HIV-1 Infection of Hematopoietic Progenitor Cells

by

Lucy Alexandra McNamara

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Doctoral Committee:

Professor Kathleen L. Collins, Chair
Professor John V. Moran
Associate Professor Akira Ono
Professor Alice Telesnitsky

Dedication

To all whose lives have been affected by HIV

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Abstract

Latent HIV infection allows virus to persist in HIV-infected individuals in spite of antiretroviral therapy. In addition to the well-studied reservoir of latent virus in resting memory $CD4^+$ T cells, we have recently proposed that hematopoietic progenitor cells (HPCs) in the bone marrow serve as a reservoir for latent HIV. Here, I first investigate whether HIV envelope tropism impacts the ability of the virus to infect different types of HPCs. I find that HIV envelopes that are able to use CXCR4 as a co-receptor permit infection of immature, multipotent HPCs defined by expression of the cell surface marker CD133, whereas CCR5-tropic HIV has a greatly reduced capacity to infect these cells. Furthermore, I find that a CXCR4-tropic HIV envelope can infect hematopoietic stem cells that support long-term, multilineage engraftment in mice. As hematopoietic stem cells can live for the entire lifespan of a person, latent HIV infection of these cells could create a very long-lived reservoir of virus. I next examine the cellular factors that promote latency in HPCs and find that HIV can establish a latent infection in multiple HPC subsets, including immature HPCs. Latent infection in these cells can be reversed by activation of the transcription factor NF-κB as well as by inhibition of histone deacetylases. Finally, I investigate whether the $CD133^+$ subset of HPCs harbors HIV genomes in HAART-treated patients with undetectable viral loads. I detect HIV genomes in CD133-sorted cell populations in 6 of 11 donors, including two donors who have had undetectable viral loads for more than 8 years. Furthermore, for at least 5 of these 6 donors I demonstrate that $CD3^+$ T cells are present at extremely low levels in the CD133-sorted populations and are therefore unlikely to contribute to the HIV DNA detected in these samples. Together, these findings illuminate the potential of long-lived,

$CD133^+$ HPCs to serve as a reservoir for latent virus in HIV-infected individuals. Further study of how latent infection in HPCs and T cells can be reversed to eliminate these viral reservoirs may lead us closer to a cure for HIV.

Chapter 1

Introduction

A major barrier to finding a cure for HIV is the existence of long-lived reservoirs of virus in the body that are not depleted by the existing treatment for HIV, highly active antiretroviral therapy (HAART). Ultimately, the goal of the work described in this dissertation is to improve our understanding of these viral reservoirs and begin to elucidate mechanisms through which these reservoirs can be eliminated. As an introduction to the research described in later chapters, this chapter will discuss the existing knowledge of HIV biology and pathogenesis and explore the viral and cellular factors that contribute to persistence of the virus during therapy. We will then examine the evidence suggesting that hematopoietic progenitor cells may serve as a reservoir for latent HIV in treated individuals. Our analysis of these topics will provide the necessary background for the characterization of HIV infection in HPCs that follows in chapters 2-4 of this dissertation.

HIV epidemiology

It is estimated that there are more than thirty-four million people living with human immunodeficiency virus type 1 (HIV-1) infection worldwide, including more than 3 million children under the age of 15 (UNAIDS 2012). In 2011, approximately 2.5 million people acquired HIV infection and more than 1.5 million died from acquired

immunodeficiency syndrome (AIDS), the clinical endpoint of HIV infection (UNAIDS 2012).

The majority of HIV-infected individuals – more than 23 million people – live in sub-Saharan Africa, where the prevalence of HIV-1 infection in adults is nearly 5% (UNAIDS 2012). However, HIV has spread to every region of the world. Although North America is far from the epicenter of the HIV pandemic, 1.4 million people on this continent are living with HIV infection; in 2011, 58,000 North Americans acquired HIV infection and 20,000 died from AIDS (UNAIDS 2012). The prevalence of HIV worldwide is summarized in **Figure 1.1**.

HIV infection can be treated with antiretroviral therapy, which suppresses viral replication and allows the immune system to recover, thereby prolonging the lifespan of infected individuals. However, antiretroviral therapy is a lifelong course of treatment rather than a cure. Although the availability of treatment has increased dramatically in recent years, fewer than 7 million people worldwide were receiving antiretroviral therapy at the end of 2010 (UNAIDS 2011). This figure represents only 47% of the people in need of therapy worldwide based on the World Health Organization's guidelines for initiating treatment (UNAIDS 2011) and only 20% of the total population with HIV. Thus, a large proportion of HIV^+ individuals remain at risk of serious morbidity and mortality as a result of HIV infection, highlighting the urgent need for a less expensive and more time-limited treatment for HIV that could be readily distributed to all of those in need.

Clinical features of HIV infection

HIV can be transmitted horizontally through sexual contact or contact with contaminated blood as well as vertically from mother to child. Within 6 weeks of becoming infected with HIV, 50-90% of individuals experience symptoms of acute infection that may include fever, rash, fatigue, and sore throat; however, others evince no sign of HIV infection (Anonymous 1984, Cooper et al. 1985, Fox et al. 1987, Ho et al. 1985, Pedersen et al. 1989, Schacker et al. 1996, Tindall et al. 1988, Tucker et al. 1985, Vanhems et al. 1997). During acute infection, HIV loads in the plasma can reach levels as high as $10^6 – 10^7$ copies of HIV RNA per ml (Piatak et al. 1993, Schacker et al. 1996). As the symptoms of acute infection, if any, subside, the plasma viral load drops to a level that varies between 10^3 and 10^6 copies of HIV RNA per ml in different individuals, then stabilizes (Daar et al. 1991, Jurriaans et al. 1994, Piatak et al. 1993). This level of viremia is known as the viral setpoint and is generally reached by 1-6 months after initial infection (Piatak et al. 1993, Jurriaans et al. 1994) (**Figure 1.2**).

$CD4^+$ T cells are the primary targets of active HIV infection, and thus $CD4^+$ T cell counts decline dramatically during acute HIV infection (Cooper et al. 1988, Pedersen et al. 1990, Zaunders et al. 1995). Within three to four weeks, however, $CD8^+$ and $CD4^+$ T cell populations expand and help to reduce viral replication to the levels described above (Cooper et al. 1988, Pedersen et al. 1990). At this point, $CD4^+$ T cell levels are high enough and viremia low enough that HIV-infected individuals generally do not evince symptoms of infection aside from possible lymphadenopathy (Crowe et al. 1991, Fox et al. 1987, Moss et al. 1988, Pedersen et al. 1987, Polk et al. 1987). However, continuing viral replication results in $CD4^+$ T cell counts that decline at a rate of approximately 100

cells/µl per year (Lyles et al. 2000) (**Figure 1.2**). When $CD4^+$ T cell counts drop below 500 cells/µl, patients may again begin to experience symptoms such as respiratory tract infections or candidiasis (Hirschtick et al. 1995, Crowe et al. 1991).

Once $CD4^+$ T cell counts have dropped below 200 cells/µl, HIV-infected persons are considered to have progressed to AIDS. AIDS generally develops an average of 8 – 10 years after infection in untreated people (Medley et al. 1987, reviewed in Feinberg 1996), but about 20% of individuals develop AIDS within 5 years of infection (Blaxhult et al. 1990). The severe immune dysfunction present at this stage allows for the development of opportunistic infections including cryptosporidiosis, active tuberculosis, and *Pneumocystis jirovecii* (formerly *P. carinii*) pneumonia (Chaisson et al. 1987, Flanigan et al. 1992, Phair et al. 1990). Additionally, certain types of cancer as well as neuropathy and dementia may occur (Crowe et al. 1991, Feingold et al. 1990, Snider et al. 1983). In untreated patients, opportunistic infections typically lead to death 1-2 years after the development of AIDS (Mellors et al. 1996).

Highly active antiretroviral therapy (HAART) for HIV suppresses viral replication, leading to very low viral loads, increased $CD4^+$ T cell counts, and greatly prolonged lifespan in treated individuals (summarized in **Figure 1.2**) (Collier et al. 1996, Gulick et al. 1997, Hammer et al. 1997)). HAART consists of a combination of at least three antiretroviral agents that inhibit viral replication through at least two mechanisms, a feature of the treatment that is imperative to prevent the development of drug resistance. Current recommendations suggest that HAART therapy consist of two nucleoside reverse transcriptase inhibitors and either a nonnucleoside reverse transcriptase inhibitor, a protease inhibitor, or an integrase inhibitor (Thompson et al. 2012). The requirement for

reverse transcriptase, protease, and integrase in the HIV life cycle is reviewed below in the section on HIV virology.

Although HAART suppresses viral replication and greatly increases the lifespan of infected individuals, it is clear that HAART is not a cure for HIV. First, although the viral loads of individuals on HAART are often clinically undetectable, sensitive assays are generally still able to detect low levels of virus in the plasma of infected individuals (Maldarelli et al. 2007). In addition, even people with viral loads that are usually undetectable with clinical assays will sometimes experience "blips" of detectable viremia at intermittent timepoints (**Figure 1.2**) (Di Mascio et al. 2003). Finally, if HAART is discontinued even after years of therapy, HIV viremia will rebound to readily detectable levels within weeks (**Figure 1.2**) (Joos et al. 2008). For all of these reasons, it is evident that although HAART is an effective treatment for HIV, it is not a cure.

While the vast majority of HIV-infected individuals progress to AIDS in the absence of antiretroviral treatment, there are rare individuals in whom disease progression does not occur or is substantially delayed. These individuals fall into two broad groups: long term non-progressors (LTNPs), who have detectable but often relatively low viral loads and maintain normal $CD4^+$ T cell counts for at least 10 years after HIV infection; and elite controllers (ECs) who maintain viral loads below the clinical limit of detection (<50 copies HIV RNA/ml) in the absence of treatment (Hubert et al. 2000, Lambotte et al. 2005, reviewed in Deeks and Walker 2007). While some LTNPs and ECs appear to be infected with HIV that is less pathogenic due to mutations or deletions in various viral genes (Alexander et al. 2000, Deacon et al. 1995, Kirchoff et al. 1995, Lum et al. 2003, Wang et al. 1996, Yamada and Iwamoto 2000), host variation

in the major histocompatibility complex (MHC) class I region of chromosome 6 also clearly contributes to the ability to control viral replication (The International HIV Controllers Study 2010). Current estimates suggest that about 5-15% of HIV-infected people have the potential to be long term non-progressors, while less than 1% are elite controllers (Buchbinder et al. 1994, Hubert et al. 2000, Lambotte et al. 2005, Muñoz et al. 1995).

HIV virology

HIV is an enveloped retrovirus of the genus *Lentivirus*. The virus is spherical in shape and approximately 110nm in diameter (Gelderblom et al. 1987). Within the lipid envelope of the virus is the protein capsid, which contains two copies of the viral genome, a positive-sense, single-stranded RNA (**Figure 1.3A**). The genome encodes the viral structural proteins Gag, Pol, and Env, the regulatory proteins Tat and Rev, and the accessory proteins Vif, Vpr, Vpu, and Nef (reviewed in Haseltine 1988). At either end of the genome is the viral long terminal repeat (LTR), which serves as the promoter for the transcription of the viral genes. In the RNA genome the 5' LTR consists of only the 5' UTR (U5) and R regions, while the 3' LTR contains the 3' UTR (U3) and R regions; the DNA provirus has the U3, R, and U5 regions at each end (reviewed in Hu and Hughes 2012). In its proviral form, the genome is approximately 9700 base pairs in length (Ratner et al. 1985). The structure of the viral genome is shown in **Figure 1.3B**.

Structural proteins and the life cycle of HIV-1. The HIV-1 life cycle (**Figure 1.3C**) can be thought of as beginning with viral assembly, which is dependent upon the expression

of HIV-1 Gag (Gheysen et al. 1989). Gag is initially translated as the p55 polyprotein; in addition, about 5% of all Gag proteins made in the cell are in fact Gag-Pol polyproteins, which are produced when the ribosome shifts frame to read through the end of the Gag polyprotein during translation (Jacks et al. 1988). The N-terminal matrix domain of p55 mediates targeting of Gag or Gag-Pol to the plasma membrane of the cell as well as membrane binding (Bryant and Ratner 1990, Fäcke et al. 1993). Meanwhile, the nucleocapsid domain of Gag and Gag-Pol mediates binding to the viral RNA genome and Gag multimerization (Burniston et al. 1999). Multimerization of Gag forms an inherently curved lattice, thereby leading to the formation of spherical viral particles (Briggs et al. 2009).

The final HIV-1 structural protein, Env, is a glycoprotein that is initially synthesized in a precursor form, gp160. Gp160 is then cleaved by one of several cellular proteases into gp120 and gp41 (Decroly et al. 1997). Then, gp120 and gp41 assemble into a complex that localizes to the plasma membrane and subsequently to the viral envelope through interactions with the matrix domain of Gag (Chan et al. 1997, Cosson 1996, Murakami and Freed 2000).

Once all of the viral structural proteins as well as two copies of the RNA genome have assembled, the virion buds from the plasma membrane (reviewed in Sundquist and Kräusslich 2012). In addition to the viral genome and proteins, these viral particles contain small cellular RNAs, including the Lys3 tRNA required for initiation of reverse transcription (see below) (Jiang et al. 1993). As the virion buds, Gag p55 is cleaved by the viral protease, which is part of Pol, to form the matrix (p17), capsid (p24), nucleocapsid (p7), and p6 proteins as well as the p2 and p1 spacer peptides (Henderson et

al. 1992, Wiegers et al. 1998). These cleavage events result in major structural changes within the virion, including the formation of the cone-shaped protein capsid around the viral proteins and RNA genome (Wiegers et al. 1998). These structural changes are required for the formation of the mature, infectious virion (**Figure 1.3A**) (Kohl et al. 1988). Finally, the viral protease also cleaves Pol into the viral enzymes required for the remaining steps of the viral life cycle: the viral protease itself; reverse transcriptase, responsible for reverse transcribing the viral RNA genome into a DNA provirus; and integrase, which catalyzes the integration of the DNA provirus into the host cell's genome (Darke et al. 1988; described in more detail below).

For the mature virion to infect a new cell, HIV Env must bind to the cellular receptor CD4 as well as a co-receptor, usually CCR5 or CXCR4 (Alkhatib et al. 1996, Choe et al. 1996, Deng et al. 1996, Doranz et al. 1996, Dragic et al. 1996, Feng et al. 1996, reviewed in Berger et al. 1999). Env then undergoes a conformational change that allows the viral envelope to fuse with the cell membrane, permitting release of the viral capsid into the cell (Furuta et al. 1998, Kowalski et al. 1987). Viral uncoating – the disassembly of the viral capsid – may occur immediately following entry into the cell (Fassati and Goff 2001); alternatively, uncoating may happen during reverse transcription and/or nuclear transport of the provirus (Dismuke and Aiken 2006, reviewed in Arhel 2010). In either case, shortly after entry the viral RNA genome is reverse transcribed to produce a double-stranded DNA provirus, using the Lys3 tRNA as the primer to initiate DNA synthesis (Ratner et al. 1985, Isel et al. 1996). Following reverse transcription, the the proviral genome associates with the viral integrase as well as other proteins to form the preintegration complex (PIC), which is imported into the nucleus of the cell through a

mechanism that has not been fully elucidated (reviewed in Craigie and Bushman 2012). Once in the nucleus, HIV-1 integrase and cellular enzymes catalyze the integration of the proviral genome into the host cell's DNA (Bushman et al. 1990). The life cycle of HIV-1 is summarized in **Figure 1.3C**.

Following integration into the host cell's genomic DNA, transcription of the HIV genes results in the production of both spliced and unspliced viral RNA. The unspliced RNA serves as the mRNA for Gag and Gag-Pol proteins as well as the viral genome for new virions (Feinberg et al. 1986). By contrast, singly or multiply spliced viral RNA serves as the mRNA for Env and the viral accessory and regulatory proteins (Feinberg et al. 1986, Malim et al. 1988, Purcell and Martin 1993). The functions of these additional proteins as well as the mechanisms controlling the transcription of the HIV genome are discussed in more detail below.

Accessory and regulatory proteins. In addition to the structural proteins highlighted above, HIV-1 also encodes accessory proteins that help the virus evade the host immune system. First, the accessory protein Vif promotes the degradation of APOBEC3G, a cellular protein that both deaminates cytidine residues in the minus strand of the HIV provirus, thereby causing G to A hypermutation in the viral genome (Mangeat et al. 2003, Marin et al. 2003), and inhibits HIV reverse transcription through a deaminase-independent mechanism (Bishop et al. 2006, Bishop et al. 2008, Chiu et al. 2005, Guo et al. 2006, Mbisa et al. 2007). In addition, Nef downregulates cell surface expression of several cellular proteins, notably CD4 and MHC Class I (Garcia and Miller 1991, Collins et al. 1998, Leonard et al. 2011). Downregulation of MHC Class I prevents infected cells

from being recognized by $CD8^+$ cytotoxic T cells and thereby promotes the survival of HIV-infected cells (Collins et al. 1998). Meanwhile, removal of CD4 from the surface promotes Env incorporation into virions and virion release while preventing superinfection of the already-infected cell, thus promoting viral spread (Benson et al. 1993, Lama et al. 1999, Ross et al. 1999). Finally, Vpu also degrades CD4 (Willey et al. 1992a, Willey et al. 1992b) and furthermore enhances the release of nascent virions from the cell membrane by counteracting the cellular factor Tetherin, which otherwise "tethers" virions to the cell (Neil et al. 2008).

The function of the accessory protein Vpr is less well understood. Vpr expression is known to lead to cell cycle arrest and apoptosis, but it is not clear why these functions are beneficial to the virus (Ayyavoo et al. 1997, Jowett et al. 1995, Re et al. 1995, Rogel et al. 1995, Stewart et al. 1997). Vpr has also been reported to assist in nuclear import of the viral pre-integration complex in non-dividing cells (Agostini et al. 2002, Heinzinger et al. 1994). In addition, we have recently reported that Vpr interacts with the host uracil glycosylase UNG2 to reduce APOBEC3G-mediated uridine incorporation in the proviral genome (Norman et al. 2011). However, this recruitment activates a DNA damage-response pathway that enhances recognition of HIV-infected cells by natural killer (NK) cells (Norman et al. 2011). Further research is needed to better understand the function of Vpr and the mechanisms through which it may promote nuclear import and/or repair of proviral DNA.

Finally, HIV-1 encodes two regulatory proteins, Rev and Tat. Rev is responsible for the export of unspliced HIV-1 RNAs from the nuclei of infected cells, an essential activity as unspliced HIV-1 RNA serves as both the template for translation of the

structural proteins and as the RNA genome for nascent virions (Malim et al. 1989, Sodroski et al. 1986). The Tat protein, on the other hand, is required for the efficient transcription of the viral genes. During transcription, Tat binds to the Tat response element (TAR) at the 5' end of viral RNAs (Berkhout et al. 1989) and recruits the cellular positive transcription elongation factor b (P-TEFb) complex to the viral genome (Wei et al. 1998) by competitively displacing inhibitory factors that otherwise bind this complex (Barboric et al. 2007, Sedore et al. 2007). The importance of Tat in promoting viral gene expression and the impact that Tat expression has on the establishment and maintenance of viral latency are discussed in more detail below.

Control of HIV gene expression

Transcription of the HIV genome immediately following integration results in the production of new viral proteins that form new virions, which can then infect additional cells and continue the viral replication cycle. Sometimes, however, viral genes are not immediately transcribed after the provirus integrates. When this happens, a latent, transcriptionally silent infection is established. Because the latently infected cell evinces no outward sign of HIV infection, neither the immune system nor current antiretroviral therapies are able to eliminate latently infected cells. Instead, the HIV-1 provirus can be maintained in the infected cell or in daughter cells from subsequent cell divisions for as long as these cells survive. However, changes in cellular conditions at a later time point can lead to reactivation of latent virus and, in the absence of antiretroviral therapy, the initiation of new cycles of viral replication. Because the potential for viral reactivation is always present, latent HIV infection represents a barrier to curing HIV.

In order to understand how latent infection can occur, it is essential to understand the factors that control the expression of HIV genes. In this section, we will explore the cellular, viral, and stochastic factors that impact HIV gene expression. Most studies of HIV gene expression have been conducted in T cell lines and primary T cells, and it is evidence from these cell types that will be focused on here. The factors that impact HIV gene expression in other cell types, such as hematopoietic progenitor cells, are less well understood and will be further investigated in Chapter 3.

Transcription factors involved in HIV gene expression. Binding sites for numerous transcription factors exist within the HIV-1 LTR (**Figure 1.4**), and many of these transcription factors have been shown to impact the transcription of HIV genes (reviewed in Pereira et al. 2000). Notably, YY1, homodimers of NF-κB p50, and Sp1 have been reported to recruit histone deacetylase 1 to the viral LTR and thereby repress viral transcription (Doetzlhofer et al. 1999, He and Margolis 2002, Williams et al. 2006). However, the Sp1 and NF-κB binding sites can also recruit activating factors, including NF-κB p65-p50 heterodimers and histone acetyltransferases, and these sites are in fact essential for efficient transcription of viral genes (Li et al. 1994, Perkins et al. 1993, reviewed in Trono et al. 2010). Additional transcription factors, including the nuclear factor of activated T cells (NF-AT) and activator protein-1 (AP-1), have also been shown to promote the transcription of viral proteins in some systems (Kinoshita et al. 1997, reviewed in Pereira et al. 2000). Overall, it is clear that cellular transcription factors are required for the transcription of HIV genes, and thus understanding the transcription

factors that promote or repress transcription in different cell types is key to furthering our understanding of latent infection.

Additional factors that impact HIV gene expression. As mentioned above, the recruitment of histone deacetylases to the HIV-1 LTR by transcription factors promotes a restricted transcriptional state, whereas histone acetyltransferase recruitment promotes transcription of viral genes. Methylation of both histones and DNA has also been shown to repress HIV gene expression and promote the establishment of latent infection (Blazkova et al. 2009, Kauder et al. 2009, Pearson et al. 2008, Tyagi et al. 2010) (**Figure 1.4**). These repressive chromatin modifications may be induced by specific recruitment of repressive factors to the HIV-1 LTR as described above; however, they can also be a consequence of the initial integration site of the HIV genome. Although HIV preferentially integrates into actively transcribed genes (Schröder et al. 2002), it has also been shown that latent infection in T cell lines is preferentially established when an HIV provirus integrates into heterochromatic chromosomal regions (Jordan et al. 2003). It is thus apparent that chromatin modifications at or near the HIV-1 LTR play a key role in the regulation of HIV gene transcription.

Production of the viral protein Tat and recruitment of P-TEFb to the nascent viral transcript are also essential for the transcription of viral genes. As noted above, Tat recruits P-TEFb to the viral TAR; once there, P-TEFb phosphorylates the C-terminal domain of RNA polymerase II (RNAPII) to stimulate transcription elongation (Marshall et al. 1996) (**Figure 1.4**). T cell receptor signaling has also been shown to promote dissociation of P-TEFb from its repressive complex with 7SK RNA and HEXIM1 (Kim

et al. 2011). In resting T cells, which as discussed below are a known reservoir for latent HIV in vivo, P-TEFb activation appears to be a major factor in the reactivation of latent virus through T cell activation.

Tat is essential for the efficient transcription of viral genes, but as a viral gene product itself it is produced at very low levels immediately following infection. Whether sufficient Tat is produced to initiate the positive feedback loop required for efficient viral gene transcription depends upon the chromatin environment and availability of the transcription factors noted above; however, stochastic variation in the amount of Tat initially produced can also influence whether viral gene transcription continues (Weinberger et al. 2005). Finally, mutations in Tat or the viral TAR can also promote latent infection in vitro (Emiliani et al. 1998, Pearson et al. 2008, Reza et al. 2003) and in vivo (Yukl et al. 2009).

There is also evidence that additional factors may impact HIV gene expression. Transcriptional interference has been proposed as a mechanism underlying HIV latency in spite of integration into actively transcribed genes (Han et al. 2008, Lenasi et al. 2008). When the viral genome is integrated in the reverse orientation as the host gene, active transcription of the host gene can result in the physical exclusion of transcription factors and RNA polymerase from the HIV-1 LTR, preventing the transcription of viral genes (Han et al. 2008, Lenasi et al. 2008). HIV gene expression may also be inhibited at the RNA level through binding of cellular microRNAs (Huang et al. 2007) or by retention of spliced viral RNA in the nucleus of resting T cells due to low levels of polypyrimidine tract binding protein (Lassen et al. 2006). Further study is required to better understand

how these factors interact with transcription factors and chromatin modifications to promote viral latency in different cell types.

HIV envelope tropism

In order for HIV to infect a cell, the HIV envelope glycoprotein must interact with the cellular receptor CD4 as well as a co-receptor, usually CXCR4 or CCR5 (reviewed in Berger et al. 1999). Although there is evidence that HIV can occasionally use alternative co-receptors, such as CCR2B or CCR3 (Doranz et al. 1996, Choe et al. 1996, reviewed in Berger et al. 1999), the use of such co-receptors is in uncommon in vivo (Zhang et al. 1998). Thus, our discussion of HIV envelope tropism will focus on the ability of the envelope to use CXCR4 and/or CCR5 as a co-receptor.

The ability of HIV Env to use CXCR4 vs. CCR5 as a co-receptor is determined in large part by the sequence of the third variable loop (V3) of the HIV envelope (De Jong et al. 1992, Raymond et al. 2008). A change in the net charge of the V3 loop, which can result from as few as one or two amino acid changes in this region, can shift the HIV envelope from CCR5-tropic to CXCR4- or dual-tropic (i.e. able to use either co-receptor) (De Jong et al. 1992, Raymond et al. 2008). As HIV has a very high mutation rate of approximately 3.4×10^{-5} substitutions per base per replication cycle (Mansky and Temin 1995), the few mutations required to permit a shift in viral tropism means that the co-receptor tropism of the virus can readily change in vivo.

CCR5-utilizing HIV predominates in most patients at early time points after infection (van't Wout et al. 1994, Zhu et al. 1993). It has been proposed that the prevalence of CCR5-tropic virus early in infection is due to a requirement for CCR5

utilization in transmission (reviewed in Margolis and Shattock, 2006). However, recent evidence suggests that CXCR4-tropic isolates can be transmitted as well and are simply less common in the individuals who are most likely to transmit (Chalmet et al. 2012), perhaps due to the association between CXCR4-utilization and disease progression noted below. Furthermore, deep sequencing of the viral populations present in recently infected individuals has demonstrated that populations of CXCR4-utilizing virus are present in 12-50% of these patients (Abbate et al. 2011, Chalmet et al. 2012, Daar et al. 2007). Thus although CCR5-using virus appears to be more common early in infection, it is clear that that many recently infected individuals also harbor CXCR4- or dual-tropic HIV.

In about half of individuals with subtype B HIV infection and at least some individuals infected with other HIV subtypes, CXCR4-using virus becomes more common later in the course of disease (Abebe et al. 1999, Cilliers et al. 2003, Juriaans et al. 1994, Shankarappa et al. 1999, Karlsson et al. 1994, Scarlatti et al. 1997, Tersmette et al. 1988). Furthermore, the emergence of CXCR4-using virus is associated with more rapid disease progression (Connor et al. 1997, Daar et al. 2007, Karlsson et al. 1994, Richman and Bozzette 1994, Schuitemaker et al. 1992, Shepherd et al. 2008, Waters et al. 2008, Weiser et al. 2008, Yu et al. 1998). The reasons for the association between the emergence of CXCR4-using virus and disease progression have not yet been elucidated. It is also not understood whether CXCR4-using virus becomes more prevalent over time in patients with fully suppressed viral loads on HAART (Delobel et al. 2005); however, it is clear that CXCR4-using virus does at least persist in such patients (Delobel et al. 2005, Seclén et al. 2010, Soulié et al. 2007). Further study is required to better understand the

relationship between HIV envelope tropism, disease progression, and the persistence of HIV during therapy.

HIV persistence during therapy

Although HAART is able to reduce viral replication to levels that are often clinically undetectable, HIV persists in spite of therapy and viremia rebounds if HAART is discontinued. The two explanations that have been proposed for the ability of HIV to persist in spite of effective antiretroviral therapy are reviewed below.

Ongoing active replication. One explanation for the persistence of HIV in spite of HAART is that HAART is unable to prevent all viral replication in vivo. This ongoing replication has been proposed to occur in sanctuary sites within the body where drug penetrance is reduced (reviewed in Dahl et al. 2010). Alternatively, cell-to-cell spread, which can result in the transfer of very large quantities of virus to an individual cell, may permit the virus to persist in spite of HAART by increasing the probability that at least one of the virions infecting an individual evades the drugs that are present (Sigal et al. 2011).

Several studies have suggested that ongoing replication does occur in vivo in spite of HAART treatment. One study found that when suppressive HAART therapy was intensified with the integrase inhibitor raltegravir, an increase in the levels of unintegrated HIV DNA was observed (Buzón et al. 2010). This finding suggested that in the patients studied, raltegravir was blocking integration of virions that would otherwise have completed a full cycle of replication in spite of HAART. However, additional

studies of intensification of HAART with raltegravir or other antiretroviral agents have failed to replicate this finding or to demonstrate any decrease in the amount of residual viremia present in the patients studied (Besson et al. 2012, Dinoso et al. 2009, Gandhi et al. 2010, Hatano et al. 2011).

Other studies have found that the residual viral sequences present in HAART-treated patients evolve over time in some individuals, which should only be possible in the context of ongoing replication (Shiu et al. 2009, Tobin et al. 2005). However, contrasting studies have not been able to find evidence of evolution in the residual viremia (Joos et al. 2008, Kieffer et al. 2004). Overall, although residual viral replication may occur in some HAART-treated patients, it does not seem to account for viral persistence in all HIV-infected individuals on HAART.

Latent infection. A latent HIV infection occurs when the HIV DNA provirus integrates into the cellular genome but the viral genes are not immediately transcribed or translated. As described above, insufficient levels of transcriptional activators such as P-TEFb or the transcription factor NF-κB as well as a restrictive chromatin state at the HIV-1 LTR can promote the establishment of latent infection. The latent virus can then be maintained throughout the lifetime of the infected cell, and proliferation of this infected cell can lead to expansion of the latent reservoir (Chomont et al. 2009).

Although latent virus is transcriptionally inactive, changes to cellular conditions can cause the virus to reactivate, resulting in the production of new virions that could contribute to the residual viremia detected during HAART and to the rebound of virus following therapy cessation. As latent virus could contribute to residual viremia without

completing full cycles of replication, viral particles derived from latent viral genomes would not be expected to evolve. Thus, latent infection provides an explanation for the non-evolving residual viral populations observed in many HAART-treated patients (Joos et al. 2008, Kieffer et al. 2004, Tobin et al. 2005).

Many studies have demonstrated that resting memory $CD4^+$ T cells can serve as a reservoir for latent HIV genomes in vivo, and that these latent genomes can give rise to replication-competent virus upon T cell activation (Chun et al. 1995, Finzi et al. 1997, Finzi et al. 1999, Zhang et al. 1999). More recent evidence has suggested that central and transitional resting memory $CD4^+$ T cells in particular are the primary T cell reservoir for latent virus (Chomont et al. 2009). HIV infection in resting T cells has been demonstrated to be blocked at reverse transcription (Zack et al. 1990); thus, it is generally believed that latent infection in resting memory T cells is established when HIV infects an activated T cell but the cell reverts to a resting state before cytotoxic viral proteins are produced. However, there is also evidence that the block to infection of resting T cells is not absolute and that HIV can infect these cells directly (Agosto et al. 2009, Cameron et al. 2010, Saleh et al. 2007, Yoder et al. 2008, Yu et al. 2009). These explanations for how the reservoir of latent virus in resting memory T cells is established are illustrated in **Figure 1.5**.

Evidence for additional reservoirs of HIV[1]. Recent data on the structure of the residual viral population suggests that in addition to latently infected resting memory T cells, there is at least one other long-lived viral reservoir in many patients. Several studies have demonstrated that many viral sequences found in the residual viremia of some HIV-infected individuals on successful HAART therapy do not match those found in the resting CD4+ T cell reservoir (Bailey et al. 2006, Sahu et al. 2009) or indeed from any peripheral blood cells (Brennan et al. 2009), though plasma viral sequences are more closely related to sequences in peripheral blood monocytes than to sequences in CD4+ T cells (Lopez et al. 2010). These data suggest that a major source of residual viremia in treated patients may be a heretofore uncharacterized reservoir of HIV.

There is also evidence that residual plasma viremia is largely homogenous, with just one or two viral clones accounting for most residual viremia in many patients on HAART (Bailey et al. 2006) or most rebounding virus during treatment interruptions (Joos et al. 2008). Given the high mutation rate of HIV, these data suggest that the major source of persistent HIV production is either a single cell or a clonal population of cells originating from a single infected cell with a substantial capacity for cell division.

Finally, a recent analysis of the decay kinetics of HIV reservoirs in HAART-treated patients suggests the existence of two long-term viral reservoirs: one with a half-life of 9-15 months, approximately consistent with the previously reported half-life of the resting T cell reservoir (6-44 months) (Siliciano et al. 2003, Finzi et al. 1999, Zhang et al.

[1] This section has been published as a section of a review article (McNamara and Collins 2011). The text has been modified slightly to incorporate recent findings and improve integration with the remaining sections of this chapter.

1999, Ramratnam et al. 2000), and a second reservoir with no appreciable decay over at least seven years (Palmer et al. 2008). This analysis suggests that not only is there an additional reservoir of latent virus aside from that in resting memory T cells, but that this reservoir may permit even more durable persistence of virus than the T cell reservoir does. The identity of this long-lived viral reservoir has yet to be established; however, as we will discuss later in this chapter, the indefinite half-life of this reservoir is consistent with the exceedingly long lifespan of hematopoietic stem cells (HSCs).

Cell types that could serve as additional reservoirs for HIV

The evidence that resting memory $CD4^+$ T cells are not the sole source of residual viremia in HAART-treated patients has led researchers to search for additional cell types that might serve as long-term reservoirs for HIV. These cells might be latently infected, or they might be cells that can endure active virion production without cytotoxicity. The evidence that various cell types can serve as either a latent or a persistent active reservoir for HIV is examined below.

Monocytes, macrophages, and dendritic cells. Although peripheral blood monocytes are resistant to HIV infection, HIV can nevertheless infect these cells at low rates (Sonza et al. 2001, Zhu et al. 2002). Furthermore, HIV readily infects mature macrophages and dendritic cells, which have a half-life that varies from days to years in vivo (reviewed in Gonzalez-Mejia and Doseff 2009, Merad and Manz 2009). Macrophages have been shown to be less susceptible to the cytotoxic effects of HIV than are $CD4^+$ T cells, and as such may remain persistently actively infected months (Ho et al. 1986, Collman et al.

1989). In addition, macrophages have been shown to support latent HIV infection in vitro (Brown et al. 2006). Although some dendritic cells can also survive the cytopathic effects of active HIV infection for up to several weeks (Popov et al. 2005), latent infection of these cells has not been reported and HIV DNA does not persist in dendritic cells of patients with suppressed viral loads on HAART (Otero et al. 2003). Thus, while both actively and latently infected macrophages may serve as reservoirs for HIV in vivo, dendritic cells are less likely to serve as a source of persistent virus.

Infection of monocytes that then cross the blood-brain barrier also provides a mechanism for the establishment of HIV infection in the central nervous system (Liu et al. 2000). Once HIV has been introduced to the central nervous system, it is able to infect microglial cells and other macrophages and may establish a persistent reservoir (Schnell et al. 2009). Of note, microglial cells are extremely long-lived cells that can persist for decades in vivo (Lassmann et al. 1993). While the ability of HIV to establish a latent infection in this cell type is poorly understood, microglia do appear to be resistant to the cytotoxic effects of HIV infection (Cosenza et al. 2004). Further study is required to better understand the contribution of persistent HIV infection in the central nervous system to long-term persistence of virus during HAART.

Mast cells[2]. Committed mast cell progenitors (prMCs) have also been shown to be susceptible to HIV infection (Bannert et al. 2001). Mast cells are tissue-resident immune

[2] This section has been published as a section of a review article (McNamara and Collins 2011). The text has been modified slightly to incorporate recent findings and improve integration with the remaining sections of this chapter.

cells important in allergy, inflammation, and helminthic infection (Casale et al. 1987, Lantz et al. 1998). Although HIV-1 cannot infect mature mast cells (Sundstrom et al. 2007), HIV-1 can infect prMCs in peripheral blood (Bannert et al. 2001). As prMCs eventually migrate to tissues, where they survive for at least ten months (Padawer et al. 1974, Gurish et al. 2002), infection of prMCs could create a reservoir of HIV-1.

Recently, placental tissue mast cells were shown to harbor latent HIV in vivo in pregnant, HAART-treated women with detectable viral loads (Sundstrom et al. 2007), suggesting that infection of prMCs creates a reservoir in mast cells of patients with ongoing viral replication. However, a recent study could not detect active HIV-1 replication in tissue mast cells from ten donors (Nelson et al. 2009), suggesting that actively infected mast cells are rapidly cleared in successfully treated patients even though HIV-1 is not cytopathic in these cells (Sundstrom et al. 2007). At present, the long-term significance of the possible mast cell reservoir is unclear, and it is not known whether a mast cell reservoir exists in HAART-treated patients with undetectable viral loads.

Hematopoietic progenitor cells (HPCs). The possibility that hematopoietic progenitor cells in the bone marrow serve as a reservoir for HIV has been debated for more than 20 years and is the focus of the research presented in chapters 2-4 of this dissertation. In this section, we will first describe hematopoiesis and the different types of hematopoietic progenitor cells that exist in humans; next, we will review the evidence for infection and particularly for latent infection in this long-lived cell type.

Hematopoiesis

Hematopoiesis begins with hematopoietic stem cells (HSCs), cells that are capable of differentiating into all of the different blood cell lineages and that have the potential for indefinite self-renewal. HSCs then differentiate into multipotent progenitor cells (MPPs), which can also give rise to all of the different blood cell lineages but have a more restricted capacity for self-renewal (Doulatov et al. 2012). The immediate progeny of MPPs are two types of cells: common myeloid progenitors (CMPs), which can give rise to all of the myeloid cell lineages, and multilymphoid progenitors (MLPs) that can differentiate into all of the lymphoid lineages as well as some monocytes and dendritic cells (Doulatov et al. 2012). These progenitor cells then differentiate into more committed progenitors and eventually into the mature blood cells found in the peripheral blood. Hematopoietic differentiation is summarized in **Figure 1.6**.

Several cell surface markers and assays are commonly used to distinguish subsets of HPCs. HPCs are often broadly characterized by the expression of the cell surface marker CD34, which is found on immature hematopoietic stem cells as well as more committed progenitors (Berenson et al. 1991, Leary et al. 1985). The cell surface marker CD133 can be used to enrich for more immature HPCs with multilineage differentiation capacity (Yin et al. 1997). Multipotency can be directly tested with the use of colony-forming assays, which assess the ability of HPCs to form colonies in methylcellulose agar. Cells are plated such that each colony will be formed from the proliferation of a single cell, then the cells are allowed to divide and differentiate for two weeks. If multiple mature blood cell lineages are found in the final colony, it is clear that the initially plated cell had the capacity for multilineage differentiation. However,

methylcellulose agar only supports the growth of myeloid lineages, and so this assay cannot be used to assess whether cells are HSCs or MPPs. Xenograft assays in immunocompromised mice remain the gold standard for determining whether an HPC is a stem cell, as only HSCs are capable of long-term multilineage engraftment in mice (Christensen and Weissman 2001, Jones et al. 1990, Uchida and Weissman 1992). The types of HPCs distinguished by these cell markers and assays are highlighted in **Figure 1.6**.

HIV infection in HPCs[3].

Evidence for HIV infection of HPCs. A proportion of $CD34^+$ cells express the HIV receptors CD4, CXCR4, and CCR5, making these cells potentially susceptible to HIV-1 infection (reviewed in Alexaki and Wigdahl 2008). Beginning more than twenty years ago, multiple studies suggested that HIV infection in $CD34^+$ cells was possible, though rare, both in vitro and in vivo (Folks et al. 1988, Stanley et al. 1992, Slobold et al. 1996, Von Laer et al. 1990, Davis et al. 1991, Zauli et al. 1992, Neal et al. 1995). However, these studies could not rule out contamination by other cell types. Furthermore, studies assessing HIV-1 infection of more immature subsets of $CD34^+$ cells, including multipotent colony-forming and $CD133^+$ HPCs, failed to detect either HIV-1 infection or

[3] This section has been published as a section of a review article (McNamara and Collins 2011). The text has been modified slightly to incorporate recent findings and improve integration with the remaining sections of this chapter.

expression of any of the three main HIV receptors in these cells (Chelucci et al. 1995, Shen et al. 1999, Hariharan et al. 1999, Weichold et al. 1998).

