

**Investigating the HIV Reservoir in Hematopoietic  
Stem and Progenitor Cells**

**by**

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# **Dedication**

To my family and to all people who have suffered because of HIV

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## Abstract

Current antiretroviral therapies are not curative because they do not eradicate long-lived cells harboring HIV proviral DNA and thus, if a patient stops therapy, circulating virus will rebound. A subset of hematopoietic stem and progenitor cells (HSPCs) express HIV receptors (CD4 and CCR5 or CXCR4) that enable both active and latent infection. Thus, HSPCs have been implicated as a source of persistent virus *in vivo*. In this dissertation, we first show that HIV genomes can be detected in CD133-sorted HSPCs from a subset of donors with long-term viral suppression and in most cases cannot be explained by contamination with CD3<sup>+</sup> T cells. In an analysis of a larger cohort of optimally treated HIV-infected donors, we wished to determine the tropism of virus in HSPCs and delineate which progenitor subsets are infected *in vivo*. In contrast to HIVs that utilize CXCR4, we found that CCR5-tropic viruses are likely to not infect hematopoietic stem cells. Instead, CCR5-tropic viruses may infect non-stem cell progenitors that may actually be long-lived *in vivo* as implicated by other recent studies. Finally, we describe a distinct CD4<sup>high</sup> HSPC subpopulation that is enriched in multipotent cells and preferentially infected by HIVs of both tropisms. In sum, these results provide evidence that HIV-infected HSPCs do persist *in vivo* and may be a relevant reservoir of the virus in HIV<sup>+</sup> people on therapy.

# Chapter 1

## Introduction<sup>1</sup>

Human Immunodeficiency Virus (HIV) infects thousands of people each year and has killed more than 39 million people over the past few decades (WHO, 2015). Over 36 million people around the world are living with HIV infection as of 2014 (UNAIDS, 2015). Without therapy, HIV infection leads to the development of AIDS and eventually death in the majority of infected people. Current therapeutic regimens effectively suppress viral replication but do not cure disease and thus lifelong therapy is required. This need for long-term treatment poses a huge economic burden for HIV-infected people and for health care systems. Only about 40% of HIV-infected people received antiretroviral drugs in 2014, while many more who were eligible for treatment, many living in middle and lower income countries, still did not have access. New treatment guidelines in 2015 recommended treatment for all people living with HIV, increasing the treatment gap even further, and thus there are now estimated to be over 22 million people living with HIV who still need antiretroviral therapy. Thus, there is an urgent need for the development

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<sup>1</sup>Sections of this chapter were previously published as: Sebastian, N T, and K L Collins (2014) Targeting HIV latency: resting memory T cells, hematopoietic progenitor cells and future directions. *Expert Review of Anti-Infective Therapy*, 12 (10), 1187–120. Content included has been updated and modified for flow.

of a therapeutic regimen that will cure the disease as opposed to just suppressing the virus.

Hope for a global cure of HIV infection has been stimulated by the documented cure of an HIV-infected man following bone marrow transplantation in Berlin and the transient 'functional cure' of an infected baby from Mississippi (Hutter et al., 2009; Persaud et al., 2013). However, there remain important questions that need to be addressed in the journey toward a cure, especially how the virus is able to persist despite suppressive treatment. This dissertation will explore further the potential reservoir of HIV in bone marrow stem and progenitor cells in HIV-infected individuals. This introductory chapter will provide an overview of HIV infection and give relevant background to my dissertation research.

## **HIV Replication**

HIV is a single-stranded positive-sense RNA virus from the Lentivirus subfamily of Retroviruses. The 9.7-kb HIV-1 genome contains three genes, *gag*, *pol*, and *env*, which encode the major structural proteins and enzymes required for HIV replication (**Figure 1-1**). These are synthesized as poly-protein precursors that are processed by viral or cellular proteases into the mature forms incorporated in viral particles (**Figure 1-1**). The Gag precursor is cleaved into matrix, capsid, nucleocapsid and other smaller peptides. A Gag-Pol polyprotein produces protease, reverse transcriptase, and integrase. Additionally, there are six genes (*vif*, *vpr*, *tat*, *rev*, *vpu*, and *nef*) that encode HIV proteins that are produced from splicing of messenger RNAs (mRNAs). Tat and Rev are important for regulating transcription

and facilitating export of HIV mRNAs from the nucleus. Vif, Vpr, Vpu, and Nef, known as accessory proteins, evolved with the virus to evade host antiviral responses and thus allow disseminated infection *in vivo* (Collins and Collins, 2014; Freed and Martin, 2013). The long terminal repeat (LTR) at either end of the genome consists of the highly structured 5' untranslated region (5' UTR), which includes *cis*-elements important to reverse transcription, transcriptional elongation of viral RNA, splicing, and dimerization and packaging of the full-length viral genome into viral particles (Freed and Martin, 2013).

### *HIV Entry*

Viral entry is mediated by fusion of the viral membrane with the cell membrane through the interaction of the viral glycoprotein Env with the CD4 receptor and then a co-receptor, typically CC chemokine receptor type 5 (CCR5) or CXC chemokine receptor type 4 (CXCR4) (Freed and Martin, 2013; Wilen et al., 2012) (**Figure 1-2**). The heavily glycosylated mature Env consists of a trimer of glycoproteins, gp120 and gp41, where gp41 anchors the complex in the viral membrane and gp120 actually binds receptors on the cell surface. In gp120, there are five conserved domains (C1-C5) interspersed with five variable loops (V1-V5), where the variable loops are areas of significant genetic diversity important for evading the host antibody response. After the virus attaches to a cell, portions of gp120 bind to CD4, an immunoglobulin superfamily member normally involved in T-cell receptor signaling. This binding induces a conformational change in Env gp41 and gp120 facilitating the V3 loop and other conserved regions to contact a co-

receptor, usually CCR5 or CXCR4. This co-receptor binding provides the potential energy needed to form the fusion complex of gp41 between the viral membrane and the host cellular membrane and fusion of the two membranes (Freed and Martin, 2013; Wilen et al., 2012). The viral nucleoprotein core is then able to enter the cytosol and proceed to reverse transcription. Several studies have indicated that signal transduction via CD4 and the co-receptor binding may be important for remodeling of the actin cytoskeleton for movement of viral complexes through the cell, especially during infection of resting CD4<sup>+</sup> T cells (Wilen et al., 2012).

### *Reverse Transcription*

After uncoating of the viral core, reverse transcription of the viral RNA genome into DNA occurs within a complex of viral proteins, including reverse transcriptase, integrase, Vpr, and likely some capsid (Freed and Martin, 2013) (**Figure 1-2**). Each virion contains two copies of the RNA genome and both are required for reverse transcription. Reverse transcriptase will switch multiple times between both strands as a template resulting in frequent recombination. This can also result in genomes with large internal deletions that may still proceed to integration (Maldarelli, 2016).

### *Integration*

A preintegration complex (PIC), which includes the double stranded DNA copy of the viral genome with LTR sequences at both ends, is then transported into the nucleus via interactions with host nucleoporins (Freed and Martin, 2013)

**(Figure 1-2).** A viral integrase molecule bound at each end of the HIV DNA in the PIC facilitates integration into the host chromosomal DNA. Integrase binds to host DNA to create 5-bp staggered cuts in each strand at the site of integration where the HIV DNA is inserted, and host enzymes can allow repair of the cut sites with ligation of the proviral genome into chromosomal DNA. Integrase has been shown to have some site preference, via binding at host DNA sequences which include 5'-AAA, 5'-TAA, or 5'AAA (Serrao et al., 2015), and binding lens epithelial-derived growth factor (LEDGF) associating the PIC with transcriptional units (Ferris et al., 2010). Recent studies with improved techniques for generating integration sites have supported a preference for integration into transcriptionally active regions of the host genome and, in patients with long-term viral suppression, there is a bias towards persistence of genomes integrated in genes associated with cell growth, such as *BACH2* and *MKL2* (Cohn et al., 2015; Maldarelli et al., 2014; Wagner et al., 2014). The study by Cohn et al provided evidence that HIV may strongly prefer to integrate near human *ALU* repeat sequences more so than transcriptionally active regions (Cohn et al., 2015). Alternatively, a portion of HIV DNAs will not be integrated and instead persist in the nucleus as circularized genomes, known as 1-LTR or 2-LTR circles, but are not transcribed to allow replication to continue.

### *Viral Transcription and Virion Production*

Following integration of the proviral DNA genome, the host transcriptional machinery is co-opted for transcription and translation of viral proteins (**Figure 1-2**). Tat or *trans* activator of transcription is an early product, which returns to the

nucleus to bind at the HIV LTR promoter and enhance transcription of viral genes. In T cells, host transcriptional factors, including nuclear factor- $\kappa$ B (NF- $\kappa$ B), the positive transcription elongation factor b complex (P-TEFb), nuclear factor of activated T cells (NFAT), activator-protein 1 (AP-1) and specificity protein 1 (SP1) have been shown to enhance HIV-1 transcription (Coiras et al., 2009; Freed and Martin, 2013). At this point in replication, the provirus may remain transcriptionally silent, resulting in a latent infection, which will be described in further detail later in this chapter. In an active infection cycle, spliced and unspliced viral mRNAs are exported into the cytoplasm via the viral Rev protein (**Figure 1-2**). The Env, Gag, and Gag-Pol polyproteins use independent pathways for processing and transport to the plasma membrane, where assembly and budding of immature viral particles occurs. Viral protease will cleave Gag and Pol during or immediately after the viral particle is released from the cell, to form the mature proteins in the virion's nucleoprotein core (Freed and Martin, 2013).

### **HIV Pathogenesis**

HIV essentially targets cells of the immune system eventually causing a profound defect in immune function that leaves the infected person vulnerable to a number of infections that would normally not cause disease. This severe state of immune insufficiency is called acquired immunodeficiency syndrome or AIDS. The virus is most often transmitted sexually, but can also have parenteral or mother-to-infant transmission (Knipe and Howley, 2013). Using simian immunodeficiency virus (SIV) models, it has been shown that CD4<sup>+</sup> T cells are the first cell types to be



infected during transmission across mucosal barriers and there is a limited time period in which the immune response or anti-retroviral drugs can block further spread of the virus in the host (Haase, 2011). Viral tropism, or the ability to infect specific cell types, is determined by the presence of CD4 and either CCR5 or CXCR4, and so the major targets of infection *in vivo* are CD4+ T cells and macrophages.

After transmission of the virus to an uninfected person, there are usually three stages of infection: acute infection, chronic infection, and AIDS (**Figure 1-3**). Acute or primary HIV infection is typically characterized by high rates of HIV replication with the median plasma viral level around  $10^6$  or  $10^7$  genome copies/mL at the peak of infection (Knipe and Howley, 2013). This massive infection is accompanied by a significant loss of CD4+ T cells. After about 6 to 12 months, viremia will drop to a mean set point of 30,000 copies/mL, where it will very slowly increase over the course of chronic infection, characterized by a clinical latency period. This period of chronic infection can range from 6 months to over 20 years, and is usually asymptomatic with a gradual CD4+ T cell decline until the individual reaches very low levels of CD4+ T cells. A patient with a severe immune depletion, defined by a CD4 cell count of less than 200 cells/uL or the onset of specific opportunistic infections or cancers, now has AIDS (Knipe and Howley, 2013).

Currently, therapies prevent disease progression by inhibiting viral enzymes, including reverse transcriptase, integrase, and protease or by blocking viral entry into a cell. When used in combinations for optimal treatment, referred to as combination antiretroviral therapy (cART), these highly potent drugs reduce plasma viral loads to levels below detection by the commonly-used clinical assays with a

limit of detection of 50 copies/mL or less (Maldarelli et al., 2007). However, single copy assays still detect viral genomes in plasma samples after 7 to 12 years of optimal treatment in HIV-infected people (Palmer et al., 2008; Riddler et al., 2016). In longitudinal sampling of 64 HIV+ individuals on cART after 4 to 12 years of viral suppression, it was shown that residual virus continues to decline at a very slow rate (Riddler et al., 2016). Even with optimal viral suppression, patients on treatment may occasionally have blips of viremia, where plasma virus transiently increases to levels that are detectable by clinical assays (Knipe and Howley, 2013). Thus, despite years of viral suppression, disruption of treatment inevitably leads to a rebound in circulating virus.

### **HIV Genetic Diversity and Tropism**

HIV-1 isolates from infected persons around the world exhibit a large amount of genetic heterogeneity, resulting in multiple clades of HIV and recombinant strains. Clade C virus is the most prevalent, commonly found in areas of Africa and India, and now accounts for almost half of all HIV infections (Freed and Martin, 2013). The best-studied subtype is clade B virus, found most commonly in the United States, Western Europe, and Australia. Even within an infected individual, HIV exists as a quasispecies, a genetically diverse and evolving population, with an estimated increase in genetic diversity of 1% per year from the initial founder viral strain during acute infection (Freed and Martin, 2013). The high genetic diversity of HIV populations are due to multiple reasons. There are many infected individuals globally, and in each of these individuals, in the absence of

therapy, there are large numbers of progeny virus being produced. With each viral replication cycle within a cell, mutations can be introduced through reverse transcriptase errors in the production of viral DNA ( $3 \times 10^{-5}$  mutations/nucleotide/replication cycle), host RNA polymerase II errors during transcription, and host factor APOBEC3G/F G-to-A hypermutation, in addition to the high rates of recombination during the process of reverse transcription (Coffin and Swanstrom, 2013; Freed and Martin, 2013). If an HIV<sup>+</sup> person does not receive therapy, the virus populations within that individual continue to increase in diversity as the virus continues to replicate (Coffin and Swanstrom, 2013). However, after treatment is initiated and viral replication is suppressed, sampling of residual viremia indicates that circulating virus is somewhat genetically uniform, likely coming from stochastic activation of few latently-infected persistent cells (Coffin and Swanstrom, 2013; Kearney et al., 2014; Simonetti and Kearney, 2015). Rebounding virus after treatment cessation is also similar to virus present before the initiation of cART (Kearney et al., 2014). Therefore, the genetically uniform circulating virus seen during cART and when cART is interrupted likely derives from a few long-lived cells that are infected pre-therapy (Eisele and Siliciano, 2012; Kearney et al., 2014; Simonetti and Kearney, 2015).

The genetic diversity of HIV also dictates viral tropism or whether that virus will infect a given cell. HIV strains were initially classified by the cell type infected, as T cell line-tropic or Macrophage-tropic, although both could infect primary T cells. It was later known that the differing tropism between these strains related to factors important for viral entry, including the required CD4 expression level, high

CD4 for T cell infection and low CD4 for macrophage infection, as well as the entry co-receptor (Berger et al., 1999). During acute infection when circulating virus peaks, the majority of virus is CCR5-tropic (Zhu et al., 1993). It is not completely understood why CCR5-tropic virus typically predominates early in infection, but may indicate a role for macrophages, where infection is less cytotoxic, to allow spread to CD4<sup>+</sup> T cells, high levels of HIV replication, and dissemination in the infected person (Collins et al., 2015; Koppensteiner et al., 2012). CXCR4- or dual (CCR5 and CXCR4)-tropic virus, on the other hand, rarely is transmitted and is usually a minority population early in infection, if present. However, CXCR4-utilizing viruses have been shown to emerge to predominance in patients without treatment and this switch has been associated with lower CD4<sup>+</sup> T cell counts and faster disease progression (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). However, it is unknown if the worsened prognosis for these individuals is due to the virus co-receptor switch, since CXCR4-tropic infection has been shown to be preferentially cytolytic in CD4<sup>+</sup> T cells by *in vitro* infection (Zhou et al., 2008), or if another factor leads to a faster progression in those individuals allowing CXCR4-tropic virus to predominate.

## **HIV Persistence**

### *HIV Latency*

The main mechanism through which HIV is believed to persist is through latent infection of long-lived cells. If the integrated proviral genome remains latent, there is little to no transcription of viral genes due to host or viral blocks. Latent infection can be established and maintained as a result of multiple factors: host transcription factor availability, epigenetic modifications, defects in the HIV Tat protein, site and orientation of integration, and post-transcriptional regulatory mechanisms (Siliciano and Greene, 2011; Trono et al., 2010; Van der Sluis et al., 2013). Current cART regimens, which target entry, reverse transcription and integration, effectively prevent new viral infections, but they do not affect integrated provirus.

### *Other Sources of Persistent Virus*

Current antiretroviral therapy may not completely block virus spread directly between cells (Sigal et al., 2011) and may also allow ongoing replication in anatomic sites with decreased drug penetration. Emerging evidence indicates that low level active infection can continue to occur in some people on effective antiretroviral treatment (Buzon et al., 2011; Buzon et al., 2010; Lewin et al., 1999; Massanella et al., 2013b; Sharkey et al., 2011; Tobin et al., 2005; Vallejo et al., 2012; Yukl et al., 2010; Zhang et al., 2000; Zhu et al., 2002a). Studies in animal models have detected viral RNA in lymphoid tissue from the gastrointestinal tract, draining lymph node, spleen and in some cases, bone marrow (North et al., 2010). Studies in

human subjects have also revealed evidence of persistent active infection in CD14<sup>+</sup> monocytes (Zhu et al., 2002a). In addition, some treatment intensification studies have detected unspliced HIV RNA in the ileum, suggesting ongoing productive infection in some HIV-infected people on ART (Yukl et al., 2010). However, a study in eight patients on cART for four to twelve years indicates that the HIV-1 reservoir in memory CD4<sup>+</sup> T cells in peripheral blood and gut contains virus that evolves minimally over the course of suppressive therapy (Josefsson et al., 2013).

Lymphoid tissue is thought to be a sanctuary site where decreased ART levels allow ongoing-replication and persistence of virus (Fletcher et al., 2014). A recent study used deep sequencing and time-calibrated phylogenetic analysis of virus in lymph nodes in conjunction with mathematical modeling to support that virus may continue to replicate and evolve in these sites with little migration of virus into the periphery and without acquisition of resistance mutations (Lorenzo-Redondo et al., 2016). Thus, infected cells in lymphoid tissue can potentially produce low levels of HIV that could re-seed the reservoir of persistent HIV. Continued virus production and infection could also lead to inflammation (Buzon et al., 2010; Massanella et al., 2013a), which may play a role in maintaining the persistent reservoir of HIV. These additional issues may also need to be addressed for effective clearance of persistent virus.

### **Viral Reservoirs**

Viral reservoirs are thought to be the cell types or anatomical sites where a replication-competent virus is able to survive outside the main pool of actively

replicating virus (Dahl et al., 2010). A clinically-significant latent reservoir is thought to be one that has the potential to produce infectious virus that can cause rebound viremia when treatment is stopped. Thus, this reservoir should have the capacity to harbor provirus for long periods of time, given that residual virus has been detected after more than 7 years of treatment (Palmer et al., 2008). Resting memory CD4<sup>+</sup> T cells are the best-studied long-lived cellular reservoir of latent HIV infection. However, recent studies implicate bone marrow hematopoietic stem and progenitors cells (HSPCs) as a potentially important latent long-lived reservoir detectable in some donors (Carter et al., 2011; Carter et al., 2010; McNamara et al., 2012).

#### *Resting CD4<sup>+</sup> T Cells*

It is well established that resting memory CD4<sup>+</sup> T cells are a stable reservoir of latent HIV infection (Finzi et al., 1999; Siliciano et al., 2003) (**Figure 1-4**). One study that estimated the size of the T cell reservoir using a viral outgrowth assay found that the CD4<sup>+</sup> T cell reservoir decays extremely slowly with a half-life of 44 months (Siliciano et al., 2003). Another study examining resting memory T cells predicted no significant loss of integrated HIV DNA over time, with a predicted half-life of roughly 25 years (Murray et al., 2014). A recent study of one patient found a unique integration site for a fully replication-competent and infectious HIV genome that was clonally expanded in peripheral blood mononuclear cells (PBMCs) (Simonetti et al., 2016), although whether functional virus can persist via clonal

expansion of CD4<sup>+</sup> T cells in the majority of patients with long-term viral suppression remains to be known.

Resting CD4<sup>+</sup> T cells contain barriers to productive viral infection, including rigid cortical actin, which inhibits transport of the preintegration complex, expression of cellular restriction factors that inhibit reverse transcription and low transcriptional activation (Pan et al., 2013). Because of these barriers to infection of resting T cells, most latent infection may occur when infected, activated T cells become quiescent. Alternatively, direct latent infection of resting T cells may be facilitated by cytokines, endothelial cells, or other environmental interactions ((Shen et al., 2013), reviewed in (Pace et al., 2011)).

The gold standard for the detection of latently infected cells utilizes an assay in which resting memory CD4<sup>+</sup> T cells are activated and viral outgrowth is measured. However, a recent study indicates that this technique potentially underestimates latent genomes in circulating resting T cells by up to 60-fold (Ho et al., 2013). In this study, Ho *et al* found a significant subset of the non-induced proviruses did not contain lethal mutations indicating that these non-induced proviruses are capable of producing new infectious virions upon reactivation. Additionally, reconstructed non-induced proviruses produced virions with similar infectivity to those reconstructed from induced proviruses. Because these proviral genomes did not appear to be activated and cleared by standard T cell activation methods, there appear to be barriers to reactivation of functional proviruses in latently infected resting T cells that are not well understood (Ho et al., 2013).



Resting memory T cells have been divided into different subtypes, including central memory ( $T_{CM}$ ), transitional memory ( $T_{TM}$ ), effector memory ( $T_{EM}$ ), and the recently-characterized stem cell memory T cells ( $T_{SCM}$ ).  $T_{CM}$  cells localize to lymph nodes and, upon stimulation, will become  $T_{EM}$  cells that can move into tissues to perform inflammatory and cytotoxic functions (Sallusto et al., 1999).  $T_{TM}$  cells show an intermediate phenotype between  $T_{CM}$  and  $T_{EM}$  cells (Riou et al., 2007). The contribution of each of these subtypes to the HIV-1 reservoir is variable (Buzon et al., 2014; Chomont et al., 2009; Gattinoni et al., 2011; Riou et al., 2007; Sallusto et al., 1999). A study by Chomont *et al.* implicated  $T_{CM}$  and  $T_{TM}$  cells as the major components of the  $CD4^+$  T cell reservoir (Chomont et al., 2009).  $T_{CM}$  cells form a reservoir of reduced size that decays slowly in HIV-infected people with normal  $CD4^+$  T cell counts who started treatment early after infection.  $T_{TM}$  cells, on the other hand, are the primary reservoir in HIV-infected people with lower  $CD4$  counts at the time of cART initiation. Evidence was presented that these latently infected cells may be maintained over time by homeostatic proliferation due to continuous immune activation (Chomont et al., 2009).

$T_{SCM}$  cells are the least differentiated T cell subset with the greatest capacity for self-renewal (Gattinoni et al., 2011). Recently, it was reported that  $T_{SCM}$  cells are also infected by HIV (Buzon et al., 2014; Flynn et al., 2014; Gattinoni et al., 2011; Tabler et al., 2014). Buzon *et al.* studied these long-lived cells in HIV-infected people with optimal viral suppression for a median of 7 years and found that latently infected  $CD4^+$   $T_{SCM}$  cells contribute a significant portion of the HIV DNA in resting memory T cells. The  $T_{SCM}$  contribution increased over the course of therapy as more

differentiated T cell subsets that initially contributed to the reservoir were lost. The authors provided a longitudinal phylogenetic analysis of plasma and resting T cell viral sequences in 3 HIV-infected people, beginning pre-therapy and continuing at multiple time points up to 13 years post-diagnosis. These data provide evidence that T<sub>SCM</sub> cells may be infected early and continue to harbor viral genomes for an extended period (Buzon et al., 2014). Thus, eradication strategies should also target T<sub>SCM</sub> cells.

Though it is widely accepted that resting CD4<sup>+</sup> T cells are an important source of latent infection, it is not clear that this is the only reservoir contributing to HIV persistence. One study of two optimally treated HIV-infected people found that sub-genomic amplicons derived from plasma virus exactly matched the same sub-genomic amplicons derived from virus produced by reactivated resting CD4<sup>+</sup> T cells (Anderson et al., 2011). However, other studies that have isolated residual plasma virus from optimally treated people with suppressed viral loads were not able to match viral genome sequences to any provirus found in circulating resting T cells (Bailey et al., 2006; Brennan et al., 2009; Sahu et al., 2009). The study by Brennan *et al.* compared provirus in resting CD4<sup>+</sup> T cells with plasma virus, and found significant compartmentalization of sequences in circulating T cells versus the plasma in 12 out of 14 optimally treated HIV-infected people (Brennan et al., 2009). Buzon *et al.* reported close relationships between plasma viral sequences and provirus from T cell subsets. However they did not report any identical viral sequences that were found in both plasma and resting CD4<sup>+</sup> T cells (Buzon et al.,

2014). Thus, there may be additional cellular reservoirs besides resting CD4<sup>+</sup> T cells that produce virus in optimally treated people.

#### *Other potential HIV reservoirs*

Other shorter-lived cell types, including monocytes/macrophages and astrocytes ((Churchill et al., 2009; Kumar et al., 2014; Narasipura et al., 2014; Zink et al., 2010), reviewed in (Koppensteiner et al., 2012; Le Douce et al., 2010)), have also been implicated. There is evidence that shorter-lived myeloid cells, including monocytes, macrophages, and dendritic cells are able to harbor integrated HIV and contribute to persistence (reviewed in (Koppensteiner et al., 2012; Le Douce et al., 2010)). Though infrequent, monocytes with integrated genomes have been recovered from HIV-infected people after many years of optimal viral suppression. Proviral genomes from these cells closely match residual plasma virus in a study of 7 HIV-infected people (Lambotte et al., 2000; Zhu et al., 2002b). Monocyte-derived cells, including perivascular macrophages, microglial cells, and astrocytes have been implicated as reservoirs in the central nervous system ((Churchill et al., 2009; Narasipura et al., 2014; Zink et al., 2010), reviewed in (Koppensteiner et al., 2012)). Because these cells are shorter-lived, their persistence may play a role in settings in which therapy is not optimal such that low-level active infection can occur.

## **Hematopoietic Stem and Progenitor Cells (HSPCs) as a Reservoir**

### *Human Hematopoiesis*

Most multipotent HPCs in the bone marrow express the cell surface marker CD34 which is found on hematopoietic stem cells (HSC) as well as more differentiated cells committed to the lymphoid or myeloid lineage (**Figure 1-5**). Thus, CD34 is commonly used as a marker of the HPC compartment (Doulatov et al., 2012). Within the CD34+ compartment there is significant heterogeneity and a defining feature of an HSC is the ability to self-renew. This means that cells are able to proliferate, without any differentiation or loss of lineage potential from the parent to daughter cells. There are two pools within the HSC population, the long term one, with life-long self-renewal, and short term HSCs, with more finite self-renewal ability (Doulatov et al., 2012).

As a hematopoietic progenitor progresses from an HSC to a MPP to a lineage-committed progenitor, it has been shown that these cells slowly exclude the potentials to form other lineages. This may include an inability to respond to specific lineage-inducing signals or the loss of necessary transcription factors (Bhandoola et al., 2007). Traditionally, there was thought to be a clear split or branch point in development from the multipotent progenitor into either a lymphoid or myeloid lineage, with the distinction of the common lymphoid and common myeloid progenitor subsets. However, recent studies have suggested that this division between the two lineages is not clear, given, for example, that myeloid lineage potential may be retained even in pre-lymphoid cells downstream of the common lymphoid progenitor. The complexity of distinguishing progenitors with restricted

potential has been attributed to a problem of determining cell fate in an experimental setting vs. a physiological one (Schlenner and Rodewald, 2010).

In recent years, additional flow cytometry markers along with functional assays, including colony formation, stromal cell cultures, and mouse xenograft models, have been used in an attempt to better define the hierarchy of human hematopoiesis. Isolation of a single hematopoietic stem cell using flow cytometric sorting is possible via the addition of CD49f to the current set of HSC markers (Notta et al., 2011). MPPs, downstream of the HSC, lack both CD49f and CD90 expression, and lose the ability for self-renewal, but can still produce all hematopoietic lineages (Benveniste et al., 2010; Doulatov et al., 2012; Kondo, 2010; Majeti et al., 2007). From the MPP, the split in lineage potential results in a lympho-myeloid and an erythro-myeloid lineage (Doulatov et al., 2012; Gorgens et al., 2013a). Thus, lymphoid potential has been shown in populations that retain some myeloid potential, resulting in the designation of a multilymphoid progenitor (MLP) (Doulatov et al., 2010). The MLP is thought to give rise to B-NK progenitors (B-NK) and likely granulocyte-monocyte progenitors (GMPs) (Doulatov et al., 2010; Doulatov et al., 2012).

