | X4 |
| 89.6 | R5X4 |
| 92HT593 | R5X4 |
| YU2 | R5 |
| 94UG114.1.6 | R5 |
| SF162 | R5 |
| BaL.01 | R5 |

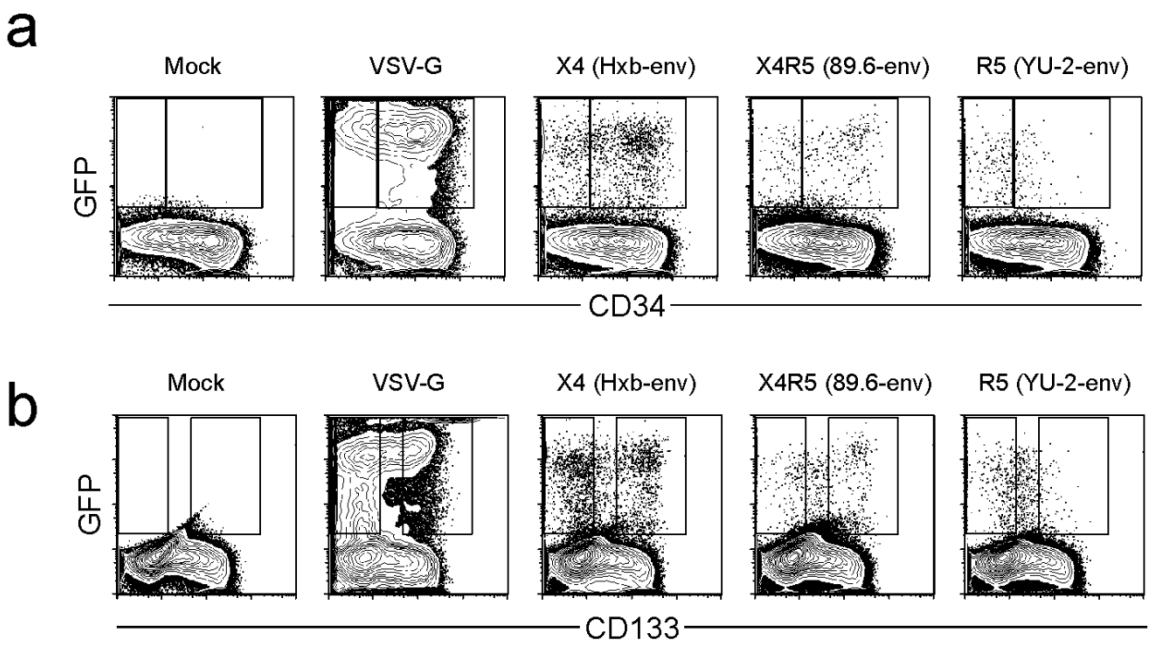



**Figure 3-1. CXCR4-tropic HIV envelopes facilitate infection of primitive phenotype HPCs.** Cord-blood derived HPCs were infected with HIV-7SF-GFP pesuedotyped with VSV-G or the HIV envelopes HXB (X4-tropic), 89.6 (dual-tropic) and YU2 (R5-tropic). Three days post-infection, cells were analyzed for GFP and CD34 expression (panel **a**) or GFP and CD133 (panel **b**).

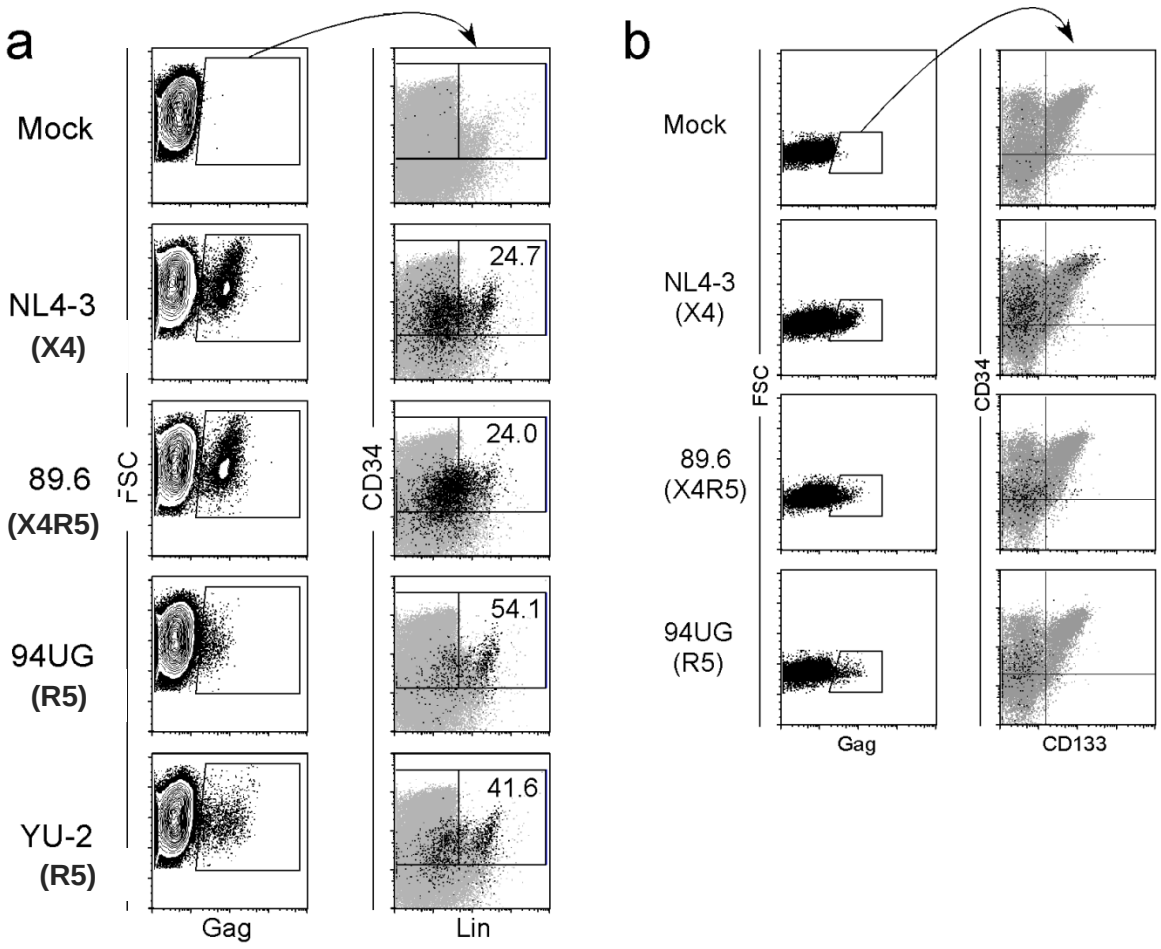



**Figure 3-2. CXCR4-tropic HIV molecular clones infect primitive phenotype HPCs.** *a*. Cord blood-derived HPC were infected with the HIV molecular clones NL4-3 (X4-tropic), 89.6 (dual-tropic), 94UG (R5-tropic) and YU2 (R5-tropic). Three days after infection, cells were analyzed for intracellular HIV Gag, CD34, and lineage markers (Lin). $Gag^+$ cells were gated in the left plots and overlayed on the Lin/CD34 plots on the right. *b*. Bone-marrow derived HPC were infected with the HIV molecular clones NL4-3, 89.6 and 94UG. Three days post-infection, cells were analyzed for intracellular HIV Gag, CD34 and CD133 expression. $Gag^+$ cells were gated in the left plots and overlayed on the CD34/CD133 plots on the right.

**Figure 3-3. CXCR4-tropic HIV envelopes infect primitive HPCs with multi-potent colony forming capacity.** Cord-blood derived HPCs were infected with HIV-7SF-GFP pseudotyped with the HIV envelopes HXB (X4-tropic) or YU2 (R5-tropic). Infected ($GFP^+$) cells were purified by FACS and 6000 cells were plated in methylcellulose medium. Cell sorting results are depicted in panel *a*. *b*. After 14 days, colonies were documented by phase contrast and epifluorescence microscopy at the indicated magnifications. *c.* Colonies were enumerated and scored for morphology (erythroid, CFU-E; myeloid, CFU-GM; or multi-lineage, GFU-GEMM). The total number of colonies from 6 replicate wells is displayed. Results are representative of three independent experiments. *d.* Cord-blood derived HPCs were infected with HIV-7SF-GFP pseudotyped with the HIV envelopes NL4-3 (X4-tropic) or BaL (R5-tropic). Cells were then sorted, plated in methylcellulose medium and analyzed as in *c*.