Based on these reports, there was a consensus that $CD34^+$ cells are not an important target of HIV-1 infection and that HIV cannot infect multipotent HPCs at all. Recently, however, improved techniques have permitted reexamination of this topic, and this reexamination has unambiguously shown that immature, multipotent HPCs are susceptible to HIV infection. First, a 2007 study investigated the ability of HIV-1 subtype C to infect multipotent $CD34^+$ HPCs in vitro and in vivo (Redd et al. 2007a). While the authors could not detect HIV-1 subtype B infection in HPCs capable of forming multilineage colonies, they found that several isolates of HIV-1C could infect multipotent cells. Furthermore, the authors were able to detect HIV proviruses in $CD34^+$ cells from the peripheral blood of 12 out of 19 donors infected with HIV-1C (Redd et al. 2007a). Importantly, the level of HIV detected in 11/12 of these $CD34^+$ samples was greater than the level observed in total peripheral blood mononuclear cells from the same patient, reducing the potential for contamination that plagued earlier studies (Redd et al. 2007a).

While this study showed that HIV-1C infects multipotent HPCs, the ability of HIV-1B to infect multipotent HPCs remained ambiguous. Redd and colleagues concluded that HIV-1B could not infect multipotent HPCs because they could not detect HIV DNA in multilineage colonies generated from HPCs exposed to HIV-1B isolates. However, the absence of HIV^+ colonies could instead indicate that although HIV-1B can infect multipotent cells, the infection is cytotoxic either immediately or upon proliferation

and differentiation of the cells, leading to cell death rather than infected colony formation.

We undertook a study to definitively assess whether HIV-1B could infect multipotent HPCs (Carter et al. 2010). Using a flow cytometric assay to detect the expression of HIV proteins in individual $CD34^+$ cells after very short incubation periods (three days), we found that a variety of HIV-1B isolates could infect CD34+ cells derived from bone marrow or umbilical cord blood (Carter et al. 2010) (**Figure 1.7A**). Because the virus was cytopathic to the cells, however, the number of infected cells declined dramatically over time. We furthermore showed that exposure of $CD34^+$ cells to a non-cytotoxic, GFP-expressing HIV-1B construct permitted the formation of multilineage colonies that were uniformly GFP^+, demonstrating that HIV-1B infects multipotent HPCs in vitro (Carter et al. 2010) (**Figure 1.7B**).

We next examined whether $CD34^+$ HPCs could support latent as well as active HIV-1 infection, a distinction that had not previously been assessed. As noted above, the initially robust infection in $CD34^+$ HPCs declined over time until active infection could no longer be observed (Carter et al. 2010). If these cells were exposed to agents that stimulated myeloid differentiation, however, we observed a resurgence of viral gene expression (Carter et al. 2010) (**Figure 1.7C**). This finding demonstrates that latent HIV-1 infection of HPCs is possible in vitro.

Finally, our study assessed the infection of $CD34^+$ bone marrow HPCs in HIV-infected patients. In a sample of HIV^+ individuals with clinically detectable viremia (>50 copies HIV-1 RNA/mL), we could directly detect HIV Gag expression in $CD34^+$, $CD133^+$ cells from a subset of donors (Carter et al. 2010) (**Figure 1.7D**). In the

remaining donors, we observed HIV Gag expression when we stimulated the $CD34^+$ cells with cytokines to induce myeloid differentiation, thus demonstrating that latent HIV infection occurs in $CD34^+$ cells in vivo (Carter et al. 2010). $CD34^-$ cells rapidly died under our culture conditions for $CD34^+$ cells, thus minimizing the potential for contamination (Carter et al. 2010). We also looked for HIV-1 proviral DNA in $CD34^+$ cells from a group of HIV-positive individuals on HARRT with clinically undetectable (<50 copies/mL) viral loads. We detected HIV-1 DNA in $CD34^+$ cells from more than 40% of these donors; in each case, we did not detect comparable amounts of HIV DNA from bone marrow depleted for CD34, indicating that infected $CD34^-$ bone marrow cells do not persist at similar levels in people on HAART (Carter et al. 2010) (**Figure 1.7E**). The sensitivity of this assay was low – we could detect HIV genomes in CD34+ cells only if at least 1 in 10,000 cells harbored an HIV genome. This limit of detection is higher than the frequency of integrated genomes that have been observed in the resting $CD4^+$ T cell reservoir in some patients (Chun et al. 1997); thus, it is likely that other patients in our cohort harbor HIV-infected $CD34^+$ cells at a lower frequency. The ability of $CD34^+$ cells to harbor HIV DNA in vivo, even in patients undergoing successful HAART treatment, demonstrates that $CD34^+$ cells can act as a long-lived reservoir of HIV.

Although we were readily able to detect HIV genomes in $CD34^+$ cells from donors with undetectable viral loads on HAART, more recent studies have been unable to replicate these results (Durand et al. 2012, Josefsson et al. 2012). Instead, these studies have suggested that contamination with T cells may account for the HIV genomes detected in CD34-sorted samples in our study (Durand et al. 2012), or that HIV^+ $CD34^+$

cells may not persist in patients who have had undetectable viral loads for many years of HAART (Josefsson et al. 2012). While we found that CD34-depleted samples in our cohort harbored very little HIV DNA, making contaminating CD34-depleted cells unlikely to serve as a source of genomes in the CD34-sorted samples, we did not directly assess whether T cells were present in the CD34-sorted samples and whether these cells could contribute to the HIV genomes observed. In chapter 4, we directly assess whether contaminating T cells might contribute to the HIV genomes detected in HPC samples and begin to examine whether there may be other differences in the methodology or donor cohorts used in these studies that can account for the discrepancies in our results.

HIV infection and hematological abnormalities. In addition to creating a latent viral reservoir, HIV infection of $CD34^+$ cells might lead to HPC death and hematopoietic abnormalities. Consistent with this possibility, many studies have reported hematopoietic defects associated with HIV infection (Adetifa et al. 2006, Calis et al. 2008, Dikshit et al. 2009, Isgrò et al. 2005, Meira et al. 2005, Mlisana et al. 2008, Moses et al. 1998, Redd et al. 2007b), including the depletion of $CD34^+$ bone marrow cells (Banda et al. 1999, Isgrò et al. 2008). The existence of HIV-associated hematologic abnormalities is well known and has been attributed to a variety of factors, including altered stromal cytokine production (Isgrò et al. 2008) and use of specific antiretrovirals (Carr et al. 2000). Recently, however, Redd and colleagues were able to show a direct association between infection of $CD34^+$ cells and anemia in their cohort (Redd et al. 2007a). This evidence suggests that in addition to creating a latent reservoir of virus, HIV-1 infection of HPCs can cause HPC death that leads to defects in hematopoiesis.

A model of HIV infection in multipotent HPCs. Based on the studies described above, we have developed the following model describing HIV infection of HPCs in vivo (**Figure 1.8**). When multipotent HPCs become infected with HIV, there are two possible outcomes: either an active infection leads to HPC death, contributing to the HIV-associated hematologic abnormalities described above, or a latent infection occurs. If latent infection occurs in a cell with self-renewal capacity, such as a hematopoietic stem cell, continued self-renewal can generate a long-lived reservoir of latent HIV. If a latently infected daughter cell is stimulated to differentiate, however, the latent virus reactivates, leading to the death of the cell and further contributing to hematologic dysfunction. Viral reactivation also results in virion release, contributing to the low-level viremia observed even in HAART-treated patients.

One consequence of this model is that defective HIV-1 proviruses incapable of reactivation should be detectable in multiple hematopoietic lineages that cannot be infected by HIV-1. The presence of such defective proviruses in $CD8^+$ T lymphocytes and granulocytes was reported in one patient (Kaneda et al. 2001); however, a second study detected only minimal integration of HIV genomes in naïve $CD8^+$ cells from patients and could not rule out contamination by other cell types (Brenchley et al. 2004). Additional studies are required to determine whether defective HIV-1 genomes can be observed in $CD8^+$ T cells and other hematopoietic lineages in multiple patients.

Summary

Although HAART therapy dramatically prolongs the life expectancy of HIV-infected people, it is unable to eradicate the virus. The ability of HIV to establish a latent infection has been proposed as the major mechanism for viral persistence in spite of HAART. Resting memory $CD4^+$ T cells are known to harbor latent HIV genomes in optimally treated individuals (Finzi et al. 1997, Finzi et al. 1999); however, there is substantial evidence suggesting that these cells are not the sole or even primary source of residual plasma virus during HAART treatment or rebounding virus upon cessation of therapy (Bailey et al. 2006, Brennan et al. 2009). Thus, it has been proposed that an additional reservoir of virus must exist.

We have recently proposed that bone marrow hematopoietic progenitor cells may serve as an additional reservoir of latent HIV. As described above, we have shown that $CD34^+$ HPCs can be actively and latently infected by multiple HIV isolates both in vitro and in vivo (Carter et al. 2010). Based on these findings, we have proposed a model of latent HIV infection in HPCs in which latent virus is maintained and expanded through the self-renewal of immature hematopoietic progenitor cells, but can be reactivated upon progenitor cell differentiation. These reactivation events would result in viral release, potentially contributing to the residual viremia observed during HAART, as well as to the death of the infected progenitor cell, potentially contributing to the hematopoietic abnormalities observed in HIV-infected patients.

To substantiate the model of HIV infection in HPCs described above, further study is required to understand whether long-lived HPCs can serve as a latent reservoir for virus. Although we have observed that HIV genomes persist in $CD34^+$ HPCs during

months of treatment, it remains to be seen whether the lifespan of these cells is sufficient to serve as a barrier to HIV eradication in vivo. To address this point, it is important to assess whether the most long-lived HPCs, hematopoietic stem cells (HSCs), are susceptible to infection by HIV. This point will be addressed in Chapter 2. Furthermore, although we have shown that HIV can infect multipotent HPCs, it is not known whether these cells can be latently infected or whether they support only active infection. This point will be addressed in Chapter 3. Finally, it is important to determine whether a more immature subset of HPCs, those expressing CD133 in addition to CD34, harbor HIV genomes in HIV^+ individuals with undetectable viral loads and whether these cells persist in patients who have had undetectable viral loads for more than 5 years. These findings would demonstrate the potential of HPCs to serve as a long-term reservoir of latent virus in vivo. Chapter 4 addresses these questions.

A better understanding of the viral and cellular factors involved in the establishment of latent infection in HPCs would help us to understand whether all HIV-infected individuals are likely to harbor latent infection in HPCs and how we might be able to reverse this latent infection to eliminate the reservoir of virus in these cells. The impact of HIV-1 envelope tropism on the infection of different subsets of HPCs will be addressed in Chapter 2, while Chapter 3 will examine the cellular factors that promote latent infection in HPCs and strategies through which latent infection in these cells can be reactivated. Together, these studies further our understanding of the potential of HPCs to serve as a latent reservoir of virus and bring us closer to discovering how latent reservoirs of virus can be eliminated to cure HIV.

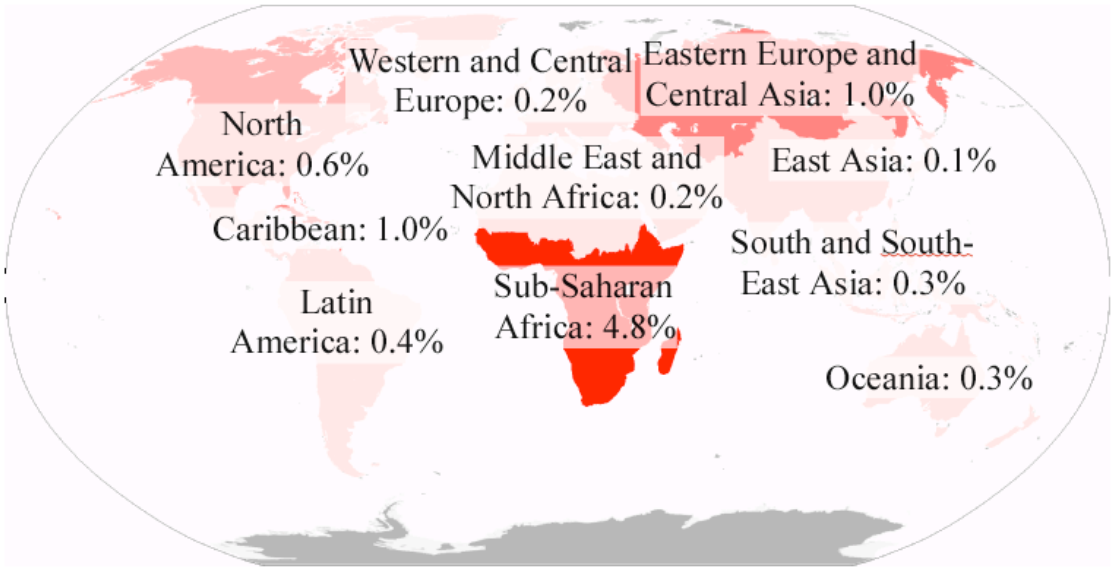

Figure 1.1. Prevalence of HIV in different regions of the world. Prevalence is expressed as the percentage of adults ages 15-49 who are infected with HIV.

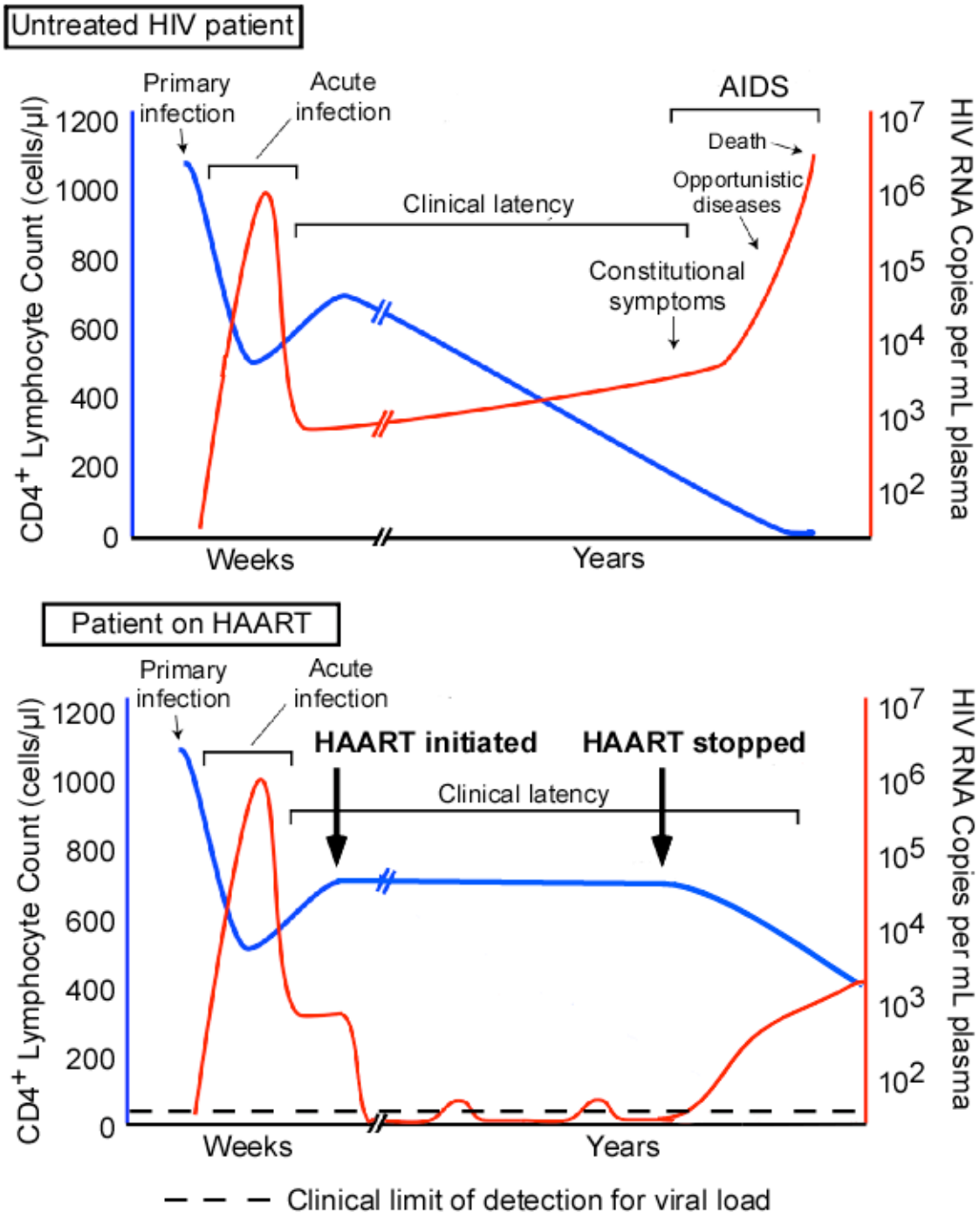

Figure 1.2. HIV disease progression. The course of HIV infection in terms of $CD4^+$ T cell counts and HIV plasma viral loads in an untreated patient (upper panel) or a patient on highly active antiretroviral therapy (HAART) (lower panel). The clinical limit of detection for viral load is approximately 50 copies per mL of plasma.

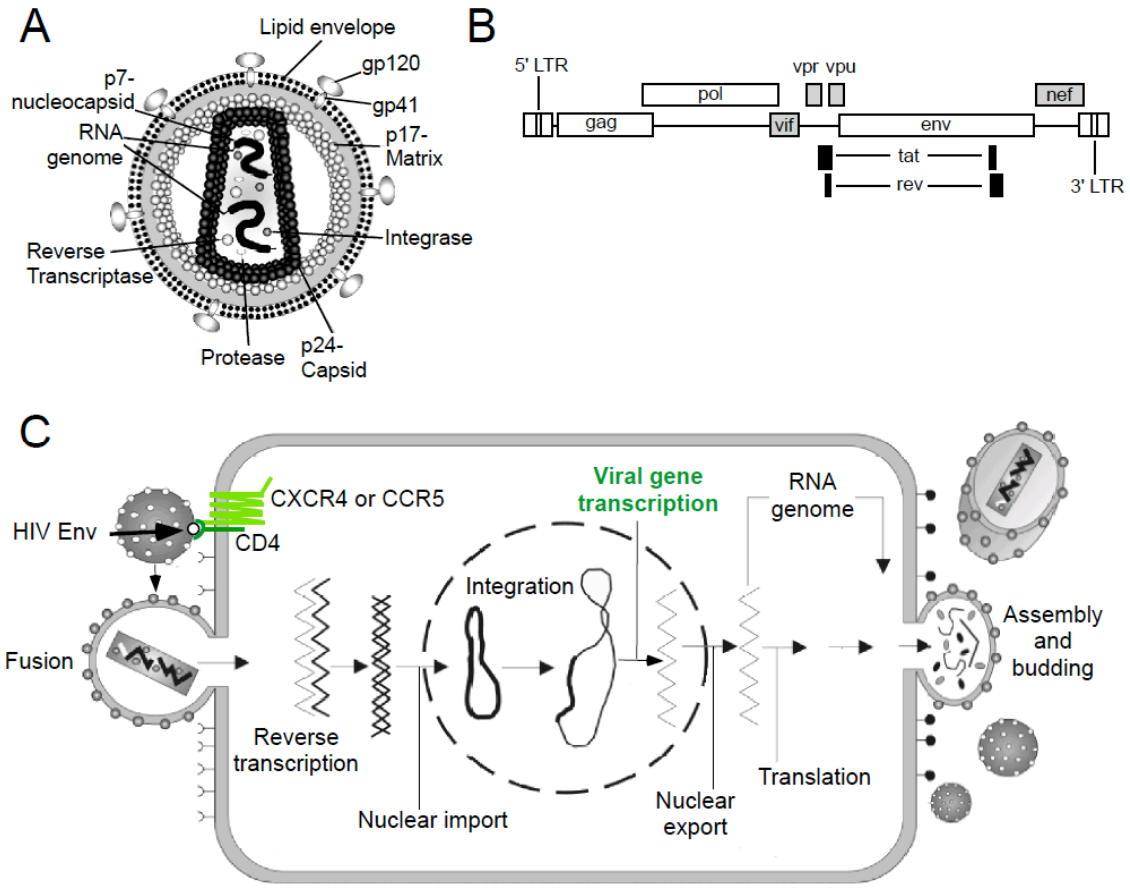

Figure 1.3. HIV structure, genome organization, and life cycle. **A**. Structure of the mature viral particle. **B**. Genome organization. The LTR and structural proteins are in white, regulatory proteins in black, and accessory proteins in grey. **C**. Generalized HIV-1 life cycle. Binding of the HIV virion to the cellular receptors CD4 and CXCR4/CCR5 as well as viral gene transcription are highlighted in green as they will be focused on in chapters 2 and 3, respectively. Parts **A** and **C** were modified from the original by Daniel Beyer under the Creative Commons Attribution-Share Alike 3.0 Unported license (original file available at: http://commons.wikimedia.org/wiki/File:HIV_gross.png; license information available at: http://creativecommons.org/licenses/by-sa/3.0/deed.en).

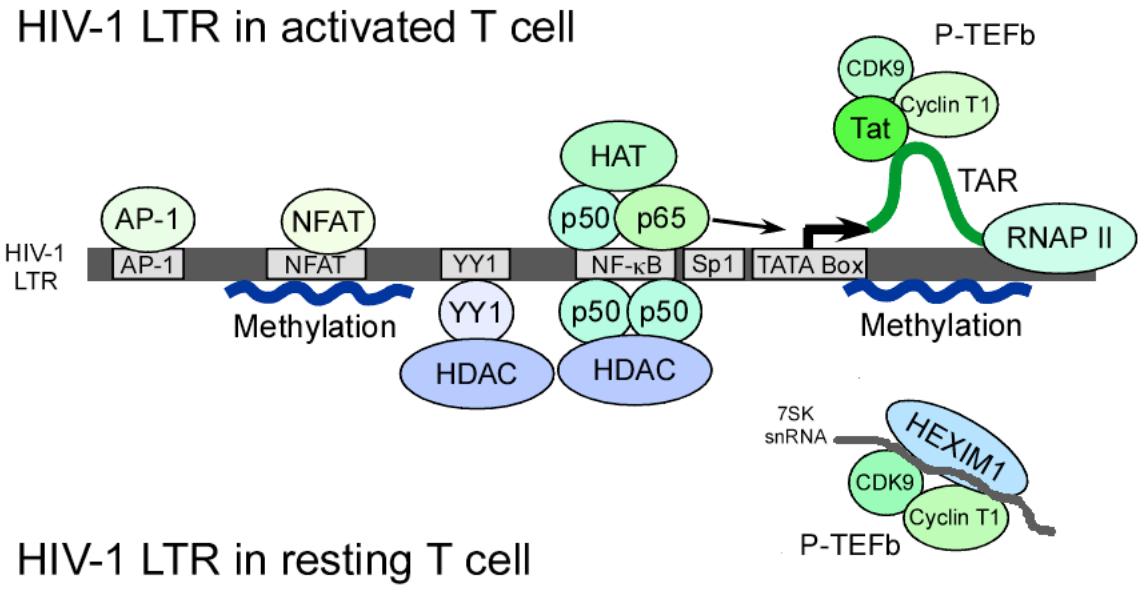

Figure 1.4. Factors associated with the HIV-1 LTR in activated vs. resting $CD4^+$ T cells. Factors associated with the LTR in activated T cells, which support high levels of HIV gene expression, are shown above the HIV-1 LTR while factors associated with the LTR in resting T cells, which support latent HIV infection and repress HIV transcription, are shown below the HIV-1 LTR. In activated T cells, the NF-κB p50/p65 heterodimer recruits histone acetyltransferases (HAT) and promotes gene expression; meanwhile, HIV-1 Tat recruits positive transcription elongation factor B (P-TEFb) to the transactivation response element (TAR) to promote transcript elongation by RNA polymerase II (RNAP II). Additional transcription factors, including activator protein-1 (AP-1) or nuclear factor of activated T cells (NFAT), may also promote viral gene expression. In resting T cells, the NF-κB p50/p50 homodimer and the transcriptional repressor YY1 recruit histone deacetylases (HDAC) and repress gene expression. P-TEFb is sequestered by HEXIM1 and the 7SK snRNA and HIV-1 Tat is absent. DNA methylation as well as histone methylation (not shown) also contribute to the repressed transcriptional state.

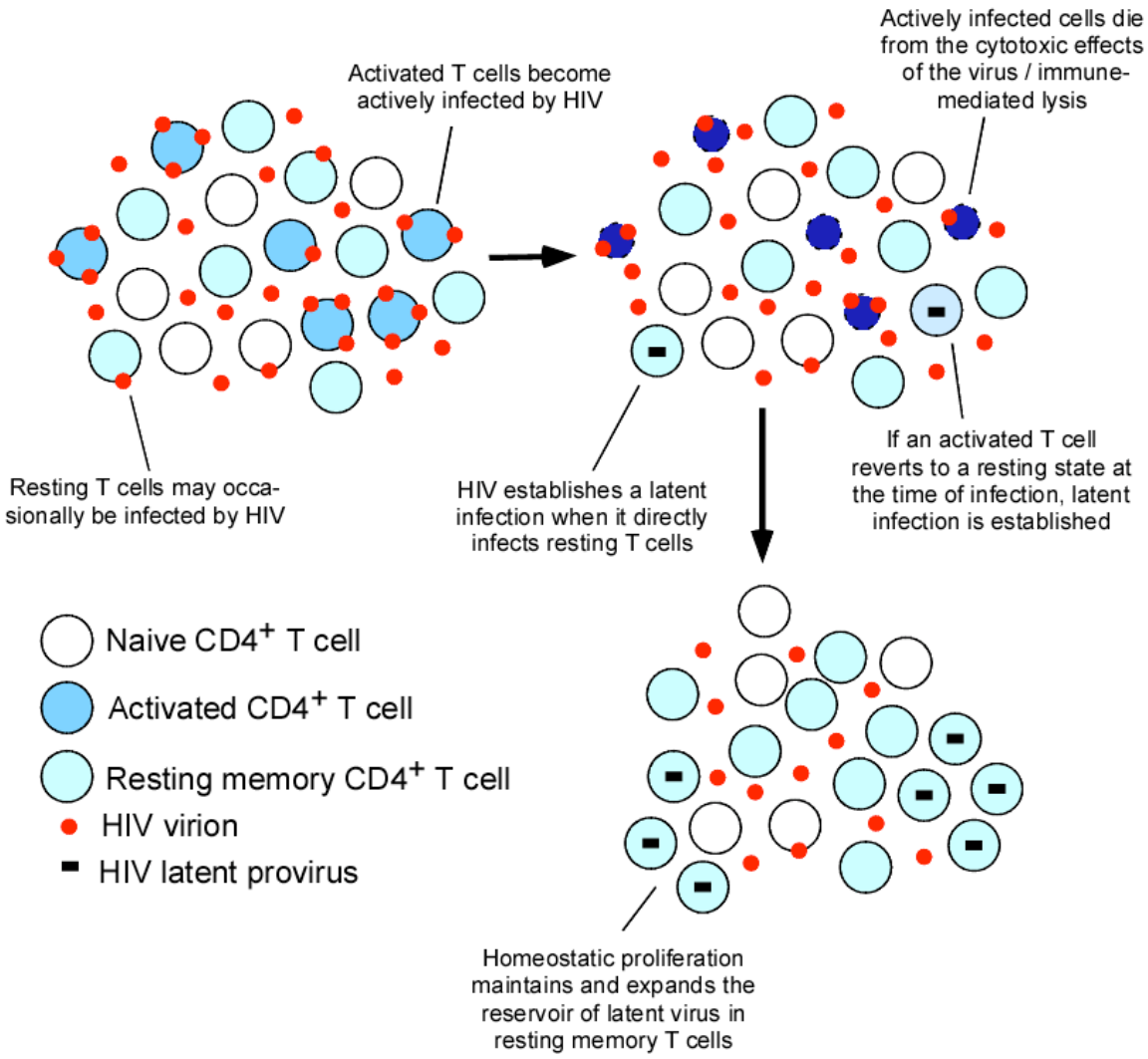

Figure 1.5. Model for the establishment of latency in resting memory T cells. Activated T cells that become infected with HIV may occasionally revert to a resting state prior to viral cytotoxicity, resulting in viral latency instead of cell death. Direct infection of resting T cells may also generate latently infected cells. Homeostatic proliferation of the latently infected cells maintains and expands this reservoir of latent virus.

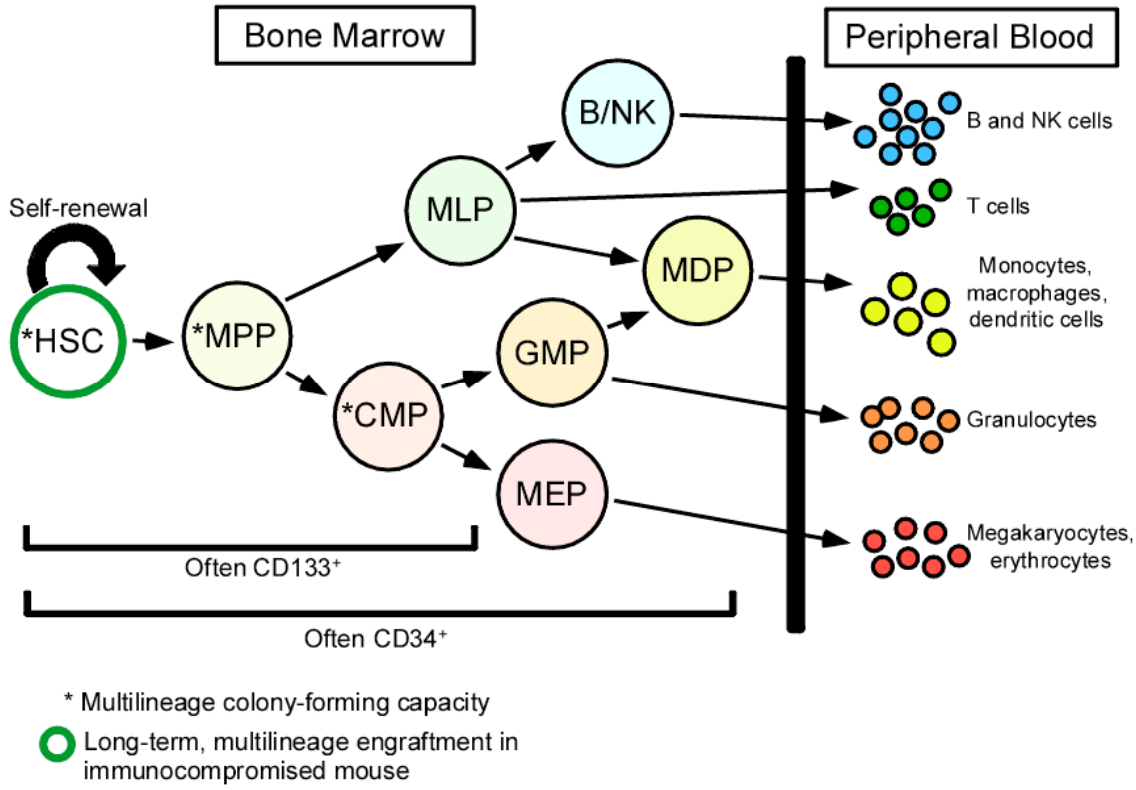

Figure 1.6. Hematopoiesis. Hematopoietic stem cells (HSC) with extensive self-renewal capacity differentiate into multipotent progenitor cells (MPP). MPPs then differentiate into either multilymphoid progenitors (MLP) or common myeloid progenitors (CMP). MLPs further differentiate into B/NK cell precursors (B/NK) or monocyte/dendritic cell precursors (MDP) or into committed T cell progenitor cells. Meanwhile, CMPs differentiate into megakaryocyte/erythrocyte progenitors (MEP) or granulocyte/monocyte progenitors (GMP), the latter of which can also differentiate into MDPs or into committed granulocyte progenitor cells. These progenitors then give rise to a series of more committed progenitor cells and eventually to the mature blood cell lineages in the peripheral blood.

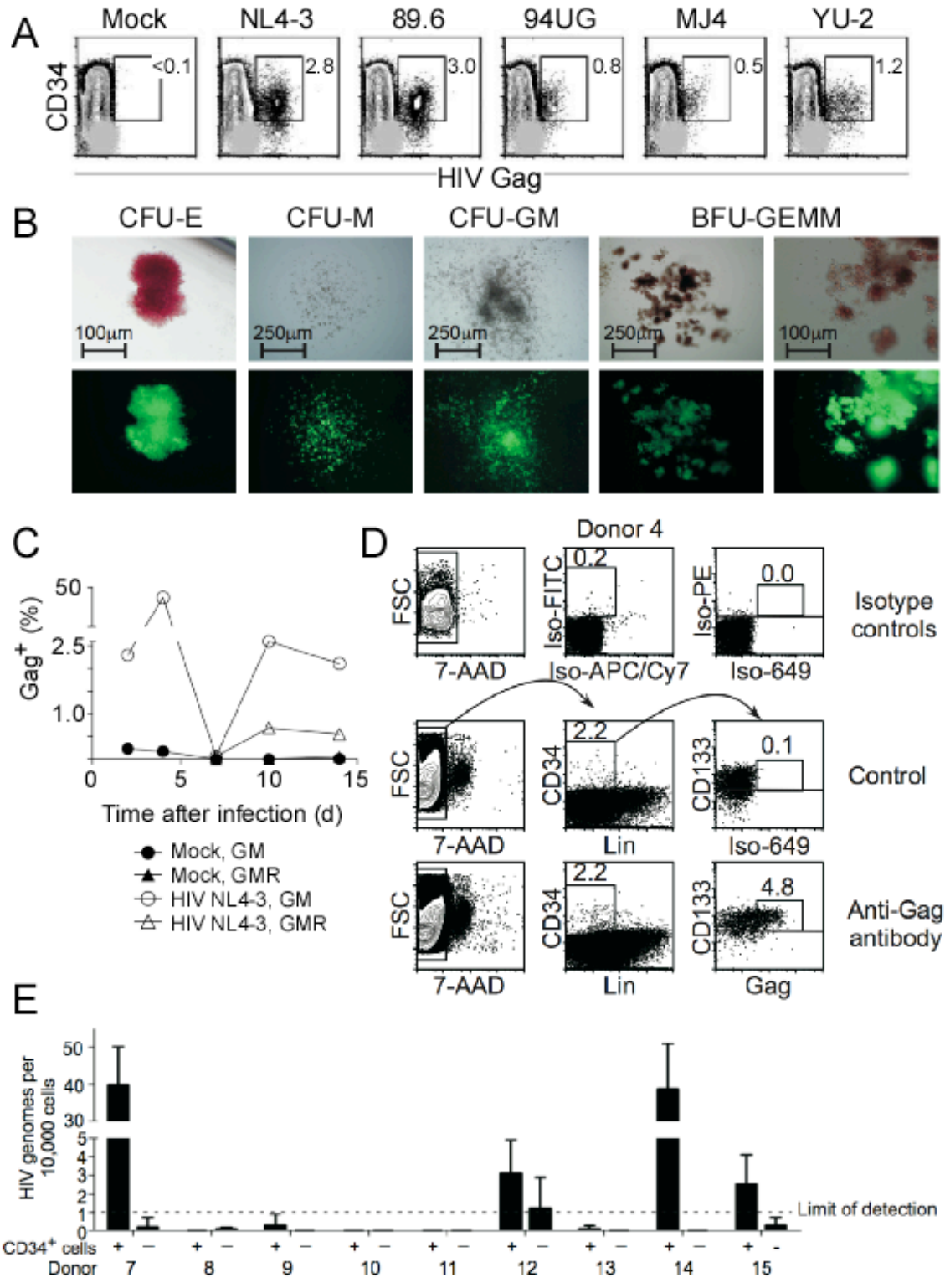

Figure 1.7. Summary of recent findings on HIV infection in HPCs. **A**. Infection of HPCs by HIV molecular clones. Umbilical cord blood (UCB)-derived $CD34^+$ HPCs were infected with NL4-3, 89.6, 94UG114.1, MJ4, or YU-2. 48 hours post-infection, cells were analyzed for CD34 and intracellular Gag expression by flow cytometry.

Numbers represent the percentage of $CD34^+$ Gag^+ cells. The shaded overlays represent mock-treated $CD34^+$ cells stained with an isotype-matched control antibody. **B**. HIV infects multipotent HPCs. $CD34^+$ UCB were infected with a highly deleted, GFP-expressing HIV construct (HIV-7SF-GFP) pseudotyped with the dual tropic 89.6 HIV envelope. Three days after infection, GFP^+ HPCs were sorted by FACS and plated in methylcellulose agar at single-cell density. Colonies were photographed 14-18 days later. CFU-E, colony forming unit-erythroid; CFU-M, colony-forming unit-macrophage; CFU-GM, colony forming unit-granulocyte/macrophage; BFU-GEMM, burst forming unit-granulocyte, erythrocyte, megakaryocyte, macrophage (multilineage colony). **C**. Latent HIV infection can be established in HPCs in vitro. $CD34^+$ UCB were infected with HIV-1 NL4-3 (0 d), then cultured in a cytokine cocktail to maintain the cells in an immature state (CC110). Gag expression in infected cells was evaluated beginning 48 hours post-infection. Cells were stained with the dead cell exclusion reagent 7-AAD, permeabilized, stained with an antibody to Gag, and analyzed by flow cytometry. When less than 0.1% of live, initially $CD34^+$ cells were Gag^+ (d7), the cells were stimulated with GM-CSF and TNF-α in the presence (GMR) or absence (GM) of the integrase inhibitor raltegravir. Gag expression in all conditions was evaluated 3 and 7 days after stimulation. **D**. Active HIV infection occurs in vivo. Flow cytometric analysis of HIV-1 Gag expression in freshly isolated adherence-depleted $Lin^-CD34^+CD133^+$ BMMCs. The middle plots show background staining with an isotype control for the Gag-specific antibody (Anti-Gag) only. Numbers are the percentage of gated cells falling within the indicated region. Lin, lineage markers (cocktail of markers for mature blood cell lineages). **E**. HIV DNA is found in $CD34^+$ HPCs from donors with clinically undetectable viral loads. Shown is the real-time PCR analysis of HIV genomes from donor $CD34^+$ or immunodepleted cells. The limit of detection was approximately one HIV genome per 10,000 cells. Means +/- s.d., n = two independent experiments with three replicates each. **A-E**, Modified from Carter et al. 2010.

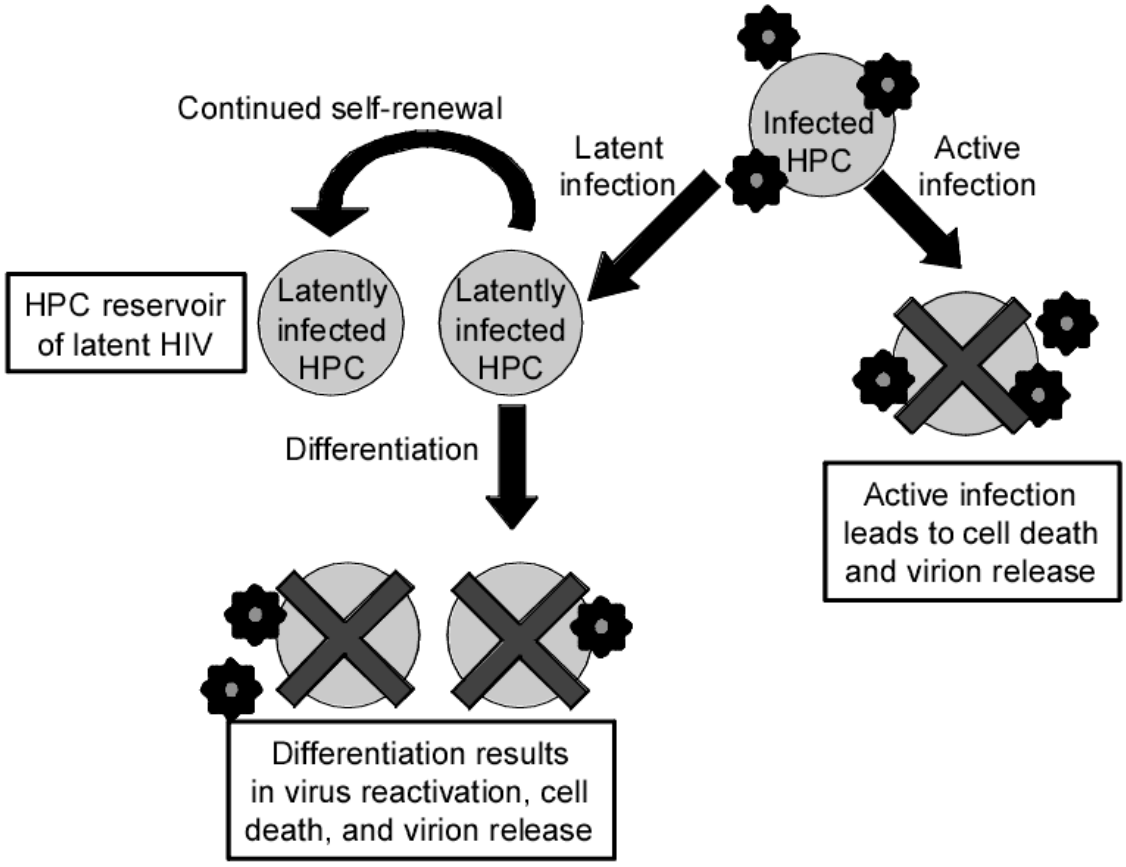

Figure 1.8. Model of HIV-1 infection in hematopoietic progenitor cells. Multipotent hematopoietic progenitor cells (HPCs) can become infected with HIV-1, leading either to active infection, cell death, and virion release, or to latent infection. Latently infected HPCs with self-renewal capacity will then continue to self-renew, generating an expanded reservoir of latent HIV-1 in these cells. If the host cell is stimulated to differentiate, the latent virus reactivates, leading to cell death and virion release. Reproduced from Collins and McNamara 2011.