On the other hand, the erythro-myeloid lineage consists of the common myeloid progenitor (CMP), which would produce a megakaryocyte-erythroid progenitor (MEP) (Akashi et al., 2000; Doulatov et al., 2012; Gorgens et al., 2013a). The GMP and its source have been controversial, as it was commonly attributed to differentiation from a CMP. A recent study seemed to resolve this difference by suggesting that granulocyte subtypes may have separate lineages: MLPs

differentiate into GMPs, which produces neutrophils, and CMPs lead to an eosinophil-basophil progenitor (EoBP) (Gorgens et al., 2013b). Additional studies have tried to further delineate the source of granulocytic, monocytic, dendritic, and erythroid cells, suggesting additional restricted progenitor populations (Lee et al., 2015; Mori et al., 2015; Xiao et al., 2015). However, a recently published study suggests a re-thinking of the classic hematopoietic hierarchy after conducting a deeper analysis of myeloid, megakaryocyte, and erythroid lineage potential in human fetal liver, umbilical cord blood, and bone marrow (Notta et al., 2016). This study suggests that within the populations previously defined by surface marker analysis, MPPs, CMPs, and MEPs, are functionally heterogeneous populations that were demarcated with additional surface markers. Additionally, CMP, GMP, and MEP progenitors downstream of the HSC may only be relevant in fetal hematopoiesis, and, in adults, HSCs may differentiate directly into unipotent progenitors which give rise to a single cell type (Notta et al., 2016). Overall, there is further study needed to elucidate the map of human hematopoiesis.

Another long-held tenet of hematopoiesis in question is that HSCs are the only bone marrow progenitors that are able to survive over years *in vivo* and are required to replenish all downstream progenitors to produce mature blood cells (Busch et al., 2015; Notta et al., 2016; Sun et al., 2014b). This was based on the fact that human HSCs are the only cells that are able to produce long-term engraftment in an immune-deficient mouse, while MPPs and other downstream progenitors only show transient engraftment in these mouse models (Benveniste et al., 2010; Majeti et al., 2007). However, in a recently-developed mouse model, mouse HSPCs were

labeled with a unique transposon insertion site *in vivo* and then mature cell types (granulocytes, B cells, T cells, and monocytes) produced over the following year were matched to both MPPs and more lineage-restricted multipotent cells opposed to the long term HSC (Sun et al., 2014a). In another study, patients with aplastic anemia, which is characterized by deficient HSCs, still had detectable myeloid progenitors, including functional subsets of CMPs, MEPs, and GMPs, similar to normal controls, with losses noted only in the megakaryocytic-erythroid functional subsets (Notta et al., 2016). Thus, multiple studies support that several non-HSC progenitors, including MPPs and myeloid-biased progenitors, may persist longer than expected and be sequentially recruited in the human bone marrow to produce mature hematopoietic cells (Busch et al., 2015; Kim et al., 2014; Notta et al., 2016; Sun et al., 2014b; Wu et al., 2014).

Mouse HSCs and their niche in the bone marrow are probably the best studied, and we can infer from these analyses that HSCs spend their time near the endosteal lining of the medullary cavities within trabecular bone (Mendelson and Frenette, 2014; Morrison and Scadden, 2014). A recent study engrafting human HSCs into mouse bones found HSCs homing to both the trabecular and long bone regions and the specific niche may expose the HSC to factors which alter function (Guezguez et al., 2013). The components of the niche supporting bone marrow progenitors are osteoblasts, CXC-chemokine ligand 12 (CXCL12)-abundant reticular (CAR) cells, osteoclasts, sympathetic neurons, and sinusoidal endothelial cells, as well as mesenchymal stem cells (MSCs) and macrophages (reviewed in (Trumpp et al., 2010)). HSCs express a variety of molecules on their surface, which will interact

with cytokines produced by the niche and mediate adhesion within the niche. CXCR4 expression by HSPCs has been shown to be important to HSC maintenance and homing by interacting with CXCL2-expressing niche cells (Mendelson and Frenette, 2014; Morrison and Scadden, 2014; Trumpp et al., 2010).

### *HIV Infection of HSPCs*

A long-lived infected HSPC could also be an important contributor to residual HIV in treated HIV-infected people as subsets of CD34+ HSPCs do express HIV receptors (Carter et al., 2011; Carter et al., 2010). Several studies looking at both mRNA and protein levels have shown that a portion of CD34+ cells will express CD4, though expression levels are noted to be less than in monocytes or CD4+ T cells (Louache et al., 1994; Muench et al., 1997; Zauli et al., 1994). CD34+ cells vary in their expression of CCR5 and CXCR4, and more primitive cells within this population tend to express CXCR4 and not CCR5 (Carter et al., 2011; Carter et al., 2010; Ishii et al., 1999; Nixon et al., 2013; Ruiz et al., 1998; Shen et al., 1999). According to another study, CXCR4 expression occurs earlier than other lymphoid markers, IL-7 receptor and terminal deoxynucleotidyl transferase (TdT), on CD34+ progenitors, and is suggested to indicate a more restricted lymphoid potential (Ishii et al., 1999). The expression of CD4 with either co-receptor does suggest that HPCs can be infected by HIV-1.

Over 20 years ago, the hematopoietic progenitor compartment in bone marrow was first investigated and it was shown that rare infection of CD34+ cells could occur both *in vitro* and *in vivo* (Davis et al., 1991; Folks et al., 1988; Neal et al.,



1995; Stanley et al., 1992). However, given the available technologies, the researchers conducting these studies could not exclude contamination from other cell types (Chelucci et al., 1995; Shen et al., 1999; Weichold et al., 1998). Because some analyses of plasma virus found that certain identical sequences predominate in circulation over multiple time points, it was proposed that latently-infected stem cells, with the capacity for self-renewal, contributed clonal virus upon intermittent activation (Palmer et al., 2008). Indeed, a number of studies have provided evidence that HIV can infect CD34<sup>+</sup> bone marrow progenitors (Bordoni et al., 2015; Carter et al., 2011; Carter et al., 2010; McNamara et al., 2012; McNamara et al., 2013; Nixon et al., 2013; Redd et al., 2007). A study of HIV-infected people in Africa revealed that HIV-1 subtype C could infect HSPCs *in vitro* and *in vivo*. Participants with HIV-infected bone marrow progenitors also had higher rates of anemia (Redd et al., 2007). More recent studies have now shown that HIV-1 subtypes B, C, and D can all infect HSPCs *in vitro* (Carter et al., 2010).

Additionally, these studies demonstrate that HIVs that use CXCR4 for entry can infect multipotent progenitors that form colonies of multiple different lineages in methylcellulose assays (Carter et al., 2011). Notably, CXCR4-tropic HIV can also infect bona fide stem cells *in vitro* based on engraftment and production of all major hematopoietic lineages in an irradiated immune-deficient mouse (Carter et al., 2011; Carter et al., 2010). However, CCR5-tropic virus seemed unable to infect multipotent progenitors, perhaps due to the low or absent CCR5 expression in these most immature subsets (Carter et al., 2011). In addition to entry restrictions due to limited receptor expression, an analysis of post-entry infection of CD34<sup>+</sup> cord blood

progenitors suggested that viral DNA synthesis and nuclear entry may be restricted in HSPCs resulting in the low infection rates seen in these cell types (Griffin and Goff, 2015).

To study latent infection in HSPCs, Carter *et al.* utilized an HIV molecular clone that expresses viral proteins under the control of the viral promoter and GFP under a constitutively-active promoter (Carter et al., 2010). Thus, it was possible to distinguish uninfected (GFP-Gag<sup>-</sup>), actively infected (GFP<sup>+</sup>Gag<sup>+</sup>) and latently infected (GFP<sup>+</sup>Gag<sup>-</sup>) cells. When latently infected HSPCs were treated with cytokines that stimulate myeloid lineage differentiation (granulocyte macrophage-colony stimulating factor [GM-CSF] and tumor necrosis factor [TNF]- $\alpha$ ), viral gene expression was induced. These studies demonstrate that HIV can infect HSPCs and cause both active and latent infection *in vitro*.

In addition, HIV Gag<sup>+</sup> CD34<sup>+</sup> progenitors were detected in bone marrow aspirates from some HIV<sup>+</sup> donors with high viral loads (Carter et al., 2010). Progenitor cells from one donor that initially lacked detectable Gag expression, expressed Gag upon culture with GM-CSF and TNF- $\alpha$ . Examination of HIV-infected individuals on cART with undetectable viral loads revealed no detectable Gag expression in HSPCs, but HIV genomes were amplified with quantitative PCR from 4 out of 9 donors (Carter et al., 2010). These initial studies provided evidence supporting the conclusion that latent HIV infection occurs in bone marrow HSPCs *in vivo*.

Two other groups have searched for latent HIV genomes in CD34<sup>+</sup> bone marrow cells from HIV<sup>+</sup> donors on long-term cART without success. Josefsson *et al.*

did not detect HIV amplicons in CD4<sup>-</sup> CD34<sup>+</sup> HSPCs in a cohort of eight virally suppressed HIV-infected people: five who initiated cART during acute or early infection and three who started cART during chronic infection (Josefsson et al., 2012). In this study, the authors removed CD4<sup>+</sup> cells to deplete the sample of T lymphocytes. However, a subset of HSPCs express CD4 and CD4 is required for HIV infection of HSPCs (Carter et al., 2011). Thus, it is possible that the negative results from this study were due to the absence of susceptible cells in the samples. The study by Durand *et al.* tested HSPCs from a cohort of 11 optimally treated HIV-infected people, 10 of whom were diagnosed prior to 2001 (Durand et al., 2012). These investigators were unable to detect HIV DNA in CD34<sup>+</sup> HSPCs by real-time PCR. Nor could they detect virus produced using a co-culture assay of HSPCs stimulated with GM-CSF and TNF- $\alpha$  plus activated CD4<sup>+</sup> lymphoblasts. Based on the latter study, some investigators suggested the possibility that CD4<sup>+</sup> T cell contamination confounded prior results (Carter et al., 2010). However, because the Durand *et al.* study was not powered to detect DNA in HSPCs from donors diagnosed after 2001, an alternative explanation is that it is harder to detect HIV infection of HSPCs in people infected decades ago, before optimal therapy was available. Indeed, all donors who tested positive in the prior study were diagnosed more recently (Carter et al., 2010).

### **Targeting HIV Reservoirs**

As discussed above, latently infected cells do not produce viral proteins that would lead to cytopathic effects and eventual cell death. In addition, latently

infected cells are not recognized and cleared by the immune system. Current anti-retroviral drugs, which target early stages of the HIV replication cycle, cannot inhibit this non-productive infection once established. Thus, to eradicate these infected cells, new latency-reversing agents (LRAs) are being developed to oppose latency and thus force the virus to reveal itself. With concurrent cART, this approach, termed 'shock and kill,' aims to eliminate the infected reservoir while blocking new infection events (Deeks et al., 2012).

Multiple factors contribute to latent HIV infection, including host transcription factors that bind the viral promoter and epigenetic changes that affect chromatin and alter accessibility of the viral promoter to transcriptional machinery (reviewed in (Trono et al., 2010), (Siliciano and Greene, 2011), and (Van der Sluis et al., 2013)). Thus, current work has focused on strategies to counteract these factors in favor of 'shock' or reactivation of latent HIV. Reactivated infected cells then need to be 'killed,' preferably by activation of cellular death pathways or through the host immune response. A few of the major strategies for reversing HIV latency in cell lines and primary T cells include altering chromatin structure with histone deacetylase inhibitors (HDACis), increasing availability of host transcription factors with the use of protein kinase C/AKT activators like disulfiram, for example, and stimulating cells with immune-modulating agents such as IL-7 and toll-like receptor agonists (reviewed extensively in (Remoli et al., 2012; Sgarbanti and Battistini, 2013; Shirakawa et al., 2013; Xing and Siliciano, 2013)). Our laboratory has investigated the mechanism of latency in HSPCs and the effect of different LRAs on latent infection in these cells (McNamara et al., 2012). In this study, a primary cell

model of HIV latency in HSPCs indicated that latency could be reversed by TNF- $\alpha$  through recruitment of NF- $\kappa$ B in these cells.

Although many LRAs show potential for antagonizing HIV latency, recent studies emphasize the need for further work to understand their clinical utility; there have been variable results when the same compound is tested side-by-side in different *in vitro* latency models and limited success thus far as sole therapies in clinical trials. Spina *et al.* (Spina et al., 2013) measured the effect of a panel of LRAs on multiple widely used models of latency compared with the standard quantitative viral outgrowth assay (QVOA) that uses patient-derived latently infected resting CD4<sup>+</sup> T cells. They found that no *in vitro* latency model recapitulates the *ex vivo* QVOA results, with many of the models seemingly biased towards reactivation by only specific classes of agents. PKC agonists generally induced latent HIV in the majority of models tested. This paper underlines the potential difficulties of using a single *in vitro* model to identify the best clinical approach for 'shocking' latent HIV.

HDACis (SAHA, romidepsin and panobinostat) and disulfiram did not induce viral outgrowth in a newly developed *ex vivo* assay that may better reflect *in vivo* conditions because it uses cells from HIV-infected people and does not employ allogeneic T cells, which may confound results (Bullen et al., 2014). Using this assay, viral outgrowth was only observed from donor CD4<sup>+</sup> T cells treated with T cell activating agents (Bullen et al., 2014). T cell activation and bryostatin-1, a PKC agonist, significantly induced HIV mRNA expression whereas the HDACis and disulfiram did not.

Methods that have demonstrated *in vitro* efficacy at reactivation of latent CD4<sup>+</sup> T cell infection have been employed in clinical trials with limited success (reviewed in (Rasmussen et al., 2013)). Thus, more research is needed to better understand this approach. Based on initially promising *in vitro* studies, SAHA, panobinostat, disulfiram, and IL-7 have been or are currently being tested in clinical trials with no clear success as yet (reviewed in (Rasmussen et al., 2013)). The first study using the ‘shock’ strategy examined the effect of the HDACi valproic acid plus a viral entry inhibitor over a three-month period (Lehrman et al., 2005). In this study, four HIV-infected individuals on cART had declines in numbers of infected CD4<sup>+</sup> T cells ranging from 68% to over 84%. However, subsequent trials of valproic acid failed to replicate these results (Archin et al., 2010; Routy et al., 2012; Sagot-Lerolle et al., 2008; Siliciano et al., 2007). In a separate study, SAHA treatment was found to increase HIV RNA expression in resting CD4<sup>+</sup> T cells, but had no detectable impact on residual plasma viremia (Archin et al., 2012). As mentioned above, a pilot study of disulfiram treatment also demonstrated no effect on the size of the circulating latent reservoir (Spivak et al., 2014). While clinical trials with single agents have not yet been successful, combinations of LRAs may prove effective in further studies (Darcis et al., 2015; Xing and Siliciano, 2013).

#### *Clearing Infection after Reversal of Latency*

Reactivating reservoirs of latent HIV is only the first step of the ‘shock and kill’ approach. Strategies to eliminate cells after reversal of latency are an equally important consideration for a cure. The two main strategies for killing a cell with

reactivated infection are activation of cell-death pathways and immune-mediated clearance. In response to viral infection, cell death pathways become activated to prevent further spread of an infection (Lamkanfi and Dixit, 2010). However, HIV encodes strategies to delay death of the cell and favor the establishment of infection (Badley et al., 2013). Further research should consider how well LRAs of interest can induce cell death in the various cell types implicated as reservoirs for latent HIV, as this effect may be cell-type dependent. One study found that *ex vivo* reactivation of latent virus with a 6-day treatment of the HDACi SAHA in PBMCs from cART-treated HIV-infected people did not reduce the number of latently-infected cells by a limiting dilution viral outgrowth assay (Shan et al., 2012). Moreover, SAHA did not promote cell death of resting CD4<sup>+</sup> T cells in an *in vitro* latency model, whereas T cell activation did (Shan et al., 2012).

Another strategy for clearing latent infection utilizes immune defenses to target and kill reactivated cells. According to the common definition of latency, there is little to no production of viral proteins, which makes them poor targets for cytotoxic T lymphocytes (CTLs). Anti-HIV CTLs limit replication of the virus, but these cells often show functional defects in the context of HIV infection (Migueles et al., 2009). A small group of HIV-infected people, referred to as elite controllers, have low levels of HIV replication without therapy, and these HIV-infected people have HIV-specific CTLs that can kill autologous resting CD4<sup>+</sup> T cells that reactivate latent infection *ex vivo* (Shan et al., 2012). In cART-treated HIV-infected people, latently-infected resting CD4<sup>+</sup> T cells reactivated with SAHA *ex vivo* are not cleared by CTLs isolated from the same patient, unless those CTLs are pre-stimulated with HIV Gag

peptides (Shan et al., 2012). The susceptibility of infected bone marrow HSPCs to immune clearance has not yet been assessed, but is certainly an important consideration for targeting this potential reservoir.

### **HIV Cure**

In discussion of a cure, two categories have been proposed: sterilizing and functional (Dieffenbach and Fauci, 2011). With a sterilizing cure, there is complete eradication of all replication-competent HIV from a patient. On the other hand, with a functional cure, there is suppression of viral replication and maintenance of CD4<sup>+</sup> T cell function without anti-retroviral therapy indefinitely.

The only confirmed case of a sterilizing HIV cure occurred with a bone marrow transplant for acute myeloid leukemia (Hutter et al., 2009). Often referred to as the Berlin patient, this 40-year-old man received an allogeneic bone marrow transplant (BMT) from a donor with a homozygous deletion in the *CCR5* gene. Thus, the donor cells were inherently resistant to HIV infection because they lacked expression of an HIV co-receptor. At the time of the transplant, the patient stopped anti-retroviral therapy, and had no detectable viremia without antiretroviral therapy for over 5 years (Allers et al., 2011; Hutter et al., 2009). Additionally, no HIV RNA or DNA was detectable in peripheral blood, bone marrow or rectal biopsies (Allers et al., 2011). Whether the donor stem cells or the bone marrow ablation strategy, or a combination of the two, led to this cure is unknown.

Thus far, there have been a few instances of functional cures when treatment was initiated early after initial infection. In one case, an HIV-infected woman from



Mississippi who did not receive pre-natal HIV treatment gave birth to a baby that immediately received cART (Persaud et al., 2013). The infant's initial viral load decayed after treatment began, and, after treatment was stopped at 18 months of age, circulating virus remained undetectable for about 2 years without any therapy. Eventually however, the child developed detectable viremia and needed to resume treatment (Martin and Siliciano, 2016; NIAID, 2014). The extended period of virological control that occurred after therapy cessation offers hope that proviral reservoirs can be reduced with early treatment. A complementary study of infants infected perinatally found lower levels and higher decay rates of PBMC provirus in four children that began cART sooner (age 0.5-2.6 years) compared with four that began cART later (age 6-14.7 years) (Luzuriaga et al., 2014).

In adults, recent studies suggest that early treatment can lead to a higher than expected rate of post treatment controllers (PTCs). PTCs refer to treated individuals who are found to have very low levels of viral replication after interrupting therapy. A group of 14 adult PTCs were identified from a cohort that started treatment early during primary HIV infection, and were able to maintain viral control at least 24 months after treatment interruption (Saez-Cirion et al., 2013). These HIV-infected people generally had small HIV reservoirs in PBMCs and less infection of long-lived subsets of resting T cells. A previous study had similar results in 5 PTCs who also initiated treatment during acute infection and, after stopping therapy, sustained viral control for a mean of 77 months (Hocqueloux et al., 2010). While complete eradication of HIV-infected cells would be ideal, it is practical to consider the goal of a functional cure, which could theoretically involve

viral suppression without therapy after clearance of just a fraction of reservoirs. Additionally, treatments to boost immune function or prevent viral immune evasion, as with a Nef inhibitor, may be the most helpful to allow a patient's own immune defenses to effectively control HIV replication.

#### *Additional HIV cure strategies*

The case of the Berlin patient renewed interest in stem cell therapy as a potential cure, though with no additional successes yet. Indeed, recent studies that have examined the impact of bone marrow transplant have not replicated the conditions that led to a cure of the Berlin patient. Cillo *et al.* detected plasma virus and HIV DNA in 10 HIV-infected people after they had received autologous BMTs (Cillo et al., 2013). Two other HIV<sup>+</sup> men experienced a decline of peripheral blood HIV reservoir after allogeneic transplants from wild type-CCR5<sup>+</sup> donors (Henrich et al., 2013). After a treatment interruption, they had undetectable viral levels for a prolonged period, but eventually both experienced viral rebound (Check Hayden, 2013; Henrich et al., 2013).

Despite the failure of bone marrow transplants as a therapy so far, an alternative approach is to transplant genetically modified hematopoietic stem cells to allow continued production of immune cells that are resistant to infection. Some studies have used genetic approaches that delete CCR5 or insert restriction factors into stem cells to prevent infection ((Walker et al., 2012), reviewed in (Zhen and Kitchen, 2014)). Gene therapy has also been used to modify T cells. In a preliminary trial, re-infusion of autologous T cells that had been edited by zinc-finger nucleases

to eliminate CCR5 gene expression was well tolerated in 12 HIV-infected people (Tebas et al., 2014).

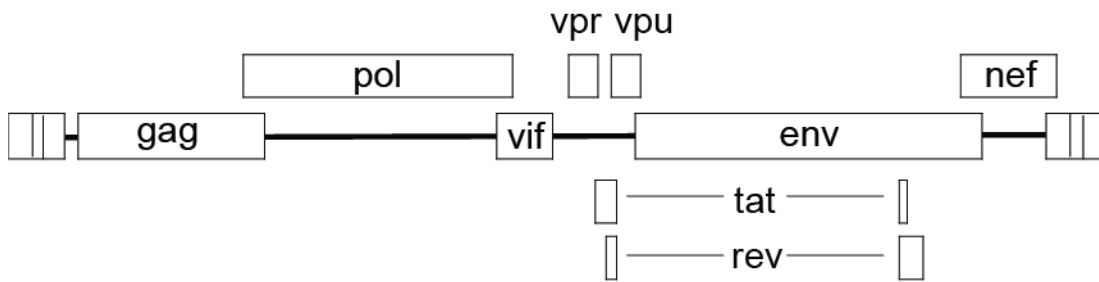
Additionally, gene therapy approaches have been utilized as a strategy to directly target latently infected cells. A recent study of interest utilized the clustered regularly interspaced short palindromic repeat (CRISPR)/ CRISPR-associated protein (CAS) 9 system to edit an integrated HIV genome and prevent transcription. This unique strategy aims to cure infection by permanently silencing proviral genomes (Ebina et al., 2013).

Other approaches to directly target latently infected cells include therapies specific to infected cells. For example, treatment with an HIV-targeted immunotoxin in combination with anti-retroviral therapy effectively kills cells with productive infection in a humanized mouse model (Denton et al., 2014). Another approach utilized radiolabeled antibodies recognizing the HIV envelope protein to selectively clear HIV-infected cells in mouse models without severe toxicity (Dadachova et al., 2012). If proven safe and effective, these therapies could be used to specifically target latently-infected cells, assuming a marker can be found that is uniquely expressed on cells with transcriptionally silent infection. One study found that CD2 expression is usually high on resting memory T cells harboring latent HIV (Iglesias-Ussel et al., 2013). However, this marker is also commonly found on uninfected cells and many infected cells were CD2<sup>-</sup>. Further characterization of which subsets of cells are infected within the resting T cell and HSPC reservoirs could reveal a targetable characteristic for cell-directed therapies.

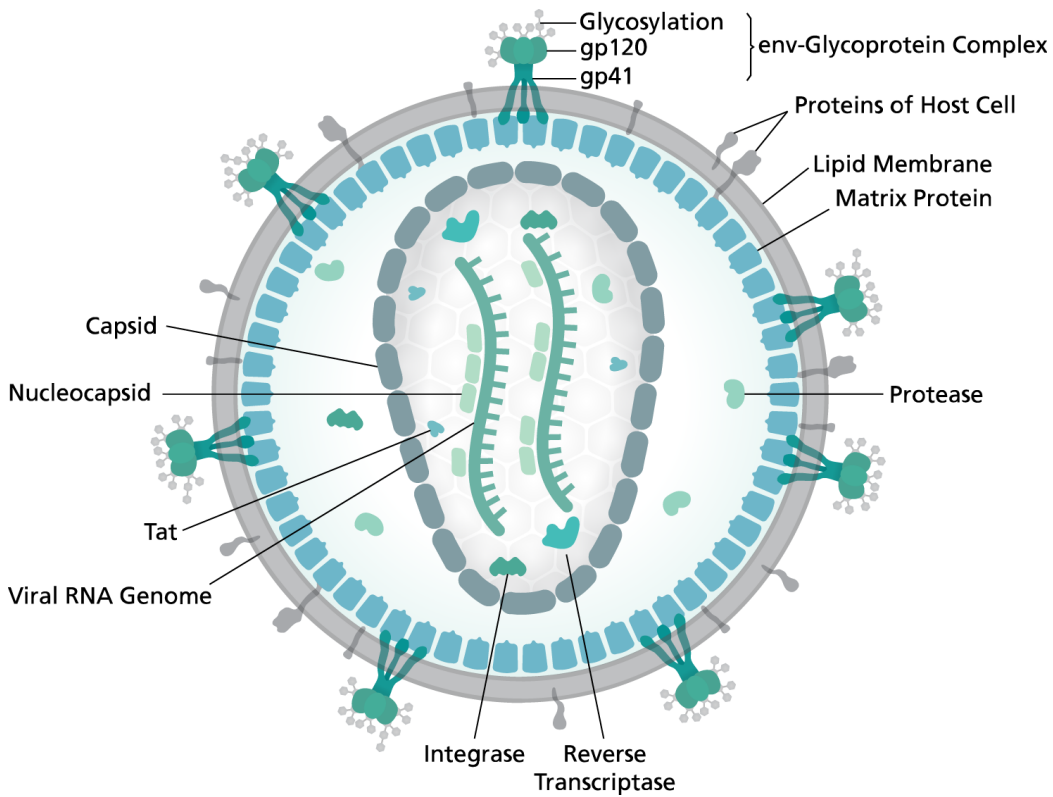
## Summary of Dissertation

The work presented in this dissertation gives insight into whether bone marrow stem and progenitor cells are truly a reservoir of HIV and if this reservoir is relevant to the cure of HIV-infected individuals. Chapter 2 presents a study of bone marrow samples from 11 HIV+ donors with suppressed viremia on therapy. Analysis of CD133-sorted progenitors from these donors indicates that a subset of donors have detectable HIV in purified HSPCs that is unlikely to be due to T cell contamination. Chapter 3 is a deeper analysis of a larger cohort of bone marrow samples from optimally-treated HIV+ donors that gives insight into the tropism of the virus *in vivo*. This study found surprisingly that CCR5-tropic virus persists in the HSPCs of these donors, even in cells populations that are depleted of true stem cells, and this supports that infected non-stem progenitors may persist *in vivo*. Chapter 4 includes a discussion of the major findings presented in chapters 2 and 3 in the context of current knowledge of HIV reservoirs, highlighting the implications and future directions of this work.

A



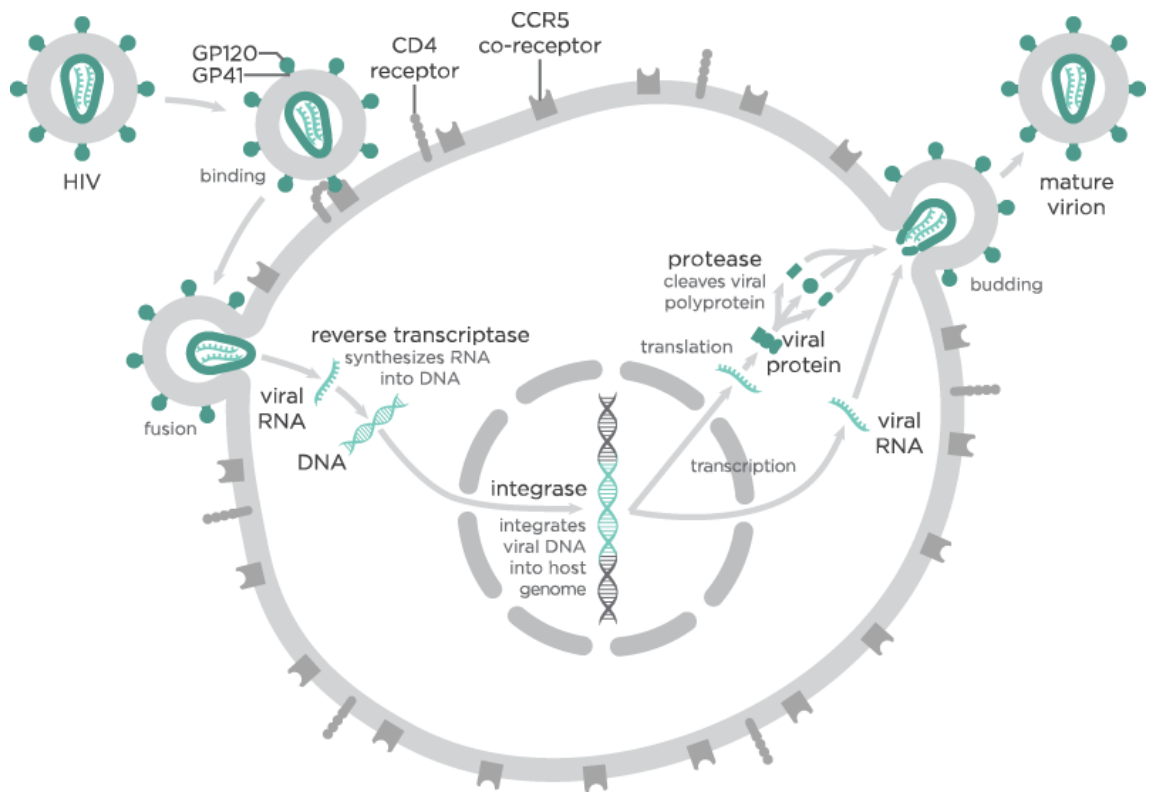
B



**Figure 1-1. HIV genome and the virion.**

(A) Organization of the HIV genome. (B) Structure of a mature viral particle.<sup>2</sup>

<sup>2</sup> Modified from the original by Thomas Splettstoesser under the Creative Commons Attribution-Share Alike 3.0 Unported license (original file and license information available at: [https://commons.wikimedia.org/wiki/File:HI-virion-structure\\_en.svg](https://commons.wikimedia.org/wiki/File:HI-virion-structure_en.svg)).