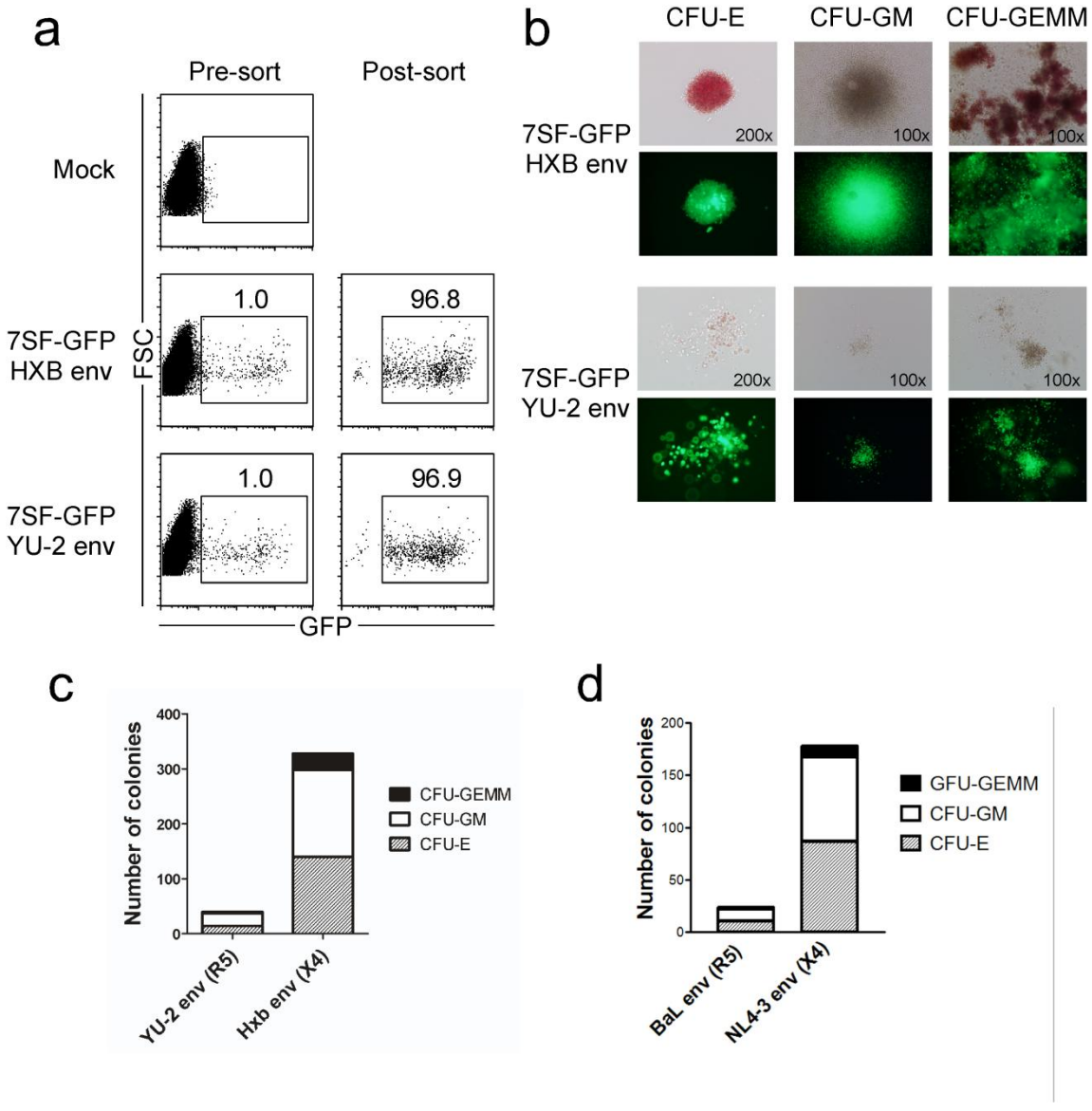

a
Pre-sort
Post-sort
Mock
7SF-GFP HXB env
7SF-GFP YU-2 env
1.0
96.8
1.0
96.9
FSC
GFP
b
CFU-E
CFU-GM
CFU-GEMM
7SF-GFP HXB env
7SF-GFP YU-2 env
200x
100x
100x
200x
100x
100x
c
Number of colonies
400
300
200
100
0
CFU-GEMM
CFU-GM
CFU-E
YU-2 env (R5)
Hxb env (X4)
d
Number of colonies
200
150
100
50
0
GFU-GEMM
CFU-GM
CFU-E
BaL env (R5)
NL4-3 env (X4)

**Figure 3-4. Infection of primitive HPCs is dependent on CD4 and CXCR4. a.** Infection of HPCs is dependent on CD4. Cord blood-derived HPCs or CEM T cells were pre-incubated with anti-CD4 antibody (L3T4, 20μg/mL) or control antibody and then infected with HIV-7SF-GFP pseudotyped with 89.6 envelope. Three days post-infection cells were analyzed for GFP expression. **b**. CD4 blockade prevents infection of primitive phenotype HPCs. Cells from panel *a* were analyzed for CD34 and CD133 expression. GFP+ events (red dots) were overlayed on CD34 vs. CD133 plots. **c**. AMD3100 inhibits CXCR4-dependent but not CCR5-dependent infection of CEM T cells. CEM –SS (X4 expressing) or CEM-R5 (X4 and R5 expressing) cells were treated with AMD3100 or DMSO and then infected with HIV-7SF-GFP pseudotyped with the dual tropic HIV envelope 92HT. Cells were analyzed for GFP expression three days post-infection. **d**. CXCR4 blockade with AMD3100 inhibits infection of colony forming HPCs. Cord-blood derived HPCs were treated with AMD3100 or DMSO and then infected with HIV-7SF-GFP pseudotyped with the dual tropic HIV envelope 89.6. Three days post-infection $GFP^+$ cells were purified by FACS and plated in methylcellulose medium. Colonies were scored and enumerated. Data are represented as number of colonies per 100 cells plated.