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Chapter 2

HIV-1 Utilizes the CXCR4 Chemokine Receptor to Infect Multipotent Hematopoietic Stem and Progenitor Cells[4]

Summary

HIV infection is characterized by gradual immune system collapse and hematopoietic dysfunction. We recently showed that HIV enters multipotent hematopoietic progenitor cells and establishes both active cytotoxic and latent infections that can be reactivated by myeloid differentiation. However, whether these multipotent progenitors include long-lived hematopoietic stem cells (HSCs) that could establish viral reservoirs for the life of the infected person remains unknown. Here we provide direct evidence that HIV targets long-lived HSCs and show that infected HSCs yield stable, multilineage engraftment in a xenograft model. Furthermore, we establish that the capacity to use the chemokine receptor CXCR4 for entry determines whether a virus will enter multipotent versus differentiated progenitor cells. Because HSCs live for the lifespan of the infected person and are crucial for hematopoietic health, these data may

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explain the poor prognosis associated with CXCR4-tropic HIV infection and suggest HSCs as long-lived cellular reservoirs of latent HIV.

Introduction

The natural course of HIV disease is characterized by progressive destruction of the host immune system, manifested as a 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 triggering the progression to AIDS remain unclear.

HIV entry into permissive cells is mediated by interactions of the HIV envelope (Env) protein with CD4 and a chemokine coreceptor (CCR5 or CXCR4 (Alkhatib et al. 1996, Deng et al. 1996, Dragic et al. 1996, Feng et al. 1996)). Initial transmission is mediated primarily by CCR5-utilizing (R5-tropic) HIV (Lathey et al. 1999, van't Wout et al. 1994) and R5-tropic isolates are more commonly detected early in disease (reviewed in Margolis and Shattock 2006), but eventually, X4-tropic isolates predominate in most infected individuals (Richman and Bozzette 1994, Shankarappa et al. 1999). The conversion of HIV Env from R5-tropic to X4-tropic requires only a small number of changes in the Env V3 region. This conversion has been associated with more rapid disease progression manifested as reduced $CD4^+$ T cell counts and a poor clinical prognosis (Connor et al. 1997, Daar et al. 2007, Karlsson et al. 1994, Scarlatti et al. 1997, Schuitemaker et al. 1992, Shepherd et al. 2008, Waters et al. 2008, Weiser et al. 2008, Yu et al. 1998, Zhou et al. 2008). Furthermore, in the rare instances when infection is

initiated by dual or X4-tropic HIV, CD4 counts decline rapidly and disease progression is sometimes accelerated (Sheppard et al. 2002, Yu et al. 1998). It is not clear whether the conversion to CXCR4-tropic virus plays a causal role in disease progression or whether other factors account for this association.

$CD4^+$ T cells, myeloid cells and subsets of HSPCs express HIV receptors (CD4 (Morrison and Weissman 1994) and CCR5 or CXCR4 (Carter et al. 2010, Ishii et al. 1999, Majka et al. 1999, Peled et al. 1999, Shen et al. 1999, Viardot et al. 1998), but whether HSPCs can be infected has been controversial in the literature (Folks et al. 1988, Redd et al. 2007, Shen et al. 1999, Stanley et al. 1992, Zhang et al. 2007) and there is evidence that these cells may be relatively resistant to infection (Shen et al. 1999, Zhang et al. 2007). Recent reports indicate that low-level infection of multi-potent HSPCs occurs *in vivo* and *in vitro* (Carter et al. 2010, Redd et al. 2007) but active infection is cytotoxic and hard to detect in long term culture (Carter et al. 2010). Importantly, the assays used in these studies could not distinguish whether infected cells were long-lived hematopoietic stem cells (HSCs) or short lived common myeloid progenitor cells. Thus, it is still unknown whether HIV infects HSCs, a subset of HSPCs defined by their ability to stably engraft and generate multiple lineages upon transplantation into immunocompromised mice. The distinction between HSCs and other multipotent hematopoietic progenitor cells (HPCs) is of key importance, as infection of the long-lived HSC population would have a greater impact on hematopoiesis and this population would have greater potential to serve as a long-term reservoir of HIV in infected people.

In this study, we provide evidence that HIV Envs can target HSCs and that integration can occur within these cells. Moreover, we show that HIV Env tropism

influences which subset(s) of HSPCs are infected: only CXCR4-tropic envelopes permit entry into multipotent HSPCs, including HSCs. These findings suggest not only that HSCs can become infected by HIV and thus have the potential to serve as a long-term reservoir of virus, but also that the association between the emergence of CXCR4-tropic isolates and declining $CD4^+$ T cell counts could be related to infection of multipotent HSPCs.

Results

Recent work has indicated that bone marrow $CD34^+$ HSPCs from HIV^+ donors are targets of HIV infection *in vivo* (Carter et al. 2010). In this study, three of six donors with high viral loads had evidence of active HIV infection of bone marrow $CD34^+$ cells. In the remaining three donors, active infection could be induced by culturing the cells in GMCSF and TNFα (Carter et al. 2010). However, $CD34^+$ cells are a heterogeneous population and it is not known whether stem cells or multipotent progenitor cells are infected in HIV^+ people. Healthy stem cells and multipotent progenitor cells are needed to maintain all hematopoietic lineages as well as normal bone marrow cellularity. Thus, it is expected that infection of primitive HSPCs by HIV would eventually be reflected in a loss of total bone marrow mononuclear cells. To examine this, we quantified the bone marrow cellularity of high viral load (>50,000 copies/ml) donors (Carter et al. 2010) who had relatively normal complete white blood cell counts (**Figure 2.1a**). Interestingly, we found a striking correlation between the number of mononuclear cells isolated from 10 ml of aspirate and the year of diagnosis (**Figure 2.1b**). This correlation was more significant in our cohort ($p<0.03$) than the correlation between CD4 cell count and year

of diagnosis (p=0.12, **Figure 2.1c**) and was significant even when an outlier with a very high cell count was excluded (p<0.02, **Figure 2.1d).** A second cohort of patients with undetectable (<48 copies/ml) viral loads on highly active antiviral therapy (HAART) was also studied (**Figure 2.1a)**. In this group, HIV genomes could be detected within $CD34^+$ cells from 40% of donors but Gag expression was only detectable after culturing the cells in GMCSF and TNFα, consistent with latent infection of this cell type (Carter et al. 2010). In this group, we observed no correlation between bone marrow cellularity and year of diagnosis (**Figure 2.1e**) or between CD4 cell count and year of diagnosis (**Figure 2.1f**). These data provide *in vivo* support for a potent effect of HIV on the bone marrow that requires active viral replication. While this effect may be due to the chronic inflammation associated with unsuppressed HIV replication, it is also consistent with direct infection of multipotent HSPCs by HIV.

X4-tropic Envs infect multipotent HSPCs. The conversion of HIV Env from R5-tropic to X4-tropic is associated with more rapid disease progression manifested as reduced $CD4^+$ T cell counts and a poor clinical prognosis (Connor et al. 1997, Daar et al. 2007, Karlsson et al. 1994, Scarlatti et al. 1997, Schuitemaker et al. 1992, Shepherd et al. 2008, Waters et al. 2008, Weiser et al. 2008, Yu et al. 1998, Zhou et al. 2008). To determine whether chemokine receptor expression influences the type of HSPC infected by HIV, we tested a panel of HIVs differing only in the Env they contained. These virus particles were generated by co-transfecting a GFP-expressing minimal HIV construct (HIV-7SF-GFP, **Figure 2.2a** (Yam et al. 2002)), which expresses GFP but no HIV proteins, along with a packaging-null/*env*-null HIV genome and an HIV *env*-expressing plasmid (Carter et al.

2010). The resulting virus particles thus contain unmodified HIV proteins, including Env, integrase, and reverse transcriptase. Once the HIV genome is integrated into the genome of the target cell, however, new HIV proteins are not transcribed and instead GFP is expressed from the constitutively active SFFV promoter. These viral supernatants were used to infect HSPCs isolated by magnetic sorting. After infection, the cell surface phenotype of GFP-positive cells was determined by flow cytometry.

Remarkably, we found that both the X4-tropic Env HXB and the R5X4-tropic Env 89.6 were able to target various cell types, including cells with a surface phenotype consistent with multipotent HSPCs ($CD34^{High}CD133^{+}$) (**Figure 2.2b-c**). Infection occurred both when the cells were infected by spin infection and when cells were pulsed with virus for 2h at 37°C (**Figure 2.3**). In contrast, the R5 tropic Envs YU-2 (**Figure 2.2b, lower panel)** and ZM53M.PB12 (**Figure 2.2c, lower panel**) infected $CD34^{High}CD133^{+}$ cells inefficiently; most infection was seen in cells with a surface phenotype consistent with less primitive cells ($CD34^{Low}CD133^{-}$). Similar results were observed in ten replicate experiments using three different CCR5-tropic Envs and sixteen replicates using three different CXCR4- or dual-tropic Envs (summarized in **Fig 2.2d**).

We performed additional experiments using full length HIVs encoding CXCR4-tropic Env (NL4-3) or CCR5-tropic Env (94UG114.1.6), except that in this case we detected infection by expression of intracellular Gag. As shown in **Figure 2.2e**, we again found that CXCR4-tropic NL4-3 was able to infect cells with a surface phenotype consistent with HSPCs ($CD34^{High}CD133^{+}$). In contrast, CCR5-tropic 94UG114.1.6 only infected cells with a surface phenotype consistent with less primitive cells ($CD34^{Low}CD133^{-}$) (**Figure 2.2e**). Collectively, these findings indicate that wild type X4-

and R5-tropic HIVs target distinct subsets of HSPCs and that X4-tropic viruses have an increased capacity to target cells with surface markers characteristic of multipotent HSPCs.

*X4-tropic HIVs infect multipotent HSPC***.** To test whether X4-tropic HIVs were capable of infecting multipotent HSPCs, we asked whether the targeted cells formed multilineage colonies in culture. For these experiments, cord-blood derived HSPCs were infected with the minimal HIV construct HIV-7SF-GFP pseudotyped with HXB Env (X4-tropic) or YU-2 Env (R5-tropic) (**Figure 2.4a**). The use of this construct was critical for these experiments because wild type HIV kills actively infected HSPCs within a few days (Carter et al. 2010), making it difficult to determine the developmental capacity of the targeted cell type by colony formation as this assay takes weeks.

Three days after infection with HIV-7SF-GFP, the GFP^+ cells were purified (>95% pure, **Figure 2.4b**), and 6000 GFP^+ cells from each infection were plated in methylcellulose medium. After two weeks, colonies were analyzed for morphology and GFP expression. We found 140 erythroid (CFU-E), 158 myeloid (CFU-GM), and 30 multilineage (CFU-GEMM) colonies generated from cells infected with HXB enveloped virus (**Figure 2.4c-d**). CFU-GEMM colonies form only from hematopoietic stem cells (HSCs), multipotent progenitor cells and common myeloid progenitors, indicating that the HXB envelope permitted entry into one of these immature HSPC types. In contrast, HSPCs infected with YU-2 enveloped virus gave rise to about ten-fold fewer colonies, primarily small granulocyte/macrophage colonies (Total of 14 CFU-E, 23 CFU-GM, and 3 CFU-GEMM; **Figure 2.4c-d**).

To determine whether the capacity to infect multipotent HSPCs was consistently associated with chemokine receptor use, we analyzed additional Envs (**Figure 2.4d**). In each case, we found X4-tropism or dual-tropism (NL4-3 and 92HT593) associated with infection of cells able to generate multilineage colonies. In contrast, cells infected using R5-tropic Env (BaL and 94UG114.1.6) largely lacked this capacity; we observed only 2 CFU-GEMM colonies generated from cells infected with HIV-7SF-GFP pseudotyped with BaL Env and none from cells infected using 94UG114.1.6 Env (**Figure 2.4d-f**). These findings demonstrate that X4-tropic Envs have the capacity to infect multipotent HSPCs, whereas R5-tropic Envs primarily infect more mature HSPCs.

CD4 and CXCR4 receptor use is required for infection of primitive HSPCs. We hypothesized that infection of primitive HSPCs with X4-tropic Envs occurred by the canonical mechanism, wherein HIV Env triggers membrane fusion after binding CD4 and CXCR4 on the target cell. While this is the most common scenario, numerous reports have documented HIV infection by CD4-independent mechanisms, usually involving the use of CXCR4 alone to facilitate entry (Endres et al. 1996, Hoxie et al. 1998, Liu et al. 2004, Saha et al. 2005, Zerhouni et al. 2004). To explore this possibility, we treated cord-blood derived HSPCs and CEM T cells with the CD4-blocking antibody L3T4 before infecting the cells with HIV-7SF-GFP pseudotyped with the dual tropic 89.6 Env. As expected, pre-treatment with CD4-blocking antibody substantially reduced infection of CEM T cells (**Figure 2.5a**). Pretreatment of HSPCs with CD4-blocking antibody inhibited infection even more robustly, causing a near-complete block in infection (**Figure 2.5a**). These data demonstrate that infection of HSPCs is strictly dependent on

CD4. Moreover, CD4 antibody treatment blocked infection of all cells tested, those with a surface phenotype consistent with primitive HSPCs ($CD34^{High}CD133^{+}$) as well as more mature progenitors, suggesting that infection of all HSPCs is CD4-dependent (**Figure 2.5b**).

As described above, infection of multipotent HSPCs by various HIVs correlates with their capacity to use CXCR4 for entry (**Figure 2.4**). These data likely reflect the expression pattern of chemokine receptors on multipotent HSPCs (i.e. that multipotent HSPCs express CXCR4 more widely than CCR5) (Carter et al. 2010). A less likely possibility was that some multipotent HSPCs express both chemokine receptors but that the X4-tropic Env is required for another function other than entry, or that signaling through CXCR4 is required for productive infection. To distinguish these possibilities, we asked whether blockade of CXCR4 would reduce infection of multipotent HSPCs by dual-tropic HIV Envs. We tested this approach using two related T cell lines: CEM-SS, which expresses only CXCR4, and CEM-R5, which expresses both CXCR4 and CCR5. These cells were treated with the small molecule CXCR4 antagonist AMD3100 (Donzella et al. 1998) and infected with HIV-7SF-GFP pseudotyped with the dual-tropic Env 92HT593. As expected, AMD3100 blocked infection of CEM-SS cells almost completely but only partially blocked infection of CEM-R5 cells, demonstrating use of CCR5 by 92HT593 Env in these cells (**Figure 2.5c**).

We then treated cord blood-derived HSPCs with AMD3100 or maraviroc (CCR5-blocking) and infected the cells with HIV-7SF-GFP pseudotyped with 89.6 Env. We found that not only did AMD3100 reduce the rate of infection in total HSPCs, it nearly eliminated infection of $CD34^{High}/CD133^{+}$ cells (**Figure 2.5d**). By contrast, maraviroc did

not impede infection of $CD34^{High}/CD133^{+}$ cells by 89.6-pseudotyped HIV-7SF-GFP (**Figure 2.5d**) but did eliminate infection of HSPCs when we used the R5-tropic Env YU-2 (**Figure 2.5e**). We further observed that when we infected HSPCs with HIV-7SF-GFP pseudotyped with 92HT593 Env, treatment with AMD3100 reduced overall infection rates in $CD34^{High}CD133^{+}$ cells to rates comparable to those observed with the R5-tropic 94UG Env (**Figure 2.5f**). Consistent with these results, we observed that $CD34^{High}CD133^{+}$ HSPCs express CXCR4 at higher levels than CCR5 (**Figure 2.5g-h**). We confirmed the low levels of CCR5 on these cells functionally with a calcium flux assay to assess response to CXCR4 and CCR5 ligands (**Figure 2.6a-b**) and with an acid wash to confirm that bound ligand was not masking CCR5 on these cells (**Figure 2.6c**). Finally, to assess whether crosslinking of CXCR4 could permit entry by CCR5-tropic virus, we infected $CD133^{+}$ HSPCs with HIV-7SF-GFP pseudotyped with YU2 Env in the presence or absence of full-length NL4-3. We then examined GFP expression in the infected populations to determine which cell types were infected by the R5-tropic virus and found that NL4-3 did not permit the CCR5-tropic virus to enter $CD133^{+}$ cells (**Figure 2.6d**).

Next, we treated cord-blood derived HSPCs with AMD3100 or maraviroc and infected with HIV-7SF-GFP pseudotyped with dual-tropic 89.6 Env. We sorted GFP^{+} cells from AMD3100-treated, maraviroc-treated, and control-treated cultures and plated the cells in methylcellulose medium. Infected control-treated and maraviroc-treated cells formed numerous erythroid, myeloid and multilineage colonies (**Figure 2.7a**), whereas infection of AMD3100-treated colony-forming cells was dramatically reduced (**Figure 2.7b**). Treatment with maraviroc (**Figure 2.7c**) or AMD3100 (**Figure 2.7d**) had no effect

on colony formation by uninfected cells. These data show that CXCR4 usage is necessary for the infection of multipotent HSPCs by HIV and that CCR5 cannot substitute.

X4-tropic HIVs infect HSCs capable of multilineage reconstitution of immunocompromised mice. Having demonstrated that HIV can infect multipotent HSPCs, we next asked whether HIV could infect hematopoietic stem cells (HSCs) capable of stably engrafting irradiated NOD/SCID IL-2Rγ^{null} mice. Stable multilineage reconstitution in immunocompromised mice can be accomplished only by engraftment of hematopoietic stem cells, and thus this is a definitive assay for infection of HSCs (Christensen and Weissman 2001, Jones et al. 1990, Osawa et al. 1996, Uchida and Weissman 1992). Because we observed little infection of multipotent HSPCs with R5-tropic HIV Envs, we used only X4-tropic Env for these experiments. We infected purified CD133^{+} cells with replication defective, minimal HIV (HIV-7SF-GFP) pseudotyped with X4-tropic HXB Env (**Figure 2.8a, upper panel**). Three days after infection, GFP^{+} cells were enriched to 40-70% purity (**Figure 2.8a, lower panel**) and intrafemorally injected into sublethally irradiated NOD/SCID IL-2Rγ^{null} mice. In all, 13 animals were injected with infected CD133^{+} cells and 4 were injected with mock-infected, unsorted HSPCs. We used a population of mixed GFP^{+} and GFP^{-} cells for two reasons: first, to minimize loss of infected cells that would occur with more stringent purification; and second, to enable us to distinguish between mice that specifically failed to engraft infected (GFP^{+}) cells and those that failed to engraft at all due to technical error.

Because the HIV genome we used was not cytotoxic and expressed GFP from a constitutively active promoter, we were able to detect infected, mature peripheral blood cells that were the progeny of the originally infected HSPCs. The use of this construct thus enabled us to evaluate the developmental potential of all infected HSPCs, whereas we have previously shown that when HSPCs are infected with replication-competent HIV, the actively infected cells die rapidly (Carter et al. 2010).

Beginning 4 weeks after transplantation and continuing monthly for 20 weeks after transplantation, peripheral blood was collected from the mice and analyzed for GFP expression by flow cytometry. Over time, we detected human cells ($HuCD45^{+}MuCD45^{-}$) in the periphery of all four mice that received mock-infected transplants (animals 1-4 in **Table 2.1** and **Figure 2.9** and animals 1-2 in **Figure 2.10).** In addition, we detected human cells in 7 of 13 mice that received infected HPC transplants (animals 5-11 in **Table 2.1** and **Figure 2.10**, animals 5-10 in **Figure 2.8b**, and animal 11 in **Figure 2.9**). Of the mice that engrafted, 71% (5/7) had GFP^{+} cells, indicating successful engraftment of cells infected with an HXB-Env bearing virus (animals 5-9 in **Table 2.1**, **Figure 2.8b**, and **Figure 2.10**). Two of the seven mice engrafted human cells that were all GFP-negative (animals 10 and 11 in **Table 2.1**, **Figure 2.8b, Figure 2.9**, and **Figure 2.10**). An example of one of the six mice that failed to engraft human cells is also shown (animal 12 in **Figure 2.8b**, animals 12-17 in **Table 2.1**). Although animals 5-11 received both GFP^{+} and GFP^{-} human cells, several animals (animals 5-7, 10-11) engrafted only GFP^{+} or only GFP^{-} human cells. This is because only a small fraction of the transplanted cells have the capacity to engraft in the mouse, and these few cells account for all of the human cells

found in the peripheral blood. Because so few cells actually engraft, it is not surprising that in some mice, all of the engrafting cells were GFP^+ or all were GFP^-.

Although the frequency of human cells varied widely in the engrafted mice (0.1-9.6% human leukocytes, **Table 2.1**, **Figure 2.8b** and **Figure 2.9**), all mice that engrafted with infected human cells had GFP^+ lymphoid ($CD3^+$ and/or $CD19^+$) and GFP^+ myeloid ($CD33^+$) cells in the periphery for at least 20 weeks after transplantation. In all cases, the frequency of GFP^+ human cells in the peripheral blood increased after 8-10 weeks post-transplant (**Figure 2.10**). As only HSCs can maintain multilineage reconstitution for more than 4-6 weeks in vivo, the increasing frequency of GFP^+ cells at later time points clearly demonstrates that infected HSCs have engrafted.

For both GFP^+ and GFP^- engraftments, T lymphocytes ($CD3^+$) were slow to appear in peripheral blood, consistent with prior reports that human thymopoiesis is inefficient in NOD/SCID/IL-2Rγ^{null} mice after stem-cell transplantation (Lan et al., 2006). For example, by 20 weeks animal 9 clearly had GFP^+CD3^+ cells, whereas these cells were not apparent at an early time point (compare **Figure 2.8c** 20 week time point for animal 9 with 16 week time point shown in **Figure 2.8b**). Additionally, animal 6 was sacrificed 26 weeks after transplantation and the tissues were examined for T cell chimerism. As shown in **Figure 2.8d**, human $CD3^+$ cells were present in both bone marrow and spleen. Thus, T cells were clearly present in at least 2 of 5 mice that stably engrafted stem cells targeted by HIV.

Discussion

The identification and eradication of long-lived cellular reservoirs is necessary to cure HIV and eliminate the need for lifelong therapy. We have previously demonstrated that HIV can infect multipotent HSPCs, establishing both active and latent infections (Carter et al. 2010). Here we demonstrate that, similar to infection of T cells, infection of multipotent HSPCs depends on CD4. However, based on the panel of Envs we tested, robust infection of primitive HSPCs capable of generating multilineage colonies in soft agar only occurs with CXCR4- or dual-tropic viruses. Blockade of CXCR4 dramatically reduced infection of multipotent hematopoietic cells by dual-tropic HIVs. In contrast, the R5-tropic HIVs we tested had only minimal infectivity in multipotent HSPCs and blockade of CCR5 had no effect on infection of multipotent cells by dual-tropic HIVs.

The simplest explanation for the inability of CCR5-bearing viruses to infect multipotent HSPCs is that CCR5 is not expressed at high enough levels to support infection. An alternative hypothesis is that engagement of R5-tropic Envs with the CCR5 chemokine receptor affects the ability of multipotent HSPC to form colonies or is toxic to the cells. This hypothesis is less likely because dual-tropic HIVs able to bind both CXCR4 and CCR5 can infect cells with a multipotent phenotype. In addition, we have detected minimal CCR5 expression and signaling in response to CCR5 ligands on human $CD34^{+}CD133^{+}$ cells.

HIV infection of multipotent HSPCs could lead to the presence of viral genomes in multiple hematopoietic lineages. However, HIV is primarily detected in myeloid and T cells, but not in B cells. This apparent enigma may be explained by the fact that active infection of HSPCs leads to the upregulation of markers of apoptosis and rapid depletion

of infected cells from the culture (Carter et al. 2010). Thus, the lack of evidence for HIV genomes in B cells in infected people may be due to the fact that active HIV infection kills early HSPCs, preventing the development of infected cells in some lineages (Carter et al. 2010). Latent infection can also occur in HSPCs, but induction of differentiation may induce viral activation and subsequent cell death (Carter et al. 2010).

We have also determined that HIV infects HSCs that are capable of stable, multilineage engraftment in irradiated NOD/SCID IL-2Rγ^{null} mice. All of the mice that were successfully transplanted with infected HSPCs demonstrated multilineage engraftment of infected, GFP^+ human cells. These results have significant implications for viral persistence because HSCs are capable of long-term self-renewal *in vivo*. Thus, latently infected HSCs would persist indefinitely, forming a long-term viral reservoir. Additionally, based on our prior results (Carter et al. 2010), active infection can trigger cell death in multipotent HSPCs. If a sufficient number of HSPCs were infected, the subsequent death of these cells could disrupt the entire hematopoietic cascade. Over many years, this disruption could impact the function of the bone marrow. Unfortunately, the toxicity of wild type HIV precluded us from generating sufficient numbers of infected cells to test whether wild type virus from patient samples infects cells *in vivo* that have the capacity to engraft. The experiments presented here were only possible with the use of replication defective HIV constructs that do not express additional cytotoxic HIV proteins following integration.

The findings in this study appear to conflict with previous reports indicating that HSCs are resistant to infection with HIV-1 as well as with lentiviral constructs pseudotyped with HIV Envs (Shen et al. 1999, Weichold et al. 1998, Zhang et al. 2007).

The mechanism by which HIV-1 infection of HSCs is purported to be blocked has been inconsistent: one study found that the block was solely at the level of entry due to insufficient expression of viral receptors and that VSVG-pseudotyped viral particles could efficiently infect HSCs (Shen et al. 1999), whereas another group found that there was a post-entry block to infection mediated by p21 (Zhang et al. 2007). The apparent difference between our findings and those of previous groups can be explained by low rates of infection (typically less than 2%) that rapidly decline over time because of the cytoxitcity of the virus. Such infection rates are too low to yield detectable infection with many of the non-flow cytometric assays used in previous studies, especially those that require that the cells be cultured for more than a couple days. The use of recently optimized culture conditions for HSPCs (Zhang et al. 2008) has allowed us to increase infection rates in these cells due to improved health of the cells. The previously described blocks to infection likely contribute to the low infection rates that we observe in HSPCs, but importantly, we show that these blocks are not absolute and that X4-tropic HIVs can infect HSCs at a low but significant rate.

In sum, we have shown that multipotent HSPCs and HSCs can be infected by HIV and that this infection is primarily accomplished by CXCR4-tropic HIVs. The infection and destruction of multipotent HSPCs may contribute to the more rapid decline in CD4 counts associated with CXCR4-tropic HIV isolate emergence. Alternatively, as infected HSCs could create an extremely long-lived reservoir of virus, preferential infection of these cells by CXCR4-tropic virus could provide a reservoir for the emergence of CXCR4-tropic isolates late in disease: as other viral reservoirs are depleted, CXCR4-tropic virus from the HSC and HSPC reservoir could begin to predominate. In

addition, our demonstration that HIV can infect cells capable of stably engrafting for months in the xenograft model indicates that HIV can infect HSCs that are capable of self-renewal and, if the integrated viral genome is latent, that it can be maintained and even expanded by cell division.

Based on these data, there should be a renewed focus on primitive hematopoietic progenitors as an important reservoir for HIV that will require eradication to improve the treatment of HIV-infected people. Our data suggesting that a subset of HSCs and other primitive hematopoietic progenitors could function as a latent reservoir for HIV raise the possibility that combining HIV therapies with approaches to activate HSCs might deplete this reservoir by triggering the apoptosis of infected HSCs.

Experimental procedures

*Antibodies and reagents***.** Antibodies to the following proteins were used for flow cytometry: CD34 (FITC-conjugated, BD biosciences), CD34 (APC-conjugated, Caltag), CD34 (647-conjugated, eBioscience), CD133 (PE-conjugated, Miltenyi Biotech), CD133 (biotin-conjugated with streptavidin-APC/Cy7 (eBioscience)), Gag (FITC-conjugated, Coulter), Gag (PE-conjugated, Coulter), CD45 (APC/Cy7-conjugated, BD Biosciences), CD33 (PE-conjugated, BD Biosciences), CD3 (PE/Cy5-conjugated, BD Biosciences), CD19 (APC-conjugated BD Biosciences), CD4 (BD Biosciences), CXCR4 (PE/Cy7–conjugated, eBioscience), CCR5 (PE-conjugated, eBioscience), mouse IgG (FITC-conjugated, Invitrogen). For receptor blocking experiments, functional grade antibody against CD4 was used (clone L3T4, eBioscience).

The *env* gene expression plasmid pcDNA-89.6*env* was created as described (Carter et al. 2010). pcDNA-94UG*env* 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. We created the *env* gene expression plasmid pEBB-NL*env* by digesting pNL4-3 with SalI and NotI and blunting the resulting fragment by Klenow treatment. The resulting fragment was then ligated into pEBB that had been digested with NotI and blunted by Klenow treatment.

*Cell culture***.** We obtained pre-existing umbilical cord blood lacking subject identifiers after scheduled cesarean section procedures. We obtained fresh whole bone marrow aspirates from a commercial source (AllCells Ltd.). We prepared BMMCs and UCB mononuclear cells by Ficoll-Paque density separation (GE Healthcare) according to the manufacturer's instructions. UCB-MNC were frequently cryopreserved in 10% DMSO in FBS. BM-MNCs were always used fresh. We prepared $CD34^+$ or $CD133^+$ cells from adherence-depleted mononuclear cells with commercially available kits (positive selection MACS, Miltenyi Biotech). After isolation we maintained HSPCs in STIF medium (StemSpan or Stemline II medium supplemented with 50ng/ml SCF, 50ng/ml TPO, 100ng/ml IGFBP-2, and 50ng/ml Flt3-L) (Zhang et al. 2008).

We conducted methylcellulose colony-forming assays according to the manufacturer's recommendation (Methocult H4034, StemCell Technologies). 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.

*HIV preparation***.** We prepared infectious supernatants by transfection of proviral plasmids into 293T cells using polyethylenimine. For pseudotyped viruses, we concentrated supernatants with high-molecular-weight polyethylene glycol precipitation (Kohno et al. 2002). Pellets were resuspended in $1/5^{th}$ to $1/10^{th}$ the original volume of StemSpan medium and stored at -80°C. 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. We conducted HIV infections with a standard spin infection technique for primary cells (1048.6 *x g* for two hours at room temperature) or by incubating the cells with virus at 37°C for two hours.

Flow cytometry. We stained cells in FACS buffer (2% FBS, 1% human serum, 2mM HEPES, 0.025% NaN3/PBS) for 10-20 minutes on ice, washed and fixed them in 2% paraformaldehyde/PBS. For intracellular Gag staining, we then incubated the cells for 5 min in 0.1% Triton X-100 in PBS at 25 °C. We incubated washed cells with anti-Gag antibody in FACS buffer for 30 minutes on ice. We analyzed the cells on a FACScan or FACSCanto flow cytometer. We excluded dead cells using 7AAD.

For analysis of murine peripheral blood, we lysed erythrocytes with IO Test 3 lysis buffer (Beckman-Coulter) and we stained leukocytes as described above. We analyzed cells on

a FacsCantoII analyzer. We excluded dead cells from analysis by DAPI uptake and we excluded cell doublets using FSC-A/FSC-H ratio.

We sorted cells with a FACSVantage SE or FACSAria cytometer (Becton Dickinson). For high-purity sorting, we used normal-R mode with a 1.0 sorted drop envelope. For cell enrichment, we sorted cells in enrich mode with a 1.0 sorted drop envelope.

For the calcium flux assay, cells were suspended in cell loading media (RPMI with 2% FBS and 25mM HEPES) and loaded with 1.5μM Indo-1 AM. Cells were incubated at 37°C for 45 minutes, then washed twice and resuspended in FACS buffer. Surface staining was conducted as described above. Following surface stain, cells were resuspended in cell loading media and equilibrated at 37°C for 30-60 minutes prior to analysis. EGTA was added 1 hour prior to analysis. Samples were analyzed using a FACSDiVa cyotmeter (BD). For the acid wash assay, cells were incubated in pH 2.7 glycine buffer for 1 minute, then washed with 9 ml PBS and stained as above except that serum-free buffer (PBS + 0.1% BSA was used).

Mice. Nonobese diabetic severe combined immunodeficiency mice lacking the interleukin-2 gamma receptor (NOD/SCID IL-2R^{null} mice, strain NOD.CB17-Prkdcscid Il2rgtm1Wjl/Szj, (Jackson Laboratory)) were maintained at the University of Michigan by the Unit for Laboratory Animal Medicine. All experiments were conducted in accordance to with research protocols approved by the University Committee on the Use and Care of Animals.

Mouse transplantation. We cultured cord blood-derived $CD133^+$ cells for 4 days in STIF medium to expand HSCs prior to infection. We then infected the cells with HIV-7SF-GFP pseudotyped with HXB2 Env. After three days, we sorted GFP-positive cells, rested them overnight in STIF medium and then transplanted them into sublethally irradiated mice (340cGy). We used a Hamilton syringe fitted with a 27Ga needle to inject 25μL of cells in PBS into the femur. We gave the transplanted mice antibiotic water (1.1gl neomycin and 0.121gl polymyxin B).

Isolation of $CD34^+$ cells from HIV-infected donors. HIV^+ individuals were recruited from the University of Michigan HIV/AIDS Treatment Program Outpatient 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. 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. The bone marrow mononuclear cells were prepared by density separation using Ficoll-Paque (GE healthcare) according to the manufacturer's instructions, and total mononuclear cells were counted.

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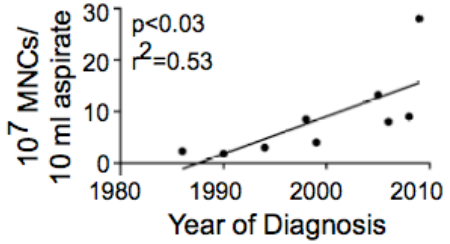
Animal	Number of cells transplanted	Condition	% Human Leukocytes	% of Human Leukocytes that are GFP+	% of GFP+ Human Leukocytes that are:		
					Myeloid	*B cells*	*T cells*
1	50,000	Mock	0.6	0.0	-	-	-
2	50,000	Mock	0.1	0.0	-	-	-
3	100,000	Mock	0.1	0.0	-	-	-
4	150,000	Mock	0.1	0.0	-	-	-
5	50,000	7SF-GFP/HXB	0.1	98	16	78	0.0
6	50,000	7SF-GFP/HXB	1.4	100	4.9	92	0.1*
7	100,000	7SF-GFP/HXB	0.4	99	19	56	0.0
8	150,000	7SF-GFP/HXB	9.6	16	6.9	88	0.0
9	150,000	7SF-GFP/HXB	0.5	46	7.7	77	0.0**
10	100,000	7SF-GFP/HXB	1.4	0.0	-	-	-
11	100,000	7SF-GFP/HXB	0.6	0.0	-	-	-
12-17	150,000	7SF-GFP/HXB (No engraftment)	0.0-0.03	0.0	-	-	-

Table 2.1. Summary of murine xenotransplantation results**.** Animals 1-4 were injected with mock-infected HPCs and successfully engrafted. Animals 5-11 were injected with virus-treated HPCs and were successfully engrafted with human cells. Animals 12-17 were injected with virus-treated HPCs but did not successfully engraft. Percents of human and GFP^+ cells are percents of total peripheral blood leukocytes at 14-18 weeks after transplant. *T cells present at 20 week time point (**Figure 2.8c**). **T cells present in bone marrow (**Figure 2.8d**). See also **Figure 2.10**.

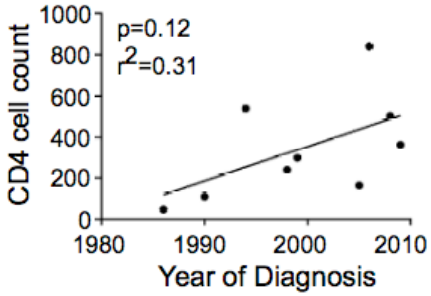
a

Patient Identifier	Year of diagnosis	CD4 count	BMMC counts ($X10^7$/10cc)	WBC ($X10^3/mm^3$)	RBC ($X10^6/mm^3$)	Platelets ($X10^3/mm^3$)
1	2006	840	8.0	5.1	4.41	173
2	1998	239	8.5	4.7	4.52	133
3	2008	504	9.0	4.3	5.38	144
4	1999	298	4.0	4.3	4.24	75
5	2009	364	28.0	7.6	4.85	295
6	2005	164	13.2	5.0	5.31	112
A1	1986	41	2.3	2.0	4.75	103
A2	1990	109	1.8	4.6	5.4	203
A3	1994	540	3.0	7.0	5.48	161
7	2006	705	5.2	7.6	4.93	180
8	1997	292	5.0	9.4	3.89	251
9	2004	263	6.6	6.1	4.64	176
10	2001	533	5.4	4.6	4.96	247
11	1987	447	3.6	4.5	3.56	298
12	2002	537	8.8	7.1	4.15	179
13	1984	612	5.1	5.8	5.19	192
14	2006	647	2.4	4.5	4.63	289
15	2001	912	10.0	5.8	4.59	268
16	1995	282	3.3	7.0	4.52	154

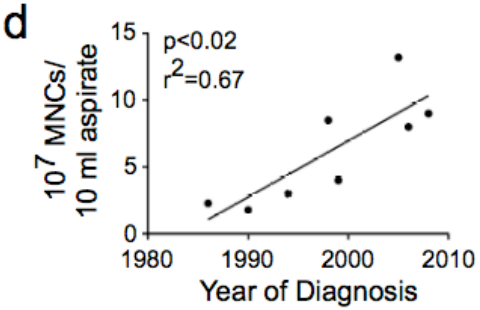
b



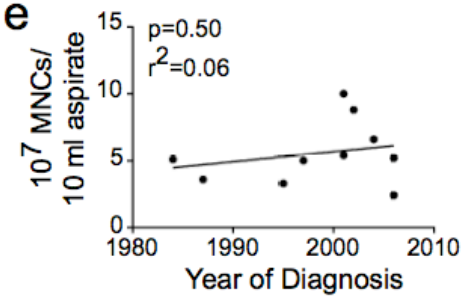
c



d



e



f

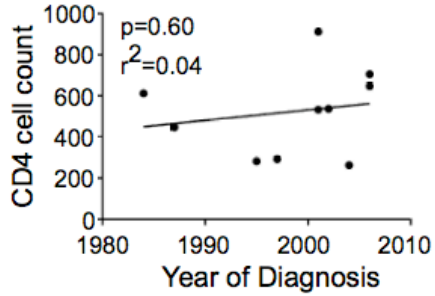


Figure 2.1. Total bone marrow mononuclear cells are correlated with year of diagnosis in HIV^+ donors with high viral loads**. (a)** Complete blood count of HIV^+ donors with high viral loads (1-6 and A1-A3, 61,000 to 202,000 copies/ml) and low viral loads (7-16, <48 copies/ml). **(b)** The number of purified bone marrow cells versus year of diagnosis for high viral load donors. **(c)** CD4 counts of high viral load donors at the time of bone marrow aspiration plotted as a function of year of diagnosis. **(d)** As **(b)**, but with an outlier with a high mononuclear cell yield (Donor 5) removed. **(e)** The number of purified bone marrow cells versus year of diagnosis for low viral load donors. **(f)** CD4 counts of low viral load donors at the time of bone marrow aspiration plotted as a function of year of diagnosis.