**Figure 1-2. HIV Replication Cycle.**<sup>3</sup>

<sup>3</sup> Modified from the original by Thomas Splettstoesser under the Creative Commons Attribution-Share Alike 3.0 Unported license (original file and license information available at: <https://commons.wikimedia.org/wiki/File:HIV-replication-cycle.svg>).

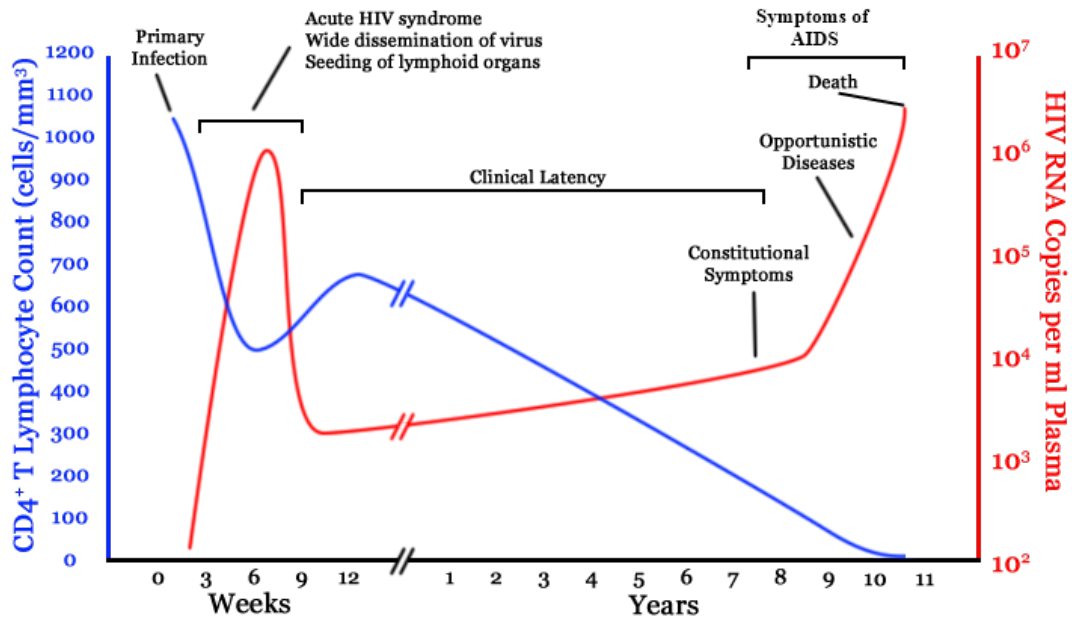
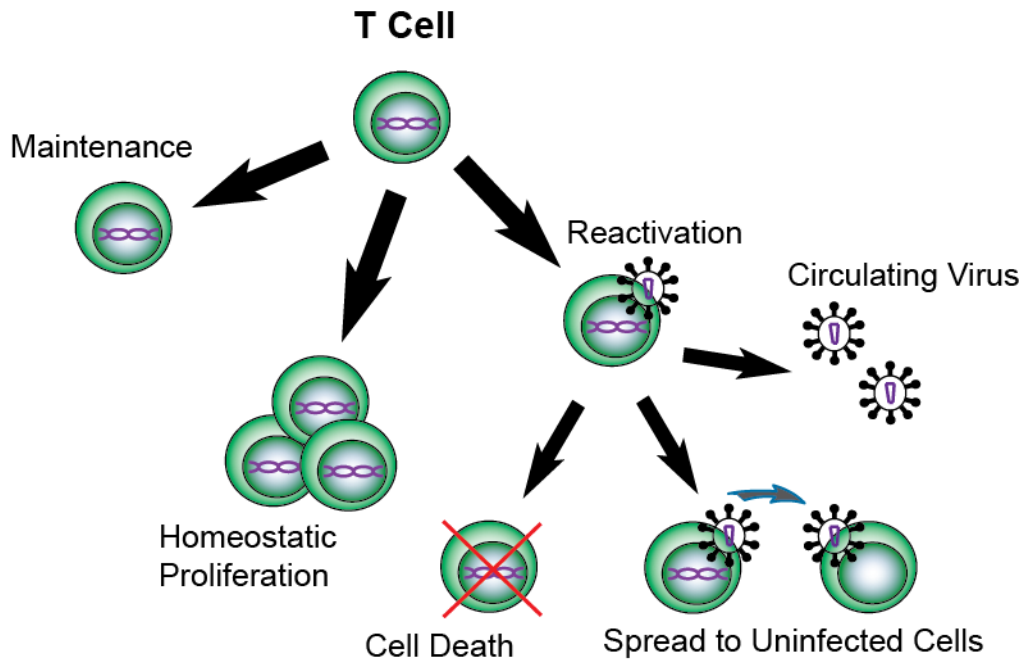


Figure 1-3. HIV infection in an untreated individual.<sup>4</sup>

<sup>4</sup> Modified from the original by Jurema Oliveira under the Creative Commons Attribution-Share Alike 3.0 Unported license (original file and license information available at: <https://commons.wikimedia.org/wiki/File:Hiv-timecourse.png>).

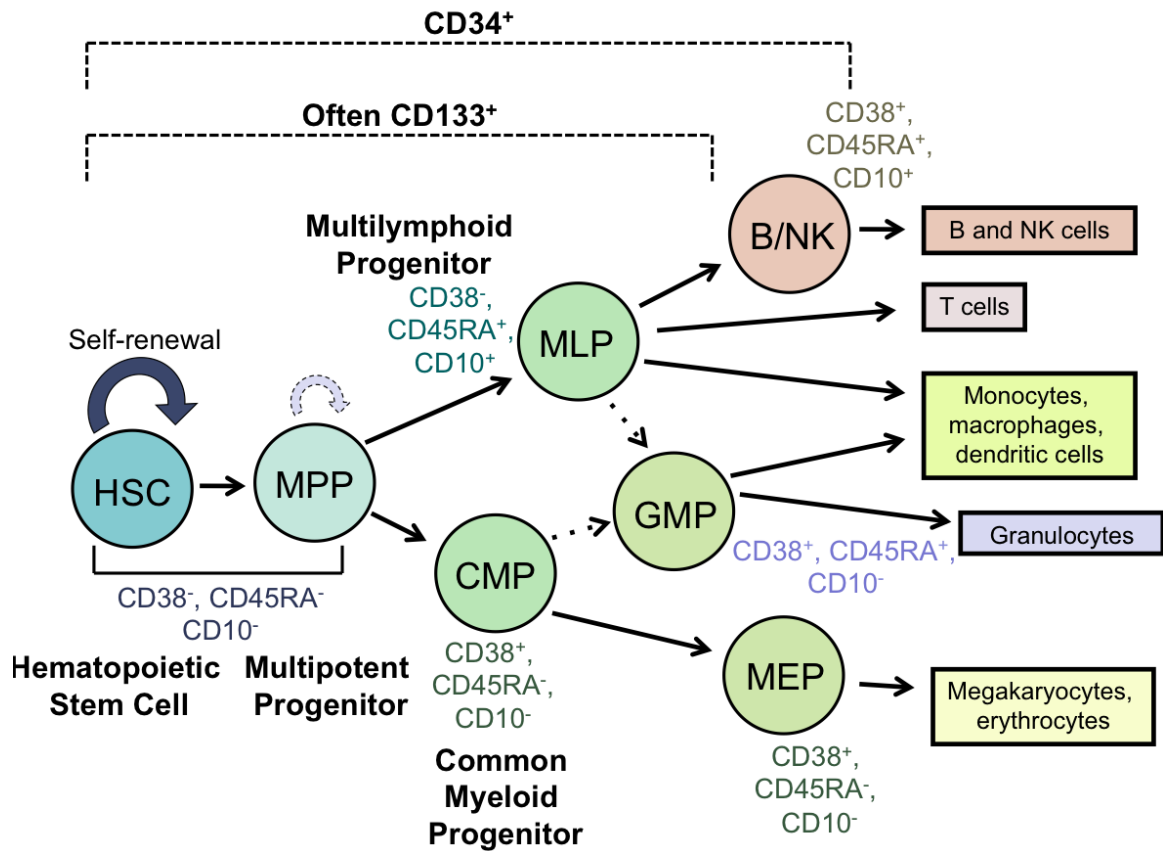


**Figure 1-4. Potential outcomes of latent infection in a T cell.**

A latently infected T cell with integrated provirus (purple) could persist through maintenance or homeostatic proliferation. With reversal of latency, the actively infected T cell could die, infect additional cells and release virus into the periphery.<sup>5</sup>

<sup>5</sup> Reproduced with permission from Sebastian, N.T., and Collins, K.L. (2014). Targeting HIV latency: resting memory T cells, hematopoietic progenitor cells and future directions. *Expert Rev Anti Infect Ther* 12, 1187-1201.





**Figure 1-5. Human hematopoiesis.**

MLP, multilymphoid progenitor; GMP, granulocyte monocyte progenitor; B/NK, B cell-NK cell progenitor; MEP, megakaryocyte erythroid progenitor. Subsets and corresponding flow cytometric markers based on (Doulatov et al., 2010; Gorgens et al., 2013b).

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## Chapter 2

# CD133+ HPCs harbor HIV genomes in a subset of optimally treated people with long-term viral suppression<sup>6</sup>

### Abstract

**Background.** Hematopoietic progenitor cells (HPCs) in the bone marrow of HIV<sup>+</sup> 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<sup>+</sup> cells represented less than 1% of cells in all CD133-sorted samples. For 5 of 6 CD133-

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sorted samples with detectable HIV DNA, the HIV genomes could not be explained by contaminating CD3<sup>+</sup> 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<sup>+</sup> 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)). The latently infected cell is thus 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<sup>+</sup> T cells are a well-studied reservoir for latent HIV, not all HIV sequences in the plasma of many successfully treated HIV<sup>+</sup> donors can be matched to sequences in peripheral blood CD4<sup>+</sup> T cells (Bailey et al., 2006;

Brennan et al., 2009; Sahu et al., 2009). These data may 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<sup>+</sup> 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 genomes per 10,000 cells (Carter et al., 2010), suggesting that HPCs might serve as a reservoir of virus. 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<sup>+</sup> HPCs from donors with undetectable viral loads (Durand et al., 2012; Josefsson et al., 2012) and have suggested that HIV genomes in CD34<sup>+</sup> samples may be due to contaminating CD3<sup>+</sup> T cells (Durand et al., 2012).

In addition to detecting HIV genomes in CD34<sup>+</sup> cells ex vivo, we have shown that CD34<sup>+</sup> 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<sup>+</sup> HPCs, are only infected by CXCR4-utilizing HIV-1 in vitro (Carter et al., 2011). However, HIV infection of CD133<sup>+</sup> cells in vivo has not been assessed.

To investigate whether CD133<sup>+</sup> HPCs harbor HIV-1 in vivo, we quantified HIV proviral genomes in CD133<sup>+</sup> HPCs from 11 HIV<sup>+</sup> donors with plasma viral loads of

<48 copies/ml for at least 6 months. We furthermore analyzed the frequency of CD3<sup>+</sup> T cells in each sample to assess whether HIV genomes could arise from contamination with CD3<sup>+</sup> cells.

## Results

**Donor characteristics.** The 11 donors 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 2-1**). Donors had viral loads of <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 2-1**).

**Magnetic sorting for CD133 minimizes T cell contamination.** Bone marrow mononuclear cells (BMMC) from each donor were subjected to magnetic sorting for CD133 (**Figure 2-1A**), a surface marker found on a subset of CD34<sup>+</sup> cells (**Figure 2-1B**). CD133 and CD3 expression on CD133-sorted and CD133-depleted populations was analyzed by flow cytometry (**Figure 2-1C**). Although the purity of the CD133-sorted populations varied from 84.4 to 98.9% among donors, less than 1% of the cells in each CD133-sorted sample were CD3<sup>+</sup> (**Figure 2-1C** and **Table 2-2**). By contrast, CD133-depleted samples contained 36-82% CD3<sup>+</sup> cells.

**CD133-sorted BMMCs contain HIV DNA.** We used quantitative PCR (qPCR) to determine the frequency of HIV genomes in CD133-sorted and CD133-depleted



samples. Samples were at limiting dilution as prepared, with no more than 1/3 of reactions yielding HIV amplification for any sample. Between 4800 and 180,000 cells were analyzed from each 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 0 - 3 total copies of HIV DNA detected per sample (**Table 2-2**). The frequency of cells containing HIV DNA varied from <0.71 to 63 per 100,000 cells for CD133-sorted samples and from <0.59 to 49 per 100,000 cells for CD133-depleted samples (**Table 2-2**).

**CD3<sup>+</sup> T cells are unlikely to account for HIV DNA in CD133-sorted samples.** If the HIV genomes detected in our samples derived from CD3<sup>+</sup> cells, we would expect to observe many more HIV genomes in the CD133-depleted samples, which contain 36-82% CD3<sup>+</sup> cells, than in the CD133-sorted samples, which contain less than 1% CD3<sup>+</sup> cells. However, we instead observed that the frequency of HIV genomes in CD133-sorted samples was higher than the frequency of HIV genomes in CD133-depleted samples for 4 of 11 donors (**Table 2-2**). To further assess the possibility that CD3<sup>+</sup> cell contamination accounted for the HIV genomes observed in CD133-sorted samples, we calculated the necessary rate of CD3<sup>+</sup> cell infection to account for all the genomes detected in each sample (**Figure 2-2A**). If all the HIV genomes derived from CD3<sup>+</sup> cells, we would expect these calculated frequencies to be similar in the CD133-depleted and CD133-sorted samples for each donor. However, we found that for five of the six positive samples, the frequency of HIV genomes in the CD3<sup>+</sup> cells from the CD133-depleted sample was approximately 100-fold too low to

support this conclusion (**Figure 2-2A**). Using Fisher's exact test, we obtained significantly discordant calculated rates of CD3<sup>+</sup> infection in the two samples for donors 304000, 305000, 312101, 313212, and 315214 ( $p < 0.0001$  (donor 315214) or  $p < 0.01$  (other donors) using mean estimates of CD3<sup>+</sup> cell number,  $p < 0.01$  (305000, 312101, and 315214) or  $p < 0.05$  (304000 and 313212) using conservative estimates; see **Figure 2-2A legend**) (**Figure 2-2A**). Donor 311000 demonstrated the same trend but statistical significance was not achieved ( $p = 0.066$  using mean estimates) (**Figure 2-2A**). 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 detected in the sorted samples comes from T cell contamination.

It is also theoretically possible that CD133<sup>-</sup>, non-T cell contaminants could account for the genomes detected in the CD133-sorted cells. However, a calculation analogous to the one described above revealed that CD133<sup>-</sup> contaminants were unlikely to account for the genomes present in CD133-sorted samples for donors 305000, 312101, and 315214 ( $p < 0.05$ ) (**Figure 2-2B**). For donor 313212, a significant difference was observed using mean ( $p < 0.05$ ) but not conservative ( $p = 0.0506$ ) estimates of CD133<sup>-</sup> cell number (see **Figure 2-2A legend**). Donors 304000 and 311000 demonstrated the same trend, but statistical significance was not achieved ( $p = 0.096$  (304000) or  $0.105$  (311000) using mean estimates) (**Figure 2-2B**). We therefore conclude that CD133<sup>-</sup> contaminants are an unlikely source of HIV genomes in the CD133-sorted samples from three of the six positive donors. Importantly, the converse is also true: CD133<sup>+</sup> cells are an unlikely source of genomes detected in the CD133-depleted sample (**Table 2-2**). Thus, these data

suggest that at least two separable cellular sources can contain HIV DNA in the bone marrow.

**HIV sequencing and assessment of contamination.** To assess whether the HIV DNA detected arose from laboratory contamination, qPCR amplicons were separated from the primers and probe by gel electrophoresis. In all cases, a distinct band of approximately 120 base pairs was observed, confirming amplification (**Figure 2-3A**). Amplicons were extracted from the gel, sequenced, and aligned to the positive control (HXB2, **Figure 2-3B**). Analysis of HXB2 DNA from three single copy reactions amplified alongside 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 sequence, the numbers of differences observed were similar to those observed in samples from the Los Alamos database (**Figure 2-3C**). Thus, it is unlikely that the positive results we obtained are due to contamination by the positive control.

**Stability of HIV DNA in HPCs over time.** As noted in **Table 2-1**, four donors had donated samples for prior studies (Carter et al., 2011; Carter et al., 2010). At the time of the previous donation, two of these donors (308103 and 312101) had high viral loads (>50,000 copies/ml) and subsequently started therapy. The other repeat donors (313212 and 315214) had undetectable viral loads at the time of previous

donation. 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 all 4 donors with detectable HIV DNA in progenitor cells were diagnosed relatively recently (2001 or later) whereas the three donors diagnosed prior to 2001 were PCR negative (Carter et al., 2010). We assessed whether this trend held in our current cohort and observed that donors with positive PCR results were diagnosed significantly more recently than donors without detectable provirus in CD133-sorted samples ( $p < 0.02$ , t-test) (**Figure 2-4A**). One sample from a recently diagnosed donor was negative in the current study (308103), but as only 14,000 cells could be analyzed from this donor (**Table 2-2**), 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 viral suppression ( $p = 0.49$ , t-test) (**Table 2-1** and **Figure 2-4B**) or from differences in the purity of CD133-sorted samples or the percentage of T cells present ( $p = 0.65$  or  $0.29$ , respectively). Neither year of diagnosis nor duration of viral suppression was related to detection of HIV DNA in CD133-depleted samples (**Figure 2-4C-D**,  $p = 0.46$  or  $0.32$ , respectively).

## Discussion

Because reservoirs of latent virus represent a barrier to curing HIV, it is essential to identify all sources of persistent virus. We previously showed that CD34<sup>+</sup> HPCs may harbor HIV genomes in donors with HIV loads of <48 copies/ml (Carter et al., 2010); however, subsequent studies suggested that contamination with CD3<sup>+</sup> cells could explain our results (Durand et al., 2012) or that HIV genomes in HPCs may not persist during years of therapy (Josefsson et al., 2012). Here, we extend our previous findings by showing that HIV can be detected in immature, CD133<sup>+</sup> HPCs from donors who have had undetectable viral loads for up to 8 years, including two donors where we had detected HIV DNA in CD34<sup>+</sup> HPCs in samples donated for our previous study 3 years earlier (Carter et al., 2010). We also demonstrate that for 5 of 6 CD133-sorted samples where HIV genomes were detected, CD3<sup>+</sup> cell contamination is a poor explanation for our results. These findings demonstrate that HPCs, including CD133<sup>+</sup> HPCs, can harbor HIV DNA during years of therapy.

We estimate that the frequency of HIV genomes in CD133<sup>+</sup> HPCs in our donors is <0.71-63 genomes per 100,000 cells. These frequencies are similar to the reported frequency of HIV genomes in peripheral blood CD4<sup>+</sup> T cells (1-100 per 100,000 cells (Chomont et al., 2009)). Consistent with reports that bone marrow CD4<sup>+</sup> T cells can also harbor provirus (Durand et al., 2012), we observed HIV DNA in some samples depleted for CD133<sup>+</sup> cells. However, our analysis was not designed to determine the type of CD133<sup>-</sup> cell that was infected.

For the two donors examined both here and in our prior study, we found higher frequencies of HIV DNA in HPCs in our prior study (Carter et al., 2010) than we did here. However, for both donors the 95% confidence intervals for the true frequency of genomes in these cell populations are overlapping. These 95% confidence intervals are very broad because of the low number of detectable genomes. Furthermore, our current study assesses the frequency of HIV DNA in CD133<sup>+</sup> cells whereas our previous study examined total CD34<sup>+</sup> cells. It is not clear whether the frequency of HIV genomes in these two HPC subsets differs. Finally, the level of variation observed between the sequential measurements from these two donors is consistent with the variation among sequential measurements of HIV frequency in resting CD4<sup>+</sup> T cells in donors with suppressed viral loads, even though this reservoir is known to decay very slowly with a half-life of ~44 months (Siliciano et al., 2003). To better compare the number of genomes in CD34<sup>+</sup> HPCs, CD133<sup>+</sup> HPCs, and peripheral blood resting memory T cells, additional studies that simultaneously compare HIV proviral DNA frequencies in all of these cell populations from the same donor are needed. Further studies examining the frequency of HIV genomes over time in the same HPC population from a larger cohort of donors are also required to understand the rate at which HIV genomes in HPCs decay over time and the contribution of these cells to long-term viral persistence.

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<sup>+</sup> 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 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 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). Alternatively, the inflammation associated with chronic high-level viremia may influence the stability of HIV genomes in HPCs. Further study is required to understand the connection between the frequency of HIV genomes found in HPCs and year of diagnosis.

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<sup>+</sup>CD34<sup>+</sup> 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<sup>+</sup>CD34<sup>+</sup> cells would exclude the CD34<sup>+</sup> 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, which could limit the sensitivity of their assay to detect variable donor HIV sequences.

In vitro, CD133<sup>+</sup> 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 (Seclen et al., 2010; 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<sup>+</sup> HPCs from a subset of donors with undetectable viral loads and that the genomes detected are not explained by contamination with CD3<sup>+</sup> 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.

## **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 was less than 80% pure and two donors where samples had been used up in prior experiments (1 sample) or lost (1 sample). We recruited HIV<sup>+</sup> 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 plasma viral loads of <48 copies/ml for at least 6 months. Twenty ml of bone marrow was aspirated from the posterior iliac crest, 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 37°C, then CD133<sup>+</sup> cells were

isolated by magnetic sorting (Miltenyi Biotec). Cells were sequentially sorted on two columns to increase purity. 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 cytometry.** 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). Healthy donor BMMCs (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.

**PCR.** Cells were lysed in MagNA Pure DNA Lysis/Binding Buffer (Roche) and DNA was extracted using a MagNA Pure Compact System (Roche). HIV-1 DNA was quantified by two-step quantitative PCR (qPCR). In the first round, 5 $\mu$ l DNA was amplified in each of 6 to 18 25 $\mu$ l reactions containing 2.5 $\mu$ l 10x Expand Long Template Buffer 2, 1.875U Expand Long Template Enzyme mix (Roche), 400nM of primers 1<sup>st</sup>-Gag-R (5'-CAATATCATACGCCGAGAGTGC GCGCTTCAGCAAG-3') (HXB2 702-718) and 2<sup>nd</sup>-LTR-F-univ (5'-GTGTIGAAAATCTCTAGCAGTGGC-3') (616-639), and 500 $\mu$ M dNTPs. In some reactions, 400nM of  $\beta$ -actin primers  $\beta$ -actin-F (5'-CCTTTTTTGTCCCCCAACTTG-3') and  $\beta$ -actin-R (5'-TGGCTGCCTCCACCCA-3') were also added. The 5' 18 bases of 1<sup>st</sup>-Gag-R are a tag used in the second round of PCR.

ACH-2 (Clouse et al., 1989) cell DNA diluted in DNA from uninfected primary HPCs or peripheral blood mononuclear cells (PBMCs) to a concentration of 10 HIV genomes/ $\mu$ l or 0.2 genomes/ $\mu$ l were used as positive controls and controls for sensitivity, respectively. DNA from uninfected HPCs or PBMCs was used as a negative control. Thermocycling was conducted using a thermocycler preheated to 93°C with the following cycling conditions: 93°C for 2 minutes; 12 cycles of 93°C for 15 seconds, 60°C for 30 seconds, 68°C for 1 minute; and a final 1 minute at 68°C.

Second round qPCR reactions were conducted in triplicate, each using 2 $\mu$ l of the first round in a 50 $\mu$ l reaction. Reactions contained 25 $\mu$ l FastStart TaqMan Probe Master 2x Master Mix (Roche), 1 $\mu$ M each of primers 2<sup>nd</sup>-LTR-F-univ and 2<sup>nd</sup>-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 with the following cycling conditions: 95°C for 10 minutes, then 45 cycles of 95°C for 15 seconds followed by 60°C for 60 seconds.

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

**DNA sequencing.** Positive qPCR reactions were run on a 2% agarose gel, the amplicon excised, and the DNA extracted (QIAquick Gel Extraction Kit, Qiagen).

Amplicons were sequenced by Sanger dideoxy sequencing and analyzed using 4Peaks (Mekentosj), EditSeq (DNASStar), and MEGA 5.05.

**Statistics.** 95% confidence intervals (CIs) for cell counts were generated using Excel 2004 (Microsoft). 95% CIs for the fraction of cells that were CD133<sup>+</sup> and CD3<sup>+</sup> were generated using a 95% CI generator for binomial distributions ([statpages.org/confint.html](http://statpages.org/confint.html)). Fisher's exact test was performed using an online calculator ([www.langsrud.com/fisher.htm](http://www.langsrud.com/fisher.htm)). Mann Whitney and t-tests were performed using GraphPad Prism 5.0a.

## **Acknowledgements**

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**Table 2-1. Donor characteristics**

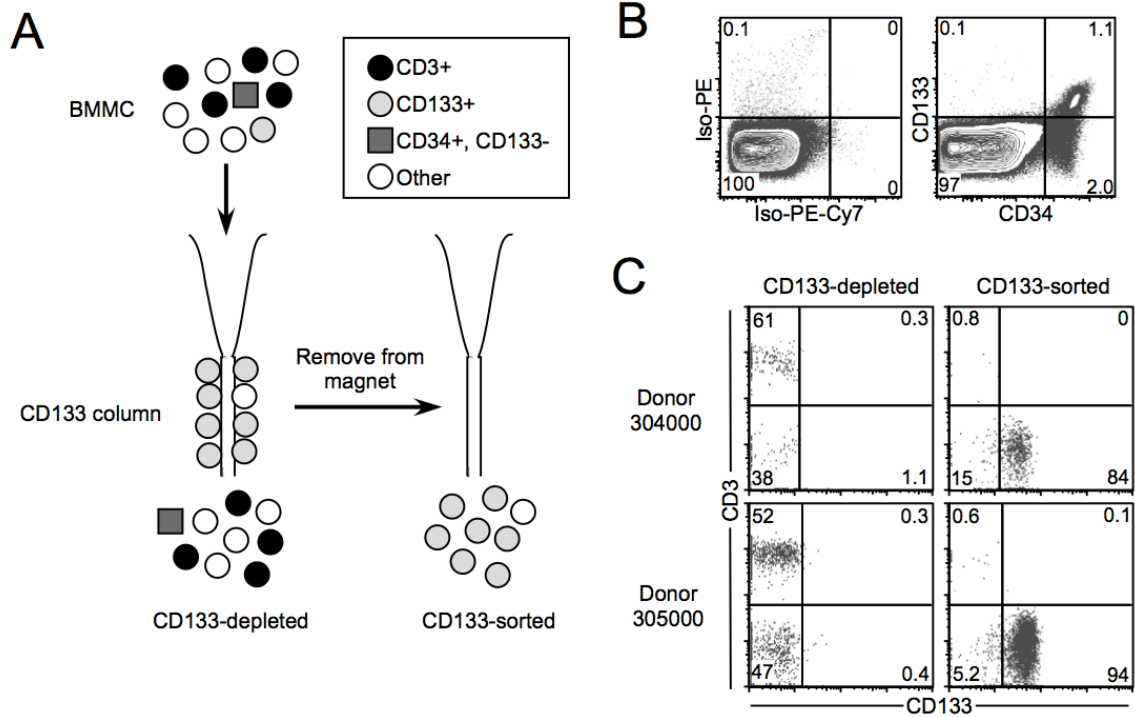
Donor identifier	Year of diagnosis	Time on ART with VL < 50 copies per ml (years)	CD4 count (cells per $\mu$ l)	WBC ( $10^9$ cells per L)	BMMC ( $10^6$ per ml)	Repeat donor? <sup>1</sup>
303000	1990	1.0	1026	4.5	15.9	No
304000	2004	3.3	533	4.8	9.35	No
305000	2007	> 0.5	1421	6.4	15.4	No
306000	1986/1987	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)

<sup>1</sup>Number in parentheses indicates the donor number in reference (Carter et al., 2010).