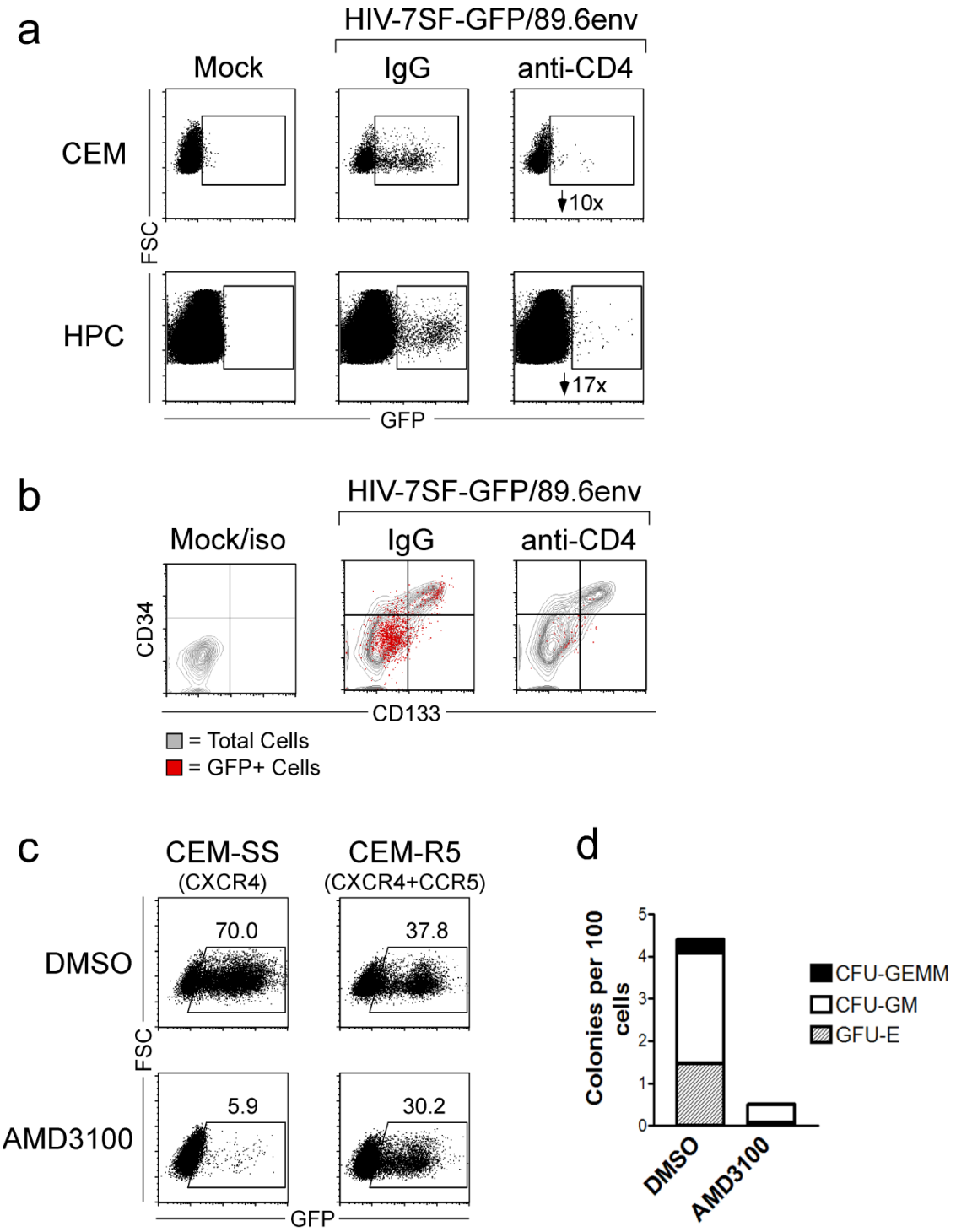
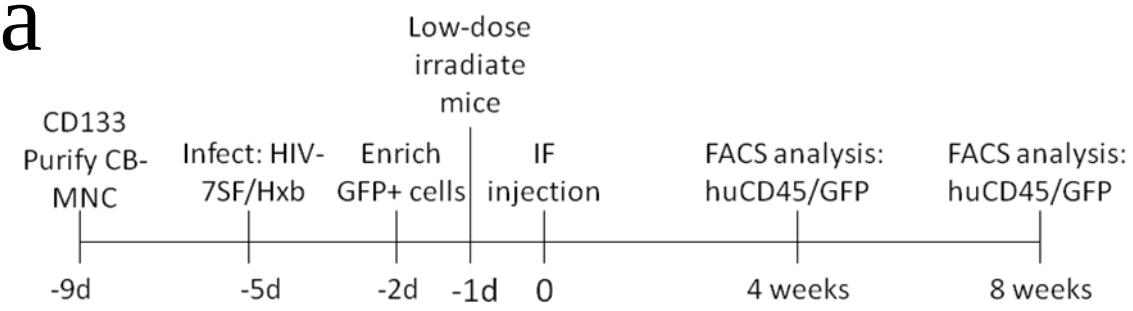
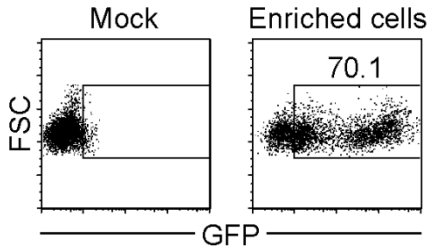

a
HIV-7SF-GFP/89.6env
Mock
IgG
anti-CD4
CEM
HPC
FSC
10x
17x
GFP
b
HIV-7SF-GFP/89.6env
Mock/iso
IgG
anti-CD4
CD34
CD133
= Total Cells
= GFP+ Cells
c
CEM-SS
(CXCR4)
CEM-R5
(CXCR4+CCR5)
DMSO
AMD3100
70.0
37.8
5.9
30.2
FSC
GFP
d
Colonies per 100 cells
0
1
2
3
4
5
DMSO
AMD3100
CFU-GEMM
CFU-GM
GFU-E

**Figure 3-5. Experimental design for NOD-SCID engraftment experiments. a.** timeline of NOD-SCID engraftment experiments. Cord blood $CD133^+$ HPCs were purified by MACS and pre-cultured for 4 days in STIF medium. Cells were then infected with HIV-7SF-GFP pseudotyped with HXB envelope. Three days post-infection, $GFP^+$ cells were enriched by FACS to 50-70% purity. 12 hours prior to transplant, NOD-SCID-γ mice were given a 340cGy radiation dose. GFP-enriched cells were then intrafemorally injected into the irradiated mice. Engraftment was analyzed by flow cytometry every 2 weeks beginning at 4 weeks post transplant. **b**. Example of FACS enrichment of infected HPCs. Cord-blood derived CD133+ HPCs were infected with HIV-7SF-GFP/HXB env and GFP+ cells were enriched by FACS. **c**. Scheme for FACS analysis of human cell engraftment in transplanted mice. Peripheral blood was lysed and stained for murine CD45, human CD45, CD33, CD3, CD19, and GFP. Viable single cells were gated by FSC/SSC profile, DAPI exclusion and FSC-H/FSC-A ratio (top panels). Human cells were then identified by muCD45/huCD45 staining, and analyzed for GFP, CD33, CD3 and CD19 expression. Human peripheral blood and murine peripheral blood from an untransplanted NOD-SCID-γ mouse are shown.

a


Low-dose irradiate mice
CD133 Purify CB-MNC
Infect: HIV-7SF/Hxb
Enrich GFP+ cells
IF injection
FACS analysis: huCD45/GFP
FACS analysis: huCD45/GFP
-9d
-5d
-2d
-1d
0
4 weeks
8 weeks

b


Mock
Enriched cells
70.1
FSC
GFP

c

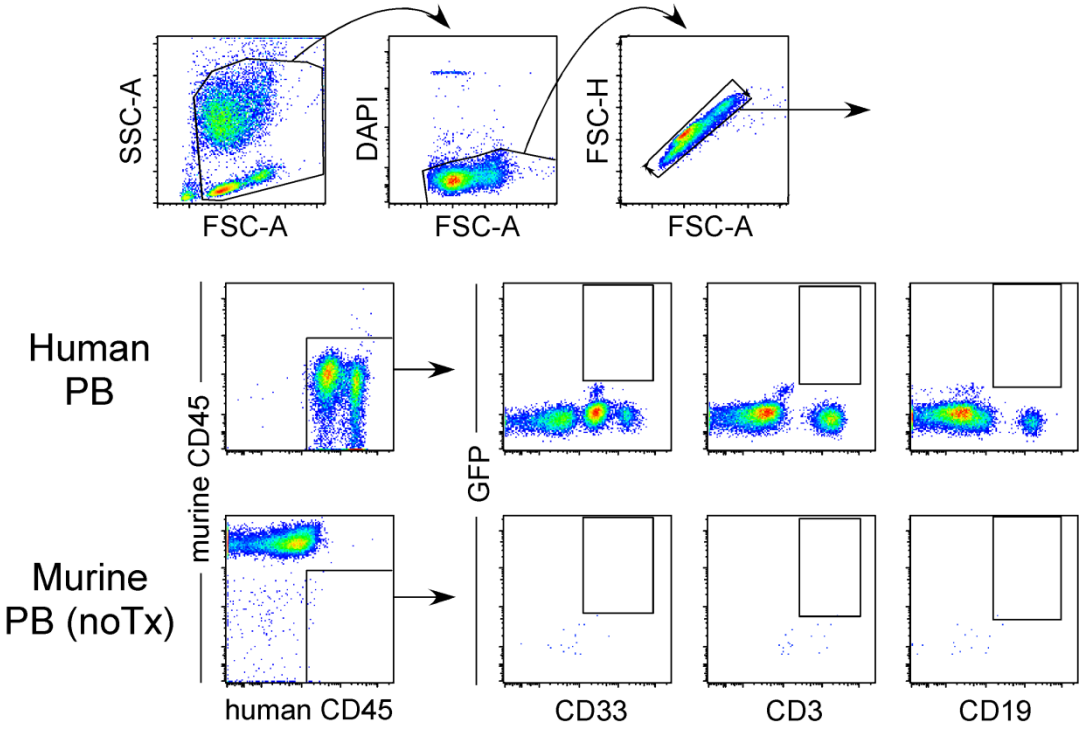

SSC-A
FSC-A
DAPI
FSC-A
FSC-H
FSC-A
Human PB
Murine PB (noTx)
murine CD45
human CD45
GFP
CD33
CD3
CD19