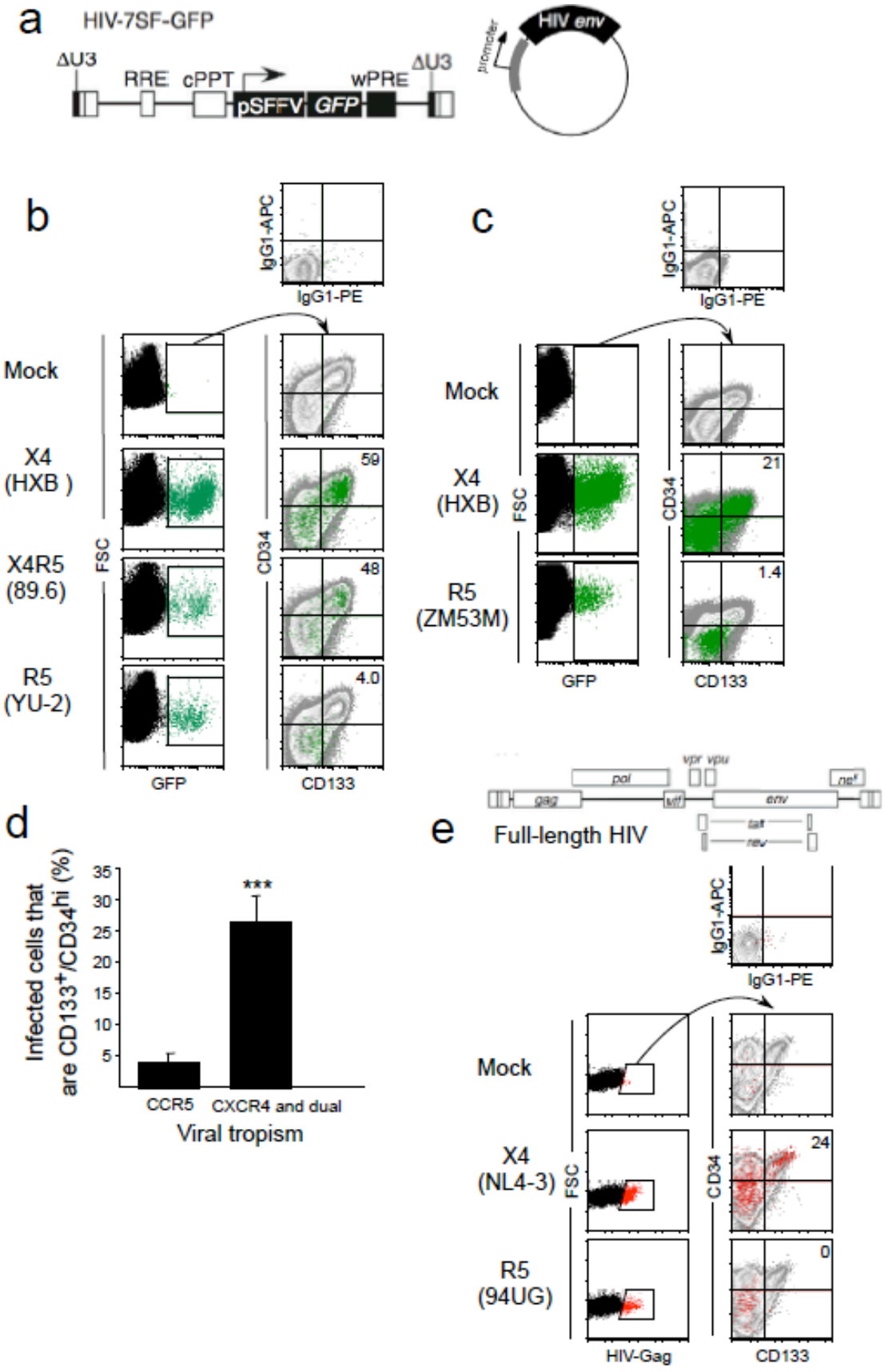

a
HIV-7SF-GFP
ΔU3
RRE
cPPT
pSFFV
GFP
wPRE
ΔU3
promoter
HIV env
b
IgG1-APC
IgG1-PE
Mock
X4
(HXB)
X4R5
(89.6)
R5
(YU-2)
FSC
CD34
59
48
4.0
GFP
CD133
c
IgG1-APC
IgG1-PE
Mock
X4
(HXB)
R5
(ZM53M)
FSC
CD34
21
1.4
GFP
CD133
d
Infected cells that are CD133+/CD34hi (%)
35
30
25
20
15
10
5
***
CCR5
CXCR4 and dual
Viral tropism
e
Full-length HIV
pol
vpr vpu
nef
gag
vif
env
tat
rev
IgG1-APC
IgG1-PE
Mock
X4
(NL4-3)
R5
(94UG)
FSC
CD34
24
0
HIV-Gag
CD133

Figure 2.2. CXCR4-tropic HIV Envs infect $CD133^+$, $CD34^+$ HSPCs**. (a)** Schematic of HIV-7SF-GFP construct and generic HIV envelope plasmid used to construct viruses used in **b-d**. (**b and c)** Flow cytometric analysis of cord-blood derived $CD133^+$ cells infected with a minimal HIV (HIV-7SF-GFP (Yam et al. 2002)) that lacked expression of HIV gene products and was pseudotyped with the indicated HIV Env. In this construct, GFP is expressed from a heterologous promoter (Yam et al. 2002). Cells were analyzed three days post-infection. The left panels show the GFP^+ gating. These events were overlaid in green on the right plots that also show staining of the total cell population in grey. Isotype control staining is shown in the top panel. The percentage of GFP^+ cells that were $CD34^{High}CD133^+$ is shown in the upper right hand corner **(d)**, Summary plot of HIV-1 Env data. Results are compiled from ten experiments with CCR5-tropic Env proteins (94UG114.1.6, 8 replicates; YU-2, one experiment; ZM53M.PB12, one experiment) and sixteen experiments with CXCR4 and dual tropic Env proteins (HXB2, 8 replicates; 89.6, 7 replicates; 92HT593, 1 experiment); error bars are standard error of the mean. ***$p<0.0001$. **(e)** Upper panel, schematic of full-length HIV used in lower panel. Lower panel, flow cytometric analysis of cord-blood derived $CD133^+$ cells three days post infection with full length, wild type HIVs. NL4-3 has an X4-tropic Env and 94UG114.1.6 has an R5-tropic Env. Gag^+ cells were gated on in the left panels, and these events (red dots) were overlaid on CD34 vs. CD133 plots in the right panels to determine the immunophenotype of the infected cells compared with the total cell population (grey dots). Isotype control staining is shown in the top panel. The percentage of Gag^+ cells that were $CD34^+CD133^+$ is shown in the upper right hand corner. See also **Figure 2.3**.

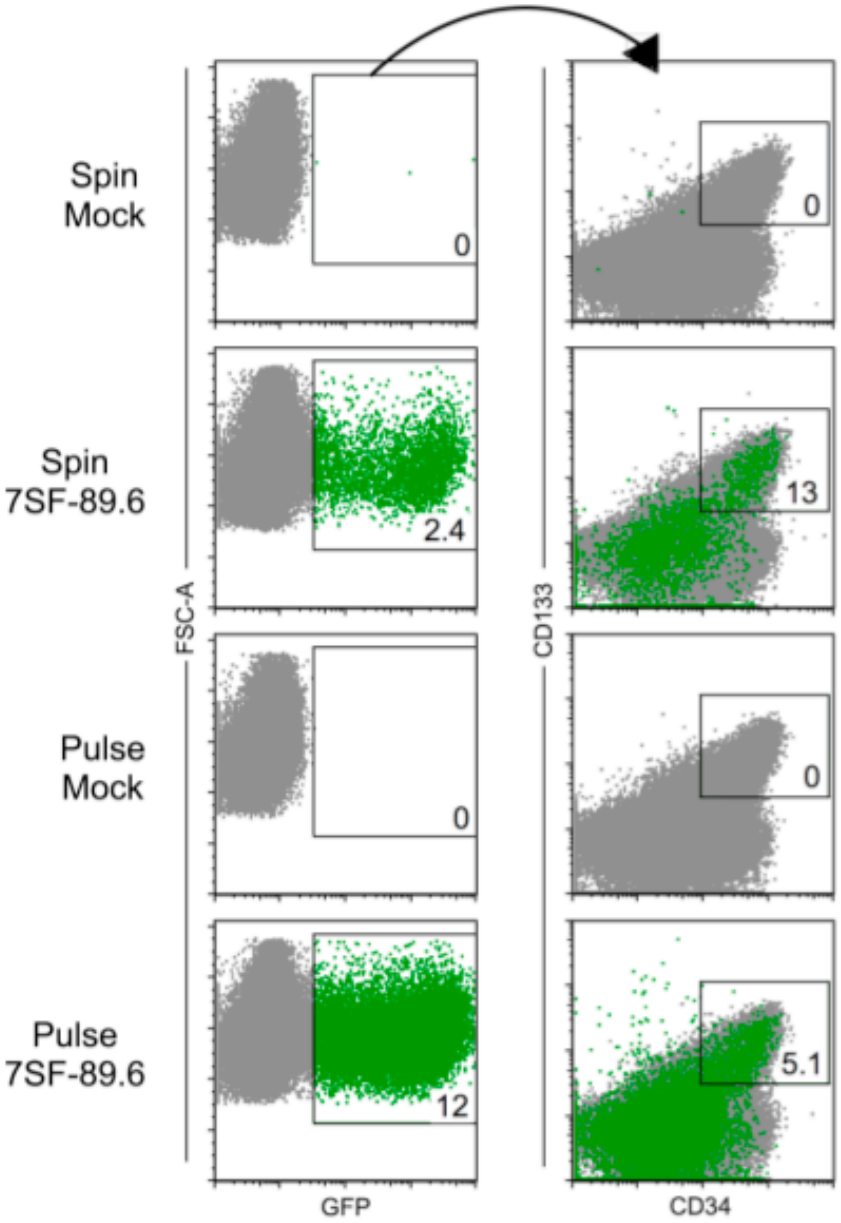
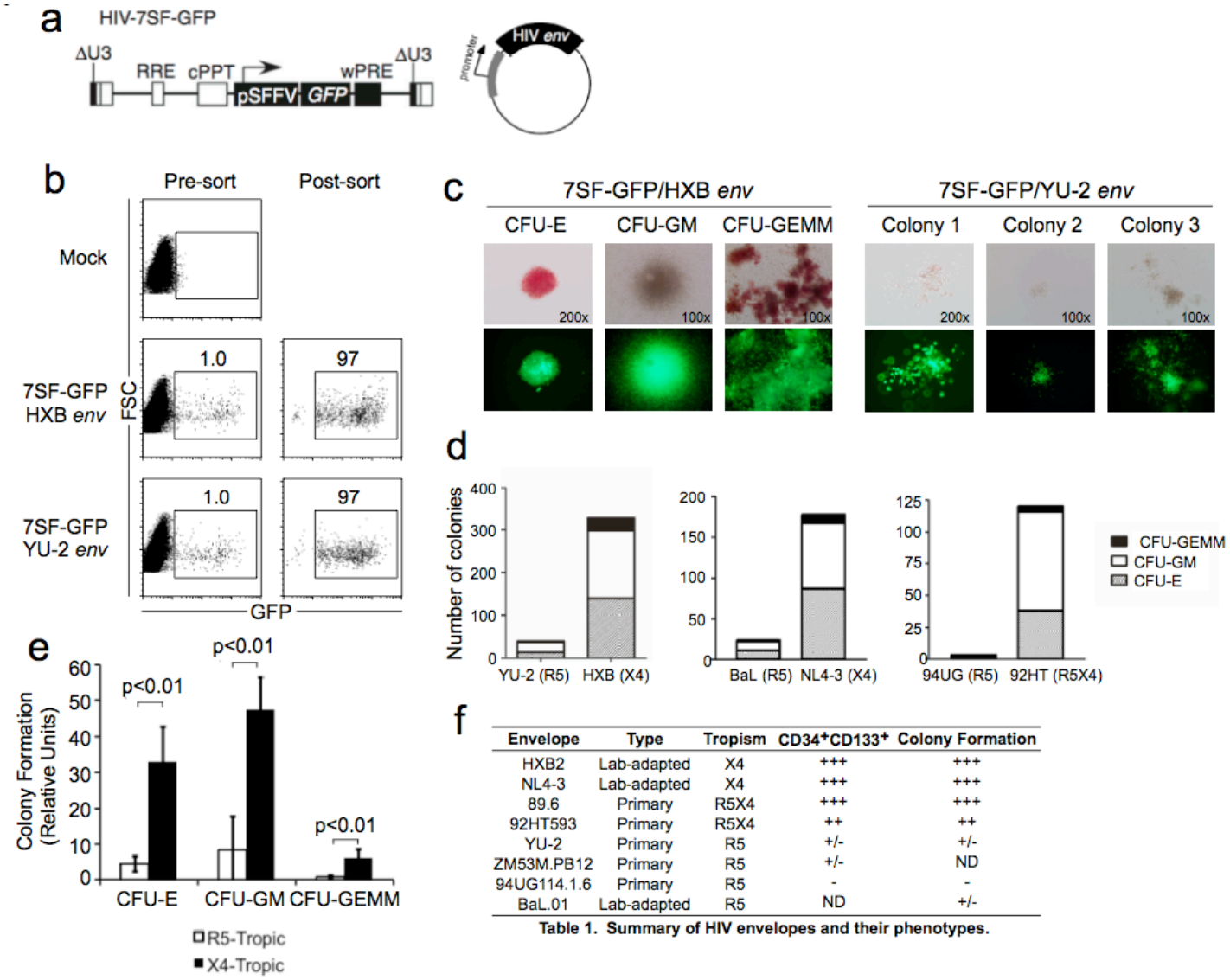


Figure 2.3. HIV can infect CD133^{+}, CD34^{High} cells without spin infection**.** Flow cytometric analysis of UCB-derived HPCs infected with HIV-7SF-GFP pseudotyped with the 89.6 dual-tropic HIV Env either by spin infection in the presence of polybrene for 2 hours at room temperature (Spin) or by incubation with virus for 2 hours at 37° (Pulse). The cells were analyzed three days after infection. GFP^{+} cells are shown on the left; in the right panel, the GFP^{+} cells were overlaid on plots of the total cell population. Numbers indicate the percentage of cells that are infected (left) or percent of infected cells falling within the gate (right).



f

Envelope	Type	Tropism	CD34⁺CD133⁺	Colony Formation
HXB2	Lab-adapted	X4	+++	+++
NL4-3	Lab-adapted	X4	+++	+++
89.6	Primary	R5X4	+++	+++
92HT593	Primary	R5X4	++	++
YU-2	Primary	R5	+/-	+/-
ZM53M.PB12	Primary	R5	+/-	ND
94UG114.1.6	Primary	R5	-	-
BaL.01	Lab-adapted	R5	ND	+/-

Table 1. Summary of HIV envelopes and their phenotypes.

Figure 2.4. CXCR4-tropic HIV Envs infect HSPCs with the capacity to form multilineage colonies. (**a)** Schematic of HIV-7SF-GFP construct and generic HIV envelope plasmid used to construct viruses used in **b-e**. **(b)** Flow cytometric analysis of cord-blood derived $CD133^{+}$ cells infected with HIV-7SF-GFP pseudotyped with HXB (X4-tropic) or YU2 (R5-tropic) Env proteins, and purified by flow sorting. (**c)** Example colonies identified after culturing cells isolated as shown in part **(b)** for 14 days. Phase contrast and epifluorescence microscopy are shown (erythroid, CFU-E; myeloid, CFU-GM; or multilineage, CFU-GEMM). (**d)** Quantification of colony formation for the experiment shown in part (**c)** and for two similar experiments using other HIV Envs as indicated (erythroid, CFU-E; myeloid, CFU-GM; or multilineage, GFU-GEMM). The total number of colonies from 6 replicate wells is displayed. (**e)** Summary of colony formation results. Data were compiled from five independent experiments using six different Env proteins. The average normalized number of colonies observed with CXCR4- or dual-tropic Env versus CCR5-tropic Env is depicted. Error bars represent standard deviation and *p*-values were determined using the two-tailed Student's T test (erythroid, CFU-E; myeloid, CFU-GM; or multilineage, GFU-GEMM). (**f)** Summary table of the ability of HIV-1 Envs of different tropism to infection $CD133^{+}CD34^{High}$ and multipotent HSPCs.

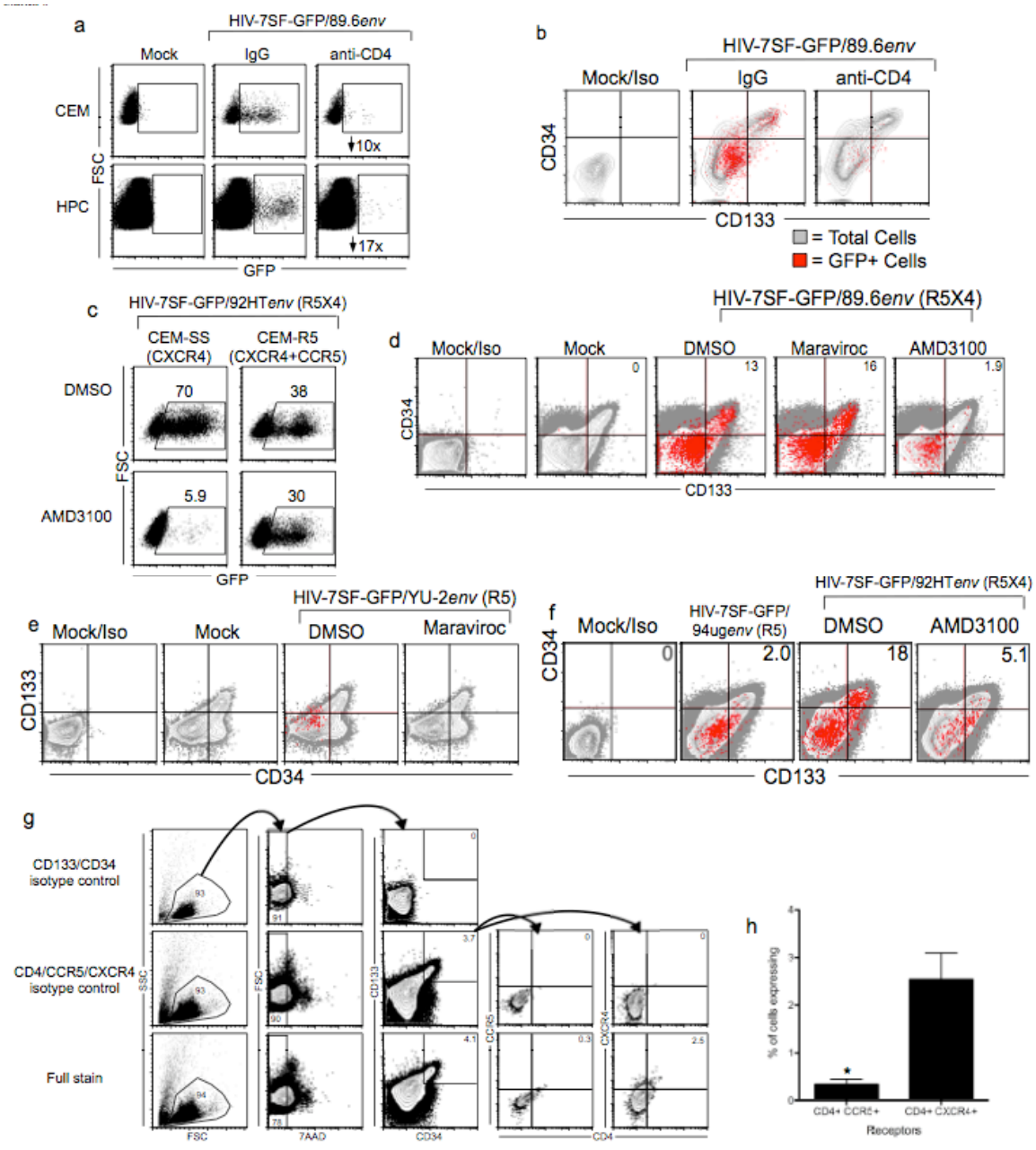


Figure 2.5. Infection of CD34^{High}CD133^{+} HSPCs is dependent on CD4 and CXCR4**. (a)** Flow cytometric analysis of cord blood-derived CD133^{+} HSPCs or CEM T cells pre-incubated with anti-CD4 antibody (L3T4, 20μg/mL) or control antibody and then infected with HIV-7SF-GFP pseudotyped with 89.6 Env. The cells were analyzed three days post-infection. The numbers in the right panels are fold inhibition of infection. **(b)** Flow cytometric analysis of cord blood-derived CD133^{+} HSPCs pre-incubated with control antibody or antibody to CD4 (L3T4, 20μg/mL) and then infected with HIV-7SF-GFP pseudotyped with 89.6 Env. The cells were analyzed three days after infection. GFP^{+} (red) cells are overlaid on the total population (grey). **(c)** Flow cytometric analysis of CEM-SS (CXCR4-expressing) or CEM-R5 (CXCR4 and CCR5 expressing) cells treated with 10μg/mL AMD3100 or an equal volume of DMSO and then infected with

HIV-7SF-GFP pseudotyped with the dual tropic HIV Env 92HT593. The cells were analyzed three days post-infection. The percent GFP^+ cells are shown in the numbers above each gate. Results are representative of two independent experiments. (**d-f)** Flow cytometric analysis of cord-blood derived $CD133^+$ cells infected with HIV-7SF-GFP pseudotyped with the dual-tropic 89.6 (**d)** or 92HT (**f)** HIV Envs or the R5-tropic YU-2 HIV env (**e**) in the presence and absence of 20μM maraviroc (R5-blocking) or 10μg/mL AMD3100 (X4-blocking). The cells were analyzed three days after infection. GFP^+ cells (red) are overlaid on the total population (grey). (**g)** Flow cytometric analysis of $CD133^+$ UCB cells expanded 4-7 days in STIF media and then stained for the indicated cell surface markers. Samples stained with isotype control antibodies for CD133 and CD34 (top) or with antibodies to CD133 and CD34 but isotype control antibodies for CD4, CXCR4, and CCR5 (middle) were included to determine gating. Numbers indicate the percent of cells falling within each gate. (**h)** Summary graph showing percent of total $CD133^+CD34^{High}$ cells that express both of the indicated receptors for three independent experiments; mean and standard deviation are shown. *$p<0.03$, paired *t* test. See also **Figure 2.6**.

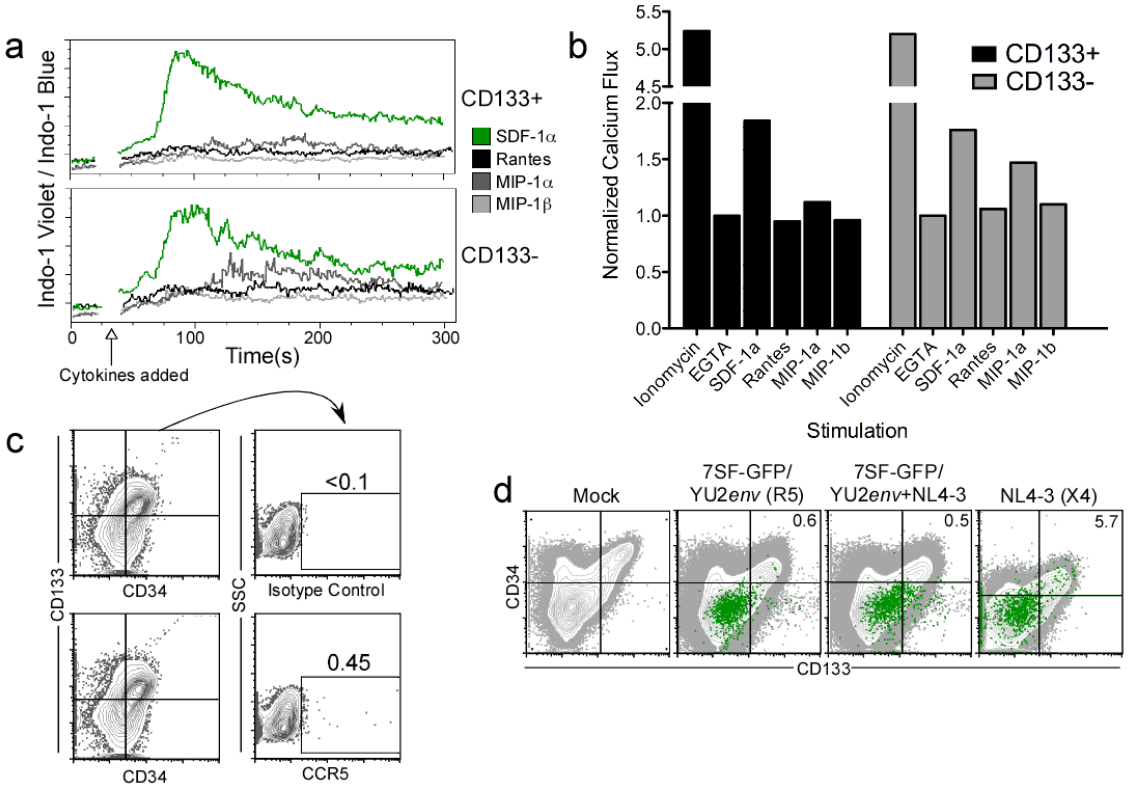


Figure 2.6. CCR5 is absent on CD133^{+}, CD34^{bright} cells. **a.** Flow cytometric analysis of Indo-1 violet (bound to calcium) normalized to Indo-1 blue (free Indo-1) in UCB CD133^{+} cells loaded with Indo-1 AM calcium sensor dye and labeled with CD133-Pe. Cytokines were added at the indicated time, each at 200ng/ml. **b.** Quantification of experiment shown in **a.** The fold increase in the Indo-1 Violet / Indo-1 Blue ratio at the peak of the calcium flux, normalized to EGTA-loaded samples (negative control) is shown. Ionomycin (1μg/ml) is a positive control for maximum calcium flux. **c.** Flow cytometric analysis of UCB CD133^{+} cells treated with acidic glycine buffer (pH 2.7) to remove bound ligand and stained for CD133, CD34, and CCR5 or isotype control in serum free buffer. 7AAD was used to exclude dead cells. CD133 vs. CD34 plots are shown on the left; CCR5 or isotype control staining for the CD133^{+}, CD34^{bright} cells is shown on the right. **d.** Flow cytometric analysis of UCB CD133^{+} cells infected with HIV-7SF-GFP pseudotyped with R5-tropic YU2*env*, full length NL4-3 (X4-tropic), or both viruses simultaneously. Three days after infection, cells were analyzed for CD34, CD133, GFP, or HIV Gag (far right panel only). Plots show the total cell population with GFP^{+} cells (left 3 panels) or Gag^{+} cells (far right panel) overlaid. The results show no increase in entry of CCR5-tropic virus with CXCR4-tropic co-infection.

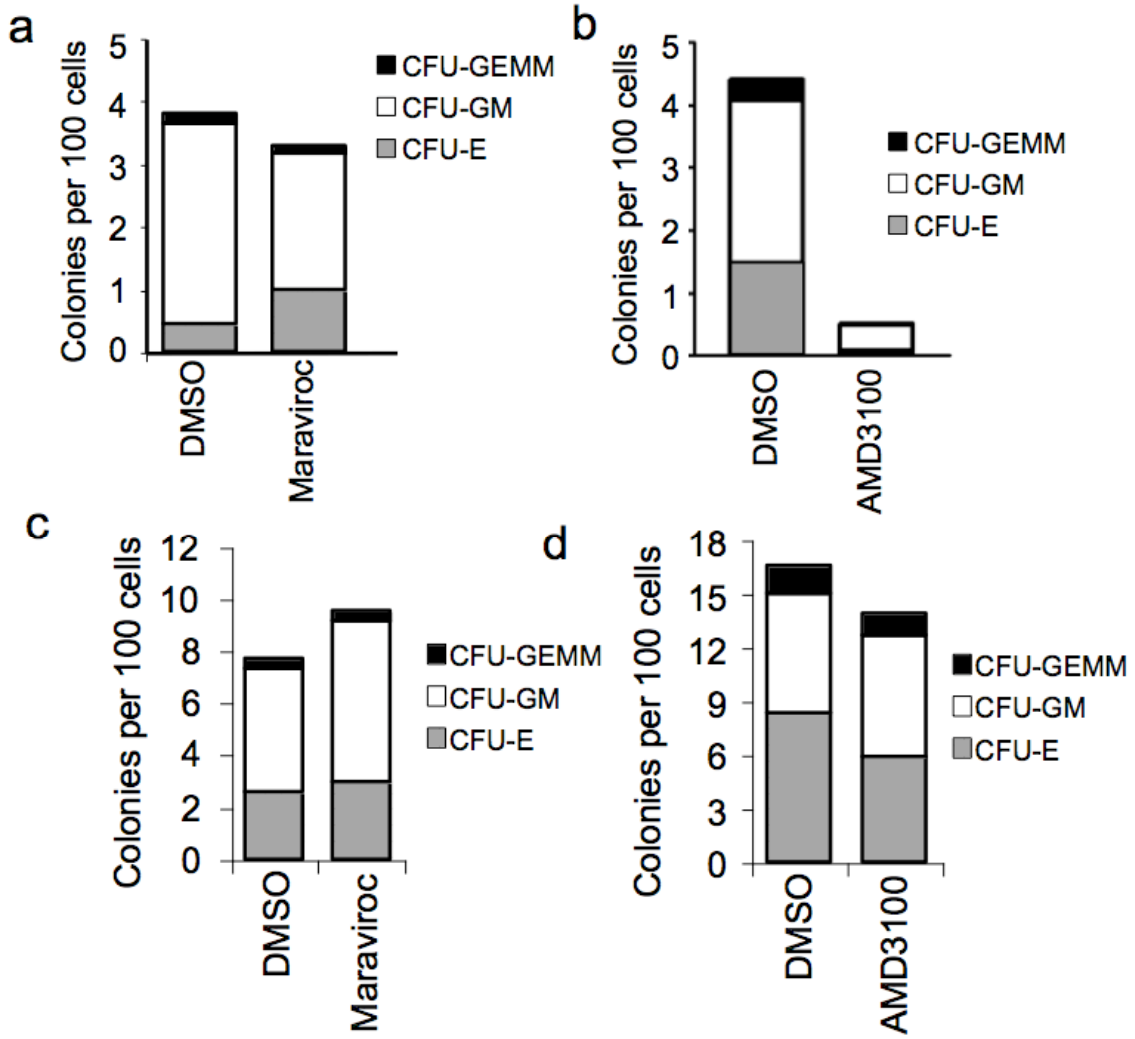


Figure 2.7. Infection of multipotent cells requires CXCR4**.** (**a and b)** Colony formation by cord blood-derived HSPCs treated with 20μM maraviroc (**a**) 10μg/mL AMD3100 (**b**) or an equal volume of DMSO, infected with HIV-7SF-GFP pseudotyped with the dual tropic HIV Env 89.6 and purified by FACS. Two blinded counters analyzed the colonies 14 to 18 days after plating in methylcellulose medium. Data are represented as number of colonies per 100 cells plated. The mean of the two scorers' counts is shown. **(c and d)** Colony formation by $CD133^{+}$ UCB mock-infected in the presence of 20μM maraviroc **(c)** or 10μg/mL AMD3100 **(d)** or an equal volume of DMSO and plated in methylcellulose three days after exposure. Colonies were scored after two weeks by two blinded counters. The mean of the two scorers' counts is shown. (Erythroid, CFU-E; myeloid, CFU-GM; or multilineage, CFU-GEMM.)

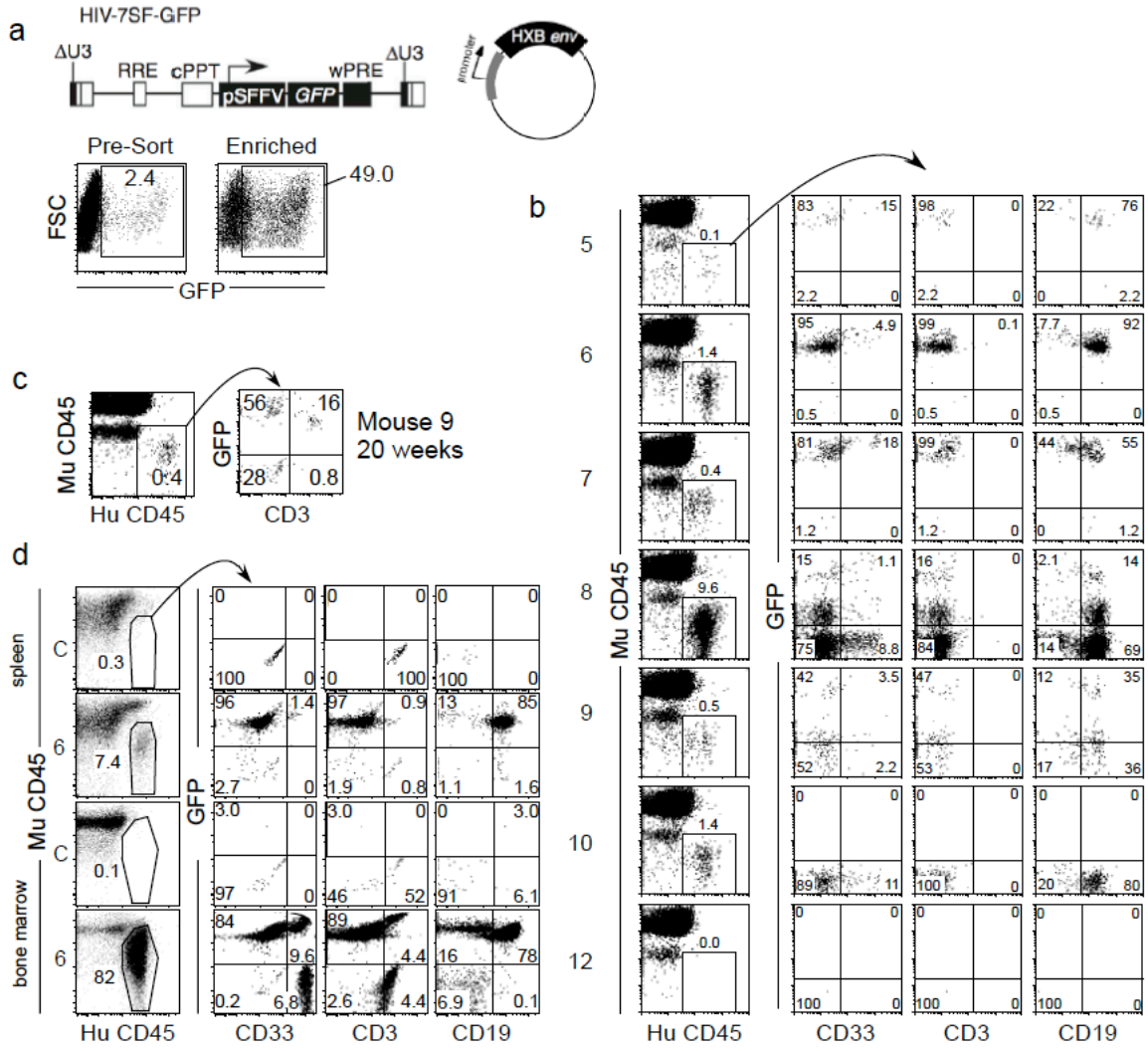


Figure 2.8. CXCR4-tropic HIV can infect HSCs that stably engraft and generate multiple lineages in NOD/SCID IL2γ^{null} mice. (**a)** Upper panel, schematic of HIV-7SF-GFP and HXB envelope plasmid used to construct virus used in lower panel. Lower panel, flow cytometric analysis of cord blood derived CD133^{+} HPCs infected with HIV-7SF-GFP pseudotyped with HXB Env after three days in culture. Cells in the right panel were sorted for GFP positivity. (**b)** Flow cytometric analysis of peripheral blood from transplanted mice 16-18 weeks post transplant. Leukocytes that were positive for human CD45 and negative for mouse CD45 were gated in the left panels. The right panels show staining of the indicated markers within this subpopulation. CD33, myeloid; CD3, T cell; CD19, B cell (**c)** Flow cytometric analysis of peripheral blood cells harvested 20 weeks post transplantation in animal 9. (**d)** Flow cytometric analysis of bone marrow and spleen from animal 6 after 26 weeks. (C: untreated control animal). See also **Figure 2.9**.

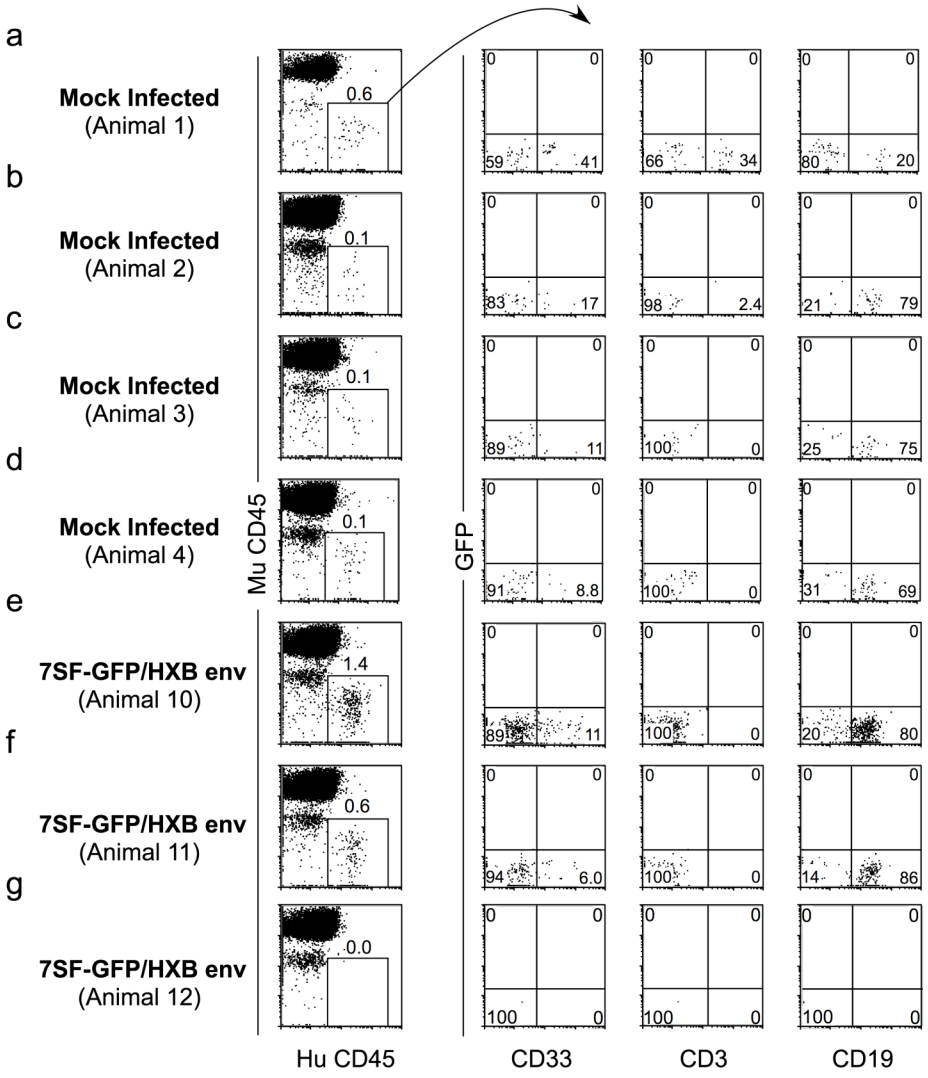


Figure 2.9. Analysis of mice that did not engraft with GFP$^+$ hematopoietic cells. Flow cytometric plots of peripheral blood from NOD/SCID IL2γ^{null} mice 14-18 weeks post-transplant with mock-infected CD133$^+$ cells or CD133$^+$ cells infected with HIV-7SF-GFP pseudotyped with Hxb Env as described in the text. Peripheral blood was analyzed for GFP; mouse CD45; and human CD45, CD33, CD3 and CD19. Human CD45$^+$ mouse CD45$^-$ leukocytes were gated on in the left panels and GFP, CD33, CD3 and CD19 expression were analyzed within this subpopulation. Note that neither the mice transplanted with mock-infected cells nor mice 10, 11, or 12 transplanted with infected cells showed detectable engraftment by GFP$^+$ cells.