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

**Table 2-2. Purity of samples and frequency of HIV genomes detected**

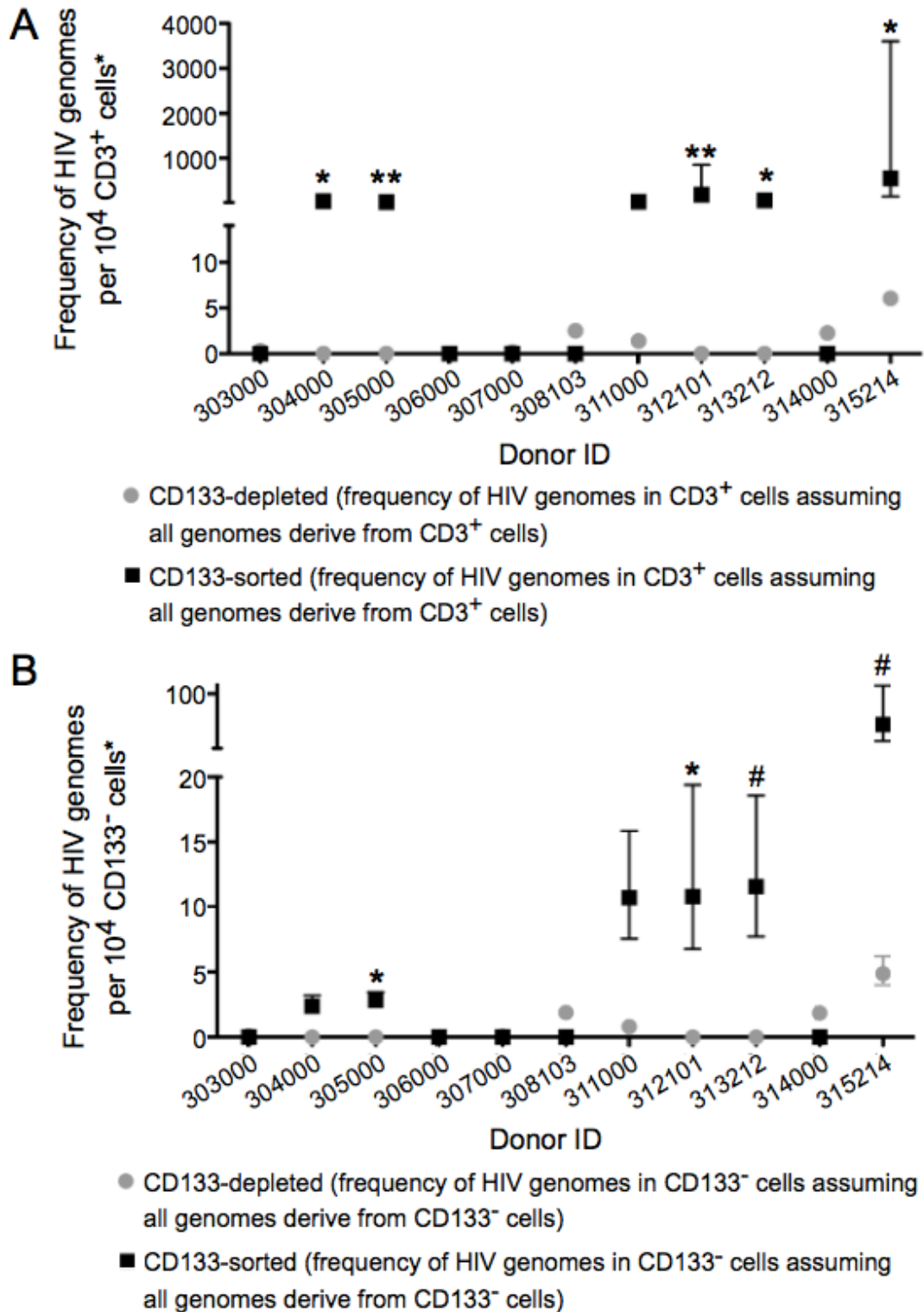
Donor identifier	10 <sup>4</sup> Cells analyzed (95% CI)	% CD133+ cells (95% CI)	%CD3+ cells (95% CI)	HIV genomes detected	Frequency of HIV genomes per 10 <sup>5</sup> cells in vivo (95% CI)
<b>CD133-sorted</b>					
303000	14 (9.7 – 17)	98.8 (98.4 – 99.0)	0.05 (0.01 – 0.14)	0	<0.71 (0 – 3.8)
304000	2.7 (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)	3	63 (10 – 250)
<b>CD133-depleted</b>					
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)	<0.19 (0 – 0.69)	75 (71 – 79)	2	19 (2.0 – 80)
311000	2.5 (2.1 – 2.9)	<0.20 (0 – 0.74)	58 (54 – 63)	2	8.1 (0.84 – 35)
312101	5.4 (4.7 – 6.0)	<0.30 (0 – 1.1)	44 (38 – 49)	0	<1.9 (0 – 7.8)
313212	3.1 (2.4 – 3.8)	<0.15 (0 – 0.55)	59 (55 – 63)	0	<3.2 (0 – 15)
314000	0.54 (0.42 – 0.66)	<0.21 (0 – 0.76)	82 (78 – 85)	1	19 (0.38 – 130)
315214	0.41 (0.32 – 0.50)	<0.22 (0 – 0.79)	81 (77 – 84)	2	49 (4.9 – 224)



**Figure 2-1. CD133<sup>+</sup> cells isolated by magnetic sorting are minimally contaminated with CD3<sup>+</sup> T cells.**

**A**, Purification protocol. CD133<sup>+</sup> 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<sup>+</sup>CD133<sup>-</sup> 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<sup>+</sup> 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 2-2. CD3<sup>+</sup> T cells are unlikely to account for HIV DNA in CD133-sorted samples.**

A, Necessary rate of infection in CD3<sup>+</sup> cells to account for all HIV genomes detected in CD133-depleted samples (gray circles) and CD133-sorted samples (black

squares). Error bars indicate 95% confidence intervals. For these calculations, the total number of HIV genomes detected in each sample was divided by the total number of CD3<sup>+</sup> cells analyzed (= (total number of cells analyzed) X (fraction of cells that are CD3<sup>+</sup>)). Fisher's exact test was used to compare these calculated frequencies using (1) a mean estimate of the number of CD3<sup>+</sup> cells analyzed as well as (2) a conservative estimate. The conservative estimate compared the top of the 95% confidence interval for the calculated infection rate in CD3<sup>+</sup> cells in the CD133-depleted sample with the bottom of the 95% confidence interval for the calculated infection rate in CD3<sup>+</sup> cells in the CD133-sorted sample to minimize the difference between these calculated infection rates. \* p < 0.01 by (1), p < 0.05 by (2); \*\* p < 0.01 by (1) and (2); \*\*\* p < 0.0001 by (1), p < 0.01 by (2). For CD133-depleted samples, the limit of detection (green circles) shows the frequency of HIV genomes in CD3<sup>+</sup> cells that would have been calculated based on an observation of 1 HIV genome in the sample. **B**, As **A**, except that it was assumed that all genomes were found in total CD133<sup>-</sup> cells. Mean (1) and conservative (2) estimates of the total number of CD133<sup>-</sup> cells in each sample were calculated as in **A**. \* p < 0.05 by (1) and (2); \*\* p < 0.01 by (1), p < 0.05 by (2); # p < 0.05 by (1) but not by (2).

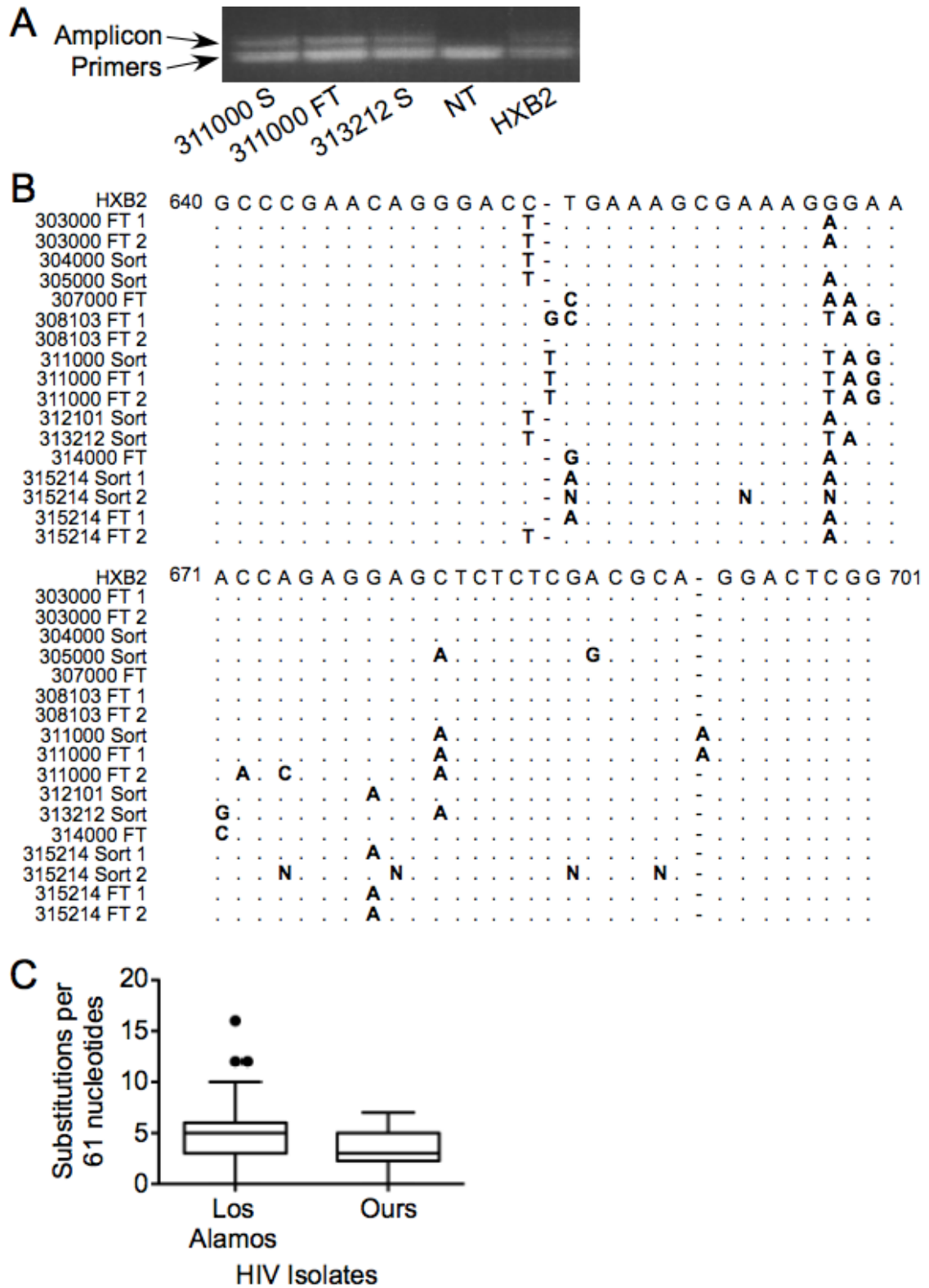


Figure 2-3. Sequence analysis of PCR products does not suggest contamination with HXB2 DNA.

**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. Because the original sequencing histogram revealed evidence of two different amplicons in the sample from 315214 Sort 2; the amplicons were cloned using standard protocols and sequencing of multiple clones confirmed the presence of two genomes (A and B). **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 median number of differences from HXB2 is not significantly different between our samples and those in the Los Alamos database ( $p = 0.07$ ) or between ours and Los Alamos subtype B sequences from the US ( $p = 0.27$ , Mann Whitney test).



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## Chapter 3

# ***In vivo* persistence of CCR5-tropic HIV in long-lived CD34<sup>+</sup> hematopoietic progenitors**

### **Abstract**

Hematopoietic stem and progenitor cells (HSPCs) express HIV receptors (CD4 and CCR5 or CXCR4) in a pattern that allows CCR5-tropic viruses to infect progenitors that were previously thought to be short-lived whereas CXCR4-tropic viruses infect both progenitors and long-lived stem cells. Here, we provide surprising evidence that HSPCs containing both types of HIV provirus endure in HIV-infected people on therapy with no detectable infectious virus for at least six months. We find that multiple subsets of HSPCs express HIV receptor RNA and that HIVs of both tropisms preferentially infect a subset of HSPCs with relatively high levels of CD4. Remarkably, we describe a case in which a multipotent HSPC became infected with a CCR5-tropic HIV *in vivo* and generated progeny of multiple lineages containing clonal copies of the provirus it harbored. Our results have implications for understanding HIV disease and pathways of hematopoiesis.

## Introduction

Long term combination anti-retroviral therapy (cART) blocks viral spread *in vivo* but is not curative as plasma virus rebounds after cART interruption. Sequence analysis of residual circulating and rebounding virus in HIV<sup>+</sup> patients indicates that virions likely come from the activation of latent provirus that had been archived since before the initiation of therapy rather than from low-level replication and spread of cART-resistant virus (Eisele and Siliciano, 2012; Kearney et al., 2014). While resting memory CD4<sup>+</sup> T cells are the best-characterized and largest reservoir, residual viral genomic sequences from plasma do not always match genomes detected in these cells suggesting that other reservoirs may exist (Bailey et al., 2006; Brennan et al., 2009; Pou et al., 2013; Sahu et al., 2009).

HIV enters cells via HIV Env interacting with CD4 plus a co-receptor, usually CCR5 or CXCR4. CXCR4-utilizing viruses differ from those that utilize CCR5 by more efficiently infecting CD133<sup>+</sup>CD34<sup>+</sup> HSPCs that are enriched for stem cells (Carter et al., 2011); Some HSPCs targeted by CXCR4-tropic viruses have stem cell-like qualities based on their ability to engraft and generate multiple lineages in a mouse xenograft model (Carter et al., 2011). In contrast, HSPCs transduced with CCR5-tropic viruses produce only small myeloid colonies (Carter et al., 2011). Consistent with these results, Nixon and colleagues elegantly demonstrated that myeloid progenitors, including common myeloid progenitors (CMPs) and granulocyte/monocyte progenitors (GMPs), express CCR5 and can be infected by HIV *in vitro* and in a humanized mouse model (Nixon et al., 2013).

HSPCs have been shown to support both active and latent infection by HIV *in vitro* and *in vivo* (Carter et al., 2010; McNamara et al., 2012). Moreover, some studies have shown that HSPCs harbor proviral DNA in viremic people (Carter et al., 2010; Redd et al., 2007) and in treated people who have no detectable circulating virus (Bordoni et al., 2015; Carter et al., 2010; McNamara et al., 2013). However, other studies reported no provirus in this cell type (Durand et al., 2012; Josefsson et al., 2012). Thus, the question of whether HIV infection of HSPCs contributes to persistent disease requires further study.

Based largely on patterns of hematopoiesis that occur following transplantation, hematopoietic progenitors, such as those targeted by CCR5-tropic HIVs, were thought to be short-lived *in vivo* (Benveniste et al., 2010; Carter et al., 2011; Nixon et al., 2013). However, *in situ* tagging experiments in mice have recently found that non-stem cell progenitors make an enduring contribution to native hematopoiesis in adults through successive recruitment of thousands of clones, each with a minimal contribution to mature progeny. Consistent with this, non-stem cell myeloid progenitors such as GMPs were found to persist in people with aplastic anemia despite dramatic losses of stem cells (Notta et al., 2016). Thus, a large number of long-lived progenitors, rather than classically defined hematopoietic stem cells, are thought to be the main drivers of steady-state hematopoiesis during adulthood (Busch et al., 2015; Sun et al., 2014).

Here, we demonstrate that hematopoietic progenitors harboring both CXCR4 and CCR5-tropic viruses persist in optimally treated people, providing evidence that non-stem cell progenitors are long-lived in people without evidence of bone marrow

disease. We also define a CD4<sup>high</sup> subset that is preferentially targeted by both HIV subtypes *in vitro*.

## Results

### CD133 marks a subset of HSPCs that is enriched for HSCs

To better understand which types of HSPCs are infected by HIV *in vivo*, we used a sequential purification protocol that separated two populations of HSPCs from bone marrow aspirates from optimally treated HIV-infected people with clinically undetectable circulating plasma virus for at least 6 months and up to 11 years (**Table 3-1**). The first purification step utilized two passes over anti-CD133 magnetic bead columns (**Figure 3-1a,b**), which isolated HSPCs with high levels of both HSPC markers, CD34 and CD133 (Sort 1, **Figure 3-1b,c**). The flowthrough from this column was passed over anti-CD34 magnetic bead columns twice to recover cells that were CD34<sup>+</sup> but had insufficient CD133 to efficiently bind to the anti-CD133 column (Sort 2, **Figure 3-1a-c**).

Compared with Sort 2, cells from Sort 1 were strikingly enriched for HSCs and MPPs and depleted for more restricted progenitors (CMPs and megakaryocyte/erythrocyte progenitors (MEPs), **Figure 3-1d-j**, **Figure 3-2a**) [Doulatov et al., 2010]. GMP/B-NKs were found at similar frequencies in both sorts (**Figure 3-1d,e**).

Methylcellulose colony formation assays, which allow the characterization of progenitor cells based on their clonogenic capacity and differentiation potential, revealed similar numbers and types of multipotent progenitors in both cell sorts

from uninfected HSPCs (**Figure 3-1k, Figure 3-2b**). Consistent with the higher frequency of MEPs in Sort 2, more erythroid colonies (CFU-E) were generated by Sort 2 cells (**Figure 3-1k, Figure 3-2b**). These results are consistent with prior studies using a similar sorting strategy and functional readouts for progenitor cells [[Freund et al., 2006](#); [Goussetis et al., 2000](#); [Matsumoto et al., 2000](#)].

To better understand which HSPC subsets are infected by HIV in vivo, we used this strategy to fractionate HSPCs from bone marrow aspirates of optimally treated, HIV-infected people who had no detectable circulating virus. To avoid confounding results from contaminating T cells, we only included donor HSPCs that contained <2% CD3<sup>+</sup> T cells (0.02-1.6%) (**Table 3-2**). Additionally, included samples were highly purified based on CD133 and CD34 staining (Sort 1: 86-99% CD133<sup>+</sup>; Sort 2: 83-99% CD34<sup>+</sup>). While similar fractions of cells in both sorts were positive for both CD34 and CD133, donor Sort 1 cells had higher mean fluorescent intensity of CD133 and CD34 (2.8-fold,  $p < 0.0001$ , and 1.4-fold,  $p < 0.0001$ , respectively,  $n = 22$ ), which is consistent with enrichment of stem cells in Sort 1.

### **HIV DNA can be amplified from purified HSPCs**

To determine which HSPCs are targeted by HIV we developed a nested PCR amplification protocol with single copy sensitivity that generates a 0.45 kb amplicon encoding the V3 region that determines co-receptor usage. For most samples, we screened at least 100,000 cells [range 20,000-700,000 (**Table 3-2**)] from each sort and we isolated up to four amplicons per sort (**Table 3-3**). Similar numbers of cells from Sort 1 and Sort 2 were screened for each donor (**Table 3-2**). In total, we

generated 41 *env* V3 amplicons from the bone marrow HSPCs of a 23 donor subset of our larger cohort of 48 donors (**Table 3-3**). We obtained bone marrow twice from three donors and three times from one donor (**Table 3-2**).

### **CD3<sup>+</sup> T cells are unlikely to account for HIV DNA in HSPCs**

If the HIV genomes detected in our samples derived from contaminating CD3<sup>+</sup> T cells, we would expect to observe many more HIV genomes in the CD133 and CD34-depleted samples (Flowthrough 1 and Flowthrough 2), which are composed of 66%–68% CD3<sup>+</sup> T cells, than in the sorted samples, which are composed of 0.0%–1.6% CD3<sup>+</sup> T cells. However, this was not observed (). We used these values to calculate the rate of CD3<sup>+</sup> T-cell contamination needed to account for all of the genomes detected in each HSPC sample and compared that to the actual frequency of CD3<sup>+</sup> T cells (McNamara et al., 2013). Using Fisher's exact test, we obtained significantly discordant calculated rates for 37 amplicons (). For one sample, we did not have T cell frequency data and, for four samples, the *p* values for this analysis values were >0.05 (). Thirty-two of the 37 had *p* values that were significant based on our most conservative estimate in which the top of the 95% confidence interval for the calculated infection rate in the CD3<sup>+</sup> T cells in the flowthrough sample was compared with the bottom of the 95% confidence interval for the calculated infection rate in CD3<sup>+</sup> T cells in the sorted sample (McNamara et al., 2013). All Sort 1 amplicons that were verified had less than or equal to 0.5% CD3<sup>+</sup> T cells. One verified Sort 2 amplicon had 1.6 % CD3<sup>+</sup> T cells and the remainder had less than or equal to 0.3% CD3<sup>+</sup> T cells (**Table 3-2**).

### **HIV provirus can be detected in HSPC populations at similar rates regardless of stem cell frequency**

Despite the fact that Sort 2 was depleted of stem cells, 13 of the 37 verified amplicons were isolated from Sort 2 (**Table 3-3**). Moreover, for 18 donors in whom we could assess cells from both sorts, there was no significant difference between the frequencies of detectable provirus (Fisher's exact test,  $p = 0.184$ ). Direct sequence analysis revealed that amplicons isolated from Sort 1 were related but not identical to each other or amplicons from Sort 2 cells for the four donors with *env* amplicons detected in both sort populations (**Figure 3-3**). Thus, Sort 2 cells containing provirus were not clonally derived from the Sort 1 cells we isolated.

### **Both CCR5 and CXCR4-tropic HIVs persist in bone marrow HSPCs**

Because prior data had indicated that CCR5-tropic HIVs do not infect stem cells and because non-stem-cell progenitors were thought to be short-lived, we expected to find only CXCR4 tropic viruses in progenitors from treated people without detectable circulating virus for at least six months. To test this hypothesis, we sequenced all the *env* amplicons directly and utilized the geno2pheno algorithm to assign tropism (Lengauer et al., 2007; Poveda et al., 2012). Surprisingly, we found that 32 total *env* amplicons from HSPCs in 20 out of the 23 donors with detectable provirus were predicted to be CCR5-utilizing (**Table 3-3**).

Additionally, we isolated CXCR4-tropic *env* amplicons from Sort 1 (6 subtype B and 1 subtype A/AG) and two amplicons from Sort 2 (both non-subtype B). We also



generated *env* amplicons from peripheral blood mononuclear cells (PBMCs) from all 23 donors. Interestingly, all donors that harbored CXCR4-tropic HIV in HSPCs also had detectable PBMC provirus predicted to be CXCR4-tropic.

Because geno2pheno was developed with subtype B sequences and predictions for non-subtype B sequences are less reliable [Garrido et al., 2008; Lee et al., 2013; Mulinge et al., 2013], we verified Env tropism with a phenotypic assay. For this analysis, we used either HSPC-derived full-length Env or a non-HSPC-derived Env with identical nucleotide or amino acid V3 region from the same donor as available (Table 3-3, Figure 3-4, Table 3-4). Our assay confirmed that all *env* co-receptor usage predictions were correct. Interestingly, infection with six Envs could not be fully inhibited by receptor blockade in Molt4-CCR5 cells or in murine 3T3 cells in which infection completely depends on expression of human CD4 and human CCR5 [Deng et al., 1997] (Table 3-3, Figure 3-4, Table 3-4). This is most likely due to resistance of patient Envs to these drugs as has been described previously [Delobel et al., 2013; Seclen et al., 2010; Soulie et al., 2008].

### **CCR5-tropic HIVs target HSPCs that are unlikely to be HSCs**

Because our studies demonstrated the surprising result that HSPCs harboring CCR5-utilizing virus persist in HIV-infected people and prior dogma indicated only bona fide stem cells persist long-term, we pursued evidence that CCR5-tropic HIVs may still target HSCs. To assess HIV receptor expression in HSCs, we used a publicly available microarray dataset of RNA expression in human bone marrow HSPCs [Rapin et al., 2014] and performed an RNA-seq analysis of mouse

bone marrow HSPCs. After confirming that the progenitor subsets expressed a developmentally appropriate set of genes (**Figure 3-5a,b**), we determined that CCR5 was expressed at low levels in HSCs of both species and was expressed to a greater extent in more differentiated progenitor subsets, although significance was not achieved in every case (**Figure 3-6a**). These results compare with published studies showing low or no expression of CCR5 protein by HSC-enriched cells [Carter et al., 2011; Carter et al., 2010] and limited expression of CCR5 protein in more differentiated progenitors [Carter et al., 2010; Nixon et al., 2013]. In addition, we found that CXCR4 and CD4 RNA was expressed across the subsets, although human HSCs had the highest CD4 expression of all the human progenitor subsets (**Figure 3-6a**).

We confirmed protein expression data by assessing infection of HSC-enriched progenitors by full-length HIV-1s (**Figure 3-6b**). CD133<sup>bright</sup> cell populations contain the vast majority of HSCs based on engraftment in NOD/SCID mice (de Wynter et al., 1998; Gorgens et al., 2013) and CD38, CD45RA and CD90 staining (**Figure 3-6c**), and thus we used the level of CD133 staining to assess the propensity of each HIV to target HSCs. To verify the co-receptor usage of our viruses in HSPCs, we demonstrated that infection by NL4-3, a CXCR4-utilizing virus, was fully blocked by AMD3100 (**Figure 3-6d**, middle panels). Additionally, maraviroc blocked infection of HSPCs by YU2, a CCR5-utilizing virus (**Figure 3-6d**, right panels). Consistent with prior experiments performed using viral pseudotypes [Carter et al., 2011], the CCR5-tropic YU2 isolate only infected progenitors that were depleted of stem cells (low levels of CD133 and CD90), whereas the CXCR4 tropic

virus targeted a wide range of progenitors, including those that are likely to be stem cells (**Figure 3-6d**); indeed, we noted 4.5 times more CD133 expression on HSPCs infected by wild type NL4-3 compared with YU2 (**Figure 3-6e,f**). To rule out latent infection in stem cells, we treated with TNF- $\alpha$ , which activates latent infection [McNamara et al., 2012] without apparent change in the pattern of infection (**Figure 3-5c,d**). Based on this analysis, CCR5-tropic HIVs are restricted to progenitors that are unlikely to be HSCs *in vitro*.

### **CCR5-tropic HIV targeting of non-stem cell progenitors is a conserved property extending to a transmitted/founder virus**

Because there is evidence that the latent reservoir *in vivo* is established during acute infection, we tested additional envelope proteins including one from a transmitted/founder virus [SVPB16 (SV16)] (Carter et al., 2011; Li et al., 2005; Yam et al., 2002) for their ability to target stem cells (**Figure 3-7a,b**). For these studies, we utilized the minimal HIV construct (HIV-7SF-GFP) pseudotyped such that each HIV Env protein decorates HIV viral-like particles containing a lentiviral genome. Again, we found that all CCR5-tropic envelopes differed from CXCR4-utilizing ones in that transduction with CCR5-tropic envelopes was restricted to HSPCs that were unlikely to be HSCs based on CD133 expression (**Figure 3-7c-e**). In contrast, viruses pseudotyped with vesicular stomatitis virus glycoprotein (VSVG), which has a broad tropism due to a ubiquitously expressed receptor [Finkelshtein et al., 2013], infected all types of HSPCs in a pattern that was similar to viruses pseudotyped with CXCR4- and dual-tropic viruses (**Figure 3-7d**). Collectively, these data extend the

conclusion that CCR5-tropic viruses, including one isolated during acute infection, are largely restricted to non-stem cell HSPCs.