**Table 3-2. Summary of NOD-SCID engraftment experiments to date.** Transplants were conducted in three separate experiments. The animal number and transplant condition are shown in the first two columns. The number of cells transplanted and the current time post transplant are displayed in the third and fourth columns. Engraftment status and the lineages of human cells observed in the peripheral blood are displayed in the fifth and sixth columns (B, B cell; M, myeloid).

| | Animal | Condition | Cell Number | Week | Engraftment | Lineages |
|---|---|---|---|---|---|---|
| | (Cg-exp-tag) | | | | | |
| Exp 1 | 1-1-R | Mock infected | 50,000 | 12 | - | - |
| | 2-1-L | Mock infected | 50,000 | 12 | - | - |
| | 1-1-N | 7SF/HXB GFP | 50,000 | 12 | **+** | B |
| | 1-1-LR | 7SF/HXB GFP | 50,000 | 12 | - | - |
| | 1-1-2L | 7SF/HXB GFP | 50,000 | 12 | - | - |
| | 1-1-2R | 7SF/HXB GFP | 50,000 | 12 | **+/-** | B |
| | | | | | | |
| Exp 2 | 2-2-LR | Mock infected | 100,000 | 10 | - | - |
| | 2-2-R | 7SF/HXB GFP | 100,000 | 10 | - | - |
| | 2-2-2L | 7SF/HXB GFP | 100,000 | 10 | - | - |
| | 2-2-2R | 7SF/HXB GFP | 100,000 | 10 | **+** | B,M |
| | 4-2-L | 7SF/HXB GFP | 100,000 | 10 | **+/-** | B |
| | | | | | | |
| Exp 3 | 4-3-R | Mock infected | 150,000 | 8 | **(+/-)** | B |
| | 3-3-L | 7SF/HXB GFP | 150,000 | 8 | - | - |
| | 3-3-N | 7SF/HXB GFP | 150,000 | 8 | **++** | B,M |
| | 3-3-2R | 7SF/HXB GFP | 150,000 | 8 | **(+)** | B,M |
| | 3-3-L2R | 7SF/HXB GFP | 150,000 | 8 | - | - |
| | 3-3-2LR | 7SF/HXB GFP | 150,000 | 8 | - | - |
| | 4-3-2L | 7SF/HXB GFP | 150,000 | 8 | **+/-** | B |

- No engraftment over background
+/- Engraftment of GFP+ cells <0.2%
+ Engraftment of GFP+ cells, >0.2%
++ Engraftment of GFP+ cells, >1.0%
(+/-) Engraftment of GFP-negative cells, <0.2%
(+) Engraftment of GFP-negative cells >0.2%

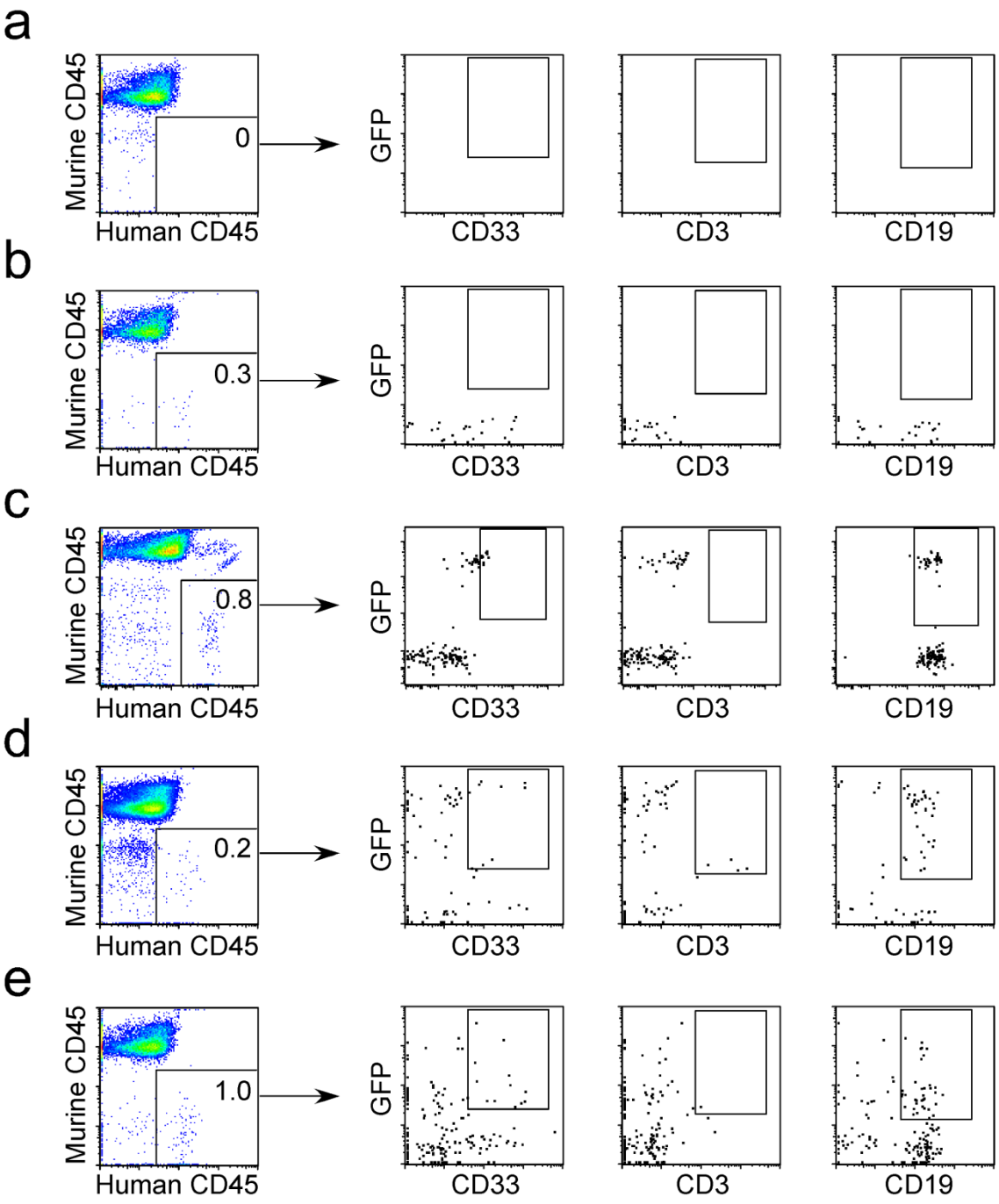



**Figure 3-6. FACS analysis of selected animals from NOD-SCID transplant experiments. a.** Analysis of animal 1-1-LR, showing no engraftment of human cells. **b**. Analysis of animal 3-3-3R, showing low-level human cell engraftment with GFP-negative B cells and myeloid cells in the peripheral blood. **c**. Analysis of animal 1-1-N, showing human cell engraftment with $GFP^+$ B cells in the peripheral blood. **d**. Analysis of animal 2-2-2R, showing engraftment of human cells with $GFP^+$ B cells and myeloid cells in the peripheral circulation. **e**. Analysis of animal 3-3-N, showing engraftment of human cells with $GFP^+$ B cells and myeloid cells in the peripheral circulation.