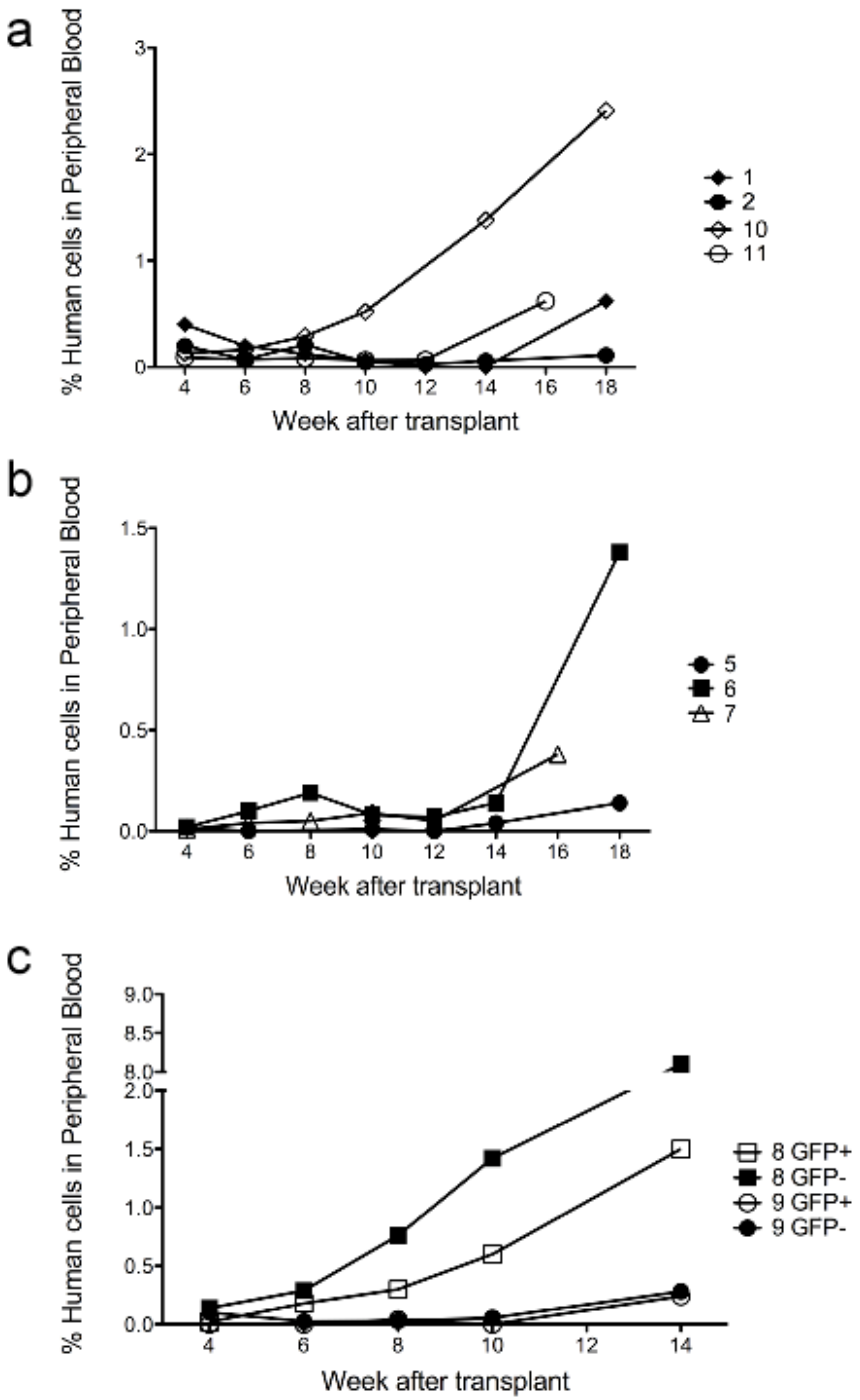


Figure 2.10. Engraftment kinetics of mice engrafted with GFP^+ and/or GFP^- human cells. The percentage of human cells in the peripheral blood of transplanted mice over time is shown. **a.** Mice that engrafted only GFP^- cells. Mice 1 and 2 were transplanted with mock-infected cells; mice 10 and 11 were transplanted with both GFP^+ and GFP^- cells but engrafted only GFP^- cells. **b.** Mice that engrafted only GFP^+ cells. **c.** Mice that engrafted both GFP^+ and GFP^- cells. In all cases, key indicates animal number used throughout paper.

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Chapter 3

Latent HIV-1 Infection Occurs in Multiple Subsets of Hematopoietic Progenitor Cells and Is Reversed by NF-κB Activation[5]

Abstract

The ability of HIV-1 to establish a latent infection presents a barrier to curing HIV. The best-studied reservoir of latent virus in vivo is in resting memory $CD4^+$ T cells, but it has recently been shown that $CD34^+$ hematopoietic progenitor cells (HPCs) can also become latently infected by HIV-1 in vitro and in vivo. $CD34^+$ cells are not homogenous, however, and it is not yet known which types of $CD34^+$ cells support a latent infection. Furthermore, the mechanisms through which latency is established in this cell type are not yet known. Here we report the development of a primary cell model for latent HIV-1 infection in HPCs. We demonstrate that in this model, latent infection can be established in all subsets of HPCs examined, including HPCs with cell surface markers consistent with immature hematopoietic stem and progenitor cells. We furthermore show that the establishment of latent infection in these cells can be reversed

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by TNF-α through an NF-κB-dependent mechanism. By contrast, we do not find evidence for a role of P-TEFb in the establishment of latent infection in HPCs. Finally, we demonstrate that prostratin and suberoylanilide hydroxamic acid (SAHA), but not hexamethylene bisacetimide (HMBA) or 5-Aza-2'-deoxycytidine (Aza-CdR), reactivate latent HIV-1 in HPCs. These findings illuminate the mechanisms through which latent infection can be established in HPCs and suggest common pathways through which latent virus could be reactivated in both HPCs and resting memory T cells to eliminate latent reservoirs of HIV-1.

Introduction

Although highly active antiretroviral therapy (HAART) for HIV-1 suppresses viral replication and extends the lifespan of infected individuals, it cannot eliminate the virus (Finzi et al. 1999, Gulick et al. 1997, Hammer et al. 1997, Zhang et al. 1999). A major barrier to viral eradication is the ability of HIV-1 to establish a latent infection (Finzi et al. 1999, Zhang et al. 1999). Latent infection occurs when a replication-competent HIV-1 provirus integrates into a host cell's genomic DNA, but viral genes are not transcribed (reviewed in Geeraert et al. 2008). Because no viral proteins are produced, the immune system cannot eliminate latently infected cells. At any point, however, cellular changes can reactivate the latent virus, leading to the production of new viral particles and, in the absence of antiretroviral therapy, the infection of additional cells (reviewed in Trono et al. 2010). Thus, reactivation of latent virus can lead to a resurgence of viremia in patients who have discontinued antiretroviral therapy.

While reactivation of latent virus can lead to increased viremia, reactivating latent virus in a patient who is receiving HAART therapy could clear latent reservoirs while preventing new infection events. Cells harboring reactivated virus would be eliminated because they produce viral proteins, leading to cell death through the action of the immune system or viral cytotoxic effects. Reactivation of virus from latently infected cells has thus been proposed as a strategy to cure HIV-1. Unfortunately, efforts to use this strategy to eliminate HIV-1 infection have thus far been unsuccessful (reviewed in Geeraert et al. 2008). There thus remains an urgent need for research to determine both the cell types that harbor latent virus as well as the mechanisms underlying the establishment and reactivation of latent infection in all latently infected cell types.

Although resting memory T cells are the best-studied and likely largest reservoir of latent HIV-1, several studies have found that not all viral genomes in the plasma can be matched to sequences in these cells (Bailey et al. 2006, Brennan et al. 2009, Sahu et al. 2009). These findings suggest that additional reservoirs of latent virus exist and contribute to residual plasma viremia. Recently, we proposed that $CD34^+$ hematopoietic progenitor cells (HPCs) in the bone marrow serve as a reservoir of HIV-1 and demonstrated that active and latent infection of these cells occur in vitro and in vivo (Carter et al. 2010). These findings are supported by additional studies demonstrating that HPCs are infected by HIV-1 in vivo (Redd et al. 2007, Stanley et al. 1992). However, there are also conflicting studies reporting that HIV-1 genomes could not be detected in $CD34^+$ cells in the bone marrow of patients with undetectable viral loads on HAART (Durand et al. 2012, Josefsson et al. 2012).

$CD34^+$ HPCs are rare cells, and only an extremely low rate of latent infection would be expected in most HAART-treated patients. This is particularly true in patients with predominantly CCR5-tropic virus, as only CXCR4-utilizing HIV is able to infect immature HPCs (Carter et al. 2011). In addition, it is difficult to rule out the possibility that contaminating $CD4^+$ T cells in HPC samples contribute to the detection of HIV genomes in these cells. Thus, it may be difficult to definitively show whether latent infection of HPCs occurs in a majority of individuals: indeed, disagreement on this point has persisted through more than two decades of study (reviewed in McNamara and Collins 2011). However, latent infection can be readily established in HPCs in vitro (Carter et al. 2010), and thus in vitro systems can be used to assess which subtypes of HPCs become latently infected. Such studies can illuminate the potential of HPCs to serve as a reservoir in vivo by demonstrating whether long-lived HPCs can be latently infected. In addition, they can identify the HPC types most likely to harbor latent infection, suggesting cell types to selectively purify in future efforts to identify latent reservoirs in vivo. Finally, in vitro studies can assess whether the mechanisms that promote the establishment and reactivation of latent infection in HPCs are comparable to those at work in $CD4^+$ T cells, and thus whether both reservoirs might be targeted and eliminated with similar reactivation strategies. For these reasons, an in vitro examination of latent infection of $CD34^+$ HPCs provides valuable information to aid in both the search for latent reservoirs and the development of strategies to reactivate and eliminate latent virus in vivo.

$CD34^+$ HPCs are not homogenous, and it is not clear which subsets of $CD34^+$ cells support latent HIV-1 infection. The most immature HPCs are hematopoietic stem

cells (HSCs), which can differentiate into all blood cell lineages and have unlimited self-renewal capacity. HSCs differentiate into multipotent progenitor cells (MPPs), which also differentiate into all blood cell lineages but have reduced self-renewal capacity (Doulatov et al. 2010). MPPs give rise to common myeloid progenitors (CMPs), which give rise to all myeloid lineages, and multilymphoid progenitors (MLPs), which generate the lymphoid lineages as well as monocytes and dendritic cells (Doulatov et al. 2010). These progenitors give rise to committed progenitor cells that eventually differentiate into mature blood cells. While we recently showed that HIV-1 is capable of infecting multipotent HPCs, including hematopoietic stem cells, the ability of HIV-1 to establish a latent as opposed to active infection in these cells is unknown (Carter et al. 2011).

The mechanisms that promote latency in HPCs have not been investigated, but models of latent infection in T cells have identified mechanisms that promote latent infection in this cell type (Bosque and Planelles 2009, Lassen et al. 2012, Saleh et al. 2007, Tyagi et al. 2010, Yang et al. 2009). In T cells, epigenetic modifications result in a heterochromatic structure at the HIV-1 long terminal repeat (LTR) (Kauder et al. 2009, Tyagi et al. 2010). Histone methylation and histone deacetylase (HDAC) recruitment contribute to the formation of this restricted transcriptional state (Tyagi et al. 2010). DNA methylation has also been associated with latent infection in both Jurkat and primary T cell models of latency (Blazkova et al. 2009, Kauder et al. 2009). Furthermore, resting memory T cells have restricted nuclear levels of nuclear factor κB (NF-κB) (Tyagi et al. 2010). NF-κB, a key transcriptional regulator in many hematopoietic cells, is sequestered in the cytoplasm of resting cells by the inhibitor of κB (IκB) (reviewed in Vallabhapurapu and Karin 2009). Upon T cell activation,

phosphorylation of IκB by IκB kinase (IKK) triggers the release of NF-κB from IkB and its translocation to the nucleus (Li et al. 1999, reviewed in Vallabhapurapu and Karin 2009). There, NF-κB can bind to the HIV-1 LTR, displacing inhibitory factors and recruiting histone acetyltransferases to relieve heterochromatic repression (Nabel and Baltimore 1987, Van Lint et al. 1996).

NF-κB activation alone is sufficient to reactivate latent virus in Jurkat cells (Duverger et al. 2009, Tyagi et al. 2010), but in primary resting memory T cells, activation of Positive Transcription Elongation Factor b (P-TEFb) is also required (Tyagi et al. 2010). P-TEFb, a complex made up of the cyclin-dependent kinase 9 (CDK9) and Cyclin T1 (CycT1), is present only at low levels in resting $CD4^+$ T cells, and much of the complex is sequestered by the 7SK small nuclear RNA and hexamethylene bisacetamide (HMBA)-induced protein (HEXIM1) (Nguyen et al. 2001, Yang et al. 2001, Yik et al. 2003). T cell activation results in increased P-TEFb mRNA transcription and protein translation (Herrmann et al. 1998) as well as the rapid phosphorylation of pre-existing CDK9 at Thr186 (Ramakrishnan et al. 2009) and dissociation of P-TEFb from 7SK RNA (Kim et al. 2011). HIV-1 Tat assists in this process by competitively displacing HEXIM1 to free P-TEFb from the inhibitory complex (Barboric et al. 2007, Sedore et al. 2007). Activated P-TEFb is then recruited to nascent HIV-1 transcripts, where it facilitates transcript elongation and thus reactivation of latent virus (Kim et al. 2011, Salerno et al. 2007, Tyagi et al. 2010, Wei et al. 1998).

Compounds that counteract the factors promoting latency in T cells have been shown to reactivate latent virus in primary T cells or cell-line models. Among these compounds are prostratin, which activates NF-κB through protein kinase C (Korin et al.

2002, Williams et al. 2004); HDAC inhibitors including suberoylanilide hydroxamic acid (SAHA) and valproic acid (VPA) (Contreras et al. 2009, Edelstein et al. 2009, Ylisastigui et al. 2004); the DNA methylation inhibitor 5-aza-2'-deoxycytidine (Aza-CdR) (Kauder et al. 2009); and hexamethylene bisacetamide (HMBA), a compound that appears to facilitate the release of P-TEFb from its inhibitory complex with 7SK and HEXIM1 (Contreras et al. 2007). Several of these compounds, notably SAHA, have been proposed as therapies to reactivate latent virus in vivo (Contreras et al. 2009, Edelstein et al. 2009, Ylisastigui et al. 2004). However, if resting memory T cells are not the sole reservoir for latent virus, these compounds will be effective therapies only if they can reactivate virus in all additional HIV reservoirs as well.

In this paper, we develop an in vitro model system of latent HIV-1 infection in HPCs that permits detailed study of the factors promoting latency in these cells. We use this model to show that HIV-1 is able to establish a latent infection in all subsets of HPCs examined, including cells with surface markers consistent with HSCs and MPPs. We furthermore show that $CD34^+$ HPCs have low nuclear levels of NF-κB and that NF-κB activation can reactivate latent virus in these cells. Meanwhile, P-TEFb is readily detectable in the nuclei of unstimulated HPCs and is not increased under conditions that reactivate latent virus. Finally, we assess the ability of compounds that reactivate latent virus in T cell systems to perform a similar function in HPCs. We find that while prostratin and SAHA can reactivate latent infection in HPCs, HMBA and Aza-CdR cannot. These findings enhance our understanding of the cellular factors required to establish a latent HIV-1 infection in HPCs and suggest common pathways in both HPCs and T cells that could be targeted to purge latent reservoirs.

Materials and Methods

Cell Isolation and Culture. Whole umbilical cord blood (CB) was obtained from the New York Blood Center and whole bone marrow (BM) was obtained commercially (AllCells Ltd.); mononuclear cells were purified by Ficoll-Hypaque centrifugation and either frozen or used fresh. Cells were adherence depleted for 1-2 hours at 37º C in StemSpan Media (STEMCELL Technologies), then $CD133^+$ cells were isolated by magnetic separation (Miltenyi Biotech). Isolated cells were cultured in STIF media (StemSpan supplemented with 100ng/ml SCF, 100ng/ml TPO, 100ng/ml Flt3L (all from STEMCELL Technologies), and 100ng/ml IGFBP-2 (R&D Systems)). Pre-sorted $CD133^+$ BM or CB cells were obtained commercially (AllCells Ltd.) and cultured as above.

Resting memory $CD4^+$ T cells were purified from buffy coats obtained from the New York Blood Center. Mononuclear cells were purified as above, then memory $CD4^+$ T cells were isolated by magnetic separation using the Memory $CD4^+$ T Cell Isolation Kit (Miltenyi Biotech). The resulting cells were incubated with biotinylated antibodies against the activation markers HLA-DR, CD69, and CD25 (eBioscience), then resting memory cells were isolated by negative selection using an anti-biotin magnetic separation kit (Miltenyi Biotech). Resting memory $CD4^+$ T cells were used immediately or cultured overnight with anti-CD3/anti-CD28 beads (Life Technologies).

U1 cells (Folks et al. 1987) and J-Lat clones 6.3, 8.4, and 9.2 (Jordan et al. 2003) were cultured in RPMI supplemented with 10% fetal bovine serum (FBS) and 2mM

penicillin, streptomycin, and glutamine (PSG). 293T cells were propagated in DMEM supplemented with 10% FBS and 2mM PSG.

HIV-1 Virus Preparation and Transductions. NL4-3-ΔGPE-GFP was generated by removing a SwaI-SwaI fragment from NL4-3-ΔE-GFP (Zhang et al. 2004) and religating.

Infectious supernatants were prepared by transfection of proviral plasmids into 293T cells using polyethylenimine. HXB-ePLAP (Chen et al. 1996) or NL4-3-ΔGPE-GFP was cotransfected with a plasmid encoding either VSV-G or HXB envelope; the helper plasmid pCMV-HIV-1 (Gasmi et al. 1999) was additionally cotransfected to permit infectious particles to be formed with NL4-3-ΔGPE-GFP. Viruses pseudotyped with HXB envelope were concentrated using high-molecular-weight polyethylene glycol precipitation as previously described (Kohno et al. 2002). Pellets were resuspended in 1/25 the original volume and used immediately. Cells were infected by spin inoculation at 1048.6 × g for 2 hours at room temperature.

Sorting and Stimulation of Latently Infected Cells. Actively infected cells from HPCs infected with HXB-ePLAP were removed by magnetic sorting using a biotin-conjugated antibody to PLAP and anti-biotin beads (Miltenyi Biotech). Actively infected cells were removed from samples infected with NL4-3-ΔGPE-GFP by flow cytometric sorting using a MoFlo XDP (Beckman Coulter), MoFlo Astrios (Beckman Coulter), or FACSAria (BD Biosciences) flow cytometer. After sorting, cells were incubated in STIF or StemSpan with 8μM Raltegravir (Selleck Chemicals) alone or with one or more of the following: GM-CSF (R&D Systems), TNF-α (Biolegend), prostratin (Sigma), SAHA (Cayman

chemical), HMBA (Sigma), Aza-CdR (Sigma), IKK2 VI (Calbiochem), or functional grade anti-TNF-α (eBioscience).

Time Course of Stimulation in Uninfected Cells. Uninfected HPCs were cultured for 5 days, then incubated in StemSpan with DMSO solvent control or TNF-α, prostratin, or SAHA for 3 days. Images of cells under these conditions were acquired using a QICAM digital camera and Q-Capture Pro 7 software (QImaging). Brightness and contrast of images were minimally adjusted using Canvas 8 (ACD Systems).

Flow Cytometry and Antibodies. Antibodies to the following proteins were used for flow cytometry: CD133 (PE-conjugated, Miltenyi), CD3 (APC- or Alexa Fluor 647-conjugated, eBioscience), CD34 (FITC, APC, Alexa Fluor 647, or biotin-conjugated, Miltenyi, eBioscience, and Invitrogen), CD45RA (PE-conjugated, eBioscience), CD38 (PE-Cy7-conjugated, eBioscience), PLAP (Serotec) as provided or conjugated to biotin using EZ-Link Sulfo-NHS Biotinylation Kit (Pierce), and HIV-1 Gag (clone KC57, FITC-conjugated, Coulter). Secondary reagents used were streptavidin (Alexa Fluor 488, PE, or Alexa Fluor 647-conjugated, Life Technologies) and antibody to mouse-IgG1 (Alexa Fluor 647-conjugated, Invitrogen). Cells were stained with 7-Aminoactinomycin D (7-AAD) to exclude dead cells. Analysis was performed using a BD FacsCanto cytometer or BD FACScan with Cytek 6-color upgrade.

For staining cell surface proteins, cells were first incubated in FACS buffer (PBS with 2% FBS, 1% human serum, 2mM HEPES, and 0.025% NaN_3) on ice for 10 minutes with directly conjugated antibodies, then washed and fixed in PBS with 2%

paraformaldeyde. For stains utilizing antibodies against PLAP, cells were incubated in 10% Fc receptor block (Accurate Chemical) in FACS buffer for 30 minutes, then incubated with antibodies against PLAP in FACS buffer with 10% Fc receptor block for 15 minutes on ice. Cells were then washed and stained with secondary antibody for an additional 5 (streptavidin) or 15 (anti-mouse-IgG1) minutes on ice. For stains using antibodies against HIV Gag, cells were first fixed, then permeabilized with 0.1% Triton X-100 in PBS for five minutes at room temperature. Cells were then washed and incubated with antibody against Gag for 30 minutes on ice.

Transcription factor enzyme-linked immunosorbent assay (ELISA). Nuclear and cytoplasmic extracts were prepared using a commercially available kit (Active Motif), then levels of activated nuclear NF-κB and AP-1 family members were determined using commercially available transcription factor ELISA assays (Active Motif). Protein content of nuclear and cytoplasmic extracts was quantified and an equal amount of protein was plated for each condition. Commercially obtained Raji nuclear cell extracts were used as a positive control for NF-κB activation and K-562 cells stimulated with 12-O-tetradecanoylphorbol-13-acetate (TPA) were used as a positive control for AP-1 activation (Active Motif). Absorbance readings from blank wells were subtracted from all sample and positive control absorbance readings. Then, readings were normalized to the positive control on each plate to control for differences in color development between plates. Normalized absorbance in cytoplasmic extracts was subtracted from the absorbance in the nuclear extracts from each sample to control for non-specific binding.

These normalized differences were designated arbitrary units (AU) and used to compare samples. In all cases, the signal from the positive control extract was set to 1 AU.

Western blotting. Nuclear extracts were collected as described above. Antibodies to the following proteins were used for Western blot analysis: Cyclin T1 (Santa Cruz sc-8127); pCDK9 (Cell Signaling #2549), and Histone H1.4 (Sigma H7665). Secondary reagents used were rabbit antibodies directed against goat IgG and conjugated to horseradish peroxidase (HRP) (Zymed) and goat antibodies directed against rabbit IgG and conjugated to HRP (Invitrogen)

Results

Development of an in vitro model of HIV-1 latency in HPCs. Although latent infection of HPCs has been previously studied in vitro (Carter et al. 2010), the methods used required relatively long culture periods. Because HPCs rapidly differentiate in culture, these methods did not permit the examination of latent infection in immature HPC subsets such as HSCs and MPPs. To better assess latent infection in immature HPCs, we developed a short-term primary cell model of latency in $CD133^+$ HPCs (**Figure 3.1A**). This short term model allowed us to examine latent infection in immature HPCs, an important benefit as these cells are the most long-lived and thus the most likely to serve as a long-term reservoir of virus in vivo. However, the short culturing times required to study these cells in an undifferentiated state meant that while we could examine factors that are

important for the establishment of latency, we could not assess whether additional factors contribute to maintenance of proviral silencing at later time points.

In our model, primary HPCs were isolated on Day 0 from umbilical cord blood (CB) or bone marrow (BM) (**Figure 3.1A**). Two days later, HPCs were infected with replication-defective HIV-1 reporter viruses, either HXB-ePLAP or NL4-3-ΔGPE-GFP (**Figure 3.1B**). These viruses both have a deletion in the *env* gene while NL4-3-ΔGPE-GFP has additional deletions in *gag* and *pol*, resulting in viruses that are capable of a single round of infection but not of continuing spread. This feature of the viruses simplified our analysis by allowing us to distinguish reactivation of latent infection from stimulation of new infection events. HXB-ePLAP encodes placental alkaline phosphatase (PLAP) as a reporter protein, which is expressed on the cell surface; NL4-3-ΔGPE-GFP expresses EGFP (shortened to GFP throughout). These viruses were pseudotyped with either VSV-G envelope or the HIV-1 envelope HXB (**Figure 3.1B**). Most experiments were conducted using VSV-G envelope to maximize the infection rate and increase the sensitivity of analysis, but major findings were confirmed using HXB envelope. The CXCR4-tropic HXB envelope was selected for these experiments because we have previously shown that only HIV-1 envelopes able to use CXCR4 as a co-receptor for entry can infect immature HPCs (Carter et al. 2011).

Three days after infection, actively infected cells expressing the reporter protein were removed by magnetic or flow cytometric sorting (**Figure 3.1C**); the remaining cells, a mixture of uninfected and latently infected cells, were resuspended in media with 8μM raltegravir (**Figure 3.1A**). The integrase inhibitor raltegravir was routinely included in these experiments to ensure that increases in reporter gene expression would stem solely

from activation of integrated virus and not from new integration events. Cells were then either incubated in STIF media, which contains a cocktail of cytokines that minimizes differentiation in culture, or were stimulated with GM-CSF and TNF-α or other compounds overnight. Finally, samples were analyzed the next day by flow cytometry (**Figure 3.1A**). In all cases, cells that remained $CD34^+$ or $CD133^+$ at this time point were gated on so that only HPCs would be included in our analysis of latent infection.

We tested our model by stimulating latently infected cells with GM-CSF and TNF-α, which we have previously shown to be capable of reactivating latent virus in HPCs in vitro and ex vivo (Carter et al. 2010). In agreement with our previous work, we found that overnight (13 hour) stimulation with GM-CSF and TNF-α resulted in a 2- to 6-fold increase in reporter protein expression compared to unstimulated controls (**Figure 3.1D**). The rate of latent infection that could be reactivated with GM-CSF and TNF-α was comparable to the rate of active infection observed prior to sorting and stimulation (compare **Figure 3.1C** and **D**). These results cannot be attributed to contamination of the culture with $CD4^+$ T cells, as $CD3^+$ cells are largely absent from our HPC cultures on the day of reactivation and are readily excluded by gating on CD34+ cells (**Figure 3.1E**).

Latent Infection Occurs in Multiple HPC Subsets. Although we have reported that HIV-1 can infect multipotent HPCs, the ability of the virus to establish a latent infection in this cell subset was not established. We therefore used our model to examine latent infection in HPCs expressing CD133, a cell surface marker found primarily on immature HPCs and associated with colony-forming capacity (Carter et al. 2011). HPCs infected with HXB-ePLAP were magnetically sorted to isolate $PLAP^-$, $CD133^+$ cells (**Figure 3.1F**),

then these cells were incubated overnight in STIF or GM-CSF and TNF-α. Compared with the rate of infection in cells immediately post-sort (0.1%, **Figure 3.1F**), we observed spontaneous induction of active infection in a subset of cells (**Figure 3.1G**), which may result from spontaneous differentiation that occurs even in STIF. Additionally, we observed a 2- to 4-fold increase in PLAP expression in $CD133^+$ cells that were stimulated with GM-CSF and TNF-α compared to the STIF-treated control (**Figure 3.1G-H**), demonstrating that HIV-1 can establish a latent infection in $CD133^+$ HPCs that can be reactivated by GM-CSF and TNF-α treatment.

To further define HPC populations in which latent HIV-1 infection occurs, we stained HPCs with antibodies against CD38 and CD45RA, cell surface markers for which expression on various HPC populations has recently been defined (Doulatov et al. 2010) (**Figure 3.2A**). HSCs and MPPs stain $CD34^+$, $CD38^-$, $CD45RA^-$, multilymphoid progenitors (MLPs) stain $CD34^+$, $CD38^-$, $CD45RA^+$, common myeloid progenitors (CMPs) and megakaryocyte/erythroid progenitors (MEPs) stain $CD34^+$, $CD38^+$, $CD45RA^-$, and finally committed granulocyte/monocytes progenitors (GMPs) and B/NK progenitors (B/NK) stain $CD34^+$, $CD38^+$, $CD45RA^+$. We infected and sorted the cells as described in Figure 1, then analyzed reactivation of latent virus in each of the populations defined by these cell surface antigens. We found that we could reactivate latent virus in each population, including the $CD34^+$, $CD38^-$, $CD45RA^-$ subset that includes the most immature, long-lived HPCs (**Figure 3.2B**). Similar results were obtained whether cells had been infected using VSV-G (**Figure 3.2C** and **E**) or HXB envelope (**Figure 3.2B** and **D**), and whether HPCs were derived from cord blood (**Figure 3.2B-D**) or bone marrow (**Figure 3.2E**). These results demonstrate that latent infection does not occur in a specific

type of HPCs but rather in all cell subsets identified, including long-lived HSCs and MPPs with the potential to serve as a long-term reservoir for latent virus in vivo.

Conditions that Reactivate Latent Virus in HPCs are Associated with Activation of NF-κB but not AP-1. We next assessed the cellular changes that occur when $CD34^+$ HPCs are stimulated with GM-CSF and TNF-α to elucidate the mechanism by which these cytokines reactivate latent HIV-1. We first examined whether nuclear levels of the transcription factors NF-κB or AP-1 are increased under these conditions. We examined these factors because both have binding sites on the HIV-1 LTR, have been shown to promote HIV-1 transcription (Canonne-Hergaux et al. 1995, Nabel and Baltimore 1987, Richman et al. 1994, Roebuck et al. 1996, Van Lint et al. 1997, reviewed in Pereira et al. 2000), and can be activated by TNF-α (reviewed in Aggarwall 2000 and Chen and Goeddel 2002).

Uninfected, cord blood-derived $CD133^+$ HPCs were expanded in STIF media for 5 days, then split into STIF vs. GM-CSF + TNF-α conditions for an overnight incubation. We isolated cytoplasmic and nuclear extracts from these cells and assessed levels of activated nuclear NF-κB and AP-1 transcription factor proteins by transcription factor enzyme-linked immunosorbent assay (ELISA). We found that levels of activated nuclear NF-κB family members p65, p50, c-Rel, and RelB were all stimulated by overnight incubation with GM-CSF and TNF-α (**Figure 3.3A**), suggesting that these cytokines activate NF-κB through the classical pathway (reviewed in Vallabhapurapu and Karin 2009). In contrast, we found that nuclear NF-κB p52, which is mainly activated through the non-classical NF-κB pathway (reviewed in Vallabhapurapu and Karin 2009), was not

upregulated by GM-CSF and TNF-α stimulation (**Figure 3.3A**). Additionally, no AP-1 family member was upregulated by GM-CSF and TNF-α (**Figure 3.3B**). Of the proteins studied, only AP-1 FosB and JunB could be consistently detected in the nuclei of HPCs incubated in STIF (**Figure 3.3B**). The finding that NF-κB is usually undetectable in the nuclei of unstimulated HPCs suggests that NF-κB restriction promotes latent infection in this cell type.

TNF-α Reactivates Latent Virus in HPCs through an NF-κB-Dependent Mechanism. In other cell types, TNF-α has been reported to activate NF-κB through the classical pathway, which requires activation of the IKKß subunit of IKK (Li et al. 1999). We therefore asked whether TNF-α alone could reactivate latent virus in HPCs and whether this reactivation required IKKß activity. To test whether TNF-α alone was sufficient to reactivate latent virus, we incubated latently infected HPCs with STIF, GM-CSF alone, TNF-α alone, or GM-CSF + TNF-α. We observed that while GM-CSF alone had no significant effect on viral gene expression, TNF-α was able to induce viral gene expression at the same level as the combination of the cytokines (**Figure 3.4A**). We confirmed that this effect was specifically due to TNF-α by stimulating cells with TNF-α in the presence and absence of antibodies to TNF-α. Induction of viral gene expression was substantially reduced in the presence of antibodies to TNF-α (**Figure 3.4B**).

To assess the dependence of TNF-α-induced viral reactivation upon activation of the classical NF-κB pathway, we stimulated latently infected HPCs with varying concentrations of TNF-α with and without an inhibitor of IKKß activity, IKK2 VI (Baxter et al. 2004). While high doses of TNF-α overwhelmed the effect of IKK2 VI, at

moderate TNF-α doses IKK2 VI had a substantial inhibitory effect (**Figure 3.4C**). IKK2 VI significantly counteracted TNF-α-induced reactivation of latent virus when cells were infected with HIV pseudotyped either with VSV-G (**Figure 3.5A-B**) or HIV (HXB) envelope (**Figure 3.5C-D**) and when either cord blood-derived (**Figure 3.5A-D**) or bone marrow-derived (**Figure 3.5E-F**) HPCs were used. Using transcription factor ELISA to assess nuclear NF-κB p50 in these samples, we observed that in samples where IKK2 VI inhibited the ability of TNF-α to reactivate latent virus, IKK2 VI also blocked TNF-α-induced NF-κB activation (**Figure 3.5G**).

In a majority of experiments, overnight incubation with IKK2 VI also reduced the level of active infection observed in cells cultured without TNF-α (**Figure 3.4C, 3.5B-C,** and **3.5F**), indicating that a portion of the low-level spontaneous reactivation observed under these conditions was also due to activation of NF-κB. This low-level spontaneous NF-κB activation is most likely a component of the spontaneous differentiation that occurs in HPCs under these culture conditions. Such NF-κB activation has been reported as a component of the differentiation of more mature hematopoietic progenitor cells (Burkly et al. 1995, Zhang et al. 1998).

Conditions that Reactivate Latent Virus in HPCs Do Not Alter P-TEFb expression. We next asked whether restricted levels of P-TEFb contribute to the establishment of latency in HPCs. To address this question, we analyzed nuclear CycT1 and phosphorylated CDK9 (pCDK9) in cord blood-derived HPCs incubated in STIF or stimulated overnight with GM-CSF and TNF-α. We compared CycT1 and pCDK9 levels in these cells to those observed in resting memory T cells or resting memory T cells that had been

stimulated overnight with anti-CD3/anti-CD28 beads. As a positive control for CycT1 and pCDK9 expression, we examined the levels of both proteins in Jurkat cells with or without TNF-α stimulation. In agreement with previous findings (Herrmann et al. 1998, Kim et al. 2011, Ramakrishnan et al. 2009, Tyagi et al. 2010), Jurkat cells had high endogenous levels of CycT1 and pCDK9 that were not affected by TNF-α stimulation, while resting memory T cells had low levels of nuclear CycT1 and pCDK9 that increased dramatically following overnight incubation with anti-CD3/anti-CD28 beads (**Figure 3.6**). Finally, we observed that both CycT1 and pCDK9 were readily detectable in unstimulated HPCs and that levels of these proteins were not affected by incubation with GM-CSF and TNF-α (**Figure 3.6**). This result demonstrates that the ability of these cytokines to induce HIV-1 gene expression in HPCs is not related to alterations in P-TEFb expression.

Prostratin and SAHA, but not HMBA, Can Reactivate Latent HIV-1 in HPCs. We next asked whether compounds that reactivate latent virus in T cell systems had similar activity in our model of latent infection in HPCs. We first tested prostratin, SAHA, and HMBA, and found that while prostratin and SAHA were able to induce viral gene expression, HMBA was not (**Figure 3.7A**). A titration of prostratin demonstrated that prostratin effectively reactivated latent virus in HPCs only at high doses (1-5μM) (**Figure 3.7A**). Although the comparative efficacy of prostratin and TNF-α varied between experiments, prostratin was on average significantly less effective than TNF-α at reactivating latent virus from HPCs at a 13 hour time point (**Figure 3.7B**). SAHA also reactivated latent virus only at high doses (2-10μM) (**Figure 3.7A**) and also induced

reactivation to a significantly lesser extent than TNF-α at 13 hours post-stimulation (**Figure 3.7B**). Finally, although HMBA could induce expression of HIV-1 Gag in the latently infected U1 cell line (**Figure 3.7C**), we did not detect an effect of HMBA on viral gene expression in HPCs at any concentration assessed (**Figure 3.7A**).

Although SAHA and prostratin were less effective than TNF-α at 13 hours post-stimulation, different results were obtained at 24 hours. At this time point, we again found that SAHA, prostratin, and TNF-α were all able to induce a statistically significant reactivation of latent virus in HPCs (**Figure 3.7D**); however, there was no significant difference between the induction of active infection caused by TNF-α and that caused by prostratin or SAHA (**Figure 3.7D**). HMBA still failed to induce significant reactivation at 24 hours (**Figure 3.7D**). The maximum reactivation achieved at 24 hours post-stimulation was somewhat lower than that observed at 13 hours post-stimulation due to an increase in spontaneous reactivation in the solvent control. Together, these results demonstrate that SAHA and prostratin, but not HMBA, are able to reactivate latent virus in HPCs; however, SAHA and prostratin reactivate virus with slower kinetics than does TNF-α.

Consistent with prostratin's established ability to activate NF-κB through protein kinase C (Williams et al. 2004), we found that a 24 hour stimulation with prostratin resulted in an increase in nuclear NF-κB p50 activity to levels comparable to that observed with a high dose of TNF-α (**Figure 3.7E**). By contrast, stimulation with HMBA or the histone deacetylase inhibitor SAHA had no effect on nuclear NF-κB activity, even though SAHA reactivated latent HIV-1 to the same extent as a 30pg/ml dose of TNF-α that did cause detectable NF-κB upregulation (**Figure 3.7E**).

Although SAHA and prostratin reactivated latent virus in our model, both compounds had additional effects on HPCs. We observed that incubating HPCs in media containing SAHA caused massive cell death after 48-72 hours (**Figure 3.7F-G**). Similar cytotoxicity was observed at SAHA concentrations as low as 800nM (data not shown). Although prostratin was not cytotoxic to HPCs (**Figure 3.7F**), it caused rapid differentiation, resulting in adherent cells after as little as 24 hours of exposure (**Figure 3.7G**). By contrast, TNF-α did not induce cellular differentiation and although somewhat cytotoxic at 72h, it was substantially less so than SAHA (**Figure 3.7F-G**).

Aza-CdR Does Not Reactivate Latent Infection in HPCs. Finally, we examined whether the methylation inhibitor Aza-CdR could induce reactivation of latent virus in HPCs. We first verified the activity of Aza-CdR in J-Lat cells, clones of Jurkat cells that are latently infected with a GFP-expressing HIV-1 reporter virus (Jordan et al. 2003). In agreement with previous findings (Kauder et al. 2009), we observed that while Aza-CdR alone had minimal ability to induce viral gene expression in three J-Lat clones, it had a synergistic effect on reactivation when combined with TNF-α (**Figure 3.8A**). However, we were unable to detect any effect of Aza-CdR on reactivation of latent virus in HPCs, either alone (**Figure 3.8B**) or in combination with TNF-α (**Figure 3.8C**).

Discussion

In this study, we developed an in vitro model to study latency in immature HPCs, allowing us to investigate the types of HPCs that support latent infection and to study the factors that promote the establishment of latency. We found that HIV-1 could establish a

latent infection in all of the subsets of HPCs that we examined, including an immature population that includes hematopoietic stem cells and multipotent progenitors. As hematopoietic stem cells and multipotent progenitors are long-lived cell types, this result has important implications for the ability of HPCs to serve as a latent reservoir in vivo. Hematopoietic stem cells in particular are capable of indefinite self-renewal, and thus latent infection in this cell type could result in a reservoir with an infinite half-life. Such a reservoir has previously been proposed based on the decay kinetics of virus in the plasma (Palmer et al. 2008).

While our results suggest that many classes of progenitor cells might serve as short-term reservoirs of latent virus, immature progenitors are more likely to serve as latent reservoirs in patients treated with antiretroviral therapy. The reason for this is that these cells have the greatest capacity to self-renew, as demonstrated by the fact that only HSCs are capable of sustaining long-term, multi-lineage engraftment in mice (Notta et al. 2011). Similarly, these immature cells are the most likely to persist in humans after months or years of treatment. Thus, efforts to assess the extent of the latent reservoir in HPCs in patients on long-term antiretroviral therapy might be most successful if they focus on the immature, $CD34^+$, $CD38^-$, $CD45RA^-$ population of HPCs to maximize the chance of finding latently infected cells.

Our analysis revealed that TNF-α-induced activation of NF-κB in the absence of P-TEFb induction reactivates latent virus in HPCs. This result differs from recent findings in resting memory T cells, where TNF-α is not sufficient to reactivate latent virus and induction of both NF-κB and P-TEFb activity appear to be required (Lassen et al. 2012, Tyagi et al. 2010, Yang et al. 2009) and is instead reminiscent of Jurkat cell line

models of latency (Duverger et al. 2009, Jordan et al. 2003, Kim et al. 2011). Thus, our analysis suggests that HPCs may have fewer factors reinforcing latent infection than do resting memory T cells. In vivo, this might result in a rate of latent infection in these cells that is lower than that observed in resting memory $CD4^+$ T cells, where about 1 in 1 million cells harbors replication-competent provirus in treated patients (Finzi et al. 1999).

In addition to TNF-α, which reactivated latent virus to the maximum level that we were able to achieve, we found that prostratin and SAHA can also reactivate latent virus in HPCs but with slower kinetics. Prostratin, like TNF-α, efficiently induces NF-κB activation; however, the two compounds activate NF-κB through distinct pathways (Ea et al. 2006, Gaide et al. 2002, Hara et al. 2003, Hsu et al. 1996, Lucas et al. 2001, McAllister-Lucas et al. 2001, Micheau and Tschopp 2003, Pobezinskaya et al. 2008, Ruefli-Brasse et al. 2003, Ruland et al. 2001, Ting et al. 1996, Wang et al. 2002, Wu et al. 2006). Thus, our finding that prostratin reactivates virus with slower kinetics than does TNF-α suggests that the pathway through which prostratin acts is less efficient in at least a subset of HPCs. Alternatively, it is possible that in addition to NF-kB, TNF-α activates another factor that promotes reactivation in HPCs at early time points. However, as we found that TNF-α treatment of HPCs does not result in AP-1 activation, the identity of such an additional factor is not clear. Overall, as TNF-α reactivates latent virus from HPCs more rapidly than prostratin does and without inducing the rapid cellular differentiation observed during prostratin stimulation, our results suggest that NF-κB activation may be a successful strategy for reactivating latent virus in HPCs but that prostratin may not be an ideal compound with which to initiate NF-κB activation in these cells.