### **CCR5- and CXCR4-utilizing viruses target a separable population of multipotent HSPCs that have high levels of CD4**

The HIV receptor CD4 is required for infection and is expressed on CD34<sup>+</sup> HSPCs, although at low levels compared to CD4<sup>+</sup> T cells (Louache et al., 1994; Zauli et al., 1994). To determine whether CD4 expression marks an HIV-susceptible subset of HSPCs, we measured CD4 expression on HSPCs transduced with a GFP-expressing lentiviral vector pseudotyped with CCR5- or CXCR4-tropic Env proteins (**Figure 3-7a,b**). Remarkably, we observed that HSPCs within a CD4<sup>high</sup> flow cytometric gate displayed 2-30 times greater infection than CD4<sup>low/-</sup> cells (**Figure 3-8a-c**). In contrast, VSVG-pseudotyped viruses demonstrated no such preference (**Figure 3-8b**). Additionally, CCR5-tropic envelopes had a significantly greater propensity to target CD4<sup>high</sup> progenitors compared to CXCR4 and dual-tropic envelopes (**Figure 3-8b,c**).

To determine whether CD4 marks a stable and separable HSPC subset, we asked whether fluorescence activated cell sorting (FACS) could separate Sort 1 and Sort 2 HSPCs into CD4<sup>high</sup> and CD4<sup>low</sup> populations. Indeed we found that these populations could be separated (**Figure 3-9a**) and we found that both populations had similar capacities to form GEMM, granulocyte/macrophage (GM), and erythroid (E) colonies (**Figure 3-9b,c**). However, CD4<sup>high</sup> HSPCs contained a significantly higher frequency of HSCs and MPPs (CD38<sup>-</sup>CD10<sup>-</sup>CD45RA<sup>-</sup>) than CD4<sup>low</sup> HSPCs

(**Figure 3-10a,b, Figure 3-11a**) and this difference was apparent for both Sort 1 and Sort 2 cells. In contrast, CD4<sup>low/-</sup> HSPCs contained a significantly higher frequency of the more differentiated progenitors [CMPs and MEPs (**Figure 3-11a**)]. Interestingly, CD133<sup>bright</sup> Sort 1 HSPCs had significantly higher levels of CD4 than CD133<sup>dim</sup> HSPCs from the same sorting protocol (**Figure 3-10c**). Thus, higher expression of CD4 and CD133 mark populations enriched for HSCs and MPPs and provide a mechanism through which HIV can target these cells.

#### **CD4-negative lineages harbor HIV proviruses, some of which are identical to HSPC provirus**

To determine whether HIV can be transmitted from infected HSPCs to progeny hematopoietic lineages via proliferation and differentiation rather than direct infection *in vivo*, we asked whether CD4-negative HSPC progeny might be recipients of clonal provirus from an infected progenitor. For this analysis, we used fluorescence activated cell sorting (FACS) to isolate CD19<sup>+</sup> B cells, CD8<sup>+</sup> T cells and CD56<sup>+</sup> natural killer (NK) cells. To reduce the possibility of contamination by CD4-expressing cells, we depleted CD4<sup>+</sup> cells using an anti-CD4 magnetic bead column prior to FACS. Post-sort analysis revealed low CD4<sup>+</sup>CD3<sup>+</sup> T cell levels after bead depletion and prior to FACS (indicated as “pre” in **Figure 3-11b**). Post-FACS, CD4<sup>+</sup>CD3<sup>+</sup> T cell contamination in the sorted lineages ranged from 0-0.12%. In addition, we confirmed that the lineage-positive cells that we isolated were CD4-negative (indicated as “post” in **Figure 3-11b**).

From approximately one million cells of each type analyzed, we detected provirus in CD4-negative cells from four of five donors with CXCR4-tropic HIV and only one of

five donors without detectable CXCR4-tropic virus. Notably, for donor 420000, we amplified an 839bp *gag* amplicon from the CD56<sup>+</sup> NK population that was identical to an amplicon isolated from 420000 Sort 1 HSPCs (Fig. 5c, Table S4). Remarkably, for donor 431000, we amplified identical *env* amplicons predicted to use CCR5 from multiple CD4-negative lineages, all of which shared identity with a CD133<sup>+</sup> HSPC from Sort 1 (**Figure 3-11c,d**). Donor 431000 is unusual in our cohort in that they were diagnosed in the 1980s and had a persistently low CD4 count despite years of suppressive therapy (**Table 3-1**).

It is important to note that we used a phylogenetic analysis to ensure that all included sequences were more similar to sequences from the same donor than to those from a different donor or lab strain. In addition, we verified that amplicons from CD4-negative lineages were unlikely to have come from contaminating CD3<sup>+</sup>CD4<sup>+</sup> T cells. However, for donor 431000 we could not rule out the possibility that the amplicons from the CD19<sup>+</sup> and CD56<sup>+</sup> populations might have come from contaminating CD8<sup>+</sup> cells.

## Discussion

To cure HIV infection, all persistent cellular reservoirs of HIV must be identified and eliminated, including potential reservoirs in bone marrow HSPCs. While prior studies suggested that only CXCR4-tropic viruses, which had the capacity to infect bona fide stem cells, potentially established long-term reservoirs in HSPCs *in vivo* [Carter et al., 2011], here we provide evidence that non-stem cell CD34<sup>+</sup> progenitors infected by CCR5-tropic viruses are also long-lived. First, we confirmed and extended the prior conclusion that CCR5-tropic viruses preferentially

target a subset of HSPCs that are unlikely to be HSCs (Carter et al., 2011; Nixon et al., 2013). Second, we demonstrated that provirus recovered from HSPCs isolated from HIV-infected people treated with cART for up to 11 years was often CCR5-tropic. Finally, we demonstrated that HIV provirus could be recovered with similar frequencies from HSPC populations that include bona fide stem cells (Sort 1) and HSPC populations depleted for stem cells (Sort 2). In sum, our results provide evidence that non-stem cell progenitors targeted by viruses of both tropisms may form long-lived reservoirs of HIV provirus in bone marrow HSPCs of optimally treated people.

While HSCs are the main drivers for reconstitution of all hematopoietic lineages in xenograft models, new insights in animal and human disease models (e.g. aplastic anemia) have shown contributions of non-stem cell progenitors to steady state hematopoiesis over long periods of time [Busch et al., 2015; Notta et al., 2016; Sun et al., 2014]. Non-stem cell progenitors appear to survive longer than previously thought in the bone marrow without contribution from HSCs, with non-stem cell clones sequentially recruited over time to produce mature blood cells (Busch et al., 2015; Kim et al., 2014; Notta et al., 2016; Sun et al., 2014; Wu et al., 2014). While we expect CCR5-tropic provirus is harbored by non-stem cell progenitors, our data also indicate that a CCR5-tropic virus can infect an HSPC that produces clonal progeny of multiple lineages.

Whether non-CD4<sup>+</sup> lineages harboring proviral genomes are capable of generating infectious virus will require further study. Because wild type HIV infection is toxic to differentiating HSPCs (Carter et al., 2010), it is possible that only

HSPC harboring defective proviral genomes will be able to generate viable progeny. Nevertheless, detection of identical proviral sequences in HSPCs and CD4-negative lineages supports our conclusions that hematopoietic progenitors capable of producing daughter cells that develop into multiple lineages (1) express HIV receptors, (2) can be infected by HIV *in vivo* and (3) endure for years.

If non-HSC progenitors persist, the prevalence of CCR5 in this compartment is not surprising. During acute infection when circulating virus peaks, the majority of virus is CCR5-tropic [Zhu et al., 1993]. Moreover, rebounding virus shows little evolution from virus present before the initiation of cART, further supporting the hypothesis that the genetically uniform populations of circulating virus seen during cART and when cART is interrupted derive from a few long-lived cells that are infected pre-therapy [Kearney et al., 2014]. However, we also detected persistent provirus that encodes Env proteins capable of utilizing CXCR4 to enter cells, which can more efficiently target HSCs [Carter et al., 2011]. Given that transmitting virus is nearly uniformly CCR5-tropic, these data suggest that a significant number of cells harboring persistent provirus are infected by a virus minority that evolved to use CXCR4 for infection by the time therapy began and was archived in both PBMCs and HSPCs.

Finally, we identified a sub-population of CD4<sup>+</sup> HSPCs that is preferentially targeted by both CXCR4-tropic and CCR5-tropic viruses and we show that this subset is enriched for HSCs and multipotent progenitors. These results are consistent with other studies investigating the lineage potential of CD4 subsets using functional assays (Louache et al., 1994; Muench et al., 1997; Zauli et al., 1994).



Two studies showed that CD34<sup>+</sup> CD4<sup>high</sup> and CD4<sup>low/-</sup> populations include clonogenic progenitors and Louache *et al* furthermore demonstrated that CD34<sup>+</sup> CD4<sup>+</sup> HSPCs are enriched for long-term culture-initiating cells (Louache et al., 1994; Zauli et al., 1994). Another study extended these results using human fetal liver to show that CD34<sup>+</sup>CD4<sup>+</sup> cells are able to engraft in an immunodeficient mouse, unlike CD34<sup>+</sup>CD4<sup>-</sup> cells (Muench et al., 1997).

The viruses tested here are shown to target a CD4<sup>high</sup> subset of HSPCs, which does correlate with other studies of the CD4 requirement of different HIV envelope proteins. CCR5-tropic envelopes that infect macrophages have been shown to use low levels of CD4 for entry (Joseph et al., 2014; Walter et al., 2005), and the envelopes here that infect HSPCs are likely using relatively low levels of CD4 as well. The subset defined here as CD4<sup>high</sup> is the subset with relatively high CD4 expression within HSPCs, which have previously been shown to be much lower in CD4 expression overall than CD4<sup>+</sup> T cells (Louache et al., 1994; Zauli et al., 1994). Thus, envelopes that have the highest infection in HSPCs do require CD4 for entry, but may have a preference for cells with low CD4 expression similar to macrophages.

Preferential infection of the CD4<sup>high</sup> subset provides an explanation for another study that failed to detect provirus in HSPCs from infected people. This small study of 8 donors (3 initiating therapy during chronic infection and 5 initiating therapy during acute infection) that failed to detect HIV in CD34<sup>+</sup> bone marrow cells isolated a CD4<sup>low/-</sup> population (Josefsson et al., 2012). Thus, we support a model in which CD4<sup>high</sup> HSPCs are an HIV reservoir. However, more studies are needed to determine the half-life of these cells.

Another study that failed to detect HIV in HSPCs in 11 long-term infected people with undetectable plasma virus may not have utilized a sufficiently sensitive assay to detect provirus in HSPC [Durand et al., 2012]. Here, we optimized PCR conditions to single copy sensitivity prior to screening cells to use donor-optimized primer sets, and we analyzed a larger cohort to better define these persistently infected cells. The possibility that our results are confounded by T cell contamination is highly unlikely based on a statistical analysis that takes into account the level of T cell contamination and the frequency of provirus in contaminating populations (**Table 3-2** and **Table 3-3**).

Additionally, our study included two donors with non-subtype B proviruses, extending the conclusion that HIVs of other clades infect HSPCs and persist in these cells in people on prolonged therapy. In particular, the detection of subtype C HIV in highly purified preparations of HSPCs establishes that these cells have significance for the most common type of HIV found worldwide. These results are consistent with a prior study reporting the presence of subtype C virus in CD34<sup>+</sup> HSPCs from viremic people in Africa [Redd et al., 2007] and another study showing active infection of CD133<sup>+</sup> HSPCs *in vitro* by subtype C and subtype D Env pseudotyped virus [Carter et al., 2011].

Interestingly, we also observed that a significant subset of persistent provirus, all from CCR5 antagonist-naïve donors, appears to be relatively resistant to receptor blockade with maraviroc. While this result is consistent with other studies that have found primary isolates that demonstrate resistance to this drug

([Delobel et al., 2013](#); [Seclen et al., 2010](#); [Soulie et al., 2008](#)), its clinical significance is unknown.

Overall, our results have important implications for understanding HSPC infection as a potential barrier to a cure for HIV globally. Moreover, the evidence presented here suggesting HIV persists in non-stem cell progenitors supports a recently revised model of normal hematopoiesis that is deserving of further study.

## **Methods**

### **Clinical Samples**

We recruited HIV-positive donors currently receiving antiretroviral therapy from the University of Michigan HIV-AIDS Treatment Program and the Henry Ford Health System. Informed consent was obtained according to a protocol approved by the University of Michigan Institutional Review Board and Henry Ford Institutional Review Board. At the time of sample acquisition, all donors were >18 years old, had normal white blood cell counts, and had plasma viral loads of <48 copies/mL for at least 6 months. 20 ml of bone marrow was aspirated from the posterior iliac crest, collected in preservative-free heparin, and processed immediately. 100 mL of peripheral blood was collected in K2-EDTA vacutainer tubes and also processed the same day. Bone marrow mononuclear cells (BMMCs) and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density separation (GE Healthcare). Sort 1 and Sort 2 HSPCs were then isolated from BMMCs as indicated below.

A fraction of each clinical sample was stained with R-phycoerythrin-

conjugated anti-CD133 (Miltenyi Biotec), fluorescein isothiocyanate-conjugated anti-CD34 (eBioscience), allophycocyanin-conjugated anti-CD3 (eBioscience), and 7-aminoactinomycin D (7-AAD). Samples were analyzed on a BD FACSCanto. The donors who provided samples analyzed in this study are a subset of a larger cohort and four of the donors included provided multiple donations with 3-9 months between donations. Only samples that were at least 80% CD133<sup>+</sup> for Sort 1 or 80% CD34<sup>+</sup> for Sort 2, with < 2.0 % CD3<sup>+</sup> contamination were considered in our analysis (**Table 3-2**).

### **Cell Isolation and Culture**

Whole umbilical cord blood (CB) was obtained from the New York Blood Center and whole bone marrow was obtained commercially (AllCells Ltd.); healthy cord blood mononuclear cells (CBMCs) or BMMCs were purified by Ficoll-Hypaque centrifugation and then used either fresh or after storage in liquid nitrogen. Adherent cells were depleted from CBMCs or BMMCs by incubation in serum-free StemSpan media (StemCell Technologies) for 1-2 hours at 37°C, and then Sort 1 (CD133<sup>+</sup> cells) was isolated by magnetic separation with a CD133 MicroBead Kit (Miltenyi Biotec) according to the manufacturer's protocol with the following modifications. To achieve higher yields of CD133<sup>+</sup> cells, 1.5x the recommended ratio of CD133 MicroBeads to cells was used for donations 451000, 453000, and 454304. CBMCs or BMMCs that flowed through the first column (CD133-depleted) were used for isolating the Sort 2 (CD133<sup>low</sup>/CD34<sup>+</sup>) fraction by magnetic sorting with the EasySep Human CD34 Positive Selection Kit (StemCell Technologies). For both

CD133 and CD34 magnetic isolations, cells were sequentially sorted on 2 columns to increase purity. For one cord blood donor included in **Figure 3-11**, lineage-positive cells were depleted using the EasySep Lineage Depletion Kit (StemCell Technologies) before proceeding to the CD133 magnetic sort.

For *in vitro* infection studies, isolated HSPCs were cultured in STIF medium (StemSpan medium supplemented with 100 ng/ml stem cell factor, 100 ng/ml thrombopoietin, 100 ng/ml Flt3 ligand [all from StemCell Technologies], and 100 ng/ml insulin-like growth factor binding protein 2 [R&D Systems]). Viruses for *in vitro* studies were generated in 293T producer cells propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco Invitrogen) and 1 U/ml penicillin, 1 µg/ml streptomycin, 292 µg/ml glutamine (Gibco Invitrogen). For tropism phenotype assays, Molt4-CCR5 cells were propagated in RPMI-1640 supplemented with 10% fetal bovine serum (R10, Gibco Invitrogen), 1 U/ml penicillin, 1 µg/ml streptomycin, 292 µg/ml glutamine (Gibco Invitrogen), and 1 mg/mL G418 (Invitrogen) and NIH-3T3 CD4<sup>+</sup>CCR5<sup>+</sup> cells were propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco Invitrogen), 1 U/ml penicillin, 1 µg/ml streptomycin, and 292 µg/ml glutamine (Gibco Invitrogen), and 3 µg/ml puromycin (Invitrogen).

Methylcellulose colony-forming assays were conducted according to the manufacturer's protocol (Methocult H4034, StemCell Technologies). HSPCs were plated at limiting dilution in cytokine-containing methylcellulose medium. Colonies were manually scored on days 14-16 in a blinded fashion by three investigators based on morphology using an inverted brightfield microscope at 40X or 100X

magnification.

### **Isolation of CD4-negative Progeny**

All solutions for thawing, staining and cell sorting contained 20 U/ml DNaseI (Roche) to reduce cell clumping. Thawed donor PBMC were depleted of CD4 expressing cells with CD4 MicroBeads (Miltenyi Biotec) according to the manufacturer's protocol modified for a bead:cell ratio of 1.5:1 and passed over two sequential LS magnetic columns. Flowthrough fractions were pooled and blocked with FACS buffer containing 40 ng/ml 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Fisher Scientific) for 20 min on ice before staining on ice for the following: CD3 (APC-H7-conjugated, BD Bioscience), CD4 (AlexaFluor488 conjugated, eBioscience), CD8 (PE conjugated, BioLegend), CD19 (APC conjugated, BD Bioscience), and CD56 (PE-Cy7 conjugated, eBioscience). Cells were washed once with FACS buffer, resuspended at  $5 \times 10^6$  c/ml in phosphate buffered saline without calcium or magnesium (PBS), then filtered through a 30  $\mu$ m pre-separation filter (Miltenyi Biotec) prior to sorting on a MoFlo Astrios flow cytometer. Sort gates were set on compensated, doublet excluded DAPI-negative viable cells for monocytes (forward scatter high, CD3<sup>-</sup>CD4<sup>dim</sup>), CD8 T cells (CD3<sup>+</sup>CD4<sup>-</sup>CD8<sup>+</sup>), B cells (CD3<sup>-</sup>CD4<sup>-</sup>CD19<sup>+</sup>CD56<sup>-</sup>), and NK cells (CD3<sup>-</sup>CD4<sup>-</sup>CD19<sup>-</sup>CD56<sup>+</sup>). Sorted populations were analyzed for purity by setting gates based on isotype controls.

To assess the frequency of amplicons in CD4<sup>+</sup> T cells, magnetically labeled CD4<sup>+</sup> cells from above were cultured overnight in R10 medium at a density of up to  $15 \times 10^6$  cells per 6-well plate. Adherence-depleted CD4<sup>+</sup> T cells were stained the

next day with DAPI, AlexaFluor488 conjugated anti-CD4, APC-conjugated anti-CD14 (BioLegend), and APC-H7-conjugated anti-CD3 as described above and acquired on a BD FACSCanto cytometer.

### **Polymerase Chain Reaction (PCR)**

Cells were lysed in MagNA Pure DNA Lysis/Binding Buffer (Roche), and DNA extracted using a MagNA Pure Compact System (Roche). A 2-step PCR assay was validated for single copy sensitivity on ACH-2 cell DNA. For each donor, first and second round primers used for HSPC DNA analysis were first verified by amplification of proviral sequences from PBMC DNA. Primer sequences are listed in **Table 3-5**. First round primer pairs for Env included 5036d plus LTR-pA-R, 5956d-f plus LTR-pA-R, or envC2F2 plus envC4R1, along with primers to amplify a region of Gag (U5-577.9662-f plus tagD4.6b-p24R1d plus or minus long1316-D4.6b depending on the patient sequence). Second round primer pairs included 5956d-f plus LTR-pA-R, envC2F2 plus envC4R1, or env1in5 [Brennan et al., 2009] plus env1in3 [Brennan et al., 2009] for *env* amplification. For *gag* amplification, second round primers were 626s [Hasegawa et al., 1985] plus D4.6b [Buszczak et al., 2014].

In the first round, 5  $\mu$ L of template DNA at limiting dilution (maximum 0.1 copies/ $\mu$ L with  $\leq 25\%$  of reactions expected positive) was amplified in 50- $\mu$ L reactions containing 10  $\mu$ L of 5X Phusion HF Buffer (ThermoFisher), 1U of Phusion Hot Start II High Fidelity DNA Polymerase (ThermoFisher), 500nM of each primer, and 200  $\mu$ M deoxyribonucleotide triphosphates (dNTPs). ACH-2 [Clouse et al., 1989] cell DNA was diluted in DNA from uninfected PBMCs to serve as a positive control

(10 HIV genomes per  $\mu\text{l}$ ) or control for sensitivity (0.2 HIV genomes per  $\mu\text{l}$ ). DNA from uninfected PBMCs was used as a negative control. Thermocycling was conducted using a BioRad C1000 thermocycler with conditions indicated in **Table 3-5**.

In the second round, 1  $\mu\text{l}$  of the first round reaction was amplified in 50- $\mu\text{l}$  reactions containing 10  $\mu\text{l}$  of 5X Phusion HF Buffer, 1U of Phusion Hot Start II High Fidelity DNA Polymerase (ThermoFisher), 500nM of each primer and 200  $\mu\text{M}$  dNTPs. Thermocycling was conducted using a BioRad C1000 thermocycler with cycling conditions as in **Table 3-5**.

### **DNA Sequencing Analysis and Cloning**

PCR reactions were run on 1.5% agarose Tris-acetate-EDTA gels with 1X GelRed (Biotium), the amplicons excised, extracted using QIAquick Gel Extraction Kit (Qiagen), and then sequenced by Sanger dideoxy sequencing. Consensus sequences were generated using SeqMan (DNASStar) and contaminants were excluded after comparison to all previously generated donor sequences and lab strains in MEGA6 [Tamura et al., 2013]. For co-receptor usage prediction, V3 nucleotide sequences were submitted to an online genotypic algorithm, Geno2pheno (<http://coreceptor.geno2pheno.org/index.php>), with a false positive rate cutoff of 10% (Lengauer et al., 2007; Poveda et al., 2012).

Molecular phylogenetic analysis was performed by maximum likelihood method using MEGA7 [Kumar et al., 2016]. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model



[Hasegawa et al., 1985]. The tree with the highest log likelihood (-2805.4022) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.8699)). Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

Full-length *env* amplicons were PCR purified using the QIAquick PCR Purification Kit (Qiagen). 3' adenine overhangs were added using Taq DNA Polymerase (New England BioLabs). Each sequence was cloned with the pcDNA3.1/V5-His-TOPO TA Expression Kit (Invitrogen) according to manufacturer's protocol and then transformed into Stbl2 (Invitrogen) competent bacteria.

### **Viral Preparation and Transductions**

Infectious supernatants were prepared by transfection of proviral plasmids into 293T cells using polyethylenimine. Plasmids with NL4-3 and YU2 full-length constructs were transfected to produce wildtype virus. HIV-7SF-GFP was co-transfected with the helper plasmid pCMV-HIV-1 [Gasmi et al., 1999] and a plasmid encoding either the vesicular stomatitis virus glycoprotein (VSVG) or an HIV

Envelope protein. Viruses pseudotyped with HIV envelope proteins were used either un-concentrated or concentrated using high-molecular-weight polyethylene glycol precipitation as described previously (Carter et al., 2011; Kohno et al., 2002). Cells were infected by spin inoculation at 1,048.6xg for 2 hours at room temperature. For infection with entry inhibitors, cells were incubated with AMD3100 (10 mg/mL) and/or maraviroc (20  $\mu$ M) for at least 15 minutes before infection, during spin inoculation, and in cell culture for 2-3 days following infection.

### **Flow cytometry and antibodies.**

Antibodies to the following proteins were used for flow cytometry: CD133 (phycoerythrin [PE] conjugated; Miltenyi Biotec), CD34 (conjugated with fluorescein isothiocyanate [FITC], allophycocyanin [APC], PE-Cy7; Miltenyi Biotec and eBioscience), CD4 (OKT4 clone unconjugated, Brilliant Violet 605 conjugated; BD Biosciences), CD45RA (APC conjugated; eBioscience), CD38 (PE-Cy7 conjugated; eBioscience), CD10 (Biotin conjugated; eBioscience), HIV-1 Gag (clone KC57, FITC conjugated; Beckman Coulter), and Human Hematopoietic Lineage Cocktail (FITC conjugated; eBioscience). The secondary reagents used were streptavidin (Brilliant Violet 421 conjugated; BD Biosciences) and anti-mouse IgG2b (Alexa Fluor 647 conjugated; Invitrogen). Cells were stained with 7-aminoactinomycin D (7-AAD) to exclude dead cells. Samples were analyzed using a BD FACSanto cytometer. Cell sorting was performed using a MoFlo XDP (Beckman Coulter), MoFlo Astrios (Beckman Coulter), or FACS Aria (BD Biosciences) flow cytometer.

For staining surface proteins, cells were first incubated in fluorescence-activated

cell sorting (FACS) buffer (phosphate-buffered saline [PBS] with 2% FBS, 1% human serum, 2 mM HEPES, and 0.025% sodium azide) on ice for 20 min with directly conjugated antibodies or primary antibodies. Cells were then washed and, if necessary, stained with a secondary antibody for an additional 20 min on ice. They were then washed and fixed in PBS with 2% paraformaldehyde. For intracellular staining of HIV Gag, cells were first fixed and then permeabilized with 0.1% Triton X-100 in PBS for 5 min at room temperature. Cells were then washed and incubated with an antibody against Gag for 30 min on ice.

### **Isolation of murine cells**

Bone marrow cells were isolated and harvested as described [Signer et al., 2014]. For flow cytometric analysis and isolation of specific hematopoietic progenitors, cells were incubated with combinations of antibodies to the following cell-surface markers conjugated to FITC, PE, PerCP-Cy5.5, APC, PE-Cy7, or biotin: CD3 $\epsilon$  (17A2), CD4 (GK1.5), CD5 (53-7.3), CD8 $\alpha$  (53-6.7), CD11b (M1/70), CD16/32 (Fc $\gamma$ RII/III; 93), CD34 (RAM34), CD43 (1B11), CD44 (IM7), CD45R (B220; RA3-6B2), CD48 (HM48-1), CD117 (c-kit; 2B8), CD127 (IL7R $\alpha$ ; A7R34), CD150 (TC15-12F12.2), Ter119 (TER-119), Sca1 (D7, E13-161.7), Gr-1 (RB6-8C5), and IgM (II/41). For isolation of CD150<sup>+</sup>CD48<sup>-</sup>Lineage<sup>-</sup>Sca-1<sup>+</sup>c-kit<sup>+</sup> (CD150<sup>+</sup>CD48<sup>-</sup>LSK) HSCs and CD150<sup>-</sup>CD48<sup>-</sup>LSK MPPs, Lineage markers included CD3, CD5, CD8, B220, Gr-1, and Ter119. For isolation of CD34<sup>+</sup>CD16/32<sup>low</sup>CD127<sup>-</sup>Sca-1<sup>-</sup>LK CMPs and CD34<sup>+</sup>CD16/32<sup>high</sup>CD127<sup>-</sup>Sca-1<sup>-</sup>LK GMPs, these Lineage markers were supplemented with antibodies against CD4 and CD11b. Biotinylated antibodies were

visualized by incubation with PE-Cy7 conjugated streptavidin. All reagents were acquired from BD Biosciences, eBiosciences, or BioLegend. All incubations were for approximately 30 minutes on ice. HSCs, MPPs, CMPs, and GMPs, were pre-enriched by selecting c-kit<sup>+</sup> cells using paramagnetic microbeads and an autoMACS magnetic separator (Miltenyi). Other sorted populations included Gr-1<sup>+</sup> cells, IgM-CD43<sup>+</sup>B220<sup>+</sup> pro-B cells, IgM-CD43<sup>+</sup>B220<sup>+</sup> pre-B cells, and unfractionated bone marrow cells. Non-viable cells were excluded from sorts and analyses using DAPI. Cell sorting was performed on a FACS Aria (BD Biosciences). All fractions were double sorted to ensure high purity.

### **Gene Expression Analysis of Isolated HSPC Subsets**

Gene expression in human bone marrow HSPC and differentiated subsets assessed by microarray analysis was extracted from a published data set (Rapin et al., 2014) accessed via the NCBI Gene Expression Omnibus database (GSE42519). For RNAseq analysis of murine hematopoietic cells, RNA was extracted from 3x10<sup>4</sup> double-sorted cells from each cell population using the mirVana miRNA isolation kit (Thermo Scientific). Total RNA was quantified using a Bioanalyzer (Agilent). To assess mRNA content, we performed RNAseq on the total RNA extracted from each cell population, adding equal amounts of 92 spiked-in RNA standards to each cell population. Since the amount of spiked-in RNA standards added to each sample was known, the relationship between RPKM values and the number of transcripts for each spiked-in RNA could be determined by regression analysis (Loven et al., 2012). RNAseq reads were aligned using Bowtie software (Langmead et al., 2009) to NCBI

build 37 (mm9) of the mouse genome with the settings: -e 70 -k 1 -m 2 - n 2. The RPKM for each RefSeq gene and synthetic spike-in RNA was calculated using RPKM\_count.py (v2.3.5) counting only exonic reads (-e option). Loess regression from R affy package was used to renormalize the RPKM values by using only the spike-in RNA to fit the loess with default parameters. Only the spike-in RNAs whose abundance could be robustly quantified (RPKM values  $\geq 1$ ) were used in the loess normalization.