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# CHAPTER IV: DISCUSSION

## MAJOR CONCLUSIONS

The findings presented in this work provide several important insights into HIV pathogenesis, providing possible explanations for HIV persistence and HIV-associated hematopoietic dysfunction. The major conclusions presented herein are outlined below:

i) HIV can establish active infection in a subset of hematopoietic progenitor cells (HPC), leading to cell death.

ii) HIV can infect primitive, multi-potent HPC.

iii) Latent HIV infection can be established in HPC, and active infection can be induced by stimuli that result in HPC maturation. A novel reporter HIV can detect latent infection of HPC as well as T cells.

iv) Infection of HPC can be detected in HIV-infected individuals. Some HIV-positive individuals have actively infected HPC, whereas others have latently infected HPC that can be induced to express HIV.

v) HIV infection of primitive HPC is facilitated by CXCR4-tropic and dual-tropic envelopes and not by CCR5-tropic envelopes

vi) CXCR4-tropic HIV envelopes facilitate the infection of NOD-SCID re-engrafting hematopoietic stem cells (HSC)

## ACTIVE INFECTION OF HPC BY HIV

In this study we sought to determine whether HIV is capable of infecting HPC in vivo and after in vitro exposure to the virus.. After exposure to HIV, a portion of CD34+ HPC expressed HIV Gag protein, indicating active infection. We employed a flow cytometric approach to detect infected cells in culture, which allowed us to directly demonstrate that infection was occurring in HPC, not in other cell types contaminating the culture. The HIV Gag protein we detected was a result of *bona fide* infection, as opposed to infection-independent delivery of viral protein because pretreatment of the cultures with the reverse transcriptase inhibitor AZT blocked infection of HPC. Additionally, we found that MHC-Class I was down-modulated in infected HPC, indicating that the HIV protein Nef was being expressed within infected cells [1, 2]. Together, these data demonstrate that active infection indeed occurs in HPC.

We also found that active infection of HPC results in cell death by apoptosis, similar to what was previously reported for infected T lymphocytes [3]. HPC in culture were rapidly lost over a period of a week and actively infected HPC displayed increased reactivity to Annexin V, demonstrating that apoptosis was being induced by infection

It has been known for some time that HIV induces bone marrow abnormalities in infected individuals. These include anemia, thrombocytopenia and neutropenia, as well as a variety of coagulopathies and hematologic neoplasms (reviewed in [4-6]).

Additionally, it is remarkable that in HIV infection the hematopoietic system cannot replace the T cells that are lost during infection. This is especially perplexing considering that the fraction of T cells infected at any moment is quite low [7]. Also, infection of natural primate hosts by the HIV ancestor SIV (simian immunodeficiency virus) leads to high levels of T cell infection, but the hematopoietic system replenishes the lost cells and immunodeficiency never occurs (reviewed in [8]). These observations clearly demonstrate the capacity of HIV to perturb the function of the hematopoietic system.

Several mechanisms have been proposed to account for the HIV-induced disruption of the hematopoietic system. Some proposed mechanisms include elevated levels of inflammatory mediators, the effects of antiretroviral drugs and effects of specific HIV proteins in circulation [9-16]. Certainly multiple mechanisms could contribute to hematopoietic dysfunction, and none of these proposed mechanisms are exclusive. Our findings suggest that direct infection of HPC by HIV could be an important mechanism of hematopoietic dysfunction. Since active infection of HPC causes cell death, infection of HPC *in vivo* could have drastic effects on the development of mature hematopoietic cells. This effect would be especially profound if primitive HPC were infected; destruction of a single HPC would effectively block the generation of hundreds of mature hematopoietic cells. This possibility is supported by the finding that primitive HPC subsets are depleted within HIV-infected individuals, although it remains to be determined whether direct infection accounts for the loss of these cells [11].

## HIV INFECTS PRIMITIVE HPC

We next wished to determine whether primitive HPC could be infected with HIV. Our observation that HIV induced cell death in actively infected HPC presented a significant obstacle, because infected cells would likely die during the long-term culture assays used to identify primitive progenitors. To overcome this problem, we employed self-inactivating viruses, which have a defective viral promoter (U3 LTR deletions), preventing the expression of viral genes. These viruses express EGFP from an internal promoter, thus allowing us to detect infected cells in the absence of active viral transcription. Using this approach, we found that HIV infection occurred at a similar frequency in total $CD34^+$ HPC and in HPC with a primitive immunophenotype ($CD34^+CD133^+CD38^-$). It has been reported that cell immunophenotype may lose reliability in identifying primitive progenitors after long culture periods [17], so it was crucial to verify these findings using a more physiologically relevant experimental system. To accomplish this, we employed self-inactivating HIV to assess the ability of HIV-infected HPC to form colonies in methylcellulose medium. We found that infected cells gave rise to single-lineage as well as multi-lineage colonies, demonstrating that HIV can infect primitive, multi-potent HPC.

The susceptibility of HPC to in-vitro HIV infection has been a topic of considerable controversy in the HIV field. Several studies reported that HIV could infect HPC [18-20], however numerous studies also reported that HPC were highly resistant to HIV infection [21-25]. Even in those studies that reported HIV infection of HPC, it was

concluded that primitive HPC were resistant to infection [20, 26]. The overall conclusion in the HIV field was that HPC are resistant to infection (reviewed in [27]).

However, several technological and scientific factors may account for the differences in experimental outcomes within these studies. Perhaps most importantly, previous studies have failed to account for the cytotoxic effect of HIV in HPC. We have found that HIV-infected HPC die even during brief culture periods, and for this reason infected cells will not be detected using experimental approaches that use replication competent virus and lengthy culture periods. In our studies we used self-inactivating HIV constructs to overcome this problem, an approach which revealed the susceptibility of HPC to HIV infection. Secondly, no studies prior to those reported here used direct approaches to detect infected HPC. The indirect approaches used, such as PCR and virus release assays, are easily confounded by contaminating cell types, giving false positive results. Paradoxically, attempts to control for this possibility could cause legitimate infection to be overlooked, especially considering the low frequency of HPC infection. To avoid these problems, we focused on techniques such as flow cytometry and colony formation assays, which allow examination of single cells and exclusion of undesired contaminant cells. These techniques allowed for the reliable observation of infected HPC for the first time. Finally, ex-vivo culture techniques to maintain HPC and HSC have only recently been developed, and inappropriate culture conditions may have contributed to the conflicting results obtained by different groups.

An interesting question that remains is whether some HPC have intrinsic resistance to HIV infection. Several studies have proposed mechanisms conferring HIV resistance to HPC. Early studies suggested that secretion of β-chemokines that bind the

CCR5 coreceptor (RANTES and MIP-1α/β) renders HPC resistant to viral entry [23, 24]. However, the presence of these factors in bone marrow has never been reported, and it is unlikely that sufficient amounts would be present to significantly block infection. A more recent study suggested that p21 expression in HPC creates a post-entry block to viral replication in primitive HPC [25]. If this were the case, then it would be expected that HIV-based gene therapy vectors and VSVG-pseudotyped HIV would also be unable to infect primitive HPC. This is in fact not the case, as it has been shown that HIV-based vectors efficiently infect HPC, including NOD-SCID re-engrafting HSCs [28]. Additionally, we have found that VSVG-pseudotyped HIV particles infect a large proportion of HPC, including those of primitive phenotype. Together, these findings argue against a post-entry block to HIV infection. Our findings suggest that the primary factor limiting infection of HPC is the expression of viral receptors, CD4, CXCR4, and CCR5. We and others have found that only a subset of HPC express the receptors needed for HIV entry, and we have found that the maximum infection frequencies we observe in *in vitro* infection experiments is similar to the proportion of cells expressing viral receptors (data not shown). Thus, it seems most likely that expression of viral entry receptors is the major factor determining the susceptibility of HPCs to HIV infection. In sum, our findings argue that an entry block exists in the majority of HPCs, conferring resistance to HIV infection. Importantly, we have found that this resistance is not absolute, and a subset of HPCs exist that express the necessary viral receptors and do undergo infection.

Our discovery of the infection of primitive HPC by HIV could have major implications for HIV pathogenesis. Firstly, the infection of primitive HPC could provide

insight into the hematopoietic dysfunction of HPC. If HIV only infected mature, differentiated HPC, the impact on the hematopoietic system would be minor, because these cells have limited proliferative capacity and could easily be regenerated if lost. On the other hand, if primitive HPC became infected, the effect on the hematopoietic system could be profound, since these cells normally give rise to hundreds or thousands of mature hematopoietic cells. The infection of primitive HPC also has implications in HIV persistence. Since differentiated HPC have a relatively short lifespan, infection of these cells would be unlikely to generate a long-lived viral reservoir. In contrast, highly primitive HPC have a long lifespan, and thus these cells to function as a long-term reservoir for the virus if infected.