Consistent with SAHA's function as a histone deacetylase inhibitor (Edelstein et al. 2009), we found that this compound reactivates latent virus in HPCs in the absence of NF-κB activation. Reactivation occurred using SAHA concentrations that are comparable to those that are effective at reactivating latent virus in T cells (Contreras et al. 2009, Edelstein et al. 2009). The relatively low efficacy of SAHA at 13 hours post-stimulation compared to NF-κB activators TNF-α and prostratin suggests that although histone acetylation enhances HIV-1 transcription in HPCs, low NF-κB levels may still restrict rapid reactivation from latency. In addition, although SAHA has been shown to be non-toxic to T cells even over long culture periods (Shan et al. 2012), we found that doses of SAHA that could reactivate latent virus were extremely toxic in HPCs. This finding suggests that SAHA may not be effective in clearing latent virus from HPCs without significant detrimental effects on hematopoiesis.

Finally, we found that neither HMBA nor Aza-CdR could reactivate latent HIV-1 in our system. These findings are not surprising given the mechanisms of action of these compounds. HMBA is believed to activate P-TEFb (Contreras et al. 2007); thus the inability of this compound to reactivate virus in HPCs confirms our finding that P-TEFb levels are not restricted in this cell type. Finally, methylation has been shown to be a late event in the silencing of an HIV-1 provirus (Blazkova et al. 2009, Duverger et al. 2009, Kauder et al. 2009); as our model is designed to examine only early events in the establishment of latency in HPCs, it is not surprising that the methylation inhibitor Aza-CdR would have no effect in this system. Unfortunately, the difficulty of maintaining HPCs in culture in an undifferentiated state precludes examination of whether methylation might similarly occur as a late event in proviral silencing in HPCs.

To maximize HPC number, viability, and immaturity in our experiments, we cultured HPCs in STIF media, which contains the cytokines stem cell factor, thrombopoietin, IGFBP-2, and Flt3L. In addition to maintaining HPCs in an immature state, this media also promotes the expansion of immature HPCs. Of note, we found that latency could be established in HPCs in spite of the fact that these cells proliferated during the course of our assay with a mean doubling time of less than 48 hours (data not shown). This observation parallels findings from resting memory T cells, where homeostatic proliferation does not reactivate latent virus and instead helps to maintain the latent reservoir (Bosque et al. 2011, Chomont et al. 2009). Similarly, our observations suggest that self-renewal of HPCs in vivo would not reactivate latent virus, but instead would help to maintain this latent reservoir. However, additional study is needed to better define the effects of cell cycling on latently infected HPCs.

While further research to understand the extent of the latent reservoir in HPCs in patients on antiretroviral therapy will be informative, the very low rate of infection anticipated in these cells may make it difficult to definitively determine the size of this reservoir in vivo. Technical issues in culturing HPCs ex vivo may also contribute to the variable results that have been obtained when these cells are examined for infection; for instance, as active HIV infection is cytotoxic to HPCs (Carter et al. 2010), the reactivation of latent virus under differentiating culture conditions may result in cell death and false negative results. Therefore, protocols that include a culturing step prior to cell purification may reduce their yield of provirus-containing cells (Durand et al. 2012). In addition, cell preparations that have been depleted for $CD4^+CD34^+$ cells may yield false negative cells as CD4 is required for infection of HPCs (Carter et al. 2011,

Josefsson et al. 2012). Conversely, false positive results could be obtained if HPC samples are contaminated with $CD4^+$ T cells. Together, these factors may make it difficult to define the role of HPCs in viral persistence.

Further complicating the detection of infected HPCs is the fact that only CXCR4-utilizing viruses can infect immature HPCs (Carter et al. 2011), which may result in widely varying infection rates in these cells in patients with different proportions of CXCR4- or dual-tropic virus. Early in the course of infection most HIV isolates utilize CCR5 (Rieder et al. 2011, van't Wout et al. 1994, Zhu et al. 1993); however, several recent reports have demonstrated that CXCR4-utilizing viral subpopulations are found in up to 10-50% of recently infected individuals (Abbate et al. 2011, Chalmet et al. 2012). In addition, CXCR4-utilizing viruses emerge in many patients later in the course of infection (Richman et al. 1994, Shankarappa et al. 1999). A substantial fraction of HIV-infected patients would thus be expected to harbor virus that is capable of infecting HPCs; however, patients for whom CXCR4-utilizing virus is a minority population would be expected to have only a very low rate of infection in these cells. Additional studies are needed to more fully understand these variables.

Nonetheless, an eradication strategy for HIV must reactivate latent virus from all latently infected cells to eliminate all reservoirs of virus in the body. Thus, our observations on the mechanisms underlying the establishment and reactivation of latency in HPCs are of great utility in designing and evaluating eradication strategies, as they allow us to assess whether strategies that are effective in T cells will be equally effective in the proposed reservoir for latent virus in HPCs. Our findings demonstrate that latent infection can occur in all HPC subsets studied, including cells with surface markers

consistent with long-lived, multipotent hematopoietic stem and progenitor cells. We found that latent infection could be reversed by NF-κB activation but that nuclear P-TEFb levels are not restricted in HPCs. Finally, we observed that TNF-α, prostratin, and SAHA are all capable of reactivating latent infection in HPCs, but that prostratin and SAHA also induce differentiation or death, respectively, in the HPC population. Together, these data show that immature HPCs have the potential to serve as a reservoir of latent virus and suggest that strategies that activate NF-κB can reactivate latent virus from these cells. Proposed reactivation strategies can be assessed in this model of latent infection in HPCs in addition to models of latently infected resting memory T cells to assess the ability of different strategies to reactivate latent virus in both cell types and thus maximize the chance of eliminating all latent reservoirs in vivo.

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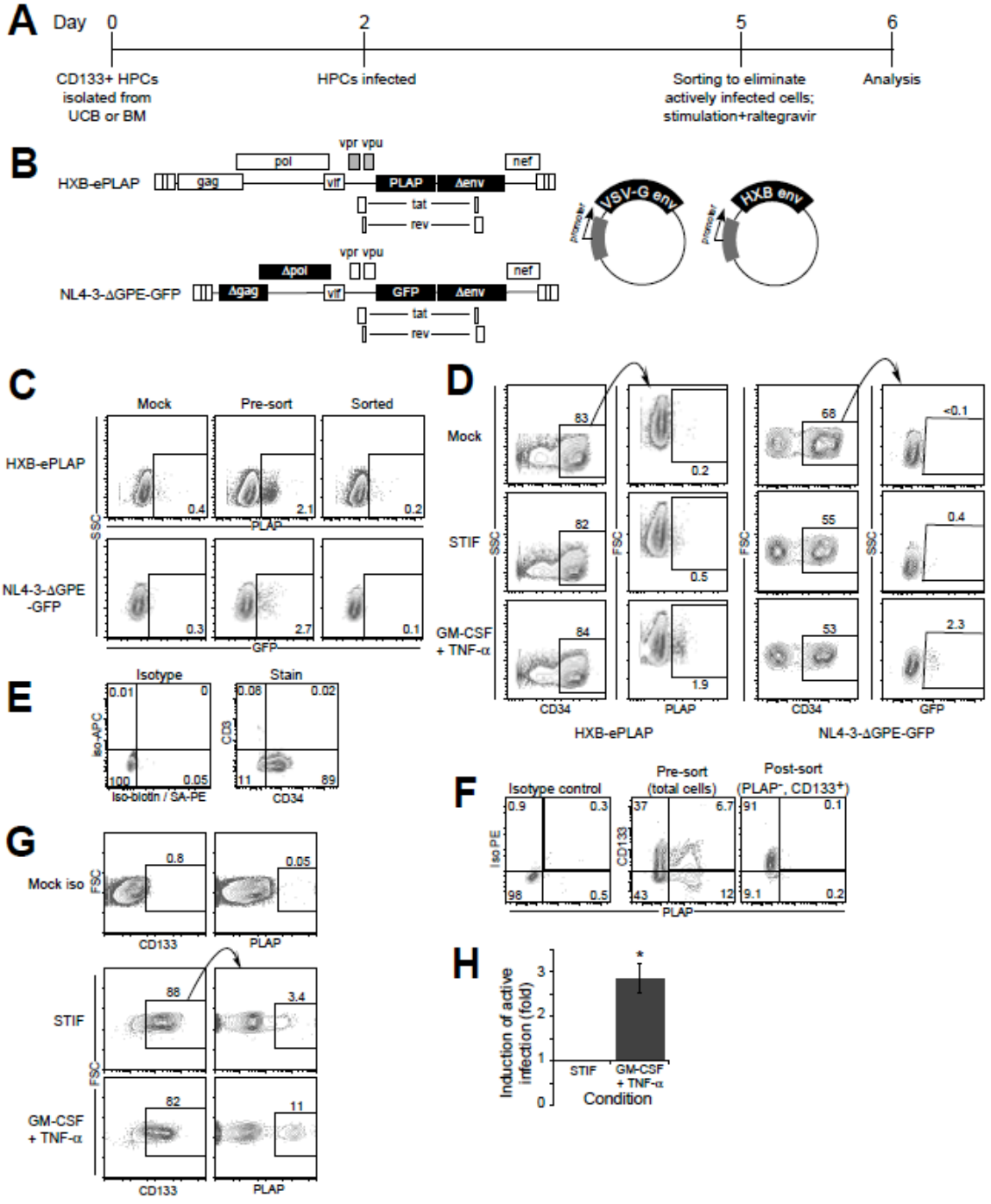

Figure 3.1. **A – D**. GM-CSF and TNF-α reactivate latent virus in a primary cell model for HIV-1 infection of $CD34^+$ HPCs. **A**. Time course for latency experiments. **B**. Schematic diagrams of HIV-1 reporter viruses used for these studies. Viruses were pseudotyped with VSV-G envelope (middle) or CXCR4-tropic HIV-1 envelope HXB (right). The gray shading of vpr and vpu in HXB-ePLAP indicates that these genes are defective in full length HXB. Black shading indicates genes that have been added or deleted compared to the wild type viral clone. **C**. Flow cytometric analysis of cells sorted

for reactivation protocols. Top, cord blood-derived HPCs infected with HXB-ePLAP/VSV-G and $PLAP^-$ cells isolated by magnetic sorting; bottom, cells infected with NL4-3-ΔGPE-GFP/VSV-G and GFP^- cells isolated by flow sorting. Numbers indicate the percentage of live cells that are $PLAP^+$ or GFP^+. Live cells were gated using FSC and SSC parameters. **D**. Flow cytometric analysis of cells shown in **C** after overnight incubation with STIF or GM-CSF (100ng/ml) and TNF-α (2.5ng/ml). $CD34^+$ cells are gated on at left, then PLAP (left) or GFP (right) expression in uninfected, STIF-treated, and GM-CSF and TNF-α-treated cells is shown at right. Numbers indicate the percent of cells within the labeled gate. Live cells were gated based on FSC, SSC, and 7-AAD. **E**. $CD3^+$ T cells are not present in the $CD34^+$ HPC population. Cord blood-derived HPCs were sorted and infected with NL4-3-ΔGPE-GFP/VSV-G as in **A**. CD3 and CD34 staining was assessed on day 6 following flow sorting and overnight incubation in STIF. Live cells were gated using FSC, SSC, and 7-AAD. Data are representative of two independent experiments, one each using cord blood and bone marrow-derived HPCs. **F – H**. Latent infection that can be reactivated with GM-CSF and TNF-α occurs in immature, $CD133^+$ HPCs. **F**. Flow cytometric analysis of $CD133^+$ cells treated with Hxb-ePLAP/VSV-G and sorted to remove actively infected ($PLAP^+$) cells. Numbers indicate the percent of live cells in each quadrant. Live cells were gated using forward scatter and side scatter. **G**. Flow cytometric analysis of cells sorted as in **F** and then stimulated overnight with STIF or GM-CSF and TNF-α. Live cells were defined by FSC, SSC, and 7-AAD. Numbers indicate the percent of cells falling within the indicated gate. **H**. Quantitation of reactivation in $CD133^+$ cells (cells sorted for CD133 prior to stimulation) relative to cells incubated in STIF. Mean and SE of 4 independent experiments are shown. *p = 0.01, 1-sample t-test vs. expected fold increase of 1.

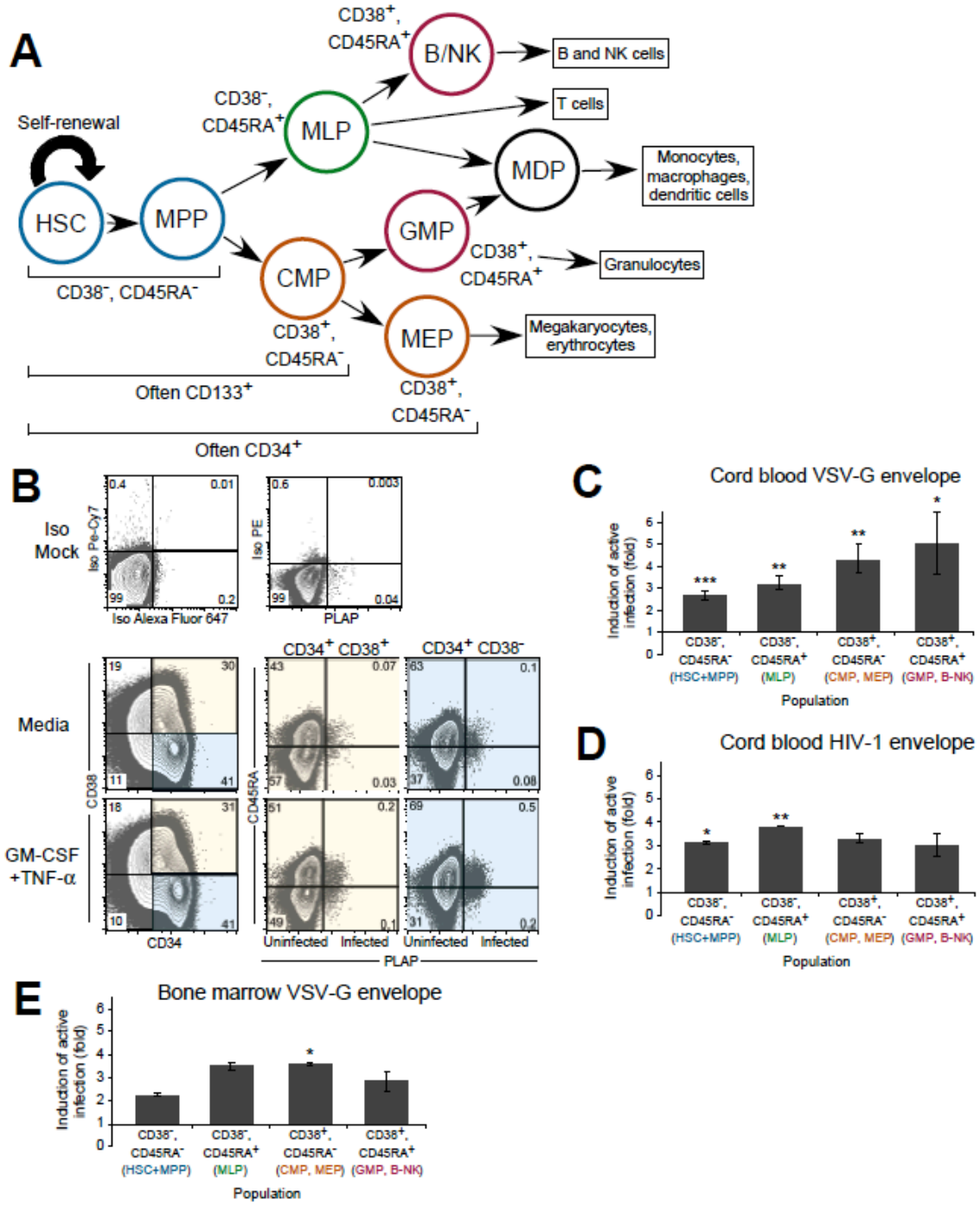

Figure 3.2. Latent infection occurs in all subsets of CD34$^+$ HPCs defined by CD38 and CD45RA expression. **A**. Diagram of hematopoiesis showing CD34, CD133, CD38 and CD45RA expression on each cell subset. HSC, hematopoietic stem cell; MPP, multipotent progenitor; MLP, multilymphoid progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte/erythrocyte progenitor; GMP, granulocyte/monocyte progenitor; B-NK, B and NK cell progenitor. **B**. Flow cytometric analysis of CD133$^+$ cells treated with HXB-ePLAP/HXB env, sorted to remove actively infected (PLAP$^+$) cells, and stimulated overnight with STIF or TNF-α. Live cells were defined using FSC,

SSC, and 7-AAD, then analyzed for CD34, CD38, CD45RA, and PLAP expression. Numbers indicate the percent of total cells in each quadrant. **C**. Summary of experiments similar to those shown in **B** except using HXB-ePLAP/VSV-G envelope to infect cells. Fold induction of active infection in cells treated with GM-CSF and TNF-α or TNF-α compared to cells incubated in media is shown. Labels are colored to match cell types in **A**. Mean and SE of 6 independent experiments are shown. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, 1-sample t-test vs. expected fold increase of 1. Differences in the fold induction of active infection between cell subsets are not significant (1-way ANOVA, $p = 0.26$). **D.** Summary of experiments shown in **B**. Fold induction of active infection in cells treated with GM-CSF and TNF-α or TNF-α vs. unstimulated cells is shown. Mean and SE of 2 independent experiments are shown. *$p<0.05$, **$p<0.01$, 1-sample t-test vs. expected fold increase of 1. Differences in the fold induction of active infection between cell subsets are not significant (1-way ANOVA, $p = 0.53$). **E**. Summary of experiments similar to those shown in **B** except using NL4-3-ΔGPE-GFP/VSV-G envelope to infect bone marrow cells. Fold induction of active infection in cells treated with TNF-α vs. unstimulated cells is shown. Mean and SE of 2 independent experiments are shown. *$p<0.05$, 1-sample t-test vs. expected fold increase of 1. Differences in the fold induction of active infection between cell subsets are not significant (1-way ANOVA, $p = 0.11$).

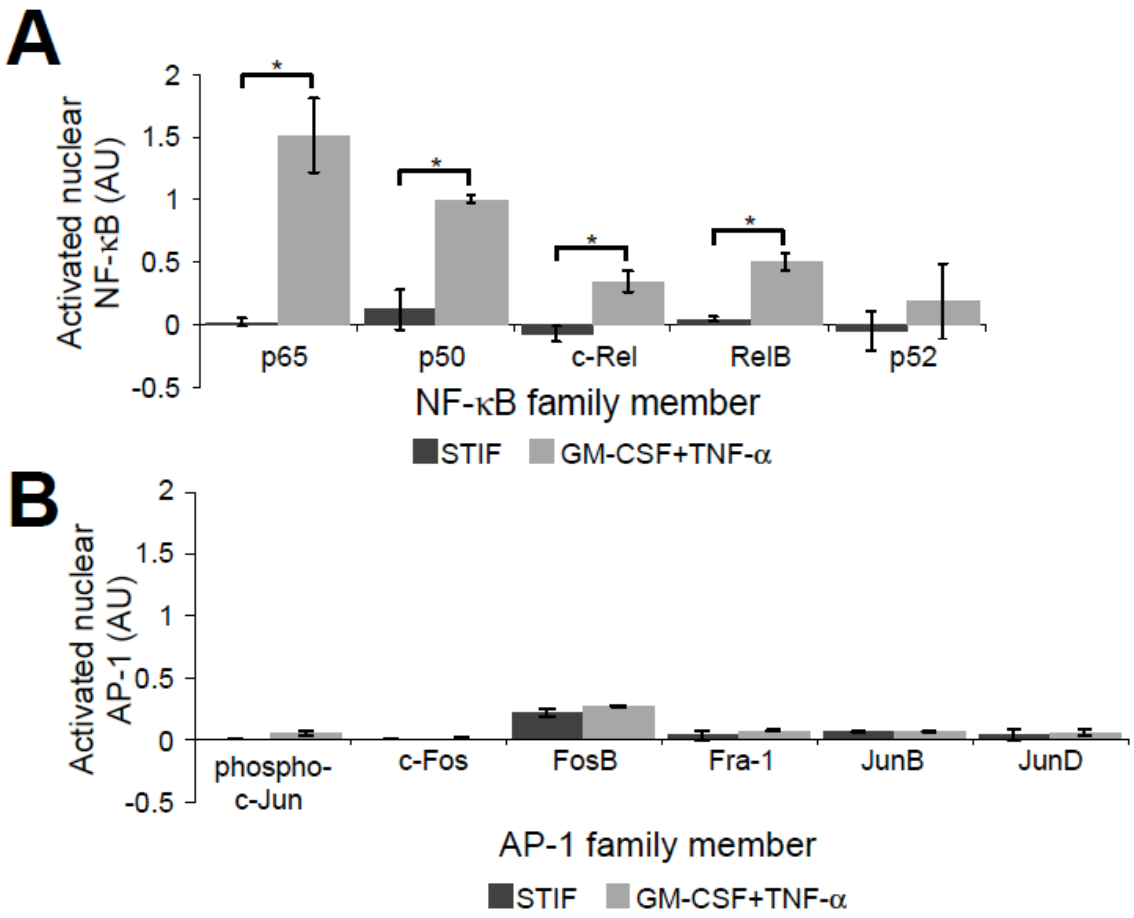

Figure 3.3. GM-CSF and TNF-α-treated HPCs have increased nuclear NF-κB DNA binding activity compared to STIF-treated cells. **A**. Quantitation of transcription factor ELISA measuring NF-κB DNA-binding activity in nuclear lysates from HPCs incubated with STIF or GM-CSF + TNF-α. Absorbance was normalized to the positive control on each plate, then non-specific activity (defined by activity obtained using cytoplasmic extracts) was subtracted from the nuclear absorbance and the difference graphed as arbitrary units (AU). AU for the positive control, Raji nuclear cell extract, was set to 1. The mean and SE of 3 independent experiments are shown. *$p < 0.05$, paired t-test. **B**. Quantitation of transcription factor ELISA measuring AP-1 DNA-binding activity in nuclear lysates from HPCs incubated with STIF or GM-CSF + TNF-α overnight. Data were analyzed as described for part **A** except that nuclear extracts of K-562 cells stimulated with 12-O-tetradecanoylphorbol-13-acetate were used as the positive control. Mean and SE of 2 independent experiments are shown. AP-1 nuclear activity was not significantly elevated by GM-CSF and TNF-α treatment ($p > 0.2$ for all subunits, paired t test); however, JunB expression in STIF-treated cells ($p < 0.05$) and FosB ($p < 0.01$) and Fra-1 ($p < 0.05$) expression in GM-CSF and TNF-α-treated cells were significantly different from 0 by 1-sample t-test.

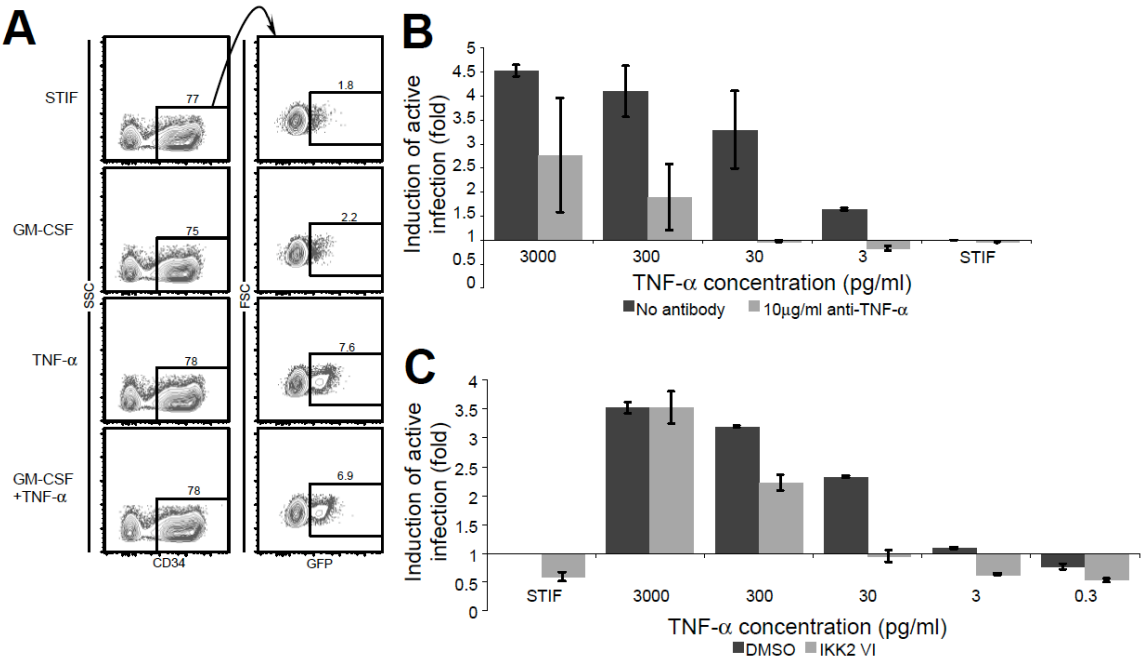

Figure 3.4. TNF-α reactivates latent infection in HPCs via an NF-κB-dependent pathway. **A**. TNF-α is sufficient to reactivate latent virus. Flow cytometric analysis of $CD133^{+}$ cells treated with NL4-3-ΔGPE-GFP/VSV-G, sorted to remove actively infected cells, and incubated overnight with the indicated cytokines. Numbers show the percent of cells falling within the indicated gate. Live cells were gated using FSC, SSC, and 7-AAD. **B**. Quantitative analysis of reactivation in cells treated as described in part **A** and incubated with ten-fold dilutions of TNF-α in the presence or absence of antibody to TNF-α. Mean fold increase in the percentage of cells expressing GFP compared to cells incubated in STIF alone is shown. Error bars represent SE of two independent experiments. **C**. Quantitation of reactivation in cells treated as described in part **B** except that cells were treated with HXB-ePLAP/VSV-G and reactivated with and without the classical NF-κB pathway inhibitor IKK2 VI (10μM). Mean fold increase in the percentage of cells expressing PLAP compared to the STIF+DMSO condition is shown. Error bars represent SE of two independent experiments.

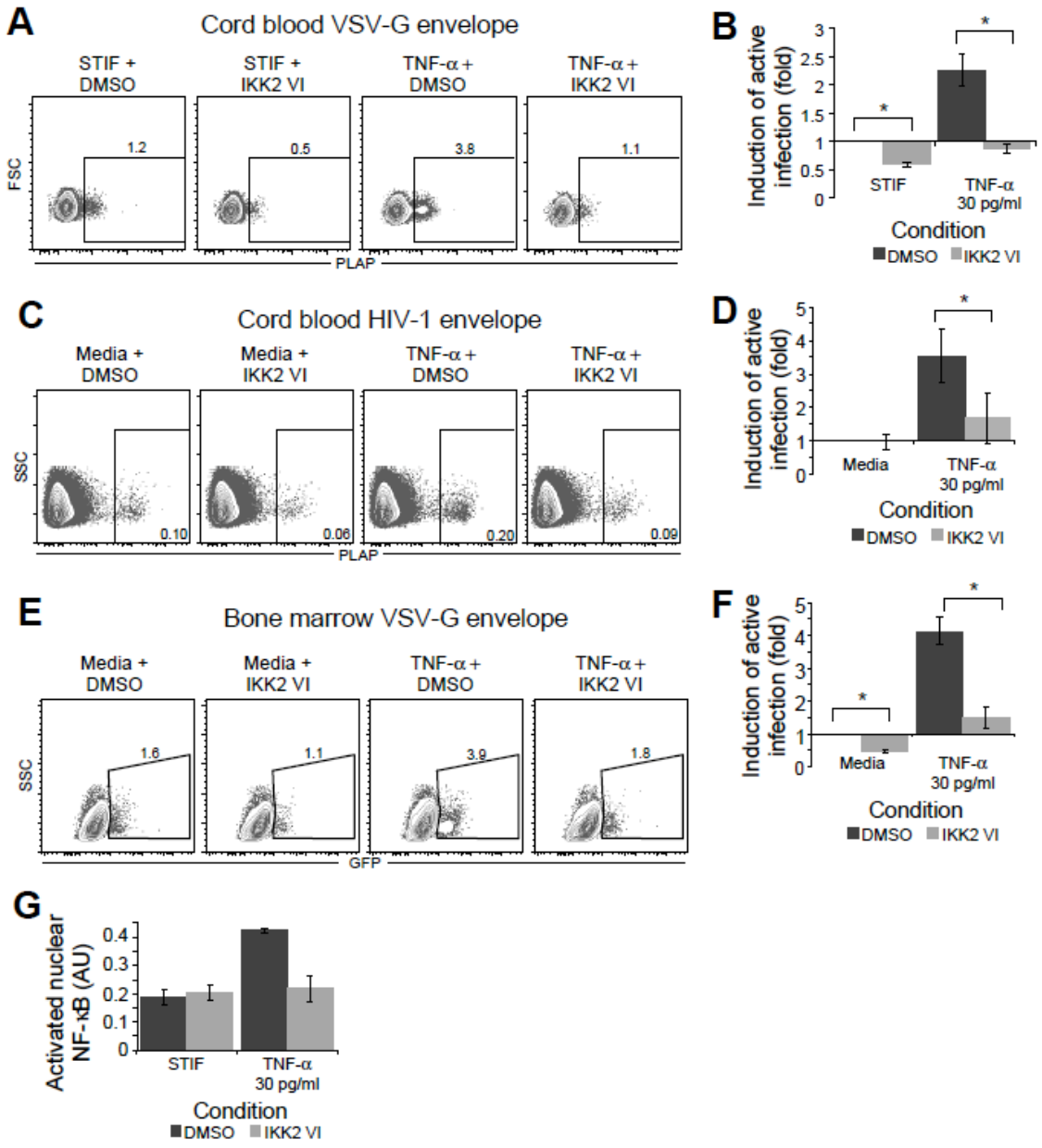

Figure 3.5. NF-κB inhibitor IKK2 VI counteracts TNF-α-induced reactivation of latent virus in HPCs. **A**, **C**, and **E**. Flow cytometric analysis of CD133^{+} cells using the indicated envelope, sorted to remove actively infected cells, and stimulated overnight as indicated. Live cells were gated using FSC, SSC, and 7-AAD; numbers indicate the percent of cells within each gate. Results are representative of 4 (**A**) or 2 (**C** and **E**) independent experiments. **B**, **D**, and **F**. Summary of reactivation for parts **A**, **C**, and **E**, respectively. Fold increase in the percentage of cells expressing PLAP compared to the STIF+DMSO condition was calculated. Mean and SE of 4 (**B**) or 2 (**D** or **F**) independent experiments are shown. *$p < 0.01$ (**B**), 0.02 (**D**), or 0.03 (**F**) by paired t-test. **G.** Quantitation of nuclear NF-kB p50 DNA binding activity for cells treated as for part **A** and analyzed as described in Fig **4A**. Mean and SE of two experiments is shown.

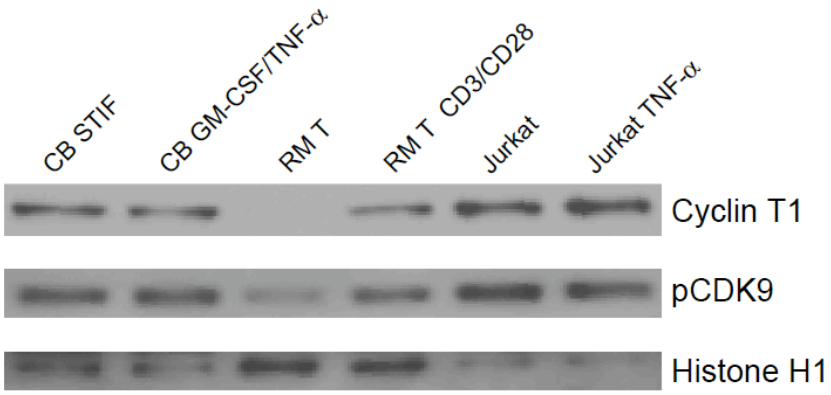

Figure 3.6. Nuclear P-TEFb in HPCs is not increased by stimulation with GM-CSF and TNF-α. Western blot analysis of CyclinT1 and pCDK9 in nuclear extracts from $CD133^+$ HPCs (CB) incubated for 5 days in STIF media and then treated with STIF or GM-CSF (10ng/ml) plus TNF-α (2.5ng/ml). Controls were nuclear extracts from resting memory T (RM T) cells isolated from peripheral blood by magnetic selection for $CD4^+$, $CD45RO^+$, $HLA\text{-}DR^-/CD25^-/CD69^-$ cells, lysed immediately or after stimulation with CD3/CD28 antibody beads overnight, and nuclear extracts from Jurkat cells incubated overnight in media or 3ng/ml TNF-α. Results are representative of two independent experiments using cells from separate donors (T cells) or separate donor pools (cord blood).

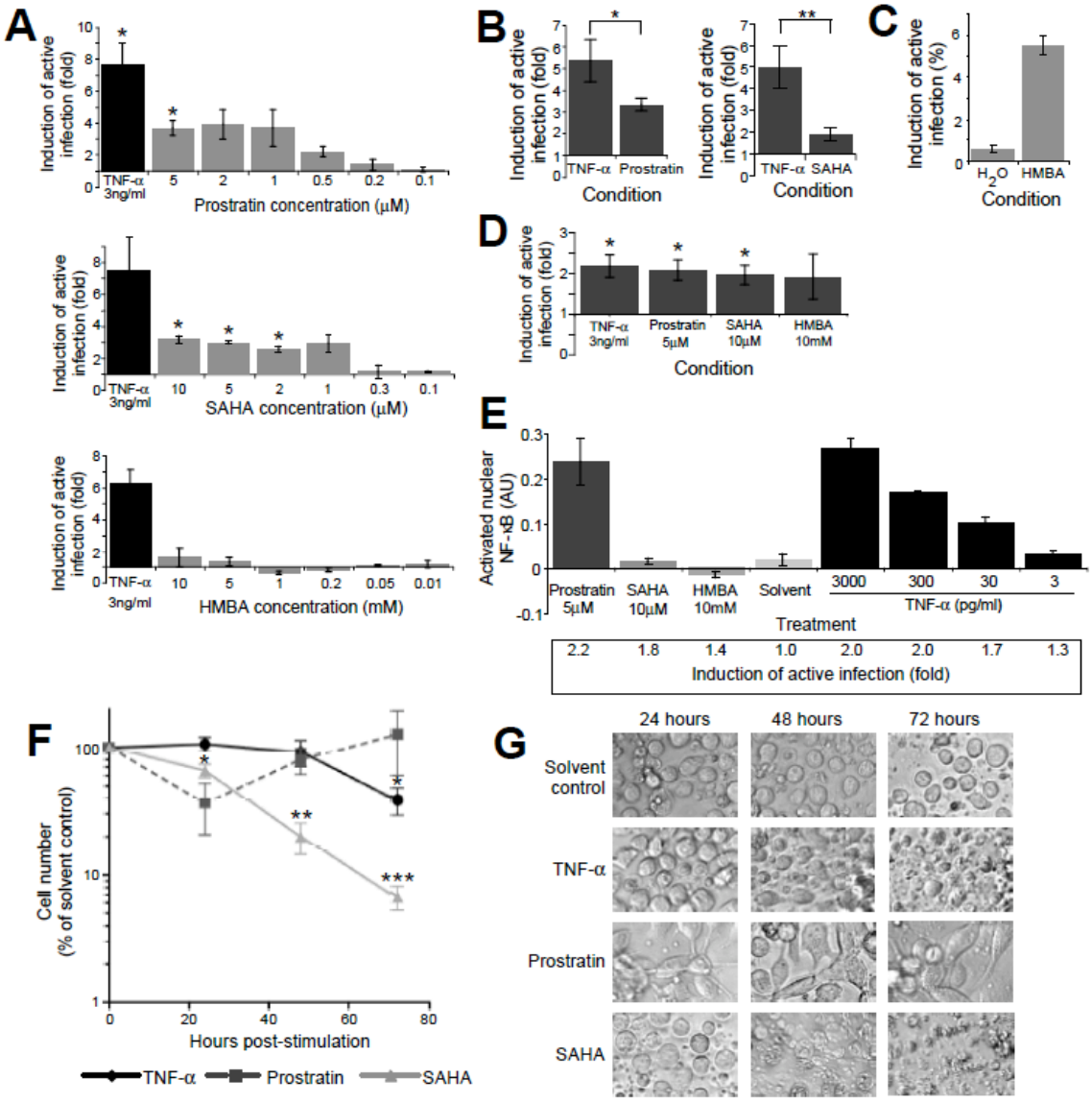

Figure 3.7. Prostratin and SAHA reactivate latent virus in CD34$^+$ HPCs. **A**. Quantitation of viral reactivation in CD133$^+$ cells treated with prostratin, SAHA, or HMBA for 13 hours. Reactivation was measured using the latency reactivation assay described in Fig **1** after infection with NL4-3-ΔGPE-GFP/VSV-G or HXB-ePLAP/VSV-G. Mean fold increase in the percentage of live, CD34$^+$ cells expressing PLAP or GFP compared to solvent control is shown. Error bars represent standard error of 3 (Prostratin, SAHA) or 2 (HMBA) independent experiments. *$p < 0.05$, 1-sample t-test vs. expected fold increase of 1. **B**. Quantitation of reactivation by cells treated with prostratin (5μM, left) and SAHA (10μM, right) for 13 hours using the reactivation assay described in Fig **1**. Mean and SE of 7 independent experiments in cord blood (6) or bone marrow (1) are shown. *p<0.05, **p<0.01, paired t-test. **C**. Quantitation of reactivation by U1 cells stimulated for 48h with 5mM HMBA or solvent control (H_2O) and analyzed by flow cytometry to assess induction of HIV-1 Gag expression. Mean and standard error of three replicates is shown. **D**. Quantitation of reactivation by cells treated with TNF-α, prostratin, SAHA, or

HMBA for 24 hours using the reactivation assay described in Fig. **1**. Mean and SE of 6 (TNF-α), 5 (HMBA), or 4 (prostratin or SAHA) independent experiments is shown. *p < 0.03, 1-sample t-test vs. expected fold increase of 1. Induction of active infection with Prostratin, SAHA, and HMBA is not significantly different from induction with TNF-α stimulation. **E**. Quantitation of transcription factor ELISA measuring nuclear NF-κB DNA binding activity in nuclear extracts from HPCs infected with NL4-3-ΔGPE-GFP/VSV-G and stimulated for 24h with the indicated compounds. ELISA data was analyzed as described in Fig **4A**. Mean and standard deviation of two replicates are shown. Fold increase in percent of cells expressing GFP compared to solvent control is indicated under the graph. Results are representative of two independent experiments using cells from separate donor pools. **F**. Time course of HPC survival when cultured with TNF-α (3ng/ml), prostratin (5μM), or SAHA (10μM). $CD133^+$ HPCs were isolated from cord blood and cultured in STIF for 5 days, then split into solvent control or the conditions shown for an additional 3 days. Cells remaining as a percentage of cells remaining in the solvent control are displayed as a function of time. Mean and SE of 3 independent experiments are shown. *p<0.05, **p<0.01, ***p<0.001, 1-sample t-test vs. expected cell count of 100% of solvent. **G**. Images of the cells analyzed in **F** obtained with light microscopy at 400x magnification. Images were collected at the indicated times post-stimulation. Data are representative of 3 independent experiments.

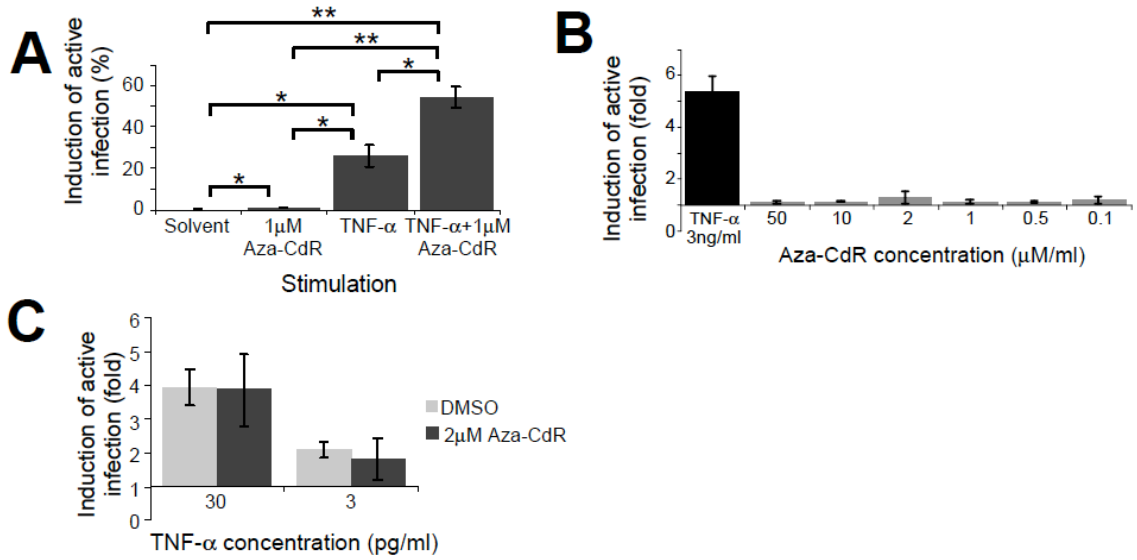

Figure 3.8. Aza-CdR does not reactivate latent virus in CD34$^+$ HPCs. **A**. Quantitation of reactivation in J-Lat cells treated with the indicated compounds and assayed by GFP expression by flow cytometry. J-Lat cells treated with 30ng/ml TNF-α and/or 1μM Aza-CdR for 24h were assayed for GFP expression (reporter for HIV-1 LTR activity) by flow cytometry after an additional 48h. Mean and SE of results from three J-Lat clones (J-Lat 8.4, J-Lat 9.2, and J-Lat 6.3) are shown. *p<0.05, **p<0.01, paired t-test. **B**. Quantitation of reactivation of viral gene expression in CD133$^+$ HPCs using the reactivation assay shown in Fig **1** following treatment with the indicated concentrations of Aza-CdR. Mean fold increase in the percentage of cells expressing GFP compared to solvent control is shown. Error bars represent standard error of 2 independent experiments. **C**. Quantitation of reactivation of viral gene expression in CD133$^+$ HPCs using the reactivation assay shown in Fig **1** following treatment with TNF-α and either Aza-CdR or DMSO solvent control. Fold increase in the percentage of cells expressing GFP compared to solvent control was calculated. Mean and standard error of 2 independent experiments are shown.