### **Statistical Analysis**

T cell contamination analysis was performed for HSPC and non-CD4<sup>+</sup> progeny sequences as described as in McNamara *et al* [McNamara et al., 2013]. All other statistical tests were performed using Excel or GraphPad Prism 5.0a.

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For this chapter, I conducted *in vitro* experiments, analyzed data, and assisted with donor sample analysis. Valeri Terry, Frances Taschuk, and Lucy McNamara processed donor samples and analyzed data. Adewunmi Onafuwa-Nuga processed donor samples. Ryan Yucha and Thomas Zaikos performed experiments. Robert Signer generated the RNA-seq data from mice, and was supervised by Sean Morrison. James Riddell and Norm Markovitz assisted with human subjects. Dale Bixby obtained bone marrow aspirates and assisted with human subjects. Kathleen Collins supervised the project and co-wrote this chapter.

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**Table 3-1. Donor Characteristics**

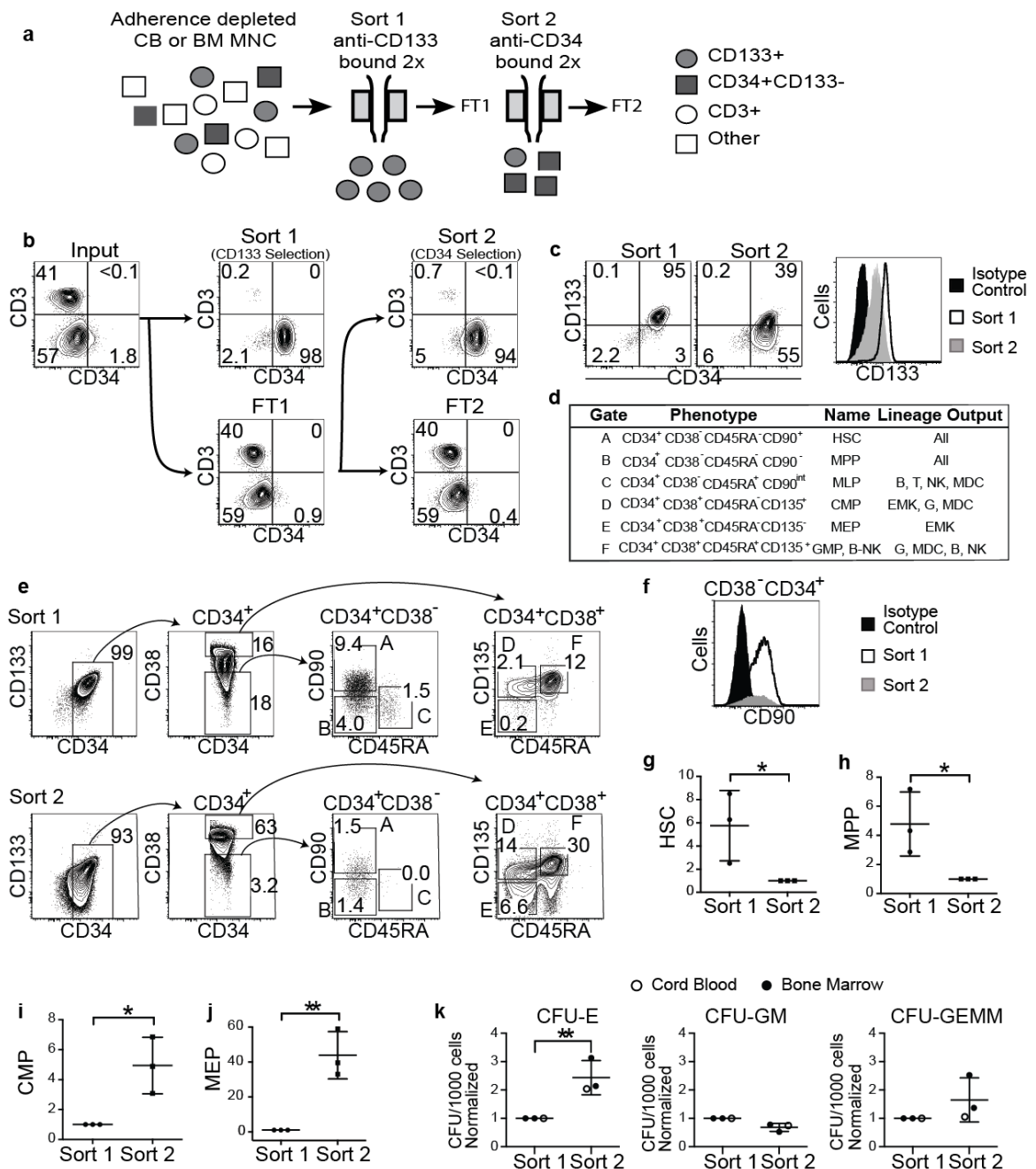
Donor ID <sup>a</sup>	Gender	Race <sup>b</sup>	Year of Diagnosis	CD4+ T-cell Count (cells/ $\mu$ l) <sup>c</sup>	Viral Load (copies/ml) <sup>d</sup>	Duration of Viral Suppression (years) <sup>c</sup>
405000	M	W-NH	1998	1341	<48	11.2
409000	M	W-NH	2010	853	<48	1.1
413402	M	B-NH	2011	321-390	<48	0.6
414000	M	W-NH	2009	749	<48	1.6
415000	M	W-H	2008	862	<48	0.5
419000	M	W-NH	1986	624	<48	2.4
420000	F	W-NH	1990	1315	<48	1.4
421000	M	W-NH	1988	775	<48	4.2
424000	M	W-NH	1995	889	<48	1.9
426000	M	W-NH	2002	1034	<40	7.1
428408	M	W-NH	2002	303-444	<40	9.2-9.6
431000	M	W-NH	mid 1980s	215	<40	3.5
432000	M	W-NH	1987	565	<40	5.2
433407	F	B-NH	2009	452-581	<48	0.6-1.4
434423	M	Other	2009	1577-1693	<40	0.9-1.2
435412406	M	W-NH	2007	1292-2060	<48	4.7-5.5
436000	F	W-NH	2006	723	<40	4.7
437000	F	W-NH	2001	418	<40	1.7
445000	M	B-NH	2009	495	<20	1.1
449000	M	W-H	1994	1392	<20	0.6
451000	M	W-NH	2010	477	<40	2.9
453000	M	W-NH	2001	704	<40	2.0
454304	M	W-NH	2004	594	<48	7.1

<sup>a</sup>First 3 digits is donation number; subsequent groups of 3 digits are ID of previous donation(s) from the same individual, if any.

<sup>b</sup>W or B indicate White or Black; H or NH indicate Hispanic or Non-Hispanic.

<sup>c</sup>Range of values are indicated for donors with multiple donations

<sup>d</sup>Based on limit of detection of clinical assay

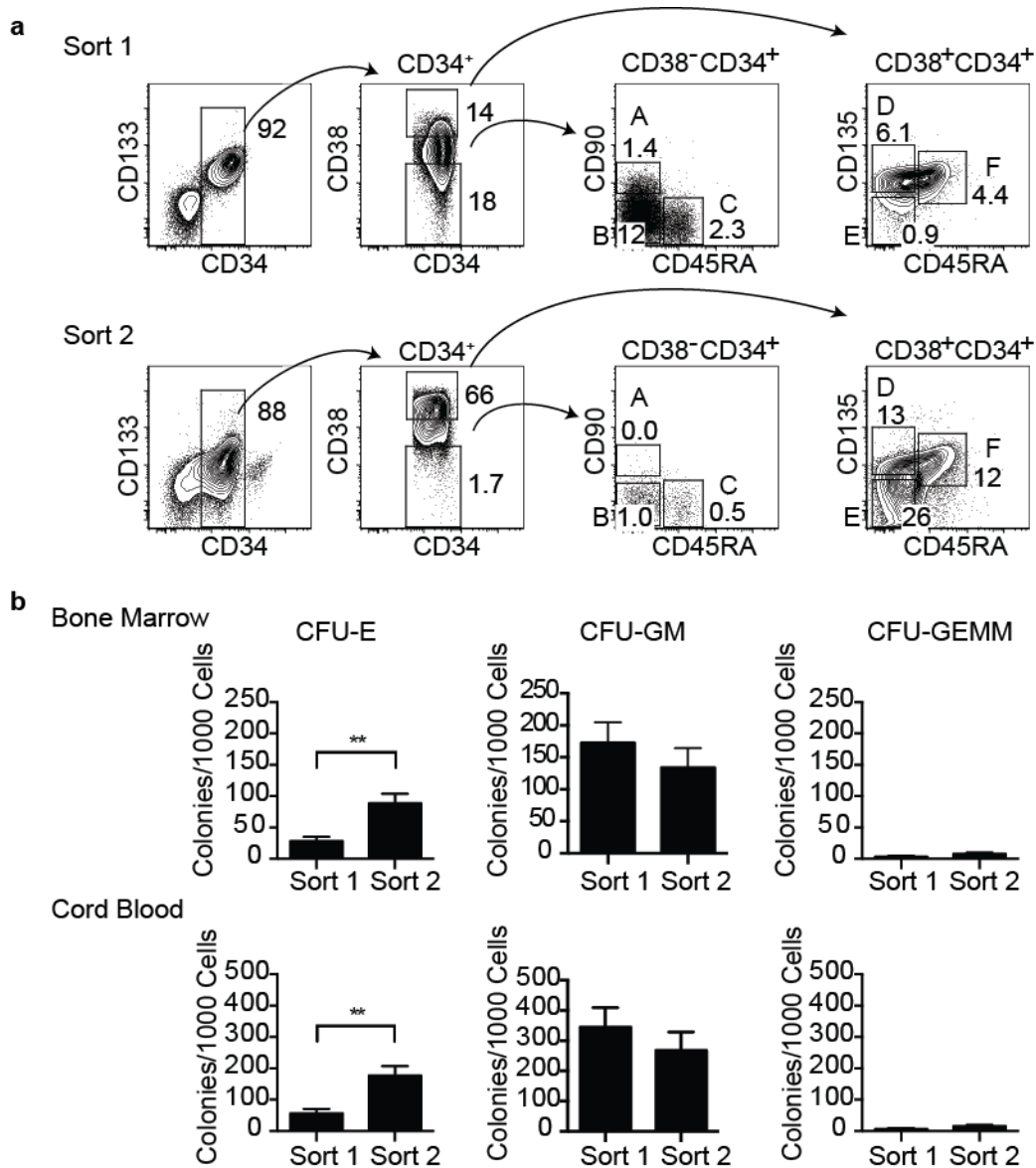


**Figure 3-1. HSPCs from Sort 2 are depleted for HSCs.**

(a) Diagrammatic representation of HSPC purification. FT, column flow through; CB, cord blood; BM MNC, bone marrow mononuclear (b) and (c) Representative flow cytometry plots of the indicated populations for a typical bone marrow aspirate from an HIV<sup>+</sup> individual. (d) Gates for each HSPC population phenotype and lineage output according to Doulatov *et al* (Doulatov *et al.*, 2010). 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; MDC,



macrophage and dendritic cell; EMK, erythroid and megakaryocyte; G, granulocyte. **(e)** Representative flow cytometric analysis of differentiation markers expressed on bone marrow HSPCs purified as described in (a and b). For the two right-most panels, numbers indicate percentage of total CD34<sup>+</sup> events from each sort falling into that gate. **(f)** Flow cytometric plot comparing relative numbers of HSCs (CD34<sup>+</sup>CD38<sup>-</sup> cells that are also CD90<sup>+</sup>) in Sort 1 versus Sort 2. **(g-j)** Summary graph showing the relative frequency of the indicated progenitor in each sort based on the analysis shown in (d), *n*=3 uninfected donors. To facilitate comparison, results were normalized to Sort 2 (g and h) or Sort 1 (i and j). Error bars represent standard deviation. **(k)** Summary plots of methylcellulose colony formation assays from three uninfected donors. Error bars represent standard deviation. CFU-E, erythroid; CFU-GM, granulocyte/macrophage and CFU-GEMM, multilineage. (\**p*<0.05 and \*\**p*<0.01, Student's t-test).



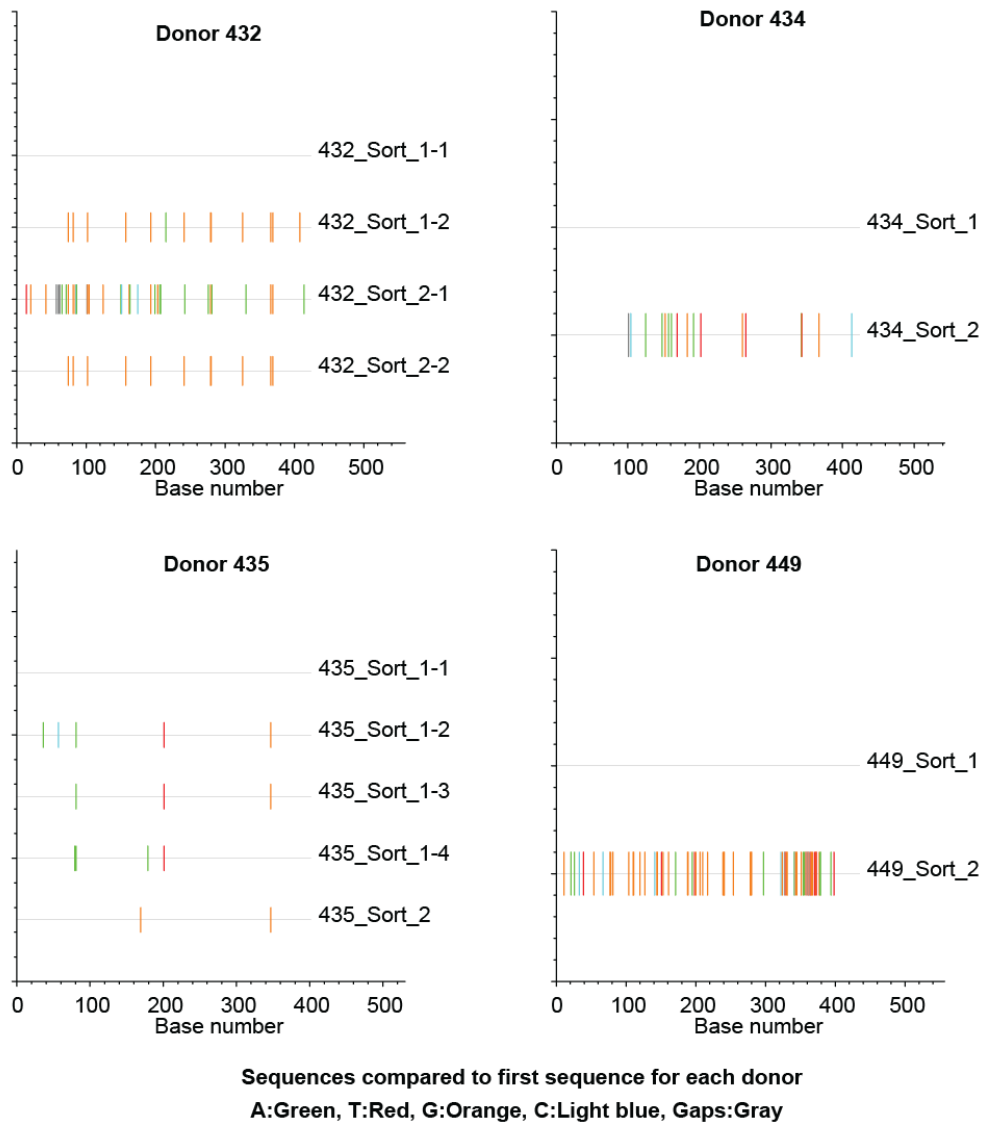
**Figure 3-2. Analysis of Sort 1 and Sort 2 HSPC subsets.**

(a) Representative flow cytometric plots of differentiation markers expressed on cord blood HSPCs purified as depicted in Fig. 1a and gated on populations listed in Fig. 1d. For the two right-most panels, numbers indicate percentage of total CD34<sup>+</sup> events falling into that gate. (b) Summary plots tabulating methylcellulose colony formation analysis of bone marrow and cord blood Sort 1 and Sort 2 HSPCs as in Fig. 1j without normalization. Error bars represent standard deviation; Student's t-test (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ ). CFU-E, erythroid; CFU-GM, granulocyte/macrophage and CFU-GEMM, multilineage.

**Table 3-2. HSPC Purity and Cells Analyzed**

Donation ID*	Sort 1			Sort 2		
	CD133+ (%)	CD3+ (%)	Cells Analyzed (x10 <sup>4</sup> )	CD34+ (%)	CD3+ (%)	Cells Analyzed (x10 <sup>4</sup> )
405000	97	NA	8.6	78	NA	NA
409000	99	0.11	9.5	98	0.07	11
413402	94	0.10	5.0	91	0.19	19
414000	95	0.22	14	85	0.33	27
415000	96	0.06	28	72	0.00	NA
419000	96	0.03	31	78	0.06	NA
420000	99	0.04	26	92	1.0	41
421000	99	0.19	17	94	0.10	37
424000	86	1.2	14	46	2.9	NA
426000	97	0.28	20	92	0.24	38
408000	95	0.61	2.0	86	0.60	3.0
428408	91	0.51	13	91	0.16	5.5
431000	93	0.23	21	89	0.15	26
432000	98	0.05	40	99	0.02	32
407000	86	2.9	NA	83	0.50	23
433407	94	0.15	9.4	90	0.13	12
423000	92	0.06	58	89	0.03	70
434423	94	0.16	67	84	0.11	53
406000	99	0.29	12	99	0.14	20
412406	96	0.40	20	83	0.06	34
435412406	95	0.22	24	94	0.66	56
436000	93	0.90	6.1	85	0.30	10
437000	94	0.16	17	92	0.04	43
445000	87	0.82	54	89	0.10	13
449000	90	0.23	29	95	0.04	60
451000	85	2.0	NA	85	1.6	19
453000	96	0.75	41	83	0.24	45
454304	92	0.41	12	87	0.21	14

\*First 3 digits is donation number; subsequent groups of 3 digits are ID of previous donation(s) from the same individual, if any.  
 Bold borders indicate multiple donations from the same individual.  
 Gray boxes indicate samples that did not meet criteria for purity.  
 Abbreviations: NA, not analyzed.



**Figure 3-3. Sort 1 and Sort 2 HSPC sequences do not share identity.** Highlighter plots of V3 *env* sequences from four donors with HSPC proviral sequence from both Sort 1 and Sort 2. Changes from the first sequence for each donor are indicated by color (see key at bottom).

**Table 3-3. Analysis of *env* amplicons isolated from HIV+ donors**

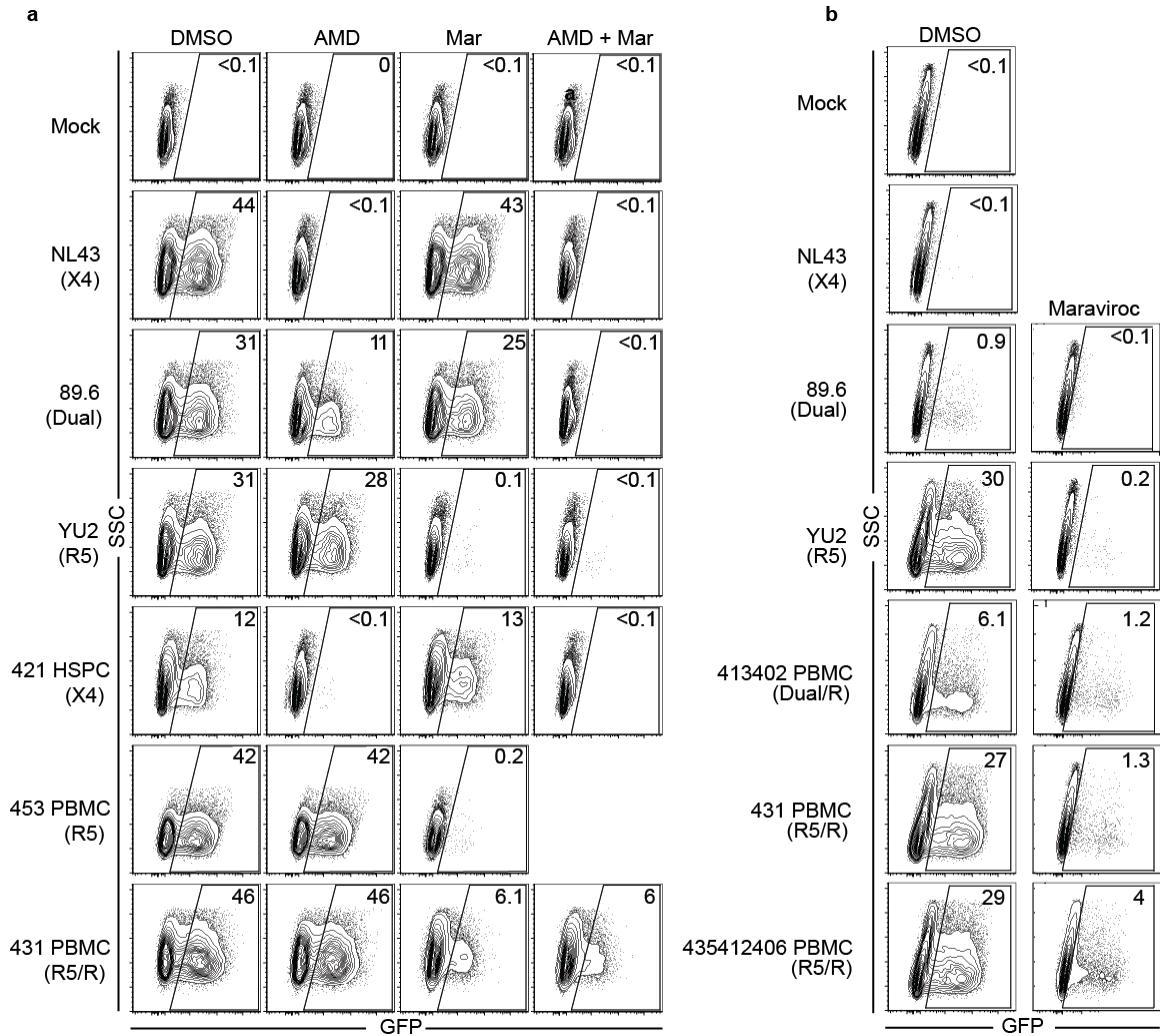
Donor ID	Clade	HSPC Sort 1 <i>env</i> amplicons				HSPC Sort 2 <i>env</i> amplicons				PBMC <i>env</i> amplicons		
		#	FPR	Geno <sup>a</sup>	Pheno <sup>b</sup>	#	FPR	Geno	Pheno	#	FPR	Geno
405000	B	1 <sup>NA</sup>	52	R5		NA				3	52	R5
409000	B B	1*	0.7	X4		ND				17 11	38-61 0.7	R5 X4
413402	B	1*	1.7	X4	Dual/R	ND				6	1.7	X4
414000	B	1*	57	R5	R5	ND				5	57	R5
415000	C	1**	71	R5	R5	NA				20	43-83	R5
419000	B	1***	89	R5		NA				21	22-99	R5
420000	B B	1**** 1****	19 0.7	R5 X4	R5 Dual/R	ND				11 7	14-73 0.5-1.7	R5 X4
421000	B B B	1*** 1*** 1***	7.4 7.8	X4 X4	X4	ND				4 5	24-94 4.7-9.6	R5 X4
424000	B	1 <sup>0.109</sup>	43	R5		NA				1	75	R5
426000	B B	ND				4****	29-60	R5		10 1	20-38 3.2	R5 X4
428408	B B	2**** 1****	83,84 1.3	R5 X4		ND				22 16	30-100 0.7-6.8	R5 X4
431000	B B	2**** 1****	75 79	R5 R5	R5/R R5/R	ND				8	38-90	R5
432000	B B AE A/AG C	1**** 1****	17 2.8	R5 X4		1**** 1****	2.8 3.4	X4 X4	NF	1 8 1 11 4	49 3.4-8.5 74 2.8-4.7 3.4-8.5	R5 X4 R5 X4 X4
433407	B	ND				1 <sup>0.079</sup>	48	R5	NF	4	27-52	R5
434423	B	1**	38	R5	R5	1**	46	R5	R5	18	38-82	R5
435412406	B B B	1** 2**** 1**	83 87 89	R5 R5 R5	R5/R R5/R R5	1*	83	R5	R5/R	10 3	42-99	R5
436000	B	ND				1*	31	R5	R5	8	31-85	R5
437000	B	1**	55	R5		ND				8	12-55	R5
445000	B B	1 <sup>0.199</sup>	73	R5		ND				16 3	17-83 3.9-6.8	R5 X4
449000	B	1*	100	R5		1**	81	R5		5	59-99	R5
451000	B	NA				1**	49	R5		5	39-41	R5
453000	B	ND				1***	74	R5	R5	6	41-86	R5
454304	B B	ND				1 <sup>0.053</sup>	31	R5		24 1	11-97 6.8	R5 X4

<sup>a</sup>genotypic prediction of co-receptor usage. We used a false positive rate (FPR) cutoff of ≤10%, which has been shown to have high predictive value to correctly distinguish CXCR4- from CCR5-tropic viruses (Lengauer et al., 2007; Poveda et al., 2012).

<sup>b</sup>phenotypic analysis of co-receptor usage; R indicates resistance to co-receptor inhibitors.

Asterisks or *p* value indicate the likelihood that amplicons did not originate from contaminating T cell DNA. *p* values were determined either using a mean cell estimate (1) or a conservative estimate (2) (McNamara et al., 2013). The conservative estimate compared the top of the 95% confidence interval for the calculated infection rate in CD3+ T cells in the CD133-depleted sample with the bottom of the 95% confidence interval for the calculated infection rate in CD3+ T cells in the CD133-sorted sample to minimize the difference between these calculated infection rates; \**p* < 0.05 by (1) only; \*\**p* < .05 by (1) and (2); \*\*\**p* < .01 by (1) and (2); \*\*\*\**p* < .001 by (1) and (2).

Abbreviations: HSPC, hematopoietic stem and progenitor cells; PBMC, peripheral blood mononuclear cells; FPR, false positive rate; NA, not analyzed; R5, CCR5; X4, CXCR4/Dual; ND, not detectable; NF, non-functional.



**Figure 3-4. Phenotypic analysis of donor Env matching the HSPC V3 region.**

Representative flow plots of MOLT4-CCR5 cells (a) and 3T3-CD4-CCR5 cells infected with HIV-7SF-GFP pseudotyped with the indicated envelope in the presence or absence of AMD3100 and/or maraviroc inhibitors and harvested for flow cytometry 3 days post-infection. Numbers indicate frequency of infected (GFP<sup>+</sup>) cells. X4, CXCR4-tropic; R5, CCR5-tropic; Dual, able to utilize both CXCR4 and CCR5; R, coreceptor inhibitor resistance.