## LATENT INFECTION OF HPC

Having found that HIV can induce an active infection in HPC, we next wished to determine if latent infection can also occur, as this could have important implications for HPC acting as a cellular reservoir for the virus. We found that stimulating cultures of HIV-exposed HPC to substances that induce cellular differentiation activated HIV gene expression, suggesting that latent virus was present that could be induced. However, as described above, it was possible that contaminating cell types could be accounting for the emergence of virus after stimulation. To rule out this possibility, we developed a novel HIV reporter to directly detect latently infected cells. This system allowed detection of latency on a single cell level for the first time. When we sought to verify this approach using T cell lines and primary T cells, we were surprised to find that populations of

latently infected cells were present immediately following infection. We employed this approach on HPC, and found that distinct populations of actively and latently infected cells exist following HIV exposure.

These findings allowed us to demonstrate that latent infection can occur in HPC following HIV exposure. The most obvious implication of these findings is that H PC may be able to function as a viral reservoir *in vivo*. This finding is particularly interesting considering recent work demonstrating the presence of unidentified cellular reservoirs in HIV patients [29]. This work demonstrated that virus is repeatedly released from a cellular reservoir distinct from the T cell and macrophage reservoirs. It is an intriguing possibility that latently infected HPC could account for the virus being released, especially since HPC have characteristics that make them uniquely suited as a viral reservoir. If primitive HPC were latently infected, viral genomes would be duplicated every time the cell divides, allowing for effective expansion of the virus without active viral replication. This unique mechanism of viral genome amplification would allow for viral propagation in a manner that is not susceptible to the immune system or antiviral drugs. If HSC with self-renewing potential became infected, the virus could be indefinitely propagated exclusively by this mechanism. Another feature of effective viral reservoirs is the potential for latent virus to be reactivated. As demonstrated by our findings, latent HIV can readily be activated as latently infected HPC mature. This process could provide a mechanism for periodic reactivation of HIV from the reservoir. Interestingly, we have found that stimulation of latently infected HPC with GMCSF and TNF alpha gives rise to actively infected macrophages and dendritic cells. These cell

types have the ability to transfer HIV to numerous T cells, which would allow even a small number of infected HPC to trigger a spreading infection.

Our experiments revealed that distinct populations of latently and actively infected HPC are present following HIV infection. This raises the question of what determines the fate of a HPC following infection, and what the mechanisms are that allow for latency. One possibility is that different types of HPC vary in their ability to support active HIV infection due to transcription factor availability. A specific set of cellular transcription factors are needed to drive transcription from the viral LTR, and different HPC may have a transcription factor milieu that is conducive to viral gene expression, whereas others may not. Some transcription factors known to activate the viral promoter are AP-1, SP1, NF-AT, NF-κB, and USF. Conversely, several cellular transcription factors have been shown to negatively regulate the viral promoter, including Oct-1, Oct-2, YY1 TDP-43 and glucocorticoid receptor (reviewed in [30]). Given this complex regulatory interplay, it is certainly conceivable that HPC of different lineages, maturity level or even cell cycle stage could be conducive or unfavorable to HIV transcription, leading to active viral gene expression or latency. In this scenario, viral gene expression could be activated during cellular maturation, as the transcription factor availability within the cell changes.

Another explanation for the establishment of latency in HPC could be the integration site of the HIV genome within host chromatin. Although it is known that HIV preferentially integrates into transcriptionally active chromatin regions [31], it has also been shown that HIV reproducibly integrates into centromeric heterochromatic in T cell lines, resulting in repression of HIV transcription [32]. Thus, it is possible that HIV

latency in HPC occurs when the viral genome integrates into inactive chromatin regions. As opposed to the transcription factor availability model, this mechanism would be largely stochastic, and would not favor particular classes of HPC. In this scenario, viral activation from latency would be dependent on chromatin remodeling occuring during HPC maturation, activating the region where the virus has integrated.

A final mechanism that could account for HIV latency within HPC is the availability of the HIV protein Tat. Tat is packaged within virions, and is required to initiate productive viral transcription. If insufficient Tat is present in the early stages of infection, transcript elongation does not occur, leading to viral latency (reviewed in [33]). It is unclear how viral gene expression would be activated in this scenario, but it is possible that under highly favorable conditions, low level Tat-independent viral transcription could occur, allowing for the *de novo* production of Tat protein and subsequent initiation of robust viral transcription. Interestingly, we have found that Tat levels contribute to latency in T cell lines, as cell lines expressing Tat constitutively show a reduced frequency of latently infected cells following HIV infection.

Our experimental approaches to studying HIV latency in HPC have also provided interesting insights into T cell latency. Current models of HIV latency in T cells propose that latency occurs when HIV infects an activated T cell but the cell reverts to a memory phenotype before the infection causes cell death. However, the plausibility of this model is questionable, because actively infected T cells die rapidly after infection and viral proteins tend to promote T cell activation rather than quiescence. Our findings show that latent infection can actually occur in activated T cells, without reversion to memory phenotype. This suggests a new possible mechanism of latency establishment which

would not be impacted by the toxicity of viral gene expression. In this scenario, latency occurs immediately, and the viral genome remains dormant throughout the normal lifespan of the activated T cell. When the activated T cell reverts to memory phenotype following activation, a long-lived reservoir would be established that could be reactivated following re-activation of the memory T cell. The mechanism for establishment of immediate latency in activated T cells is an interesting question for future study. It is unlikely that transcription factor availability accounts for this type of latency, as the transcription factors needed for viral transcription are present at high levels in activated T cells. It is more likely that viral integration site or HIV Tat availability accounts for immediate latency in activated T cells.

## HIV INFECTED HPC IN VIVO

To determine whether HIV infection of HPC occurs *in vivo*, we have begun a study to examine HIV infection of HPC from ten infected individuals. We collected bone marrow aspirates from donors with viral loads greater than 50,000 RNA copies per milliliter and purified $CD34^+$ HPC from the marrow specimens. Remarkably, flow cytometric analysis revealed that the majority of subjects (4/7) had infected CD34+ HPC detectable immediately after isolation. In 2 of the 3 donors that that initially displayed no HPC infection, HIV could be detected under conditions that induced myeloid differention of HPCs. The final donor's HPCs failed to grow in culture so no conclusions could be reached.

These findings demonstrate that HPC do become infected in HIV-positive individuals. We were surprised to find that active infection was detectable in several donors immediately after isolation of the cells. This confirms our findings of active infection after *in vitro* exposure of HPCs to HIV, and gives substantial support to a model of HIV-induced hematopoietic failure that includes direct infection of HPC as a contributing mechanism. Interestingly, in donors who did not have actively infected HPC present at the time of isolation, virus outgrowth was observed after the cells were stimulated to differentiate. This indicates that latently infected HPC reside in the marrow of infected individuals, and can be induced as infected cells mature. This finding mirrors our *in vitro* findings, where some HPC undergo active infection, whereas others undergo latent, inducible infection. The finding of latently infected HPC *in vivo* shows that HPC can indeed contribute to the latent HIV reservoir found in infected individuals.

The presence of infected HPC in vivo has been a topic of controversy within the HIV field, as was the question of general susceptibility of HPC to HIV infection in vitro, discussed above. One study failed to detect HIV in $CD34^+$ HPCs from infected persons using PCR. The authors also performed methylcellulose assays and did not detect HIV within the resulting colonies [34]. Another study, however, did detect HIV infected HPC in a subset of infected individuals [35]. Both of these studies were hindered by the indirect approaches used, as discussed earlier. The first study could have failed to detect infected cells due to lack of assay sensitivity, which is especially problematic when using PCR: the HIV genomic sequence is so highly variable, a single set of PCR oligos could fail to detect many variants. Additionally, as discussed above, cytotoxic effects of the virus would likely prevent the growth of hematopoietic colonies from infected HPC.

Even the second study could potentially over- or underestimate the frequency of infected HPC as small numbers of contaminant cells in the HPC preparations could yield false positive results in the PCR assay used. In an attempt to control for this possibility, the authors only considered subjects to have HPC infection if the $CD34^+$ cells were PCR-positive and the $CD34^-$ bone marrow mononuclear cells were PCR-negative. This approach is quite stringent, but could easily underestimate the proportion of subjects with infected HPC. Indeed, we have found that many donors with infected $CD34^+$ HPC also have infected cells within the $CD34^-$ fraction. To overcome these problems, we examined HPC for infection on a single cell level using flow cytometry. This approach allowed for the first time the detection of infected HPC without the potentially confounding variables that plagued earlier studies.