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Chapter 4

HIV genomes in CD133$^+$ hematopoietic progenitors in optimally treated people with long-term viral suppression cannot be explained by CD3$^+$ T cell contamination

Abstract

Background. Hematopoietic progenitor cells (HPCs) in the bone marrow of HIV$^+$ individuals have been proposed as a persistent reservoir of virus. However, some studies have suggested that HIV genomes detected in HPCs arise from T cell contamination.

Methods. CD133-sorted HPCs and CD133-depleted bone marrow cells were purified from 11 antiretroviral-treated donors with a viral load of <48 copies per ml for at least 6 months. CD133 and CD3 expression on the cells was assessed by flow cytometry. HIV DNA was quantified by real time PCR.

Results. HIV genomes were detected in CD133-sorted samples from 6 donors, including two with undetectable viral loads for more than 8 years. CD3$^+$ cells represented less than 1% of cells in all CD133-sorted samples. For 5 of 6 CD133-sorted samples with detectable HIV DNA, the HIV genomes could not be explained by contaminating CD3$^+$ cells. Donors with detectable HIV DNA in HPCs were diagnosed significantly more

recently than the remaining donors but had had undetectable viral loads for similar periods of time.

Conclusions. HIV genomes can be detected in CD133-sorted cells from a subset of donors with long-term viral suppression and in most cases cannot be explained by contamination with $CD3^+$ cells.

Introduction

Latent HIV infection represents a major barrier to curing HIV (Finzi et al. 1999, Zhang et al. 1999). When HIV establishes a latent infection within a cell, the DNA provirus integrates into the host cell's genome but viral genes are not transcribed (reviewed in Geeraert et al. 2008). As a result, the latently infected cell is indistinguishable from an uninfected cell and cannot be targeted by the immune system or current antiretroviral therapies. The HIV provirus can persist in this state for the lifetime of the cell; however, it can also be reactivated if cellular conditions change, leading to the production of new virions and potentially new infection events (reviewed in Trono et al. 2010). Thus, HIV replication will resume if antiretroviral therapy is discontinued unless all latent reservoirs of virus are eliminated.

Although resting memory $CD4^+$ T cells are a well-studied reservoir for latent HIV, not all HIV sequences in the plasma of many successfully treated HIV^+ donors can be matched to sequences in peripheral blood $CD4^+$ T cells (Bailey et al. 2006, Brennan et al. 2009, Sahu et al. 2009). These data suggest that additional reservoirs of virus exist

and contribute to residual viremia during treatment as well as to viral rebound upon treatment interruption (Sahu et al. 2009).

Recently, we proposed that hematopoietic progenitor cells (HPCs) in the bone marrow serve as a reservoir for latent HIV. We assessed HIV-1 infection in $CD34^+$ HPCs from nine HIV-infected donors with undetectable viral loads on highly active antiretroviral therapy (HAART) for at least 6 months (Carter et al. 2010). In four of nine donors, we detected HIV-1 proviral genomes in CD34-sorted cells at a frequency of 3-40 HIV genomes per 10,000 cells (Carter et al. 2010), suggesting that these cells could serve as a reservoir of virus in optimally treated individuals. Comparable amounts of HIV DNA were not observed in bone marrow cells immunodepleted for CD34 (Carter et al. 2010). However, subsequent studies have not detected HIV genomes in $CD34^+$ HPCs from donors with undetectable viral loads (Durand et al. 2012, Josefsson et al. 2012) and have instead suggested that HIV genomes in $CD34^+$ samples may be due to contamination with $CD3^+$ T cells (Durand et al. 2012).

In addition to detecting HIV genomes in $CD34^+$ cells ex vivo, we have shown that $CD34^+$ cells can be infected by CCR5- and CXCR4-tropic HIV in vitro (Carter et al. 2010). We also demonstrated that HPCs expressing CD133, a marker for an immature subset of $CD34^+$ HPCs, can only be infected by CXCR4-utilizing HIV-1 in vitro (Carter et al. 2011). However, HIV infection of $CD133^+$ cells in vivo has not been assessed.

To investigate whether $CD133^+$ HPCs are a reservoir for HIV-1 in vivo, we quantified HIV proviral genomes in $CD133^+$ HPCs from 11 HIV^+ donors with plasma viral loads of <48 copies per ml for at least 6 months. We furthermore analyzed the

frequency of $CD3^+$ T cells in each sample to assess the possibility that HIV genomes could arise from contamination with $CD3^+$ cells.

Methods

Clinical Samples. The donor samples analyzed in this study are a consecutive subset of our cohort, excluding two donors where the CD133-sorted sample did not meet our cutoff of 80% overall purity and two donors where samples had been used up in prior experiments (1 sample) or lost (1 sample). We recruited HIV^+ donors currently on antiretroviral therapy from the University of Michigan HIV-AIDS Treatment Program and obtained informed consent according to a protocol approved by the University of Michigan Institutional Review Board. At the time of aspiration, all donors were over the age of 18, had normal white blood cell counts, and had had a plasma viral load of <48 copies per ml for at least 6 months. Twenty ml of bone marrow was aspirated from the posterior iliac crest of each donor, collected in preservative-free heparin, and processed immediately.

Isolation of CD133-sorted and CD133-depleted cells. Bone marrow mononuclear cells (BMMC) were isolated by Ficoll-Paque density separation (GE Healthcare). Adherent cells were depleted by incubation in serum-free StemSpan media (STEMCELL Technologies) for two hours at 37ºC, then $CD133^+$ cells were isolated by positive selection using a commercially available magnetic sorting kit (Miltenyi Biotec). Cells were sequentially sorted on two columns to increase purity of the CD133-sorted population. BMMCs that flowed through the first column were collected as the CD133-

depleted fraction. Samples were cryopreserved in 10% DMSO in fetal bovine serum until analysis.

Flow cytometric analysis. A fraction of each clinical sample was stained with R-phycoerythrin-conjugated anti-CD133 (Miltenyi Biotec), allophycocyanin-conjugated anti-CD3 (eBioscience), and 7-Aminoactinomycin D (7-AAD). BMMCs from a healthy donor (AllCells) were stained with R-phycoerythrin-conjugated anti-CD133, R-phycoerythrin-Cy7-conjugated anti-CD34 (BD), and 7-AAD. Samples were analyzed on a BD FACSCanto.

DNA amplification. Cells were lysed in MagNA Pure DNA Lysis/Binding Buffer (Roche) and DNA extracted using a MagNA Pure Compact System (Roche). HIV-1 DNA was quantified using a two-step real-time PCR assay. In the first round, 5µl of DNA was amplified in each of 6 to 18 25µl reactions containing 2.5µl 10x Expand Long Template Buffer 2 and 1.875 U Expand Long Template Enzyme mix (Roche), 400nM of primers 1st-Gag-R (5'-CAATATCATACGCCGAGAGTGCGCGCTTCAGCAAG-3') (702-714 in HXB2) and 2nd-LTR-F-univ (5'-GTGTIGAAAATCTCTAGCAGTGGC-3') (616-639), and 500µM dNTPs. In some reactions, 400nM of ß-actin primers ß-actin-F (5'-CCTTTTTTGTCCCCCAACTTG-3') and ß-actin-R (5'-TGGCTGCCTCCACCCA-3') were also added. The 13 bases at the 3' end of the 1st-Gag-R primer are homologous to HIV-1 Gag, while the remaining bases form a tag used in the second round of the PCR.

DNA from ACH-2 cells (Clouse et al. 1989) diluted in DNA from uninfected primary HPCs or peripheral blood mononuclear cells (PBMCs) to a concentration of 10

HIV genomes per µl or 0.2 genomes per µl were used as a positive control and a control for sensitivity, respectively. DNA from uninfected HPCs or PBMCs was used as a negative control. Thermocycling was conducted using a thermocycler preheated to 93ºC with the following cycling conditions: 93ºC for 2 minutes; 12 cycles of 93ºC for 15 seconds, 60ºC for 30 seconds, 68ºC for 1 minute; and a final 1 minute at 68ºC.

Second round quantitative PCR reactions were conducted in triplicate, each using 2µl of the first round reaction in a 50µl reaction. Reactions contained 25µl FastStart TaqMan Probe Master 2x Master Mix (Roche), 1µM each of primers 2nd-LTR-F-univ and 2nd-Tag-R (5'-CAATATCATACGCCGAGAGTGC-3'), and 250nM Gag-probe-2 (5'-FAM-CGCTTCAGCAAGCCGAGTCCTGC-BHQ-1-3') (Biosearch Technologies). Reactions were run and analyzed on an Applied Biosystems 7300 thermocycler (Applied Biosystems) with the following cycling conditions: 95ºC for 10 minutes, then 45 cycles of 95ºC for 15 seconds followed by 60ºC for 60 seconds.

Second round quantitative PCR reactions to amplify ß-actin were conducted to validate sample cell counts. Conditions were identical to those listed for the HIV-1 quantitative PCR except that the ß-actin-F and ß-actin-R primers and a ß-actin probe (5'-FAM-CCCAGGGAGACCAAAAGCCTTCATACA-BHQ-1-3') (Biosearch Technologies) were used.

DNA sequencing. All positive qPCR reactions were run on a 2% agarose gel, then the amplicon was excised from the gel and the DNA extracted using the QIAquick Gel Extraction Kit (Qiagen). Amplicons were sequenced by Sanger dideoxy sequencing and analyzed using 4Peaks (Mekentosj), EditSeq (DNAStar), and MEGA 5.05.

Statistical analysis. 95% confidence intervals for cell counts were generated using Excel 2004 (Microsoft). 95% confidence intervals for the fraction of cells that were $CD133^+$ and $CD3^+$ were generated using a 95% confidence interval generator for binomial distributions (statpages.org/confint.html). Fisher's exact test was performed using an online calculator (www.langsrud.com/fisher.htm). The Mann Whitney test was performed using GraphPad Prism version 5.0a.

Results

Donor characteristics. The 11 donors included in this study had been diagnosed with HIV infection for an average of 11.9 years (standard deviation [s.d.] 8.1 years, range 3 – 24 years) (**Table 4.1**). Donors had viral loads of less than 48 copies per ml for at least 6 months (mean 4.1 years, s.d. 2.7 years, range <1 – 8.4 years) and were being treated with at least 3 active antiretroviral agents at the time of bone marrow aspiration (**Table 4.1**).

Magnetic sorting for CD133 minimizes contamination with $CD3^+$ T cells. Bone marrow mononuclear cells (BMMC) from each donor were subjected to magnetic sorting for CD133 (**Figure 4.1A**), a cell surface marker found on a subset of $CD34^+$ cells (**Figure 4.1B**). CD133 and CD3 expression was analyzed on the CD133-sorted and CD133-depleted populations by flow cytometry (**Figure 4.1C**). Although the purity of the CD133-sorted populations varied from 84.4 – 98.9% for different donors, less than 1% of the cells in each CD133-sorted sample were positive for CD3 (**Figure 4.1C** and **Table 4.2**). By contrast, CD133-depleted samples contained 36-82% $CD3^+$ cells.

HIV DNA is detected in CD133-sorted BMMCs. We used a two-step, quantitative PCR assay to determine the frequency of HIV genomes in CD133-sorted and CD133-depleted samples. Donor samples were at limiting dilution as prepared, with no more than 1/3 of reactions yielding HIV amplification for any sample. HIV-1 DNA was detected in CD133-sorted samples for 6 of 11 donors and in CD133-depleted samples for 6 of 11 donors with between 0 and 2 total copies of HIV DNA detected per sample (**Table 4.2**). The frequency of cells containing HIV DNA varied from 0 to 42 per 100,000 cells for CD133-sorted samples and from 0 to 49 per 100,000 cells for CD133-depleted samples (**Table 4.2**).

$CD3^+$ T cells are unlikely to account for HIV DNAin CD133-sorted samples. If the HIV genomes detected in our samples derived from $CD3^+$ T cells, we would expect to observe many more HIV genomes in the CD133-depleted samples, which contain 36-82% $CD3^+$ cells, than in the CD133-sorted samples, which contain less than 1% $CD3^+$ cells. However, we instead observed that the frequency of HIV genomes in the CD133-sorted samples was higher than the frequency of HIV genomes in the CD133-depleted samples for 4 of 11 donors (**Table 4.2**). To further assess the possibility that contamination with $CD3^+$ cells accounted for the HIV genomes observed in CD133-sorted samples, we calculated what the frequency of HIV genomes in the $CD3^+$ cells in each CD133-sorted and CD133-depleted sample would be if all genomes detected derived from $CD3^+$ cells (**Figure 4.2A**). If all of the HIV genomes were derived from $CD3^+$ cells, then we would expect these calculated frequencies to be similar in the CD133-depleted and CD133-

sorted samples for each donor. Instead, we found that for each donor where an HIV genome was detected in the CD133-sorted sample, the calculated frequency of HIV genomes in the $CD3^+$ cells from the CD133-sorted sample was much higher than the calculated frequency of HIV genomes in the $CD3^+$ cells from the CD133-depleted sample (**Figure 4.2A**). Using Fisher's exact test, we found that the calculated frequency of HIV genomes in the $CD3^+$ cells in the CD133-sorted sample was significantly greater than the calculated frequency of HIV genomes in the $CD3^+$ cells in the CD133-depleted sample for donors 304000, 305000, 312101, 313212, and 315214 ($p < 0.01$ using mean estimates of $CD3^+$ cell number, $p < 0.01$ (donors 305000 and 312101) or $p < 0.05$ (donors 304000, 313212, and 315214) using conservative estimates; see legend for **Figure 4.2A**) (**Figure 4.2A**). For donor 311000 the same trend was observed but statistical significance was not achieved ($p = 0.066$ using mean estimates) (**Figure 4.2A**). We therefore conclude that for at least 5 of the 6 donors with detectable HIV DNA in CD133-sorted cells, it is unlikely that the HIV DNA we detected in the sorted samples comes from T cell contamination.

We used an analogous calculation to determine whether total contaminating $CD133^-$ cells could account for the HIV DNA observed in CD133-sorted samples. For this analysis, we calculated what the frequency of HIV genomes in the $CD133^-$ cells from the CD133-sorted and CD133-depleted samples would be if all genomes derived from $CD133^-$ cells. Using this analysis, we found that the frequencies of HIV genomes calculated to be present in the $CD133^-$ cells in the CD133-sorted and CD133-depleted samples were significantly different for donors 305000 and 312101 ($p < 0.05$) (**Figure 4.2B**). For donors 313212 and 315214, a significant difference was observed using mean

estimates ($p < 0.05$) but not conservative estimates ($p = 0.0506$ or 0.136, respectively) of $CD133^-$ cell number (see **Figure 4.2A** legend). Donors 304000 and 311000 demonstrated the same trend, but statistical significance was not achieved ($p = 0.096$ (donor 31025) or 0.105 (donor 31031) using mean estimates) (**Figure 4.2B**). We therefore conclude that total $CD133^-$ contaminants are unlikely to be the source of HIV genomes in the CD133-sorted samples from donors 305000 and 312101. Furthermore, for donors 304000, 313212, and 315214 we were able to rule out $CD3^+$ T cell contamination as the source of the HIV genomes in the CD133-sorted samples, making $CD133^+$ progenitors the most likely source of the genomes detected based on current knowledge of the cell types that can serve as long-lived reservoirs for HIV.

HIV sequencing and assessment of contamination. To assess whether the HIV DNA detected may have arisen from laboratory contamination, amplicons from the quantitative PCR reaction were separated from the primers and probe on a 2% agarose gel. In all cases, a distinct band of approximately 120 base pairs was observed, confirming successful amplification (**Figure 4.3A**). PCR products were extracted from the gel and sequenced, then aligned to the positive control (HXB2 DNA, **Figure 4.3B**). An analysis of HXB2 DNA from three single copy reactions amplified alongside the donor samples revealed that all three sequences were identical and agreed with the HXB2 reference sequence. In contrast, no two donors samples yielded identical sequences. Unsurprisingly, we did observe identical sequences within the CD133-sorted and CD133-depleted fractions from the same donor (311000 and 315214). Compared with the HXB2 reference sequence, the number of differences we observed was similar to that observed

in samples from the Los Alamos database (**Figure 4.3C**). Thus, it is unlikely that the positive results we obtained are due to contamination by the positive control template.

*Stability of HIV DNA in HPCs over time***.** As noted in **Table 4.1**, four donors had donated samples for prior published studies (Carter et al. 2010, Carter et al. 2011). At the time of the previous donation, two of these donors had high viral loads (>50,000 copies/ml; donors 308103 and 312101) and subsequently started therapy. The other repeat donors had undetectable viral loads at the time of previous donation (donors 313212 and 315214). In the prior study, HIV DNA was detected in HPC samples from both of these donors (previously referred to as donors 12 and 14, respectively (Carter et al. 2010)). In concordance with these results, we also detected HIV DNA in the current HPC samples, which were collected after an additional 3.3 years of suppressive therapy. Thus, HIV infection of HPCs can be consistently detected in the same donors after years of suppressed viral replication.

Year of diagnosis is associated with detection of HIV DNA in CD133-sorted cells. In our prior study we had noted that of the 9 total donors, all 4 donors with detectable HIV DNA in progenitor cells were diagnosed relatively recently (in 2001 or later) whereas all three donors that had been diagnosed prior to 2001 were PCR negative. We assessed whether this trend held in our current cohort and observed that donors with a positive PCR result were diagnosed significantly more recently than donors that did not have detectable provirus in CD133-sorted samples ($p < 0.02$, t-test) (**Figure 4.4A**). One sample from a recently diagnosed donor was negative in the current study (308103). However, only

14,000 cells could be analyzed from this donor (**Table 4.2**) and thus this may be a false negative result. The association between infection of HPCs and year of diagnosis does not result from differences in the duration of time these donors had received fully suppressive HAART ($p = 0.49$, t-test) (**Table 4.1** and **Figure 4.4B**) or from differences in the purity of the CD133-sorted samples or the percentage of T cells present ($p = 0.65$ or 0.29, respectively.

Discussion

Because reservoirs of latent virus represent a barrier to curing HIV, it is essential to identify all sources of persistent virus. We have previously shown that $CD34^+$ HPCs may serve as a persistent reservoir of virus in donors with HIV viral loads of <48 copies per ml (Carter et al. 2010); however, subsequent studies suggested that contamination with $CD3^+$ cells may account for our positive results (Durand et al. 2012) or that HIV genomes in HPCs may not persist during years of therapy (Josefsson et al. 2012). In the current study, we extend our previous findings by showing that HIV can be detected in the immature, $CD133^+$ subset of HPCs from donors with undetectable viral loads, including two donors where we had detected HIV DNA in $CD34^+$ HPCs in samples donated for our previous study 3 years earlier (Carter et al. 2010). We furthermore demonstrate that for 5 of 6 CD133-sorted samples where HIV genomes were detected, $CD3^+$ cell contamination does not provide a good explanation for our positive results. Finally, we demonstrate that HIV DNA can be detected in HPCs from antiretroviral-treated patients who have had suppressed viral loads for up to 8 years. Together, these

findings demonstrate that HPCs, including $CD133^+$ HPCs, are a persistent reservoir of HIV DNA during therapy.

The mean reported frequencies of HIV DNA in the $CD34^+$ cells examined in our prior study (Carter et al. 2010) were higher than those reported for the $CD133^+$ cells examined here. However, the 95% confidence intervals for the true frequency of genomes in these cell populations are overlapping in the two donors who donated samples in both studies (data not shown). These 95% confidence intervals are very broad because of the low number of detectable genomes. To better quantify the number of genomes in each cell population and to permit comparison with the frequency of HIV genomes in peripheral blood resting memory T cells, additional studies are needed that use larger cell numbers and that simultaneously compare HIV proviral DNA frequencies in all of these cell populations from the same donor.

We report here that donors with evidence of infected HPCs were diagnosed significantly more recently than donors without evidence of infection. This result cannot be explained by a shorter period of suppressive therapy or by the number of contaminating $CD3^+$ cells. Instead, we hypothesize that individuals with high levels of HIV infection in HPCs are less likely to have survived or maintained low viral loads until the present if they were diagnosed prior to the advent of HAART. This reduced survival might be due to higher levels of CXCR4-tropic virus, which we have previously shown to be required for infection of immature HPCs in vitro (Carter et al. 2011) and which is associated with more rapid disease progression (Connor et al. 1997, Karlsson et al. 1994, Scarlatti et al. 1997, Shepherd et al. 2008, Waters et al. 2008, Weiser et al. 2008, Yu et al. 1998), or to an as-yet uncharacterized factor.

In a recent study by Durand et al., which failed to detect evidence of infection of HPCs in optimally treated donors, 10 of 11 total donors were diagnosed prior to 2001 and 5 of 11 donors were diagnosed during the 1980s (Durand et al. 2012). Based on the results reported here, it is not surprising that positive results were not achieved in this study. In addition, the protocol used by Durand et al. included an overnight incubation in serum-containing media whereas our one-day protocol utilized media optimized to maintain progenitors in an undifferentiated state that preserves latent infection (McNamara et al. 2012).

A second study authored by Josefsson et al., also failed to detect evidence of HIV infection of HPCs in eight optimally treated donors who were more recently diagnosed. However, methodological differences may have contributed to these negative results. Josefsson and colleagues excluded $CD4^+CD34^+$ cells from the HPC population studied (Josefsson et al. 2012). Because we have previously shown that CD4 expression on HPCs is required for infection of HPCs in vitro (Carter et al. 2011), the exclusion of $CD4^+CD34^+$ cells would exclude the $CD34^+$ cell population most likely to contain HIV genomes. Furthermore, the primers used in our PCR analysis are substantially more conserved than those used by Josefsson and colleagues (data not shown), which could limit the sensitivity of their assay to detect variable donor HIV sequences.

In vitro, $CD133^+$ HPCs are almost exclusively infected by HIV-1 envelopes that use CXCR4 as a coreceptor (Carter et al. 2011). We would therefore expect that at least a minor population of CXCR4-tropic virus exists in the 6 donors for whom we detected HIV DNA in CD133-sorted cells. This is consistent with studies showing that isolates predicted to use CXCR4 can be detected as a minor population in 12-50% of recently-

infected patients (Abbate et al. 2011, Chalmet et al. 2012, Daar et al. 2007); furthermore, CXCR4-utilizing virus persists in patients on suppressive therapy (Seclén et al. 2012, Soulie et al. 2007) and may become more prevalent during therapy in some patients (Delobel et al. 2005, Hunt et al. 2006). Further study to assess HIV envelope tropism in our cohort is needed to confirm the role of HIV envelope tropism in the infection of HPCs in vivo.

Our results demonstrate that HIV genomes can be detected in $CD133^+$ HPCs from a subset of donors with undetectable viral loads and that the genomes detected are not explained by contamination with $CD3^+$ T cells. While these findings do not prove that HPCs serve as a reservoir for HIV in these donors, as the genomes detected may be defective, they do indicate that HPCs can retain HIV DNA during years of successful antiretroviral therapy. We are currently investigating the contribution of HIV genomes in HPCs to residual viremia in treated donors. Meanwhile, strategies to reactivate latent virus from HPCs should be considered alongside strategies that reactivate virus in resting memory T cells to develop therapies with the best chance of eliminating all reservoirs of persistent HIV.

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Table 4.1. Donor characteristics

Donor identifier	Year of diagnosis	Time on ART with VL < 50 copies per ml (years)	CD4 count (cells per µl)	WBC (10^9 cells per L)	BMMC (10^6 per ml)	Repeat donor?[1]
303000	1990	1.0	1026	4.5	15.9	No
304000	2004	3.3	533	4.8	9.35	No
305000	2007	> 0.5	1421	6.4	15.4	No
306000	1986/1987	3.4	1039	9.4	11.4	No
307000	1992	5.8	829	8.0	20.7	No
308103	2008	1.6	852	5.3	5.05	Yes (3)
311000	1999	8.2	564	7.2	6.25	No
312101	2006	2.2	812	7.5	8.75	Yes (1)
313212	2002	8.4	466	4.0	9.00	Yes (12)
314000	Late 1980's	5.1	543	7.6	4.22	No
315214	2006	5.0	850	6.3	4.11	Yes (14)

[1]Number in parentheses indicates the donor number in Carter et al. 2010.

Note: VL = viral load; WBC = white blood cells; BMMC = bone marrow mononuclear cells.

Table 4.2. Purity of samples and frequency of HIV genomes detected

Donor identifier	10^4 Cells analyzed (95% CI)	% CD133+ cells (95% CI)	%CD3+ cells (95% CI)	HIV genomes detected	Frequency of HIV genomes per 10^5 cells in vivo (95% CI)
CD133-sorted samples					
303000	14 (9.7 – 17)	98.8 (98.4 – 99.0)	0.05 (0.01 – 0.14)	0	<0.71 (0 – 3.8)
304000	2.7 (2.4 – 3.0)	84.4 (81.6 – 86.9)	0.80 (0.29 – 1.7)	1	3.7 (0.084 – 23)
305000	6.0 (5.6 – 6.4)	94.1 (93.3 – 94.9)	0.59 (0.36 – 0.89)	1	1.7 (0.040 – 9.9)
306000	5.4 (4.8 – 6.0)	94.3 (93.5 – 95.0)	0.62 (0.39 – 0.94)	0	<1.9 (0 – 7.7)
307000	12 (11 – 13)	93.2 (92.5 – 93.8)	0.22 (0.12 – 0.38)	0	<0.83 (0 – 3.3)
308103	1.4 (1.0 – 1.7)	92.1 (90.2 – 93.8)	0.23 (0.03 – 0.82)	0	<7.1 (0 – 37)
311000	4.1 (3.6 – 4.5)	97.7 (97.1 – 98.3)	0.83 (0.51 – 1.3)	1	2.5 (0.057 – 15)
312101	8.4 (5.9 – 11)	98.9 (98.7 – 99.1)	0.06 (0.02 – 0.15)	1	1.2 (0.023 – 9.5)
313212	5.4 (4.3 – 6.5)	98.4 (98.0 – 98.7)	0.29 (0.16 – 0.48)	1	1.9 (0.039 – 13)
314000	0.96 (0.83 – 1.1)	85.8 (81.4 – 89.5)	0.65 (0.08 – 2.3)	0	<10.4 (0 – 44)
315214	0.48 (0.35 – 0.61)	92.4 (89.4 – 94.8)	0.76 (0.16 – 2.2)	2	42 (4 – 210)
CD133-depleted samples					
303000	18 (13 – 23)	5.0 (4.0 – 6.2)	36 (34 – 38)	2	1.1 (0.11 – 5.7)
304000	4.0 (3.6 – 4.3)	0.18 (0.01 – 1.5)	61 (56 – 66)	0	<2.5 (0 – 10)
305000	17 (14 – 20)	0.13 (0.03 – 0.43)	52 (50 – 54)	0	<0.59 (0 – 2.7)
306000	1.8 (1.7 – 2.0)	0.31 (0.08 – 1.1)	73 (70 – 76)	0	<5.6 (0 – 22)
307000	13 (12 – 14)	1.6 (1.0 – 2.4)	43 (40 – 46)	1	0.77 (0.017 – 4.8)
308103	1.1 (0.90 – 1.2)	0 (0 – 0.69)	75 (71 – 79)	2	19 (2.0 – 80)
311000	2.5 (2.1 – 2.9)	0 (0 – 0.74)	58 (54 – 63)	2	8.1 (0.84 – 35)
312101	5.4 (4.7 – 6.0)	0 (0 – 1.1)	44 (38 – 49)	0	<1.9 (0 – 7.8)
313212	3.1 (2.4 – 3.8)	0 (0 – 0.55)	59 (55 – 63)	0	<3.2 (0 – 15)
314000	0.54 (0.42 – 0.66)	0 (0 – 0.76)	82 (78 – 85)	1	19 (0.38 – 130)
315214	0.41 (0.32 – 0.50)	0 (0 – 0.79)	81 (77 – 84)	2	49 (4.9 – 224)

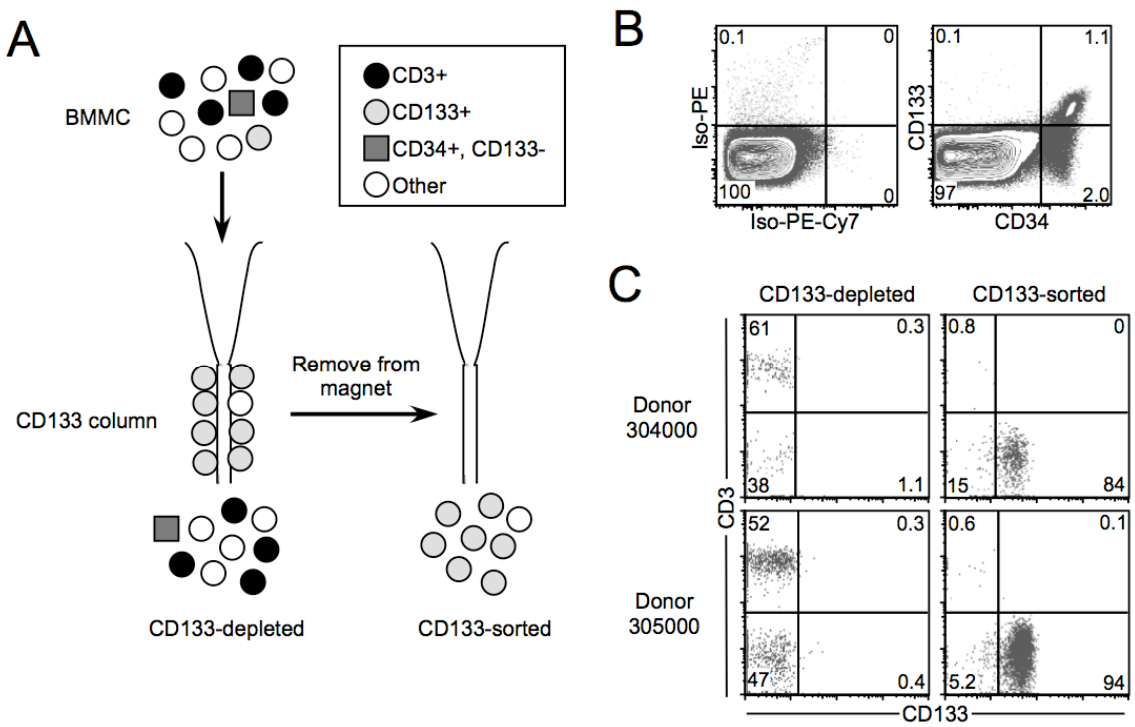

Figure 4.1. CD133$^+$ cells isolated by magnetic sorting are minimally contaminated with CD3$^+$ T cells. **A**, Purification protocol. CD133$^+$ hematopoietic progenitor cells (HPCs) were isolated from total bone marrow mononuclear cells (BMMC) using anti-CD133-conjugated magnetic beads. Cells were sequentially sorted on two columns to maximize the purity of the CD133-sorted population. Bone marrow cells not expressing CD133, including more mature CD34$^+$CD133$^-$ HPCs, were collected in the CD133-depleted fraction. **B**, Example of CD133 and CD34 staining on adherence-depleted bone marrow cells from a healthy donor. Live cells were gated based on forward scatter (FSC), side scatter (SSC), and 7-aminoactinomycin D (7-AAD) uptake. Numbers indicate the percent of the population falling into each quadrant. The percentage of BMMCs that are CD34$^+$ ranges from 0.1 to 5% between donors. **C**, Flow cytometric analysis of CD133 and CD3 expression in CD133-sorted and CD133-depleted samples. Live cells were gated based on FSC, SSC, and 7-AAD uptake. Numbers indicate the percent of the population falling into each quadrant.

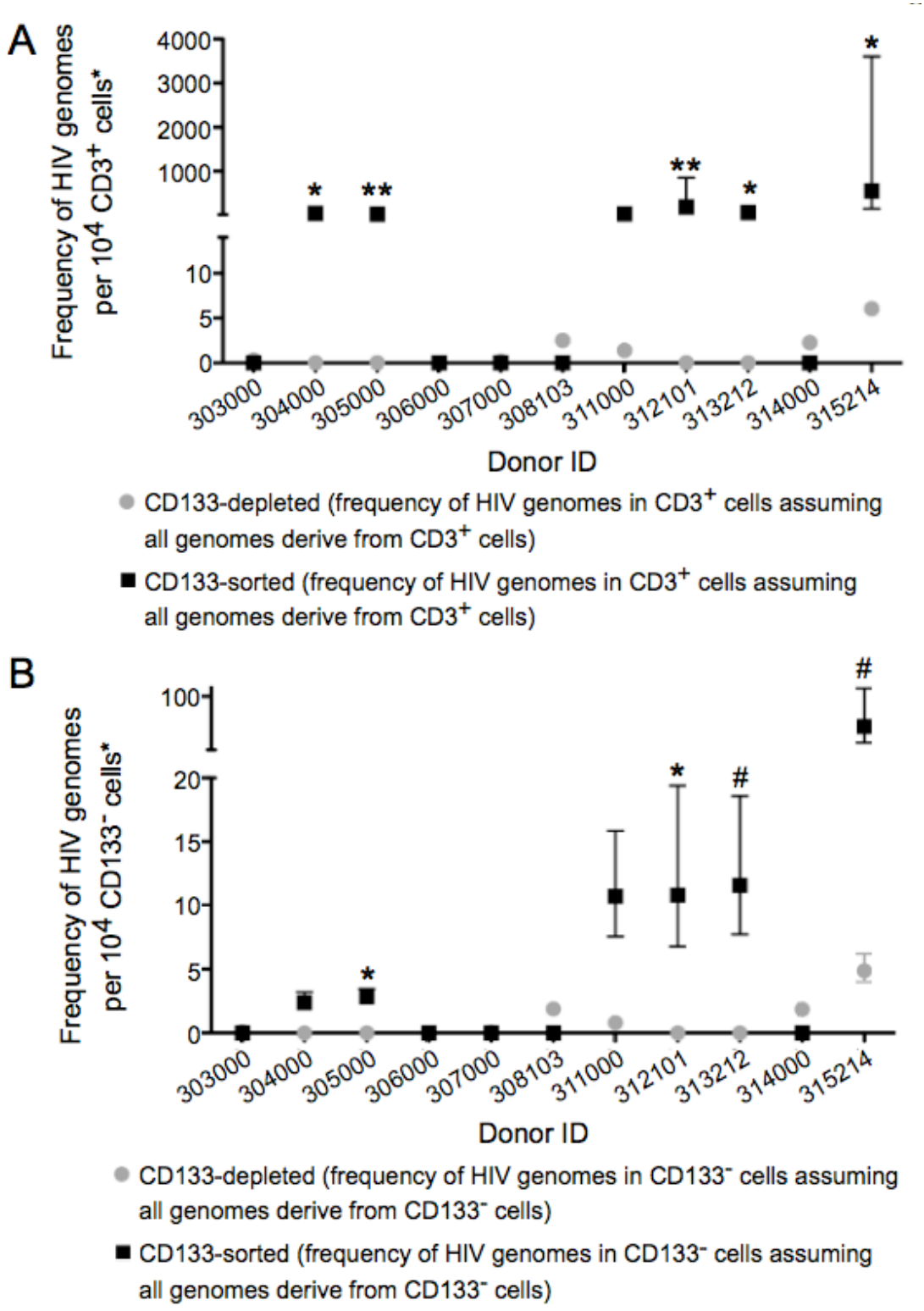

Figure 4.2. HIV genomes detected in CD133-sorted samples are not attributable to contamination with $CD3^+$ cells for a majority of samples. **A**, Comparison of the frequency of HIV genomes in $CD3^+$ cells from CD133-depleted samples (round symbols) with the frequency (square symbols) of HIV genomes in $CD3^+$ cells in CD133-sorted

samples under the assumption that all HIV genomes are found in $CD3^+$ cells. Error bars indicate 95% confidence intervals for the frequency of HIV genomes in these cells. For these calculations, the total number of HIV genomes detected in each sample was divided by the total number of $CD3^+$ cells analyzed in each sample (= (total number of cells analyzed) X (fraction of cells that are $CD3^+$)). Fisher's exact test was used to compare these calculated frequencies using (1) a mean estimate of the number of $CD3^+$ cells analyzed as well as (2) a conservative estimate. The conservative estimate used the upper bounds of the 95% confidence intervals for the observed number of $CD3^+$ cells analyzed in the CD133-sorted samples and the lower bounds of the 95% confidence intervals for the number of $CD3^+$ cells analyzed in the CD133-depleted samples. * $p < 0.01$ by (1), $p < 0.05$ by (2); ** $p < 0.01$ by (1) and (2). **B**, As **A**, except that it was assumed that all genomes were found in total $CD133^-$ cells. Mean (1) and conservative (2) estimates of the total number of $CD133^-$ cells in each sample were calculated as in **A**. * $p < 0.05$ by (1) and (2); # $p < 0.05$ by (1) but not by (2).

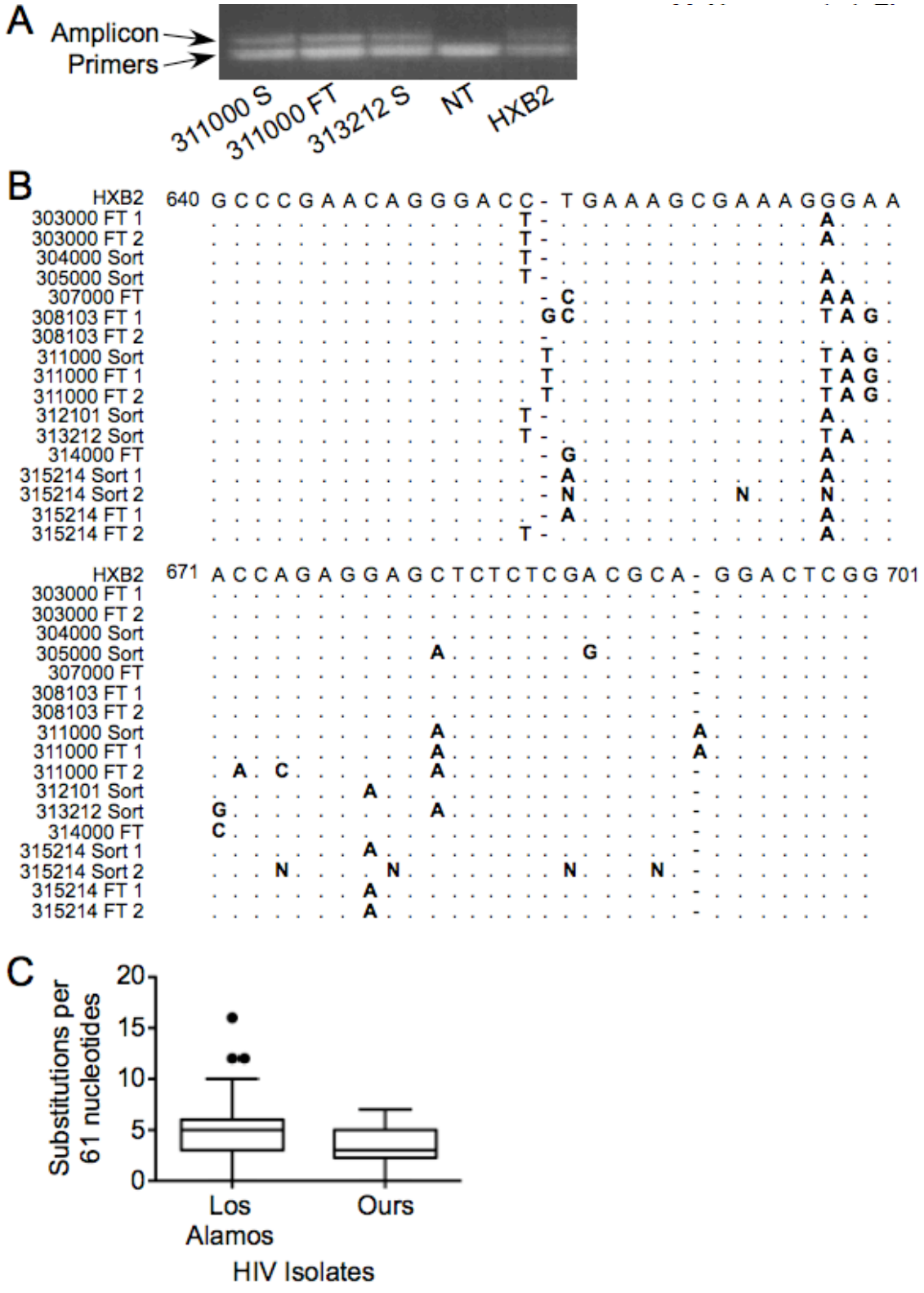

Figure 4.3. Sequence analysis of PCR products does not suggest contamination with HXB2 DNA. **A**, Example of agarose gel analysis and purification of qPCR products. S, CD133-sorted sample from the listed donor; FT, CD133-depleted (flowthrough); NT, no-

template PCR control; HXB2, single copy of HXB2 HIV-1 DNA amplified from ACH-2 cells. **B**, Alignment of donor sequences with HXB2. Numbers indicate coordinates in the HXB2 reference sequence. The HXB2 sequence was obtained by sequencing 3 single-copy qPCR reactions of HXB2 genomes from ACH-2 cells. All 3 sequences were identical to the HXB2 reference sequence. N's in the 315214 Sort 2 sequence are due largely to double peaks, suggesting that more than one HIV genome was present. **C**, Comparison of the number of differences from HXB2 in our donor sequences and the number of differences from HXB2 in all subtype B isolates in the Los Alamos database that have been sequenced through the region of our 61 nucleotide qPCR amplicon (n = 378, 1 sequence per patient). Each base pair change, insertion, and deletion was counted as 1 difference. Box plots indicate median, first and third quartiles, and minimum and maximum excluding outliers; outliers are indicated with dots. The 315214 Sort 2 sequence was excluded due to the ambiguities in the sequence. The median number of differences from HXB2 is not significantly different in the two samples ($p = 0.22$, Mann Whitney test).