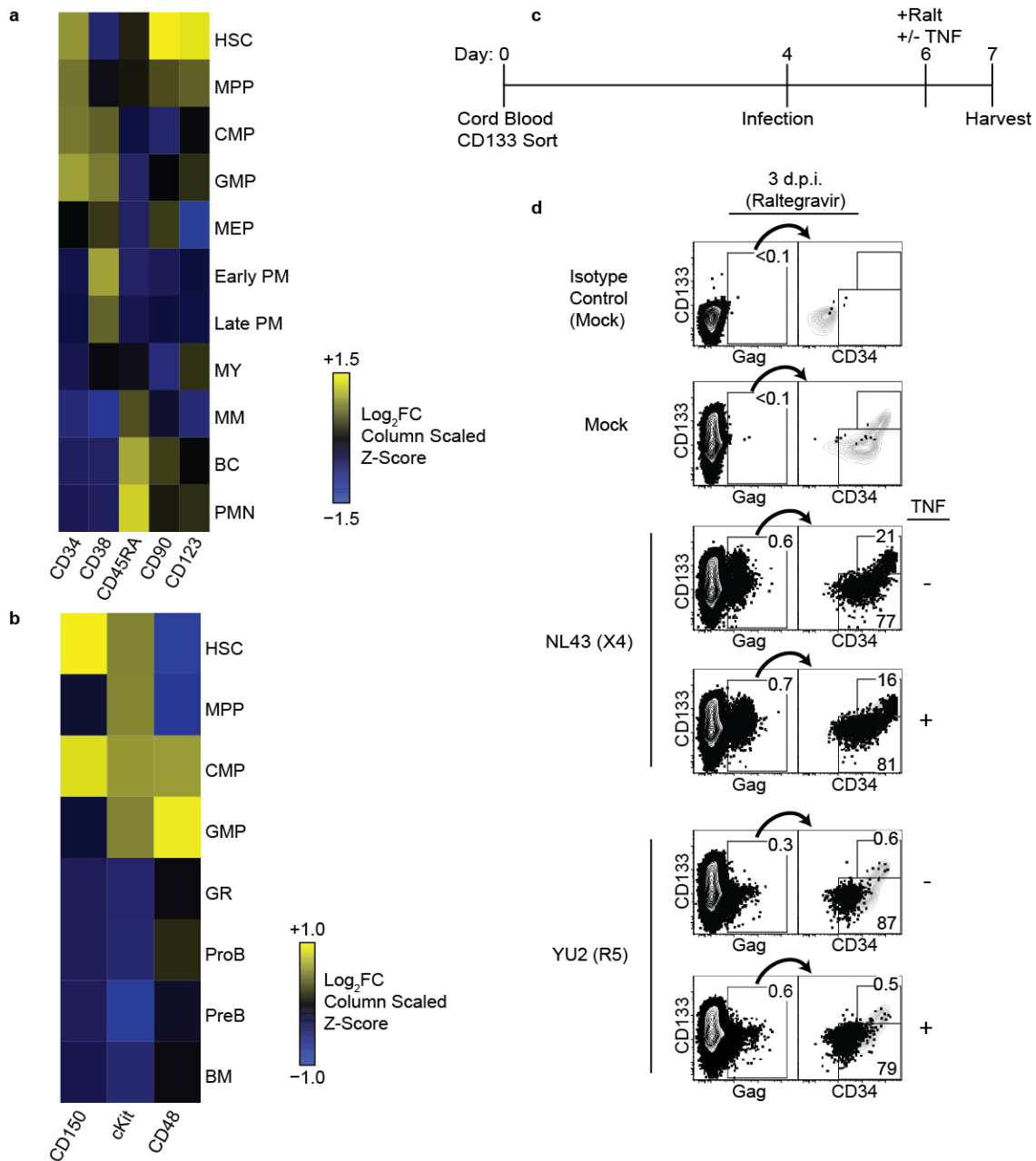
**Table 3-4. Env Phenotype**

Donor ID	Clade	Source of FL Env Amplicon	Source of Matching V3 Amplicon	FPR	Genotype	Phenotype
413402	B	PBMC	Sort 1	1.7	X4/Dual	Dual/R**
414000	B	PBMC	Sort 1	57	R5	R5
415000	C	HSPC	Sort 1	71	R5	R5
420000	B	PBMC	Sort 1	19	R5	R5
420000**	B	PBMC	Sort 1	0.7	X4/Dual	Dual
421000	B	HSPC	Sort 1	7.8	X4/Dual	X4
431000	B	HSPC	Sort 1	75	R5	R5/R
431000	B	Sort 1 FT	Sort 1	79	R5	R5/R
432000	A/AG	HSPC	Sort 1	2.8	X4/Dual	X4
432000	A/AG	HSPC	Sort 2	2.8	X4/Dual	NF
433407	B	HSPC	Sort 2	48	R5	NF
434423	B	PBMC	Sort 1	38	R5	R5
434423	B	PBMC	Sort 2	46	R5	R5
435412406**	B	PBMC	Sort 1, Sort 2	83	R5	R5/R
435412406	B	PBMC	Sort 1	87	R5	R5/R
435412406	B	PBMC	Sort 1	89	R5	R5
436000**	B	PBMC	Sort 2	31	R5	R5
453000	B	PBMC	Sort 2	74	R5	R5

\*indicates more than one V3 sequence identical to same V3 amplicon from an HSPC, both have same tropism; number of asterisks indicates number of FL Env proteins tested

\*\*R indicates resistance to co-receptor inhibitors.

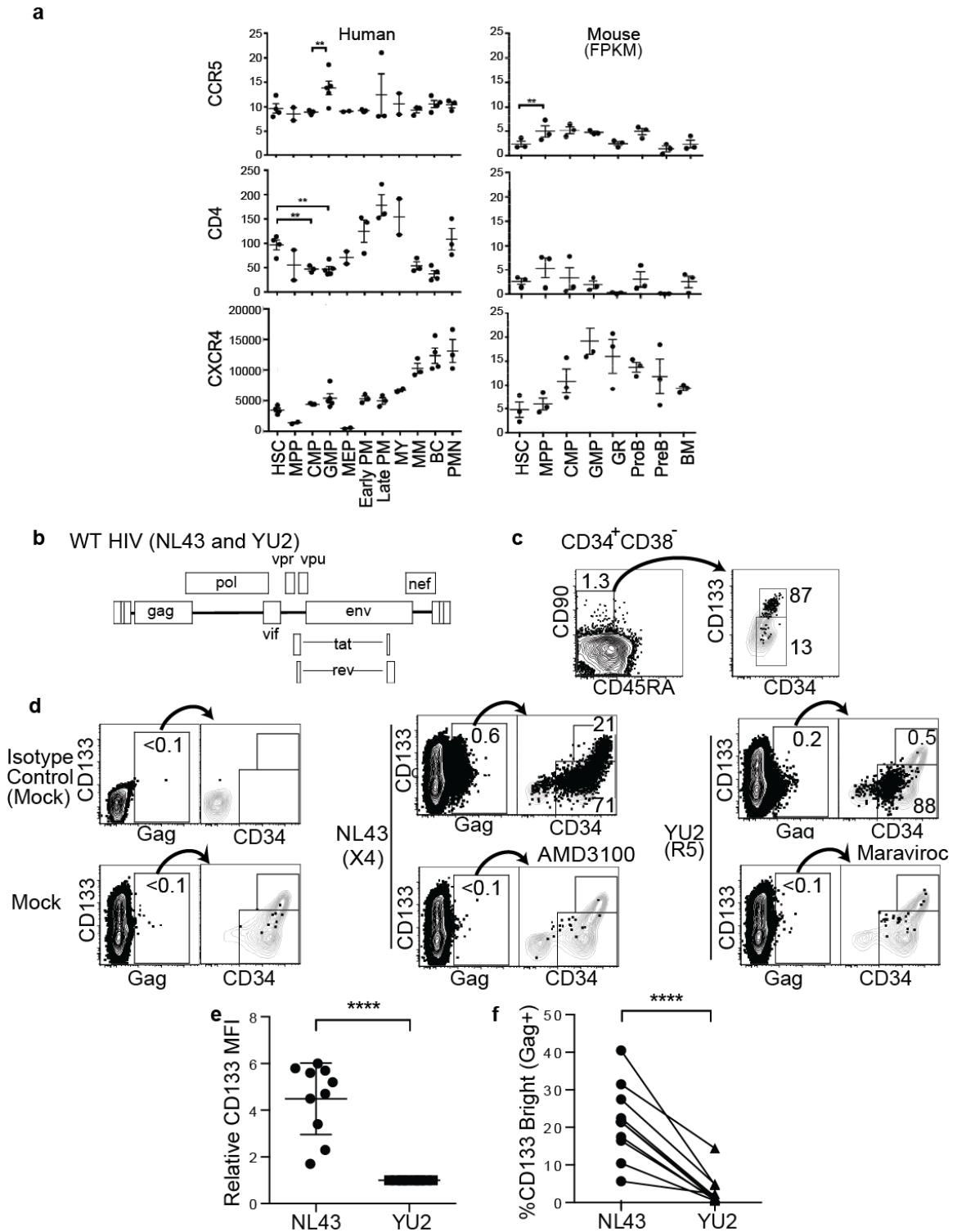
Abbreviations: FL, full-length; FT, flowthrough; NF, non-functional virus.



**Figure 3-5. CCR5-tropic virus targets HSPCs that are unlikely to be HSCs.** (a) Heat map of gene expression data of human bone marrow and differentiated cell subsets from microarray analysis as in Fig. 2a. FC, fold-change. HSC, hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; GMP, granulocyte-macrophage progenitor; PM, promyelocyte; MEP, megakaryocyte-erythroid progenitor; MY, myelocyte; PMN, polymorphonuclear; BC, band cell; MM, metamyelocyte. (b) Heat map of gene expression data of mouse bone marrow and differentiated subsets from RNA-seq analysis as in Fig. 2b. FC, fold-change; GR, granulocyte; BM, bone marrow; ProB, pro-B cell; PreB, pre-B cell. (c) Schematic diagram of experimental set up for (d). (d) Representative flow plots of cord blood-



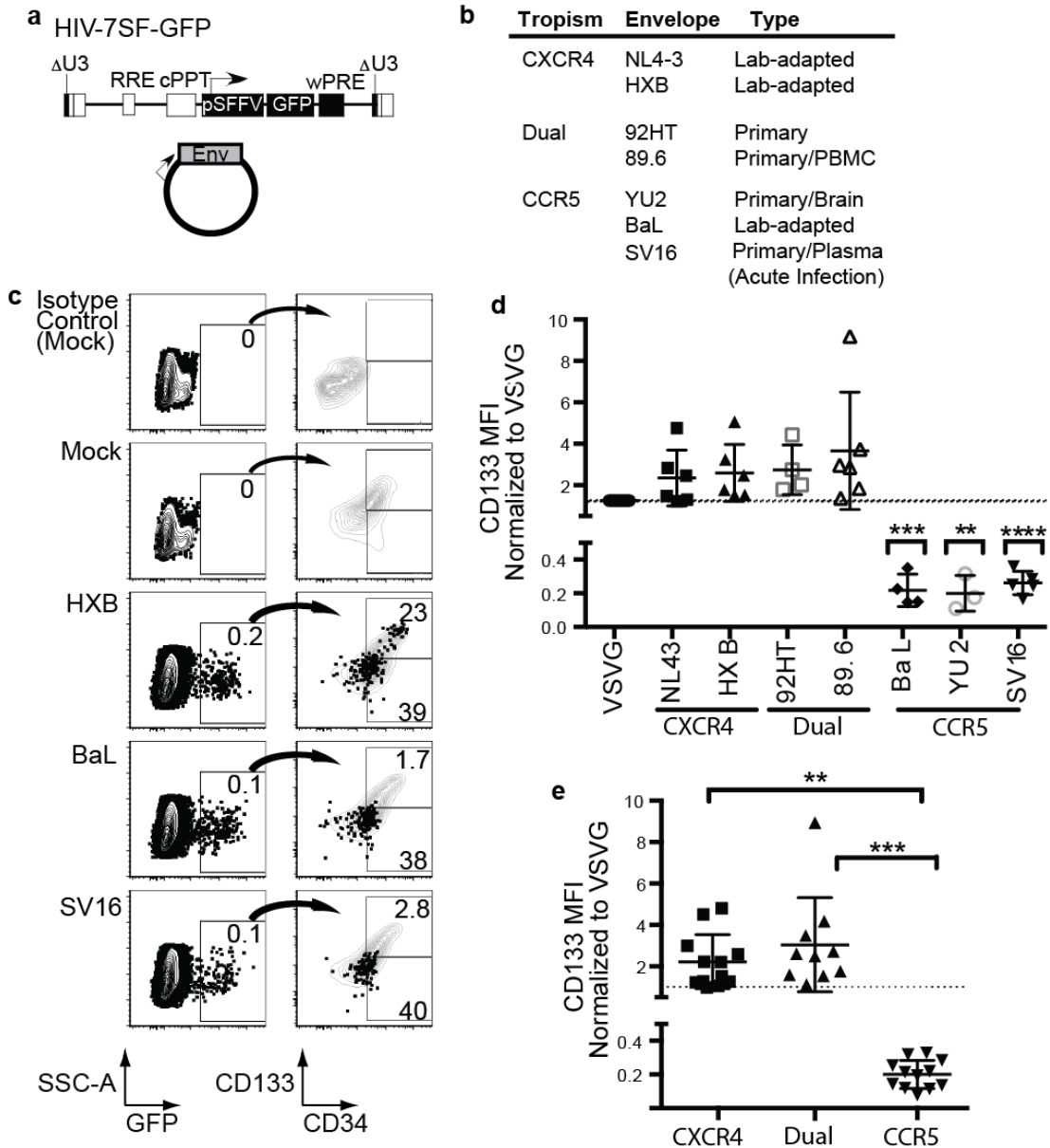
derived CD133-sorted cells infected with wild-type HIVs, NL4-3 and YU-2 and harvested 3 days post-infection. Where indicated, cells were incubated with Raltegravir +/- TNF- $\alpha$  for 16-20 hours before harvest. In the right panels, Gag<sup>+</sup> cells are overlaid onto plots of the total live cell population and the percentage of Gag<sup>+</sup> cells in the CD133<sup>high</sup> and CD133<sup>low</sup> regions is indicated in the overlay.



**Figure 3-6. CCR5 expression and targeting of progenitors by HIV**

(a) Gene expression in human and mouse bone marrow stem and progenitor subsets, along with differentiated cell populations by microarray analysis. (human) or RNAseq (mouse). Error bars represent standard error of the mean (SEM). HSC,

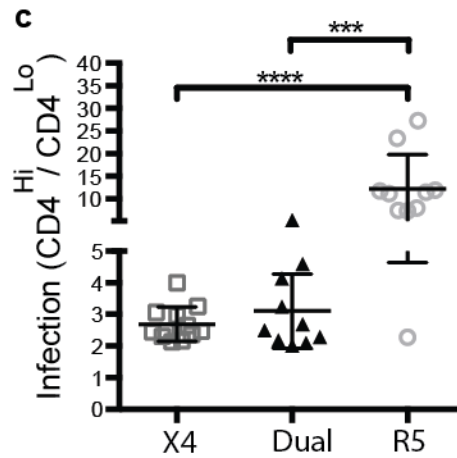
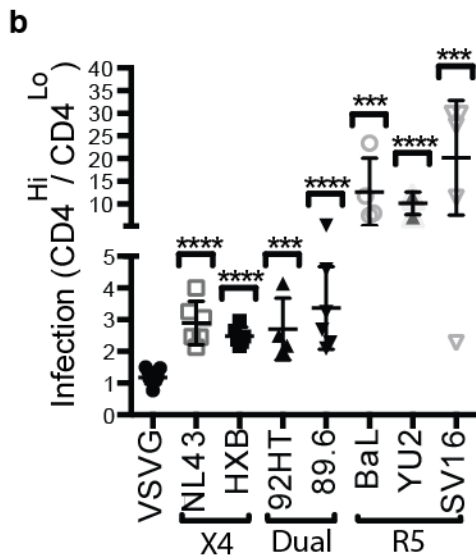
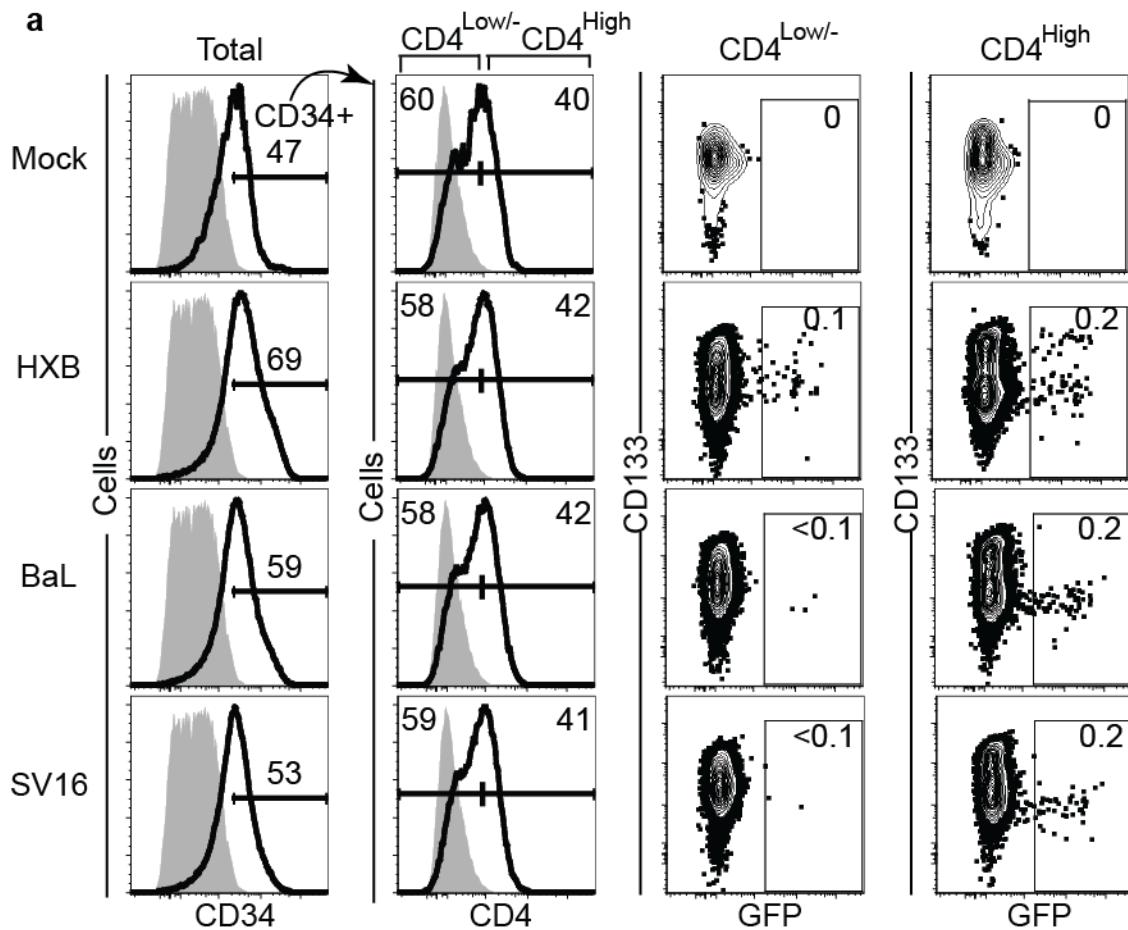
hematopoietic stem cell; MPP, multipotent progenitor; MLP, multilymphoid progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-monocyte progenitor; PM, promyelocyte; MY, myelocyte; MM, metamyelocytes; BC, band cell; PMN, polymorphonuclear cells. (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , unpaired t test). FPKM, Fragments Per Kilobase of Exon Per Million Fragments Mapped; GR, granulocyte; ProB, pro-B cell; PreB, pre-B cell; BM, unfractionated bone marrow. (\* $p < 0.05$ , \*\* $p < 0.01$ , paired ratio t test). **(b)** Schematic of full-length HIV used in (d-f). **(c)** Flow cytometric plots of CD133-sorted cord blood HSPCs cultured for 7 days and stained as in Fig. 1d. Cells gated in left plot were overlaid on the total live population on the right. **(d)** Representative flow cytometric plots of cord blood-derived CD133-sorted cells expanded for four days, infected with the indicated virus and harvested 2 days post-infection. Gag<sup>+</sup> cells are overlaid onto CD34 versus CD133 plots for the total live cell population and the percentage of Gag<sup>+</sup> cells in the CD133<sup>high</sup> and CD133<sup>low</sup> regions is indicated in the overlay. **(e)** Summary graph of relative CD133 expression by mean fluorescence intensity (MFI) on HSPCs infected with the indicated HIV. Results were normalized to those for YU-2 infected cells for each experiment. **(f)** Summary graph showing the frequency of Gag<sup>+</sup> cells falling into CD133<sup>bright</sup> gate shown in (e). Error bars represent standard deviation. (\*\*\*\* $p < 0.0001$ , paired t-test).



**Figure 3-7. Targeting of intermediate progenitors by CCR5-tropic Envs is a conserved property extending to a transmitted/founder virus**

(a) Schematic of HIV-7SF-GFP construct and HIV envelope plasmid used to construct pseudotyped viruses used in (c-e). (b) Summary table of envelope proteins used to pseudotype HIV-7SF-GFP virus. (c) Representative flow plots of cord blood-derived CD133-sorted cells expanded for four days, transduced with the indicated virus and harvested 3 days post-infection for flow cytometric analysis. In each right panel, GFP<sup>+</sup> cells were overlaid onto plots of the total cell population and the percentage of GFP<sup>+</sup> cells in the CD133<sup>high</sup> and CD133<sup>low</sup> regions is indicated. Gates were determined based on isotype control antibody staining (top panel). (d) Summary graph of CD133 MFI for experiments performed as in (c). Results are compiled using cells from 11 uninfected donors. Error bars represent standard deviation. Student's

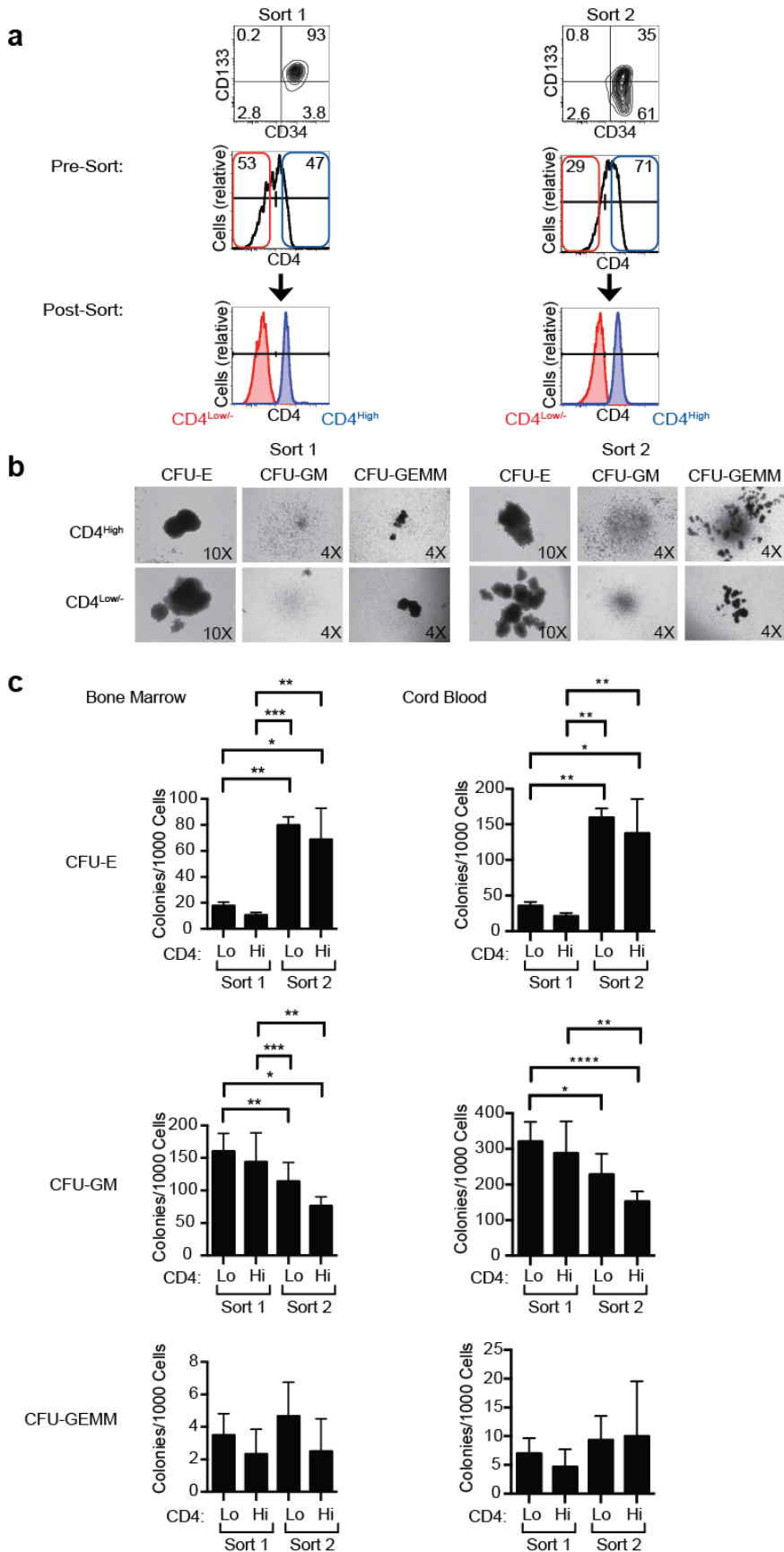
t-test indicates significance for each HIV envelope with respect to VSVG (\*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ). (e) Data from (d) compiled by tropism. Error bars represent standard deviation; one-way ANOVA,  $p = 0.0002$ , with Tukey's Multiple Comparisons Test indicated (\*\* $p < 0.01$  and \*\*\* $p < 0.001$ ).



**Figure 3-8. HSPCs with greater CD4 expression are preferentially infected by HIV Envs.**

(a) Representative flow cytometry plots and gating strategy for cord blood-derived CD133-sorted cells infected with virus containing the indicated envelope protein

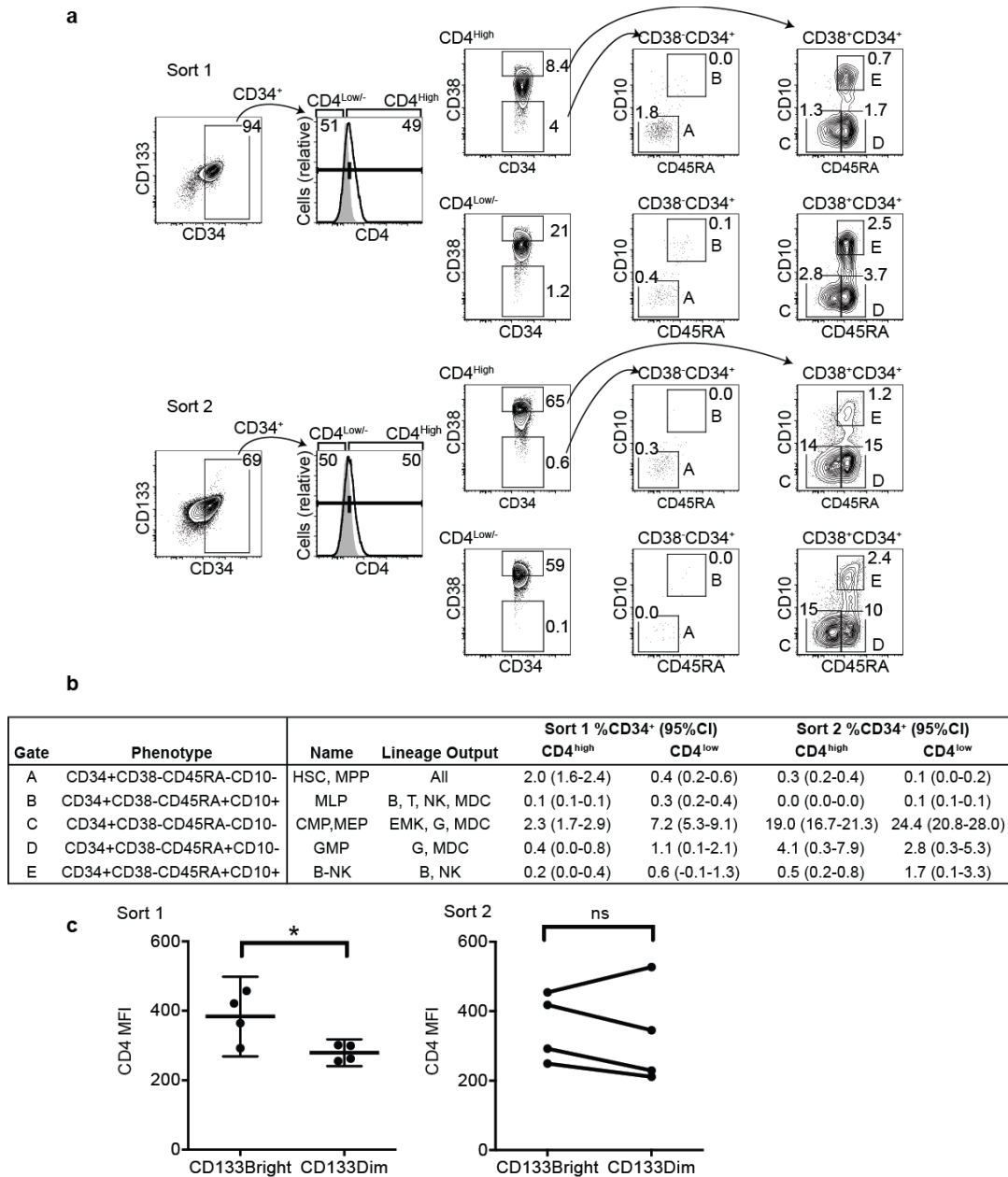
and harvested 3 days post-infection. Gating for CD4 was determined by the inclusion of 1% of cells stained with an isotype control antibody (gray). For GFP plots, numbers indicate the percentage of GFP<sup>+</sup> events. **(b)** Summary graphs depicting the ratio of infected cells in CD4<sup>high</sup> versus CD4<sup>low/-</sup> subsets of cord blood-derived HSPCs infected and analyzed as in part (b). For SV16, two replicates had 0.0% infection in the CD4<sup>low/-</sup> gate leading to an undefined ratio, so 30.0 was used as a conservative estimate of the ratio. Error bars indicate standard deviation. Results were compared to infection by VSVG pseudotyped viruses and *p* values were determined using Student's t-test (\*\**p*<0.001, \*\*\*\**p*<0.0001). **(c)** Data from (b) but compiled by tropism. Error bars represent standard deviation; one-way ANOVA, *p*<0.0001, with Tukey's Multiple Comparisons Test indicated (\*\**p*<0.001 and \*\*\*\**p*<0.0001).