## CXCR4-TROPIC HIV ENVELOPES INFECT PRIMITIVE HPC

Our findings suggest that the primary mechanism that determines the susceptibility of HPC to infection is the expression of receptors for the HIV envelope. This raised the question whether different HIV envelopes vary in their ability to infect HPC. To explore this question we assembled a panel of HIV envelopes, and tested their ability to infect HPC using self-inactivating HIV constructs. We found that CXCR4-tropic and dual-tropic (CXCR4+CCR5) HIV envelopes allowed infection of primitive HPC, assessed by immunophenotype and clonogenic capacity. In contrast, CCR5-tropic HIV envelopes were largely incapable of infecting primitive HPC. Receptor blocking

experiments confirmed that CXCR4 was indeed the co-receptor being utilized for primitive HPC infection, and also demonstrated that infection of HPC is CD4-dependent.

HIV infection in general is thought to be dependent on the interaction between CD4 expressed on the host cell and the HIV envelope. However, this dogma is not absolute, and several studies have reported HIV envelopes that infect target cells without using CD4 [36-40]. For this reason, it was important to determine if infection of HPC occurred through a CD4-dependent pathway. We found that CD4 receptor blockade dramatically reduced HIV infection, demonstrating CD4 dependence of HPC infection. It is not surprising that CD4-dependent infection can occur in HPC, as several groups have found that CD4 is expressed on subsets of HPC [41, 42]. Our findings imply that a wide repertoire of HIV envelopes should be capable of infecting HPC, whereas if infection was CD4-independent, only a small subset of unusual HIV envelopes would be able to infect HPC.

In addition to CD4 usage, we have found that CXCR4 co-receptor usage is crucial for the efficient infection of primitive HPC. Interestingly, both strictly X4-tropic as well as dual (R5X4)-tropic enveloeps were able to infect primitive HPC. It is perhaps not surprising that CXCR4 expression facilitates infection of primitive HPC, as this receptor is expressed on primitive HPC and [43], including common lymphoid progenitors [44]. Indeed, the CXCR4 receptor is crucial for normal hematopoietic physiology, as it binds stromal derived factor 1 (SDF-1), facilitating bone marrow homing of HPC [45, 46].

The reasons why CCR5-tropic HIV envelopes fail to infect primitive HPC are less clear. It is possible that primitive HPC simply do not express CCR5, or that CCR5 and CD4 are not co-expressed on these cells. Indeed, we have been unable to detect

significant expression of CCR5 on HPCs (data not shown). Another possibility is that CCR5 is indeed expressed and functional on primitive HPC, but that ligation of the receptor by HIV envelope induces changes in cell physiology that alter differentiation or proliferation. In T cells, HIV envelope binding to CCR5 activates signal transduction pathways that trigger intracellular calcium mobilization and activation of Pyk2 and FAK 125, which are regulators of apoptosis and cell survival (reviewed in [47]). Thus, it is possible that envelope-mediated CCR5 signaling in HPC could trigger apoptosis or perturb HPC physiology in such a way that infected primitive HPC cannot be detected by our assays. This possibility is difficult to evaluate experimentally, because attempts to block the effects of CCR5 signaling could have unforeseen effects on normal cell physiology. If this is the case, however, CCR5-tropic HIV isolates could have profound effects on hematopoietic physiology *in vivo* simply by ligating and activating CCR5.

The implications of CXCR4-mediated infection of HPC with regards to disease pathogenesis are substantial. It has been known for quite some time that the emergence of CXCR4-tropic HIV isolates within patients corresponds to disease progression and carries a poor prognosis. Individuals who develop CXCR4-tropic isolates or in the rare case when a CXCR4-tropic isolate initiates infection, the host shows rapid T cell count drops and immune system failure. The explanation for this observation remains unclear (reviewed in [48]). Our findings point to a novel mechanism for the progression of disease associated with CXCR4-tropic HIV emergence. If CCR5-tropic HIV isolates in fact cannot infect highly primitive HPC, then the effect on the hematopoietic system would be less severe during the early stages of disease, when these isolates predominate. As CXCR4-tropic isolates emerge, infection of highly primitive HPC could occur, and

the impact on the hematopoietic system would be far more profound. This model could help explain the precipitous drop in lymphocyte counts associated with CXCR4-tropic HIV emergence: if common lymphocyte progenitors (which express CXCR4) became infected and were depleted by HIV, the ability of the hematopoietic compartment to replenish lost CD4+ T cells would be compromised. Such an occurrence would certainly shift the balance of viral replication and immune control in favor of the virus. If confirmed, this model could explain a major mystery in HIV disease pathogenesis, and would certainly argue for the employment of CXCR4-targeted HIV fusion inhibitors in the clinical setting of patients with CXCR4-tropic isolates.

## EVALUATION OF INFECTED HPC IN THE NOD-SCID MODEL OF HEMATOPOIESIS

Having found that primitive colony forming HPC can be infected by HIV, we wished to determine if hematopoietic stem cells (HSC) can be infected by HIV. To answer this question, we performed murine xenotransplantation assays using infected HPCs. In this system, human HPC are transplanted into immunocompromised NOD-SCID-IL2R$\gamma^{-/-}$ (NSG) mice. The peripheral blood is then monitored for the presence of human leukocytes. If an HSC is injected into the mouse and engrafts in the bone marrow, a sustained release of human cells of multiple hematopoietic lineages will be observed in the peripheral blood. If a long-term reconstituting HSC engrafts, the production of human cells should persist for many months, and the bone marrow from the engrafted

mouse should be able to induce multi-lineage engraftment when transplanted into a second mouse.

Since R5-tropic envelopes largely failed to infect colony forming HPCs, we believe it is unlikely that HSCs would be susceptible to R5-tropic HIV infection. For this reason, we focused CXCR4-tropic HIV envelopes in these studies. We infected HPCs with a GFP-reporter HIV pseudotyped with X4-tropic HIV envelope, enriched GFP+ HPCs, and injected the cells into the bone marrow cavity of NSG mice. We began analyzing the transplanted mice at 4 weeks post-transplant and are currently continuing these analyses. To date, several animals have shown interesting preliminary findings. One animal had GFP+ B cells present in the peripheral blood that were detected a 6 weeks post-transplant. The frequency of these cells peaked at 8 weeks post-transplant and then gradually declined. This observation is most consistent with the engraftment of a primitive lymphoid-committed HPC or a multi-potent HPC. Our previous experiments showed infection of multipotent myeloid/erythroid HPC, but in vitro culture assays do not exist to evaluate infection of primitive lymphoid HPCs. The murine xenograft approach allowed us to address this issue, and the results from the animal mentioned above suggest that primitive lymphoid HPC can indeed be infected. The infection of a primitive lymphoid HPC would not constitute an effective long-term reservoir because these cells lack self-renewal capacity, but could have serious implications for disease pathogenesis: if lymphoid HPC were infected in vivo, this could reduce the ability of the marrow to replenish T cells lost to viral infection.

Two other animals have shown engraftment of human cells, with GFP+ B cells and myeloid cells in the peripheral blood. These findings are exciting because two

distinct lineages of cells are seen in the peripheral blood, which is consistent with the engraftment of HSCs. However, these animals are currently only 8-10 weeks post-transplant, and engraftment levels are modest. It will be necessary to monitor these animals over time to determine if HSC engraftment has indeed occurred. If multi-lineage engraftment is stable over time, the next step will be to perform secondary transplants, using the marrow from these animals to engraft into secondary donors. This approach will allow us to determine if long-term reconstituting HSCs can truly be infected by HIV.

If we find that HSCs can be infected by HIV, this will have profound implications in HIV pathogenesis. Firstly, long lifespan of these cells would make them suited as a long-term viral reservoir. Indeed, if a HSC became latently infected, the cell could act as a reservoir for the lifespan of the host. If HSCs became actively infected, the effect could be equally profound, as loss of HSCs would substantially impair the hematopoietic system.