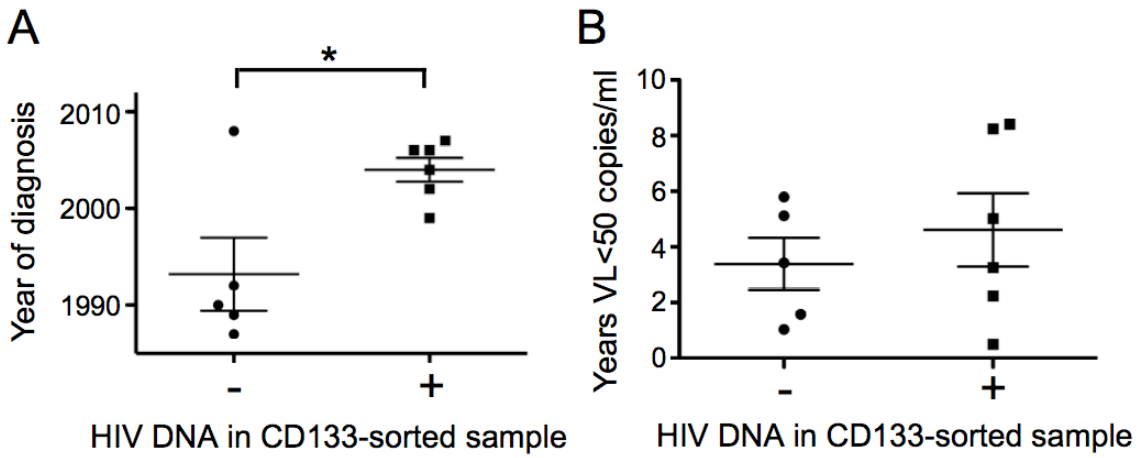

Figure 4.4. Donors with detectable HIV DNA in CD133-sorted samples were diagnosed significantly more recently than donors without detectable HIV DNA in CD133-sorted cells. **A**, Comparison of the mean year of diagnosis of donors with or without detectable HIV DNA in CD133-sorted cells. * $p < 0.02$, t-test. Lines indicate mean and standard error; symbols indicate individual values. Conservative estimates of the year of diagnosis were used in cases where the year of diagnosis was not known precisely (1987 for donor 306000 and 1989 for donor 314000). **B**, Comparison of the mean length of time that viral load in the plasma has been undetectable in donors with or without detectable HIV DNA in CD133-sorted cells. The difference between the two groups is not significant ($p = 0.49$, t-test). Lines indicate the mean and standard error; symbols indicate individual values. A conservative estimate of 0.5 years for time that the viral load in the plasma had been undetectable was used for donor 305000.

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Chapter 5

Discussion

Latent HIV infection establishes reservoirs of virus that are a barrier to curing HIV. In addition to the well-characterized reservoir in resting memory $CD4^+$ T cells, we have proposed that hematopoietic progenitor cells (HPCs) in the bone marrow also serve as a reservoir for HIV (Carter et al. 2010). In this dissertation, we have characterized this potential reservoir by examining the viral and cellular characteristics that promote active and latent HIV infection in HPCs as well as the extent of infection in HPCs from HIV-infected donors. In chapter 2, we demonstrated that CXCR4- and dual- but not CCR5-tropic HIV can infect immature, multipotent HPCs. We furthermore showed that CXCR4-tropic HIV was capable of infecting the most immature HPCs, hematopoietic stem cells (HSCs). In chapter 3, we found that active as well as latent HIV infection could be established in HPCs with cell surface markers of multipotent cells, and that this latent infection could be reactivated by activation of the transcription factor NF-κB or by inhibition of histone deacetylases (HDACs). Finally, in chapter 4, we demonstrated that HIV DNA can be detected in the immature, $CD133^+$ subset of HPCs from donors who have had undetectable viral loads for up to 8 years. Together, these findings demonstrate that immature HPCs have the potential to serve as a persistent reservoir of virus in vivo.

In this chapter, we will discuss the implications of these findings and situate them within the body of research on HIV pathogenesis and persistence during therapy. We

will then explore new questions that are raised by these findings and discuss how they can be addressed in further studies. Ultimately, this discussion will clarify how the data presented in this dissertation can contribute to the search for a cure for HIV.

Summary of findings

Multipotent HPCs are infected almost exclusively by CXCR4-using virus. In chapter 2, we demonstrated that although CCR5-, dual-, and CXCR4-tropic HIV-1 envelopes all permit the infection of $CD34^+$ HPCs, multipotent, $CD133^+CD34^{bright}$ HPCs can be readily infected only by CXCR4-using envelopes. We confirmed that infection of these cells required use of the CXCR4 co-receptor through receptor-blocking experiments and demonstrated that CXCR4 is expressed on a larger fraction of $CD133^+$ HPCs than is CCR5. Together, these findings suggest that the more rapid disease progression associated with the emergence of CXCR4-using HIV isolates (Connor et al. 1997, Daar et al. 2007, Karlsson et al. 1994, Richman and Bozzette 1994, Schuitemaker et al. 1992, Shepherd et al. 2008, Waters et al. 2008, Weiser et al. 2008, Yu et al. 1998) could be related to the improved ability of these isolates to infect HPCs. Possible mechanisms through which CXCR4-mediated infection of HPCs could contribute to disease progression are explored later in this chapter.

The finding that CCR5-tropic HIV has only a limited ability to infect immature HPCs suggests that these cells may not be a major target of viral infection in individuals who harbor only CCR5-tropic virus. However, it is not clear how common it is for CXCR4-using virus to be entirely absent from an infected individual. Although CCR5-

tropic virus is more common than CXCR4-utilizing virus among recently infected individuals, new data suggest that 12-50% of recently infected individuals harbor at least minor populations of CXCR4-using virus (Abbate et al. 2011, Chalmet et al. 2012, Daar et al. 2007). Furthermore, CXCR4-using virus often emerges later in the course of disease in patients who initially appear to harbor only CCR5-tropic strains (Karlsson et al. 1994, Scarlatti et al. 1997, Shankarappa et al. 1999). Finally, the HIV Env protein can switch from CCR5- to CXCR4- or dual-tropic with a change of only one to two amino acids (De Jong et al. 1992, Raymond et al. 2008), and thus it would not be surprising if very small populations of CXCR4-using virus exist even in patients where only CCR5-tropic virus is detected. Thus, CXCR4-mediated infection of HPCs is expected to be relevant for a large proportion of HIV-infected individuals; however, the rates of infection in vivo would be expected to vary widely based on the prevalence of CXCR4-using virus in each person. This is consistent with the heterogeneity of infection rates in HPCs observed in our previous study (Carter et al. 2010) and in chapter 4.

HSCs can be infected with CXCR4-tropic HIV. We also showed that using a CXCR4-tropic HIV envelope, we were able to infect HSCs capable of stable, multilineage engraftment in irradiated mice. This finding may be of key importance to understanding HIV persistence: as HSCs can live for the lifetime of a person, a latent infection in this cell could create a reservoir of virus with an essentially infinite half-life. The presence of such a reservoir was suggested by a recent analysis of the decay kinetics of low-level viremia in HAART-treated patients (Palmer et al. 2008). Furthermore, active HIV infection and killing of HSCs could explain why HIV infection is associated with

depletion of multiple blood cell lineages, including many that are not targets of HIV infection (Adetifa et al. 2006, Dikshit et al. 2009, Meira et al. 2005, Mlisana et al. 2008, Redd et al. 2007a, Redd et al. 2007b).

Although several studies have reported HIV infection of HPCs (Carter et al. 2010, Davis et al. 1991, Folks et al. 1998, Neal et al. 1995, Redd et al. 2007a, Ruiz et al. 1998, Slobold et al. 1996, Stanley et al. 1992, Von Laer et al. 1990, Zauli et al. 1992), previous studies that focused specifically on HIV infection of HSCs were unable to detect HIV infection in this cell type (Shen et al. 1999, Weichold et al. 1998, Zhang et al. 2007). There are a number of methodological differences that could account for the negative results of previous studies, including different culturing conditions and different assays for HIV infection. Due to the very low rates of infection that are possible in HSCs, highly sensitive assays for HSC infection, such as the flow cytometric assays that we employed, are essential to permit the detection of infected cells. In addition, our cycling conditions promote the proliferation of HSCs whereas at least one prior study focused only on non-cycling cells (Shen et al. 1999); this could be an important factor in the discrepancy in our results if HSCs are more readily infected when they are cycling rather than quiescent. Finally, it is important to note that previous studies have also conflicted with each other as to the mechanism by which HIV infection of HSCs is blocked. One previous study reported solely an entry-level block that was overcome when virions were pseudotyped with VSV-G envelope (Shen et al. 1999), whereas another study suggested that the block to infection of these cells occurs at integration (Zhang et al. 2007). Although our data clearly demonstrate that these blocks to infection are not absolute, they may still contribute to the low rates of infection that we observe in HSCs and other types

of HPCs. Indeed, we do observe much higher rates of infection in HPCs when we use virions pseudotyped with VSV-G envelope.

Latent HIV-1 infection occurs in diverse HPC subsets. While the findings presented in chapter 2 showed that immature HPCs, including HSCs, could be infected by HIV, it was not clear whether a latent HIV infection could be established in these cells. In chapter 3, we expanded upon the findings of chapter 2 by demonstrating that HIV can establish a latent infection in both $CD133^{+}CD34^{bright}$ and $CD34^{+}CD38^{-}CD45RA^{-}$ HPC subsets. $CD133^{+}CD34^{bright}$ cells are an HPC subset that contains stem cells and multipotent progenitors (MPPs) as well as granulocyte/monocyte progenitor cells (Yin et al. 1997), while the $CD34^{+}CD38^{-}CD45RA^{-}$ subset has recently been shown to consist primarily of stem cells and MPPs (Doulatov et al. 2010). Thus, our findings demonstrate that latent HIV infection can occur in HPCs that have the cell surface markers of immature, multipotent cells.

An interesting aspect of our findings is that both active and latent infection could be established in the same HPC population with no indication that active or latent infection occurred preferentially in a specific HPC type. This finding suggests that the establishment of latency in our model depends to some extent on stochastic factors, such as the integration site of the virus (Jordan et al. 2003) or variation in the level of Tat produced immediately following integration (Weinberger et al. 2005). Further studies to evaluate the impact of exogenous Tat on the establishment or maintenance of latency in this system or to identify the integration sites of active vs. latent HIV genomes would clarify the role of stochastic factors in latent infection of HPCs.

A limitation of our findings in chapter 3 is that we were unable to determine whether latent HIV infection could occur specifically in bona fide HSCs. Although several cell surface markers have recently been proposed to more specifically identify human HSCs, notably Thy-1 (Majeti et al. 2007) and CD49f (Notta et al. 2011), we were unable to detect sufficient expression of these markers in our $CD34^+$ HPCs to analyze in our study (data not shown). The stability of these markers in culture has not been demonstrated and in our experiments the expression of these markers seemed to change substantially over our 6-day culture period. We found that initially detectable Thy-1 declined to undetectable levels while CD49f became highly expressed on the more differentiated cells in our culture, as assessed by (lack of) CD34 staining and colony-forming ability (data not shown). Thus, it presently does not seem feasible to identify HSCs by cell surface markers under our culture conditions.

The ability of HIV to establish a latent infection in HSCs might be better assessed via a xenograft assay. HPCs could be infected with a non-cytotoxic reporter virus expressing GFP under the control of the HIV-1 LTR, then the GFP^- subset that contains both uninfected and latently infected cells could be sorted out and transplanted into mice. Mature peripheral blood cells could then be screened for GFP expression or, as we have not confirmed that lymphoid differentiation results in the reactivation of latent HIV, HIV genomes could be detected by PCR. Long-term reconstitution of both the myeloid and lymphoid lineages with human GFP^+ or HIV DNA-containing cells would indicate the engraftment of a latently infected stem cell in the mouse, thereby clarifying the ability of HIV to establish a latent infection in this cell type.

Latent HIV-1 infection in HPCs can be reversed by NF-κB activation or histone deacetylase inhibition. In HPC populations that supported the establishment of latent HIV infection, we found that levels of nuclear NF-κB family members were uniformly low whereas positive transcription elongation factor b (P-TEFb) was readily detected. Furthermore, conditions that reactivated latent virus resulted in a distinct upregulation of most NF-κB family members in the nucleus but did not affect P-TEFb expression. These findings contrast with observations in resting T cells, where TNF-α-induced NF-κB activation is insufficient to reactivate latent virus while upregulation of both nuclear NF-κB and P-TEFb through T cell receptor signaling reactivates latent virus efficiently (Tyagi et al. 2010).

The finding that P-TEFb restriction is not associated with the establishment of latency in HPCs may indicate that there are fewer factors reinforcing latency in this cell type than in T cells. This reduced reinforcement of latent infection could potentially result in more frequent reactivation of latent virus in these cells. Such an increased rate of reactivation might lead to reduced stability and more rapid decay of this reservoir. Alternatively, if the reservoir is maintained through HPC proliferation in spite of increased rates of reactivation, this reactivation might lead this reservoir to contribute disproportionately to the residual viremia detected in HAART-treated patients. This latter possibility is consistent with studies demonstrating that most viral sequences in the plasma of many HAART-treated patients cannot be matched to sequences found in peripheral blood resting memory T cells (Bailey et al. 2006, Brennan et al. 2009, Lopez et al. 2010, Sahu et al. 2009), and might suggest that HPCs could contribute

disproportionately to residual viremia even if very few HPCs harbor latent viral genomes in vivo.

We found that NF-κB activation by both prostratin and TNF-α reactivated latent virus in our HPC model system. Prostratin was not toxic to our cells but did induce substantial differentiation that could have negative consequences in vivo. TNF-α was also non-toxic to our cells and, unlike prostratin, did not induce cell differentiation; however, this cytokine has been associated with significant toxicity in vivo (reviewed in Beutler and Cerami 1986). Interestingly, it has been shown that activation of the heat shock response in mice can reduce TNF-α-related toxicity without negating this cytokine's anti-tumor effects (Van Molle et al. 2002, Van Molle et al. 2007). If a similar strategy can reduce TNF-α-related toxicity in humans, TNF-α could potentially be used therapeutically to reactivate latent virus in HPCs and eliminate this viral reservoir. However, the ability of the heat shock response to modify TNF-α toxicity in humans has not yet been investigated and it is not clear how this response could be safely and effectively induced.

We also found that the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA) reactivates latent virus in HPCs whereas hexamethylene bisacetimde (HMBA) and the methylation inhibitor 5-aza-2'-deoxycytidine (Aza-CdR) does not. The findings that HMBA and Aza-CdR are unable to reactivate latent virus in HPCs may not be surprising. HMBA is purported to act by activating P-TEFb (Contreras et al. 2007), which is not restricted in HPCs. Methylation, meanwhile, is thought to be a late event in the maintenance of viral latency (Blazkova et al. 2009, Duverger et al. 2009, Kauder et al. 2009); however, our short-term model system could examine only early factors in latency

establishment. Nevertheless, our findings suggest that as in T cells, histone deacetylases may be recruited to the latent HIV LTR in HPCs; thus, inhibition of histone deacetylases may provide a common mechanism whereby latent infection could be eliminated in both cell types. Unfortunately, we observed that SAHA was toxic to HPCs at doses that reactivated latent virus, suggesting that this compound could not be effective in vivo without significant toxicity. Further research is required to determine whether other histone deacetylase inhibitors can reactivate latent virus with less cytotoxicity.

$CD133^+$ HPCs are a reservoir for HIV DNA in patients with undetectable viral loads. Finally, in chapter 4 we investigated whether $CD133^+$ HPCs harbor HIV DNA in HAART-treated donors who have had clinically undetectable viral loads for at least 6 months. In 6 out of 11 donors, we were able to detect HIV DNA in CD133-sorted samples at a frequency of 1.2 - 42 genomes per 100,000 cells. Furthermore, we were able to demonstrate that for at least 5 of these 6 donors, contamination with $CD3^+$ T cells was not a good explanation for the genomes detected in the sample. These results confirm our previous finding that HPCs from HAART-treated donors contain HIV-1 DNA (Carter et al. 2010) and extend these findings to demonstrate that the immature, $CD133^+$ subset of HPCs also harbors HIV-1 DNA after up to 8 years of viral suppression.

We also found that the mean year of diagnosis for donors where we were able to detect HIV genomes in CD133-sorted samples was significantly more recent than the mean year of diagnosis for donors where we could not detect HIV genomes in these cells. This association could not be explained by the decay of this viral reservoir during treatment, as the mean length of time that donors had had undetectable viral loads was

similar in the two groups. Although it is not yet clear why year of diagnosis would be associated with infection of HPCs, this finding may partially explain the discrepancy between our results and those recently reported by Durand and colleagues, who were unable to detect HIV DNA in HPC samples from a cohort of 11 HAART-treated donors (Durand et al. 2012). Six of the 11 donors in this cohort had been diagnosed in 1990 or earlier, and thus based on the association we observed it may not be surprising that these donors did not show evidence for HIV infection in HPCs.

Another recent study was also unable to detect HIV DNA in hematopoietic progenitor cells. This study focused on 8 donors, of whom 5 had been treated during acute HIV infection (Josefsson et al. 2012). Such donors would be expected to have very low levels of HIV DNA in any cell type; furthermore, the observation that CXCR4-tropic virus usually constitutes a relatively small fraction of the total virus found in recently-infected donors (Abbate et al. 2011) suggests that infection rates in HPCs would be even lower than those in T cells. Although such a low infection rate could still be physiologically relevant, as a single latently infected HSC could serve as a lifelong viral reservoir, it would be extremely difficult to detect HIV infection of HPCs in these donors.

The negative results from the remaining donors in these two studies might be explained in part by heterogeneity in the frequency of HIV infection in HPCs in different donors, which we have observed to vary widely (chapter 4 and Carter et al. 2010). Furthermore, methodological differences between these studies and ours could impact the detection of HPCs. In particular, Josefsson and colleagues isolated $CD34^+$ HPCs through a sorting protocol that specifically eliminated $CD4^+$ cells from the CD34-sorted population (Josefsson et al. 2012). However, as we showed in chapter 2, CD4 is required

for infection of HPCs by HIV; thus this sorting protocol may have removed those HPCs most likely to contain HIV DNA from the samples analyzed. In addition, the primers used by Josefsson et al. to detect HIV DNA are less conserved in subtype B HIV isolates than those we used (data not shown), which may also have decreased the sensitivity of their assay. Finally, Durand and colleagues cultured their cells in serum-containing medium overnight prior to sorting. The effects that this protocol might have on the survival or CD34 expression of HIV-infection HPCs are unknown.

A final possible explanation for the discrepancy between our results and those of other groups is that some of the genomes detected in our CD133-sorted samples are due to contaminating cell types. Although we showed that $CD3^+$ T cell contamination is unlikely to explain the HIV DNA detected in 5 out of 6 of our positive CD133-sorted samples, we were able to rule out total contamination as an explanation for the HIV DNA detected for only 2 of 6 samples. It is possible that long-lived macrophages or mast cells in the bone marrow could contain HIV DNA. As these cells express Fc receptors, they might non-specifically bind the antibodies used in our sorting protocols and thus end up in our CD133-sorted populations. However, bone marrow macrophages or mast cells have never been reported to harbor HIV genomes in donors with undetectable viral loads. Furthermore, if contamination were the explanation for the detection of HIV DNA in our sorted samples, we would expect to see an association between sample purity and genome detection in our cohort; however, we did not detect such an association. Finally, it should be noted that the samples analyzed by Josefsson et al. were only 76.7% pure on average (Josefsson et al. 2012) whereas all of the CD133-sorted samples analyzed in our

study were more than 80% $CD133^+$. Thus, it is unlikely that contamination would serve as a source of HIV genomes in our HPC samples but not in theirs.

In sum, there are many possible explanations for the discrepancies between our findings and those observed by other groups. Hopefully, additional research on this topic will clarify the reasons that other studies have not detected HIV genomes in HPCs and will allow us to better understand the importance of this potential viral reservoir in vivo.

Future directions

The studies described in this dissertation raise exciting new questions about the role of HPCs in HIV pathogenesis and persistence. In this section, we will outline some of these questions and discuss how they might be addressed in future studies. Specifically, we will discuss how infection of HPCs might contribute to the more rapid disease progression associated with the emergence of CXCR4-tropic virus. We will also discuss additional factors that may affect the establishment or maintenance of latent HIV infection in HPCs and examine how we can develop strategies that may be able to reactivate latent virus in this potential viral reservoir. Finally, we will discuss possible explanations for the heterogeneous rates of HIV infection observed in HPCs in vivo and outline the additional research that is needed to understand the role of these cells in the persistence of HIV during treatment. Through these additional studies, some of which are already ongoing in our lab, we will enhance our understanding of latent infection of HPCs and move one step closer to a cure for HIV.

Contribution of HPC infection to disease progression associated with the emergence of CXCR4-tropic virus. It is not yet clear whether our finding that only CXCR4-tropic HIV is able to infect immature HPCs may contribute to the association between the emergence of CXCR4-using virus and more rapid disease progression (Connor et al. 1997, Daar et al. 2007, Karlsson et al. 1994, Richman and Bozzette 1994, Schuitemaker et al. 1992, Shepherd et al. 2008, Waters et al. 2008, Weiser et al. 2008, Yu et al. 1998). The enhanced ability of CXCR4-tropic HIV to infect immature HPCs suggests that one explanation for the association between the emergence of CXCR4-tropic virus and disease progression could be the infection and destruction of immature HPCs. By killing HPCs, CXCR4-tropic virus could hasten disease progression by diminishing production not only of $CD4^+$ T cells, but also of the $CD8^+$ T cells and other immune system cells that help to control viral replication.

This possibility is supported by data showing that the emergence of CXCR4-tropic virus is associated not only with a more rapid decline in $CD4^+$ T cell counts (Connor et al. 1997, Daar et al. 2007, Karlsson et al. 1994, Richman and Bozzette 1994), but also with a loss of T cell homeostasis that results in a decline in total T cell numbers (Maas et al. 2000, Shankarappa et al. 1999, Shepherd et al. 2008), including $CD8^+$ T cells (Hazenberg et al. 2003, Yu et al. 1998). A decline only in $CD4^+$ T cell counts could be explained by the enhanced infection rate and cytotoxicity of CXCR4-using virus in these cells (Grivel and Margolis 1999, Grivel et al. 2000, Jekle et al. 2002, Jekle et al. 2003, Kreisberg et al. 2001, Schramm et al. 2000, Schuitemaker et al. 1992), but the observed decline in $CD8^+$ T cells, which do not express CD4, cannot be explained by direct

infection of the mature lymphocytes. Infection of hematopoietic progenitor cells could account for the loss of both T cell subsets.

Depletion of both $CD4^+$ and $CD8^+$ T cells could also be accounted for by infection and killing of committed T-cell progenitors in the thymus, some of which have been reported to express high levels of CXCR4 (Berkowitz et al. 1998). Indeed, HIV-induced apoptosis of thymocyte progenitors by CXCR4-tropic HIV has been reported (Choudhary et al. 2006, Su et al. 1995), and one study has suggested that CXCR4-tropic virus replication in the thymus depletes thymic progenitors to a greater extent than CCR5-tropic virus does (Berkowitz et al. 2000). By contrast, depletion of more immature HPCs by CXCR4-tropic virus should result in a loss of additional mature blood cell types. Although numerous hematological abnormalities have been associated with HIV infection, including anemia, neutropenia, thrombocytopenia, leucopenia, and depletion of bone marrow B cells (Adetifa et al. 2006, Dikshit et al. 2009, Meira et al. 2005, Mlisana et al. 2008, Redd et al. 2007a, Redd et al. 2007b), the association of these hematologic abnormalities with viral envelope tropism was addressed only in one study in which the frequency of patients with CXCR4-tropic virus was too low to draw definitive conclusions (Calis et al. 2008). One additional study did observe an association between hematopoietic progenitor infection and anemia, but in this study viral envelope tropism was not assessed (Redd et al. 2007a). Additional studies assessing viral envelope tropism, hematologic abnormalities, and infection rates in hematopoietic progenitors in treatment-naïve, HIV-infected individuals would clarify how CXCR4-mediated infection of HPCs might contribute to cytopenias in vivo.

Finding new ways to reactivate latent HIV. Using the assay for reactivation of latent virus in HPCs that we developed in chapter 3, we are continuing to screen additional compounds for the ability to reactivate latent virus in this cell type. Thus far our efforts have focused on disulfiram, an FDA-approved compound that reactivates latent virus in a T cell model (Xing et al. 2011), as well as several HDAC inhibitors that might be more active or less toxic than SAHA (Nakajima et al. 1998, Rai et al. 2008, Thomas et al. 2008). We also plan to investigate whether inhibitors of specific histone deacetylases might reactivate latent virus with reduced cytotoxicity. A recent study demonstrated that specific inhibition of Class II HDACs reactivated latent virus in the ACH-2 latently infected T cell line (Clouse et al. 1989, Palmisano et al. 2012) and that the toxicity of this treatment was less than that of pan-HDAC inhibition with trichostatin A (Palmisano et al. 2012). Inhibition of Class II HDACs did not reactivate latent virus in the latently infected pro-monocytic U1 cell line (Folks 1987, Palmisano et al. 2012), however, highlighting the importance of investigating whether different components of the chromatin modification machinery contribute to HIV latency in different cell types. We will thus continue to use our assay to screen compounds that have been demonstrated to reactivate latent HIV in other cell types as well as to test novel compounds for the ability to reactivate latent HIV.

Although the use of primary HPCs ensures that our model of latent infection accurately reflects HPC-specific factors that impact latency in this cell type, the use of primary cells also limits the speed with which we can screen novel compounds for the ability to reactivate virus. Because of this, we have attempted to develop a cell-line model for latency in HPCs using the $CD34^+$ KG-1 and KG-1a cell lines. Unfortunately,

we were not able to establish a latent HIV infection in these cells. However, additional $CD34^+$ cell lines exist (Lorenzana et al. 1993, Rajotte et al. 1996, Simmons et al. 1992) and may be more permissive to the establishment of latent infection. The identification of an HPC-like cell line in which latent HIV infection could be established would allow us to perform high-throughput screening to identify novel compounds that reactivate HIV in this cell type. The activity of these compounds in primary cells could then be verified in our existing model. The combination of these two methods could provide a powerful way to identify new compounds that reactivate latent HIV in HPCs.

Impact of HPC cycling on the establishment and maintenance of latency. In our model of latent infection in HPCs, we were able to establish a latent infection in spite of the fact that the cells were cycling during the course of our assays with a mean doubling time of 24-48 hours (data not shown). However, we also observed spontaneous reactivation in a proportion of latently infected cells. This low-level spontaneous reactivation is likely related to the ongoing differentiation that unavoidably occurs in cultures of immature HPCs; additionally, it could be related to the proliferation of the cells. Such low-level reactivation of latent virus has been observed in latently infected T cells induced to proliferate via IL-7 stimulation (Bosque et al. 2011).

Based on this observation, we are currently investigating the role of cell cycling in the establishment and maintenance of latent infection in HPCs. Our initial approach to this question is to determine whether the rates of active or latent infection or of spontaneous reactivation are altered when HPCs are cultured with or without cytokines that induce proliferation. We will also use carboxyfluorescein succinimidyl ester (CFSE)

to track cell cycling and investigate whether latency or spontaneous reactivation occurs specifically in dividing or non-dividing cells. These studies will not only help us to better understand the factors controlling the establishment and maintenance of latent infection in our model, they will further our understanding of HIV infection of HSCs, which are found predominantly in a non-cycling state in vivo (Cheng et al. 2000).

Impact of HPC differentiation on latent HIV. Although we have demonstrated that induction of myeloid differentiation with the cytokines GM-CSF and TNF-α results in reactivation of latent virus (Carter et al. 2010), we have not evaluated the impact of lymphoid differentiation on latently infected HPCs. B and T cell differentiation both involve the activation of NF-κB (Liou et al. 1994, Voll et al. 2000), which we have shown to reactivate latent virus in HPCs. Thus, we would expect differentiation along these pathways to reactivate latent virus, resulting in cell death and an absence of HIV-infected B cells in the periphery. The importance of NF-κB in natural killer (NK) cell differentiation is not as well understood. Although activated NF-κB is present in immature NK cells, levels of NF-κB are relatively low compared to those found in B cells (Samson et al. 2004). Furthermore, the differentiation of NK cells appears to be less sensitive to NF-κB levels than is B or T cell differentiation (Samson et al. 2004). As a result, it is not clear whether inducing HPCs to differentiate down the NK cell lineage would result in reactivation of latent virus.

Conditions to differentiate $CD34^+$ HPCs into NK cells in vitro have been developed (Grzywacz et al. 2011) and could be integrated with our reactivation assay to assess whether NK cell differentiation reactivates latent HIV. Investigating the fate of

latently infected HPCs induced to differentiate into NK cells would illuminate whether these cells might be expected to harbor HIV genomes in vivo, a topic which has not been directly addressed. Furthermore, if latent infection is reactivated during NK cell differentiation, the mechanism of this reactivation may differ from that observed during myeloid differentiation. Thus, studying the effects of NK cell differentiation on latently infected HPCs may allow us to identify novel mechanisms through which latent virus can be reactivated in these cells.

Heterogeneity in the frequency of HIV infection in HPCs in vivo. As noted above, we found an association between year of diagnosis and detection of HIV genomes in HPC samples in our donor cohort. This association could not be explained by the decay of the viral reservoir during treatment, as there was no difference in the length of time that donors had had suppressed viral loads between donors for whom we could detect HIV genomes in HPCs and donors for whom we could not. An alternative explanation is that donors with high rates of HIV infection in HPCs who were diagnosed prior to the advent of combination antiretroviral therapy in 1995-6 (Hogg et al. 1997) may have been less likely to survive to the present day or to maintain suppressed viral loads. We demonstrated in chapter 2 that CXCR4-tropic viruses, which are associated with more rapid disease progression (Connor et al. 1997, Daar et al. 2007, Karlsson et al. 1994, Richman and Bozzette 1994, Schuitemaker et al. 1992, Shepherd et al. 2008, Waters et al. 2008, Weiser et al. 2008, Yu et al. 1998), have a greatly enhanced ability to infect immature HPCs compared to CCR5-tropic HIV. Thus, donors with high levels of CXCR4-tropic virus would be expected to be more likely to both harbor HIV DNA in

HPCs and to experience mortality if they were diagnosed prior to the advent of HAART. Further research to assess the envelope tropism of the virus in our donors could reveal whether the presence of CXCR4-using virus in the plasma or in HPCs is associated with HPC infection in vivo.

A second possibility is that inflammation induces HSC cycling that results in increased HIV infection in these cells. Biomarkers of inflammation are increased among HIV-infected patients with higher viral loads (Eastburn et al. 2011, Kuller et al. 2008), and higher viral loads are associated with more rapid disease progression (Mellors et al. 1995, Mellors et al. 1996). Thus, HIV-infected individuals with high levels of inflammation might again be less likely to have survived until the present if they were diagnosed prior to the development of HAART. Meanwhile, HSCs have been shown to proliferate in response to inflammation (Baldridge et al. 2010, Essers et al. 2009, Takizawa et al. 2011), which might alter how permissive these cells are to HIV infection. The role of HPC cycling in HIV infection and latency will be investigated through the experiments described above. In addition, the possible role of inflammation in the infection of HPCs in vivo could be assessed by measuring viral loads, markers of inflammation, and levels of HIV DNA and protein in HPCs in untreated HIV-infected patients. If an increased viral load and increased inflammation promotes infection of HPCs, as proposed here, an increased infection rate in these cells would be observed in donors with increased viral loads and biomarkers for inflammation. This investigation would shed light on the observed association between year of diagnosis and HIV infection in HPCs and would help to explain the heterogeneous rates of infection observed in this cell subset.

Do HIV genomes in HPCs contribute to residual viremia in treated patients? To truly understand the role of HIV-infected HPCs in the persistence of HIV, it is essential to determine whether HIV genomes found in HPCs contribute to residual viremia during HAART. To assess this question, we will amplify HIV sequences from plasma, peripheral blood resting memory T cells, and purified HPC populations at limiting dilution so that we can assess the variation among sequences within each compartment. We will then use a phylogenetic analysis to assess whether there are viral sequences in the plasma that are more related to the sequences found in HPCs than to those in T cells. As previously noted, a large proportion of residual viral sequences in the plasma of HAART-treated individuals are not closely related to those found in peripheral blood T cells (Bailey et al. 2006, Brennan et al. 2009, Lopez et al. 2010, Sahu et al. 2009). Thus, the finding that some or all of the sequences that cannot be matched to those in resting memory T cells are similar to those found in HPCs would provide strong evidence for a role of HPC infection in the persistence of HIV during HAART.

It is also possible that the genomes detected in HPCs will be highly similar to those found in $CD4^+$ T cells. This finding would demonstrate that there must be yet another, as yet uncharacterized reservoir for latent HIV in HAART-treated patients that contributes substantially to residual viremia. Either way, the results of this study will provide important new information on the source of residual virus in effectively treated patients, which will allow us to better assess which cell types need to be targeted to eliminate this residual virus and cure HIV.

Conclusions

In this dissertation, we have investigated the role of HPCs in the pathogenesis and persistence of HIV infection. We found that CXCR4- but not CCR5-tropic HIV is able to infect immature, multipotent hematopoietic progenitor cells. We furthermore showed that even extremely long-lived HSCs are susceptible to infection with CXCR4-tropic HIV, providing a mechanism for lifelong persistence of HIV in vivo. In addition, we demonstrated that immature, $CD133^+$ HPCs could be latently infected by HIV in vitro and that these cells may serve as a reservoir for latent HIV in vivo that persists through at least 8 years of suppressive antiretroviral therapy. An updated model of HIV infection in HPCs that incorporates these findings is shown in **Figure 5.1**.

These findings suggest that we need to look beyond T cells when developing approaches to eradicate reservoirs of latent HIV. Through the development of a primary cell model for latent HIV infection in HPCs, we have provided the first data on strategies that may eliminate latent virus from this cell type. We found that both activation of NF-κB and inhibition of histone deacetylases reactivate latent virus from these cells. In combination with strategies that reactivate latent virus from T cells, modulation of these cellular factors might provide a means of eliminating latent reservoirs of virus from HIV-infected people. The findings described in this dissertation thus provide a foundation for further research that will lead us closer to a cure for HIV.

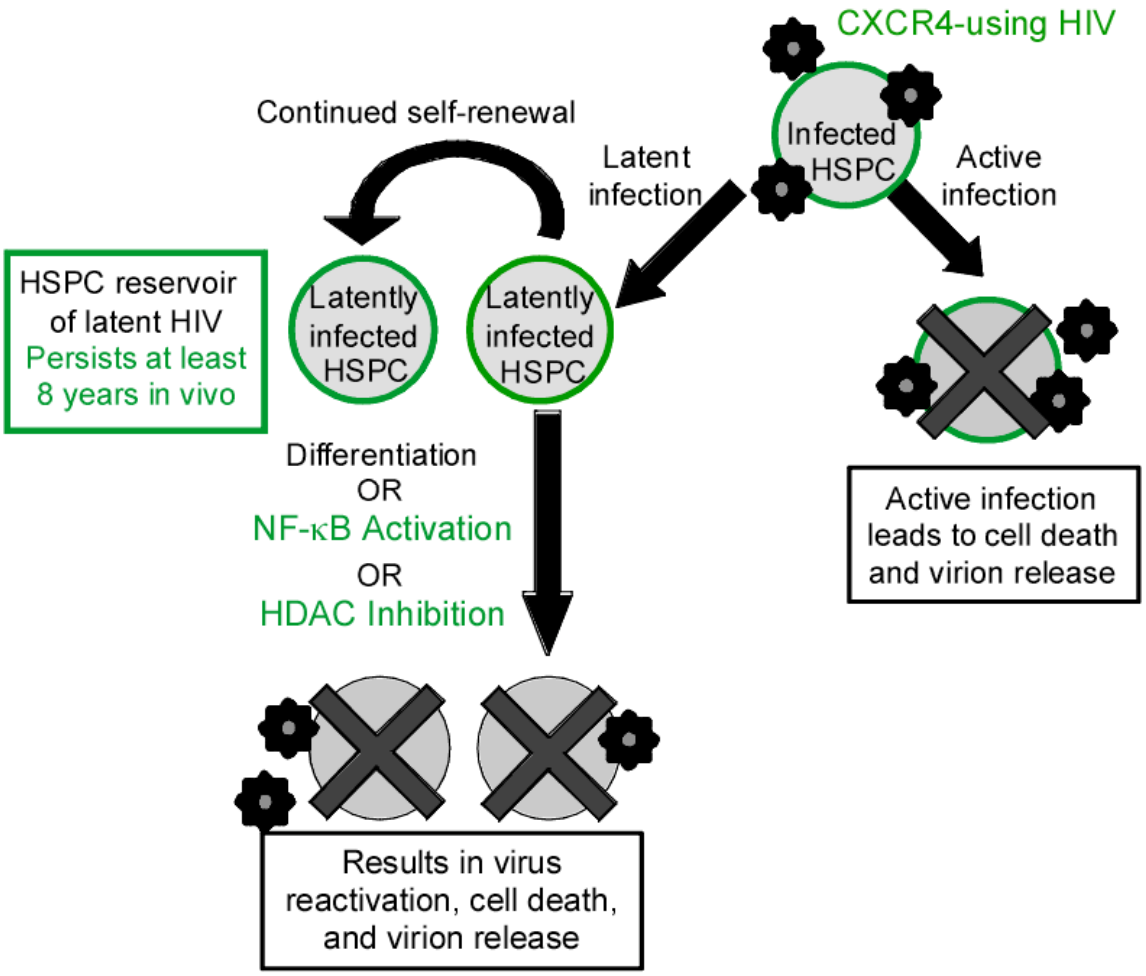

Figure 5.1. Updated model of HIV-1 infection in hematopoietic progenitor cells. Modifications to the model based on the work shown in this dissertation are highlighted in green. Immature, $CD133^{+}$ hematopoietic stem and progenitor cells (HSPCs) can be infected by CXCR4-utilizing HIV-1 (chapter 2), leading either to active infection, cell death, and virion release, or to latent infection (chapter 3). Latently infected HSPCs with self-renewal capacity can continue to self-renew, generating an expanded reservoir of latent HIV-1 in these cells that persists for at least 8 years during highly active antiretroviral therapy (chapter 4). If the latently infected HSPC is induced to differentiate or stimulated with compounds that activate NF-κB or inhibit histone deacetylases (HDAC), the latent virus can reactivate (chapter 3), leading to cell death and virion release. Modified from Collins and McNamara 2011.

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