## FUTURE DIRECTIONS

The studies described in this work have raised numerous important questions about the interactions of HIV with the hematopoietic system. The following section outlines approaches to address some of these questions.

*Contribution of infected HPC to the in vivo HIV reservoir*. In the studies described in this work, we have demonstrated that primitive HPC are susceptible to infection by HIV, and that in some cells the infection is latent. We have also shown that

HIV-positive individuals have infected HPC detectable within the bone marrow. Given these findings, the crucial next step is to understand the contribution of these infected HPC to viral persistence. To address this question, we plan to use a genetic approach to determine whether infected HPC release virus into the plasma of patients on anti-retroviral therapy. In this approach, CD34+ HPC and blood plasma will be collected from subjects with low viral loads. Genomic DNA from the HPC and viral RNA from the plasma will then be purified and sequenced, and viral sequences from the HPC and the plasma will be compared. Active viral replication invariably leads to mutations in subsequent viral progeny, so if identical sequences are found in HPC genomic DNA and in the plasma, it can be concluded that the plasma virus was released from HPC. Thus, this approach will allow us to conclusively determine whether virus is being released from an HPC reservoir.

Subjects with plasma virus originating from an HPC reservoir would be followed over time, to determine if the same sequence emerges repeatedly in the plasma, which will allow us to determine the longevity of the HPC reservoir. Also, the virus that emerges from the HPC reservoir can be sequenced for antiretroviral drug resistance mutations to estimate the age of the reservoir. For instance, if the subject has been effectively treated with protease inhibitors (PIs) for 3 years, but the HPC-derived HIV has no protease inhibitor resistance mutations, in can be concluded that the HPC became infected prior to PI initiation, and thus has been archived for at least three years. Such experiments will be crucial in understanding the clinical significance of HPC infection in HIV persistence.

*Assessment of stem cell infection in vivo*. Another interesting question to be addressed is whether long-term re-engrafting HSC are infected in HIV-infected individuals. To answer this question, we will purify HPC from infected individuals and transplant the cells into NOD-SCID-IL2R$\gamma^{-/-}$ mice. The mice will be monitored over time for the emergence of human cells in the peripheral blood, and human peripheral blood cells will be assessed for HIV infection by PCR. If the subject had latently infected HSC, we would expect to see HIV-infected human cells released into the blood repeatedly over time. Although murine cells do not support HIV replication, it is possible that viral replication could occur in the human cells that are produced within the transplanted mouse. For this reason, we will sequence the viral genomes from the infected human blood cells. If the infected cells found in the animal's peripheral blood are indeed the progeny of an infected HSC, then the identical HIV sequence should be found repeatedly over time. This genome could then be sequenced to reveal the characteristics of the virus that infected the HSC. For instance, we could sequence the envelope gene from such a virus and determine whether it is CXCR4 or CCR5-tropic, providing additional insight into the role of viral tropism in infecting HSC.

*Mechanism of latency in HPC*. If HPC constitute an important viral reservoir *in vivo*, it will be important to understand the mechanisms that cause latency within these cells, in order to develop therapeutic approaches to target the reservoir. As mentioned above, three mechanisms most likely cause latency within infected cells: 1) Integration of HIV into heterochromatin regions, 2) insufficient levels of HIV Tat protein to initiate

HIV transcription and 3) Lack of necessary cellular transcription factors needed to drive HIV transcription.

To begin addressing the mechanism of latency in HPC we will evaluate the HIV integration site in latently infected HPC. To accomplish this, HPC will be infected with the latency reporter virus described in Chapter 2, and actively and latently infected cells will be purified by FACS. We will then use nested PCR to detect integration near heterochromatin as has been described in the literature [32]. Briefly, genomic DNA will be subjected to first round PCR with oligos specific for the HIV LTR and for alphoid repeats in the human genome, which are found in centromeric heterochromatin. The product of the first round product will then be quantified by real-time PCR using nested oligos in the HIV LTR. If latency occurs by integration into heterochromatin regions, then latently infected cells would display a higher frequency of alphoid-adjacent integrants than their actively infected counterparts. As an alternative approach, the regions adjacent to integration sites of latently infected cells could be directly sequenced, which would reveal if integration occurred near heterochromatin alphoid elements or in centromeric DNA. Latently infected HPC could also be treated with histone deacetylase inhibitors (e.g. Trichostatin A) or methylase inhibitors (e.g. aza-dC) to determine if chromatin remodeling would re-activate the latent virus. However, in such an approach it is difficult to determine whether viral re-activation occurred as a direct result of chromatin remodeling or as an indirect effect of other cellular genes being activated following inhibitor treatment.

A second possible mechanism of latency is insufficient Tat expression during the early phase of infection, which would prevent productive viral transcription from

initiating. To evaluate this, we will infect HPC with the latency reporter virus described in Chapter 2. Cells would then be treated with soluble Tat protein, both wild type and a transactivation-null mutant. Soluble HIV Tat has a remarkable ability to enter cells when present in the extracellular environment, and following cell entry the protein localizes to the nucleus and can enhance viral transcription [49]. Thus, if Tat insufficiency is responsible for HIV latency in HPC, treatment with active Tat should reduce the frequency of latently infected HPC.

The final mechanism for HIV latency in HPC is transcription factor availability. The simplest approach to test this possibility is to treat latently infected cells with chemical activators of transcription factors, such as TPA to activate NF-κB, and determine if HIV is activated within those cells. However, with this approach it is difficult to determine if HIV activation occurred as a direct result of the transcription factor targeted, or whether activation of the transcription factor induced other changes in the cell that subsequently activated viral gene expression. An alternate approach would be to use the latency reporter virus to infect HPC, and to then perform intracellular staining and flow cytometry for active forms of relevant transcription factors. Such approaches have been used in immunobiology to assay the activation of numerous signaling pathways on a single cell level (reviewed in [50]). This approach would allow us to determine whether the presence or activation of certain transcription factors corresponds with latent or active infection.

*Pharmacologic agents to induce latent HIV from HPC.* In order to eradicate HIV from infected individuals, it will be necessary to target viral reservoirs. Recently, several

groups have begun studying compounds that induce latent HIV. The ultimate goal in this approach is to treat infected individuals with HAART until viral load is undetectable, and to then add a latency-activating drug. If effective, latently infected cells would convert to active infection, killing the infected cell. In the presence of HAART, the virus produced would be unable to infect new target cells, and the virus could theoretically be eradicated from the body. In order for this approach to be effective, the activation of latently infected cells must be 100% complete, for even a few remaining latent cells could later re-establish infection if HAART therapy is discontinued. For this reason, it is crucial to develop latency-activating compounds that are effective on all viral reservoirs.

Initially, it would be interesting to evaluate existing compounds that have the ability to induce latent HIV from resting T cells. One such agent is valproic acid. Valproic acid acts as an HDAC inhibitor, relaxing heterochromatic and activating viral transcription. Another interesting compound to evaluate would be prostratin, a phorbol ester that can activate transcription of latent provirus (reviewed in [51]). These compounds can be evaluated in primary HPC using the latency reporter virus system. HPC will be infected with latency reporter virus and treated with the compounds of interest, and the frequency of latently infected cells assayed by flow cytometry.

It will also be important to develop novel compounds to induce viral expression from latently infected HPC. In order to assay large numbers of compounds, it will be necessary to develop a cell-line based model for HIV latency that can be used in high-throughput assays. We have found that the CD34+ erythromyelogenous leukemia cell line KG-1 could be suitable for such an assay system. KG-1 cell lines express viral gene products poorly after infection, but treatment of infected KG-1 with PMA and ionomycin

induces robust gene expression, much like the induction of HIV by PMA in primary HPC (Chapter 2). To facilitate a high-throughput assay, the latency reporter virus will be modified so that red fluorescent protein (mCherry) is expressed from the viral LTR and GFP is expressed from an LTR-independent internal promoter. KG-1 cells will be infected with this virus and latently infected cells ($GFP^{+}mCherry^{-}$) will be purified. The cells will then be screened with a chemical compound library. The cells will then be assayed for induction of mCherry expression by high-throughput flow cytometry. Compounds that succeed in activating HIV expression in the high-throughput screen will then be assayed in primary cells. This approach could reveal novel pharmacologic agents capable of inducing latent HIV form infected HPC.

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