**Figure 3-9. CD4<sup>high</sup> HSPCs include progenitors with multi-lineage potential.**

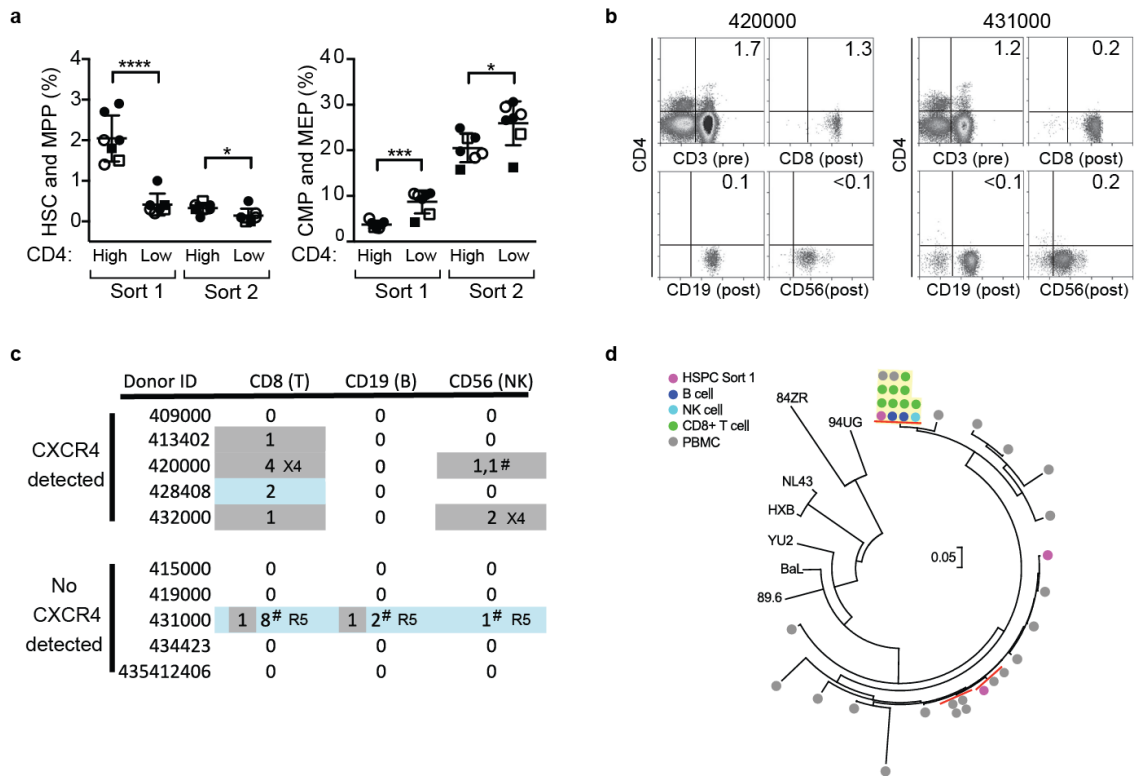
(a) Representative flow plots of Sort 1 and Sort 2 bone marrow-derived HSPCs sorted using fluorescence activated cell sorting into CD4<sup>high</sup> and CD4<sup>low/-</sup> subsets. (b) and (c) Sorted HSPCs were analyzed by methylcellulose colony formation assays. Error bars represent standard deviation; Student's t-test (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ). CFU-E, erythroid; CFU-GM, granulocyte/macrophage and CFU-GEMM, multilineage.



**Figure 3-10. CD4 marks HSPCs enriched for HSCs and MPPs.**

(a) Flow cytometric analysis of differentiation markers expressed on bone marrow HSPCs purified as described in **Figure 3-1a**. For the two right-most panels, numbers indicate percentage of total CD34<sup>+</sup> events in each sort falling into that gate. (b) Summary table of frequencies for each phenotypic gate as shown in (a). Lineage outputs based upon Doulatov *et al* (Doulatov et al., 2010). Results are based on data from 7 uninfected donors (5 cord blood and 2 bone marrow). 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; MDC, macrophage and dendritic cell; EMK, erythroid and megakaryocyte. (c) CD4 mean

fluorescence intensity of CD133bright versus CD133dim subsets for Sort 1 and Sort 2 from experiments in **Figure 3-11a**. Error bars represent standard deviation; Student's t-test ( $*p < 0.05$ ).



**Figure 3-11. CD4 marks HSPCs enriched for HSCs and MPPs.**

(a) Summary graphs depicting the percentage of each subset of the total CD34<sup>+</sup> cells in each sort. Cells were isolated from cord blood (circles) or bone marrow (squares). For three experiments (2 cord blood and 1 bone marrow), lineage-positive cells were physically or analytically excluded from analysis (open symbols). Error bars represent standard deviation; Student's t-test (\* $p < 0.05$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ).

(b) Flow cytometric plots showing purity of CD4-negative lineages for donor 431000, see also Table S4. "Pre" indicates the cell population post CD4-bead depletion and prior to fluorescence activated cell sorting (FACS). "Post" indicates the cell populations following FACS.

(c) Summary table showing HIV amplicons (*gag* or *env*, see Table S4 for details) detected in CD4-negative lineages. # indicates sequences identical to an HSPC sequence from that donor. Gray highlights detected provirus, blue denotes clonal sequences within each donor. All amplicons were unlikely to have resulted from contaminating CD4<sup>+</sup> T cells (see also Table S4) as described for Table 1, Table S2, and in Methods. Where indicated, *env* amplicons were predicted to be CXCR4-tropic (X4) or CCR5-tropic (R5).

(d) Phylogenetic tree showing genetic relationships of amplicons from donor 431000. Highlighted area indicates location of clonal amplicons from CD4-negative lineages. Red lines indicate identical sequences. Scale indicates nucleotide substitutions per site. 89.6, Bal, YU-2, HXB and NL4-3 are subtype B HIV molecular clones. 84ZR and 94UG are subtype D HIV molecular clone outgroups (Signer et al., 2014).

**Table 3-5. PCR Primer Sequences and Cycling Conditions**

Amplicon	PCR Round	Primer	Orientation	HXB2 Location	Sequence
env	1 <sup>st</sup>	5036d	Forward	5036-5059	5'-GGAYTATGGAAAACAGATGGCAGG-3'
env	1 <sup>st</sup> or 2 <sup>nd</sup>	LTR-pA-R	Reverse	9625-9600	5'-AGGCAAGCTTTATTGAGGCTTAAGC A-3'
env	1 <sup>st</sup> or 2 <sup>nd</sup>	5956d-f	Forward	5956-5983	5'-CTTAGGCATYTCCTATGGCAGGAAG AAG-3'
env	1 <sup>st</sup> or 2 <sup>nd</sup>	envC2F2	Forward	6950-6976	5'-CAGCACAGTACAATGTACACATGGA AT-3'
env	1 <sup>st</sup> or 2 <sup>nd</sup>	envC4R1	Reverse	7540-7520	5'-ATGGGAGGGGCATACATTGC T-3'
env	2 <sup>nd</sup>	env1in5	Forward	7060-7081	5'-ACAATGCTAAAACCATAATA GT-3'
env	2 <sup>nd</sup>	env1in3	Reverse	7530-7511	5'-CATACATTGCTTTTCCTACT-3'
gag	1 <sup>st</sup>	U5-577.9662-f	Forward	577-603	5'-GACTCTGGTAACTAGAGATC CCTCA GA-3'
gag	1 <sup>st</sup>	long1316-D4.6b	Reverse	1322-1316	5'-atcttgccgctctgtgTGGGGTGGCTCCTTCTG-3'
gag	1 <sup>st</sup>	tagD4.6b-p24R1d	Reverse	1500-1480	5'-atcttgccgctctgtgTGCTATGTCACTTCCCCTTGG-3'
gag	2 <sup>nd</sup>	626s	Forward	626-651	5'-TCTCTAGCAGTGGCGCCCGAACAGGG-3'
gag	2 <sup>nd</sup>	<b>D4.6b</b>	<b>Reverse</b>	<b>N/A</b>	<b>5'-atcttgccgctctgtg-3'</b>

Primer 1	Primer 2	Cycling Conditions
<b>*5036d</b>	LTR-pA-R	98°C for 30 seconds; 35 cycles of 98°C for 10 seconds, 68° C for 10 seconds, and 72°C for 2 minutes; 72°C for 10 minutes
<b>*5956d-f</b>	LTR-pA-R	98°C for 30 seconds; 35 cycles of 98°C for 10 seconds, 71° C for 10 seconds, and 72°C for 2 minutes; 72°C for 10 minutes
<b>*envC2F2</b>	envC4R1	98°C for 30 seconds; 35 cycles of 98°C for 10 seconds, 68° C for 10 seconds, and 72°C for 25 seconds; 72°C for 5 minutes
<b>env1in5</b>	env1in3	98°C for 30 seconds; 40 cycles of 98°C for 10 seconds, 56° C for 10 seconds, and 72°C for 15 seconds; 72°C for 5 minutes
<b>626s</b>	D4.6b	98°C for 30 seconds; 35 cycles of 98°C for 10 seconds, 70° C for 10 seconds, and 72°C for 40 seconds; 72°C for 10 minutes

**\*Conditions optimized for 1<sup>st</sup> round of multiplex PCR with gag primers (U5-577.9662-f plus tagD4.6b-p24R1d plus or minus long1316-D4.6b)**

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## Chapter 4

### Conclusion

The discoveries presented in this dissertation provide significant insight into the potential reservoir of HIV in hematopoietic stem and progenitor cells (HSPCs) and HIV pathogenesis, as well as steady-state human hematopoiesis. The major conclusions to be discussed here include:

- (a) Bone marrow HSPCs do harbor provirus in HIV+ people on optimal therapy.
- (b) A subset of HIV+ donors have a detectable reservoir in bone marrow HSPCs.
- (c) CCR5- and CXCR4-tropic virus persists in HSPCs.
- (d) HIV may persist in CD4-expressing HSCs and non-stem cell progenitors *in vivo*.
- (e) Non-stem cell progenitors may be an enduring population *in vivo*.
- (f) Infected HSPCs can differentiate into mature lymphoid cells with provirus *in vivo*.

#### *Bone marrow HSPCs do harbor provirus in HIV+ people on optimal therapy*

The analysis presented here of CD133<sup>+</sup> and CD34<sup>+</sup> HSPCs from HIV<sup>+</sup> donors with at least 6 months of suppressed viremia indicates that there is a detectable proviral reservoir in these cells that is unlikely to come from CD4<sup>+</sup> T cell contamination in the analysis. Our laboratory's initial study of HSPCs demonstrated

that CD34<sup>+</sup> HSPCs could be infected *in vitro* and HIV was detectable in a small study of HIV<sup>+</sup> donors with high viral loads and treated donors with low levels of viremia (Carter et al., 2010). However, two studies were recently published which disputed that HIV persists in HSPCs as they were unable to detect HIV in small cohorts of donors with suppressed plasma virus beginning therapy during chronic or acute infection (see Chapter 2 for discussion of key differences between these two studies and ours) (Durand et al., 2012; Josefsson et al., 2012). They asserted that HIV does not persist in CD34<sup>+</sup> HSPCs from individuals on treatment, and the previous findings were likely due to contamination by bone marrow CD4<sup>+</sup> T cells which did have detectable provirus by their analysis. In Chapter 2, we tested this statement in a cohort of 11 treated donors with suppressed viremia using a sensitive quantitative PCR assay for a conserved HIV region to detect provirus in CD133-sorted bone marrow mononuclear cells (BM MNCs). In this analysis, we measured the frequency of provirus in HSPCs with high CD133 purity and in the CD133-depleted fraction of BM MNCs. Provirus was detected in 6 of the 11 donors and in 5 of those samples the provirus was unlikely to be due to CD3<sup>+</sup> T cell contaminants. This was determined using the percentage of CD3<sup>+</sup> cells in the CD133-sorted samples and the measured proviral frequency in the CD3<sup>+</sup> populations in the CD133-depleted sample to predict the proviral frequency in HSPCs if all amplicons came from these contaminating populations. Comparing the predicted frequency due to contaminating cells to the measured frequency in the CD133-sorted population, by both a mean estimate and a more conservative estimate (see Chapter 2), the measured proviral frequency in the CD133-sorted sample was significantly higher than the predicted contribution from

CD3<sup>+</sup> BM MNCs. This analysis provides initial evidence that HSPCs do harbor provirus *in vivo*, but was limited by the cohort size and cell numbers assessed.

Chapter 3 extended this analysis to a larger cohort of donors with a single-copy assay amplifying larger regions of genomic sequence, and a portion of *env* was amplified from purified HSPCs in 23 HIV<sup>+</sup> individuals on treatment with optimal viral suppression. In the two HSPC populations analyzed in this study, the majority of amplicons (36 out of 41 total *env* amplicons) were unlikely to be due to CD3<sup>+</sup> T cells by the same comparison of expected frequency due to contaminants versus the measured frequency in the HSPC population used in Chapter 2. A broader analysis of the frequency of *env* and *gag* amplicons in 27 donors of the ongoing cohort of 44 donors presented in the Appendix indicates that when detecting either portion of the HIV genome, most HSPC provirus is unlikely to be due to T cells. Thus, bone marrow HSPCs are a discrete reservoir of provirus in a portion of optimally treated HIV<sup>+</sup> donors.

#### *A subset of HIV<sup>+</sup> donors have a detectable reservoir in bone marrow HSPCs*

In addition to showing that the provirus is distinct from the infection of bone marrow T cells, we wished to know if HSPCs are a reservoir of HIV in all or just a subset of HIV-infected individuals. In the cohort in Chapter 2, donors with detectable provirus in their HSPCs were more likely to have been diagnosed with HIV around the year 2000 or later, consistent with the negative findings in the previous study where 10 out of 11 analyzed donors were diagnosed before 2001 (Durand et al., 2012). Given that effective cART regimens became available around

that time, perhaps there was something different in the clinical course of those donors diagnosed in the 1980's and 1990's and able to survive until cART was accessible. However, in the larger cohort discussed in Chapter 3, 8 of the 23 donors with detectable *env* were diagnosed before 2001, between the mid 1980's and 1998. The Chapter 3 analysis included the Sort 2 (CD34-sorted, CD133-depleted) HSPC population not analyzed in Chapter 2, but this does not provide an explanation of the discrepancy: 6 of the 8 donors with years of diagnosis before 2001 had detectable amplicons in Sort 1 (CD133-sorted) only and the other 2 donors had detectable amplicons in both Sort 1 and Sort 2 (CD34-sorted, CD133-depleted). Compared to the study in Chapter 2, Chapter 3 did include more Sort 1 and Sort 2 HSPCs in the analysis, with greater than 100,000 cells analyzed from the majority of each sorted population.

Sequencing of *env* amplicons from donor HSPCs and peripheral blood mononuclear cells (PBMCs) in Chapter 3 allowed us to determine the HIV subtype and tropism of virus in donors with detectable HIV in HSPCs. While the majority of donors had subtype B virus, HSPC provirus was identified in two donors with non-subtype B provirus, one with all subtype C in HSPCs and PBMCs and the other with a mix of subtypes (A/AG, AE, B, and C) in both bone marrow and peripheral blood. HSPC infection by these subtypes *in vivo* was supported by a previous study where Subtype C virus was detected in peripheral blood-derived CD34+ HSPCs from a treatment-naïve patient cohort in Africa (Redd et al., 2007) and a subsequent study indicating limited infection of colony-forming PBMCs with subtype A and D infected patients not on therapy (Mullis et al., 2012). Our research group had previously



shown that subtype C and D Env-pseudotyped viruses could infect CD133-sorted cord blood HSPCs (Carter et al., 2011). Additionally, the tropism analysis of the virus in donors with HIV in HSPCs indicated that donors with both CCR5- and CXCR4-utilizing provirus in PBMCs had persistent HIV in bone marrow HSPCs. Although our previous study predicted that CXCR4-tropic virus would be needed to establish a persistent HSPC reservoir of HIV in HSPCs (Carter et al., 2011), CXCR4-tropic and CCR5-tropic viruses could persist in HSPCs similar to PBMCs from the same donor, as will be discussed below. Given the current analysis, there is no donor characteristic as yet that identifies the subset of donors who are likely to harbor provirus in bone marrow HSPCs. Additionally, there remains the possibility that with further sampling or more sensitive assays, we may find HIV in additional donors that had previously undetectable virus in HSPCs.

#### *CCR5- and CXCR4-tropic virus persists in HSPCs*

In HIV+ individuals with infected HSPCs, our findings indicate that both CCR5-tropic and CXCR4-tropic virus persist *in vivo*. With the assumption that only infection of bona fide hematopoietic stem cells (HSCs) would allow the virus to persist for longer periods of time in an HIV-infected person, the previous study in our laboratory investigated the *in vitro* infection of CD133-sorted cord blood and found that CXCR4-utilizing viruses were most likely to infect true HSCs (Carter et al., 2011). In these experiments, CXCR4-tropic viruses were best able to infect CD133+ cord blood progenitors *in vitro*, and HPCs infected with a non-cytotoxic HIV reporter virus with a CXCR4 tropic envelope were capable of long-term

reconstitution of hematopoietic lineages in an immune-deficient mouse. In colony-formation assays, the CXCR4-tropic GFP-expressing reporter virus infection was able to produce all colony types expressing GFP (multilineage (GEMM), granulocyte/macrophage (GM), and erythroid (E) colonies), while the CCR5-tropic reporter virus infection only produced small GFP<sup>+</sup> GM colonies. Thus, it seemed likely that CXCR4-tropic virus would infect long-lived cells that could persist *in vivo*, while CCR5-tropic virus may allow only minimal infection in progenitors that were not thought to persist long-term. Surprisingly, in the study in Chapter 3, 32 *env* amplicons from HSPCs in 20 donors were predicted by genotype and/or phenotype to use CCR5 for entry out of 41 total amplicons isolated in 23 donors with detectable provirus. The other 9 *env* amplicons were predicted to use CXCR4 entry, which could have come from the infection of CXCR4- or dual-tropic envelopes. Thus, it seems that the majority of virus that persists in HSPCs is predicted to have come from infection using CCR5. Given that latent reservoirs are thought to be established early on during infection (Kearney et al., 2014; Simonetti and Kearney, 2015), when infected-people have high viral titers and before plasma viremia is suppressed with cART, establishment of latent infection in HSPCs would likely occur when CCR5-tropic viruses are predominant early in infection. Indeed, as shown in Chapter 3, we tested a transmitted/founder Env derived from a patient during acute infection in pseudotyped virus experiments and saw infection of HSPCs in a pattern similar to the other CCR5-tropic envelope proteins. CXCR4-tropic virus is typically a minority population, if present during early stages of infection, but given their greater propensity for infection of HSPCs *in vitro*, as demonstrated in Chapter 3, it

follows that CXCR4-tropic virus may infect HSPCs during that period. Additionally, all donors with CXCR4-tropic virus in HSPCs also had at least a minority of CXCR4-tropic virus detected in PBMCs, indicating that these types of viruses were likely present early in infection as the switch from CCR5-tropic to non-CCR5-tropic virus is rare in people with clinically undetectable virus during cART (Lee et al., 2014). Therefore, we present evidence that both CCR5- and CXCR4- tropic virus are able to infect and persist in HSPCs; however, it was unknown whether the infected cells are HSCs or another progenitor cell which had the capability to survive in the bone marrow during therapy.

*HIV may persist in CD4-expressing HSCs and non-stem cell progenitors in vivo*

Examination of the receptor and co-receptor requirement of the viruses from HSPCs allowed us to also determine the subsets of HSPCs which are most likely to form a persistent reservoir of HIV *in vivo*. Our initial work showing that HSPCs can be actively and latently infected *in vitro* and also *in vivo* focused on CD34<sup>+</sup> HSPCs, which is a heterogeneous population including hematopoietic stem cells to more lineage-restricted progenitors (Carter et al., 2010). This study also demonstrated that CD4 was required for HIV infection of HSPCs, as it was blocked by a CD4 blocking antibody. However, it was unknown what level of CD4 expression is needed for HSPC infection or if CD4 was expressed on a subset of HSPCs that was functionally distinct. The main HIV receptor, CD4, is expressed, although at a level much lower than CD4<sup>+</sup> T cells in the CD34<sup>+</sup> population, but there have been few studies of this protein's expression on HSPCs (Louache et al., 1994; Muench et al.,

1997; Zauli et al., 1994). These studies pointed towards CD4 being expressed on hematopoietic cells that survive in long-term culture initiating cell assays and fetal liver derived HSPCs engraft that can engraft in an immune-deficient mouse (Louache et al., 1994; Muench et al., 1997).

In Chapter 3, my analysis of Sort 1 and 2 HSPCs that are relatively CD4<sup>high</sup> and CD4<sup>low</sup> indicates that both of these populations include cells that form multilineage colonies, HSCs, MPPs, and CMPs. However, using a panel of flow cytometry markers that differentiates immature populations (HSC, MPP) from more differentiated populations (MLP, CMP, MEP, GMP, and B-NK), I found that HSCs and MPPs are enriched in CD4<sup>high</sup> populations, while the CD4<sup>low/-</sup> populations had higher numbers of the differentiated progenitor populations (**Figure 4-1**). Another set of experiments with a variety of CCR5- and CXCR4-tropic Env-pseudotyped viruses showed that the CD4<sup>high</sup> population is preferentially infected by envelope proteins of both tropisms. CCR5-tropic envelopes had an even greater preference for CD4<sup>high</sup> HSPCs than the CXCR4- and dual-tropic envelopes. Thus, these viruses likely target cells with high CD4 expression, which include more immature cells like HSC and MPP, but a minority of the differentiated subsets can also express high CD4.

This led us to the question of whether HSCs or downstream progenitor subsets are infected by HIV *in vivo*. A study in our lab of latent HIV infection in HSPCs used CD38 and CD45RA to distinguish infection of four HSPC subsets (HSC,MPP; MLP; CMP,MEP; GMP,B-NK) and found that reversible latent infection occurred in all four subsets (McNamara et al., 2012). However, this study only used HXB Env pseudotyped virus, which is CXCR4-utilizing for entry. In another of our

previous studies, as described above, experimental results advocated that CXCR4-tropic viruses are capable of infecting true HSCs, while it is unlikely that a CCR5-tropic virus can infect this same population (Carter et al., 2011). The results presented in Chapter 3 using wildtype and Env-pseudotyped viruses agreed with this conclusion by showing that CXCR4-tropic viruses are able to target a CD133-bright population that had significantly less infection by CCR5-tropic viruses. Given that HSCs are typically CD133-bright (de Wynter et al., 1998; Gorgens et al., 2013), it seemed likely that CXCR4-tropic viruses would target HSCs, in addition to more differentiated progenitors, but CCR5-tropic viruses would rarely target an HSC. However, CCR5-tropic viruses do have a greater preference for the CD4<sup>high</sup> populations which are enriched for HSCs according to the flow cytometric stains in Chapter 3, so there remains the possibility that an HSC could rarely be infected with CCR5-tropic virus. However, in our isolation of two populations of HSPCs from the donor cohort in Chapter 3, we were able to compare the number of donors with provirus detected in the Sort 1 population, which was enriched for HSC and MPP, to the Sort 2 population, which was depleted of HSC and MPP, but enriched for CMP, MEP, and other differentiated progenitors. Provirus was detected at a similar rate in both populations (no preference for the HSC-enriched Sort 1 population), and, given that the majority of provirus in both sorts was CCR5-tropic, it is likely that non-stem cell progenitors in Sort 2 and perhaps Sort 1 are infected by CCR5-tropic virus.

*Non-stem cell progenitors may be an enduring hematopoietic population in vivo*

When we found that the majority of provirus detected from donor HSPCs was CCR5-tropic and it was unlikely that CCR5 would only infect HSCs, we hypothesized that HSPCs persisting *in vivo* with provirus included progenitors downstream of an HSC. Interestingly, a number of recent studies, using models such as mice with transposon-tagged HSPCs or analysis of diseased human bone marrow, have provided evidence that non-stem cell progenitors are important to steady-state hematopoiesis without contribution from HSCs (Busch et al., 2015; Kim et al., 2014; Notta et al., 2016; Sun et al., 2014; Wu et al., 2014). These progenitors, including MPPs and myeloid-lineage progenitors, are thought to survive for longer in bone marrow than previously thought, contradicting the traditional view of the hematopoietic hierarchy where only HSCs persist *in vivo* (Busch et al., 2015; Notta et al., 2016; Sun et al., 2014). Based on previous studies supporting that latently-infected reservoirs are established before viral suppression with cART (Kearney et al., 2014; Simonetti and Kearney, 2015) and that at least a portion of the provirus that we have detected in our donor HSPCs is maintained in these non-stem cell progenitors, our work supports that these cells can persist for years in HIV+ people on therapy. In Chapter 2, we had two treated donors with suppressed plasma virus who had donated again after initially donating in our first *in vivo* study, with samples taken approximately 3.3 years apart (Carter et al., 2010). They both had detectable HIV in our initial study of CD34+ HSPCs and in the study presented here of CD133+ HSPCs, in which they had 5.0 and 8.4 years of viral suppression before bone marrow was collected. However, we did not have proviral sequence to determine tropism in these two donors. In Chapter 3, we had a 20 donors with

detectable CCR5-tropic provirus, and all but two of the Sort 2 amplicons were CCR5-tropic. Of our donors with the longest periods of viral suppression, two donors with 9.6 or 11.2 years of viral suppression before analysis had CCR5-tropic amplicons detected in Sort 1 and two suppressed for 7.1 years each had CCR5-tropic amplicons in Sort 2. Interestingly, one of these donors with 7.1 years of viral suppression had also donated in the Chapter 2 study, with 3.75 years between donations. This donor, 454304, had detectable HIV in the CD133-sorted HSPCs from the initial donation, and, as mentioned, only a CCR5-tropic amplicon in Sort 2. This donor had a majority of CCR5-tropic virus archived in PBMCs, with just 1 out of 25 PBMC *env* sequences predicted to use CXCR4, so the virus detected in the Chapter 2 study could still have been CXCR4-tropic. Overall, our results predict that HIV provirus may persist for years in an infected individual on therapy, and non-stem cell progenitors, which are likely infected in these people, can thus endure for years in the bone marrow.

*Infected HSPCs can differentiate into mature lymphoid cells with provirus in vivo*

In an interesting experiment presented in Chapter 3, we searched for HIV provirus in purified populations of CD4-negative cells, including CD8<sup>+</sup> T Cells, B cells and NK cells, from PBMCs in 10 donors: 5 donors with at least one CXCR4-tropic provirus detected in HSPCs and 5 donors with only CCR5-tropic virus in HSPCs. Except for CD8<sup>+</sup> T cells, which undergo a brief CD8<sup>+</sup>CD4<sup>+</sup> stage during differentiation, these cell types lack the surface receptors for HIV infection and are typically uninfected in peripheral blood. Interestingly, in 4 out of the 5 donors with some CXCR4-tropic virus in HSPCs, we amplified *gag* and/or *env* amplicons from

one or more of these CD4-negative subsets. When *env* was detected, our genotypic predictions indicated that the provirus was often CXCR4-utilizing, but some sequences were predicted to be CCR5-tropic. In contrast, we only found 1 out of the 5 CCR5-tropic only donors to have detectable HIV in the differentiated populations and all *env* amplicons were CCR5-tropic. Thus, there seems to be a difference in the HSPCs infected by CXCR4-tropic versus CCR5-tropic viruses that more often allows the cells with CXCR4-tropic provirus to differentiate to a mature hematopoietic cell with maintenance of integrated provirus. However, CCR5-tropic provirus was present in differentiated subsets in at least two of the donors, which supports that these viruses of different tropism could infect the same progenitor populations.

Sequence analysis of the one donor with CCR5-tropic provirus in HSPCs and in CD8<sup>+</sup> T cells, B cells, and NK cells produced a striking result indicating that all the *env* amplicons in the differentiated subsets, 8 from CD8<sup>+</sup> T Cell, 2 from B cells, and 1 from NK cells, had identical nucleotide sequence to an HSPC Sort 1 amplicon from that donor. Without comparison of the entire viral genomic sequence and the integration site for the provirus in each of the differentiated cells, it cannot be known for sure that the differentiated subsets came from the same HSC or an early progenitor with multi-lymphoid lineage potential. However, these cells are extremely unlikely to be infected by circulating virus and *env* has a high level of genetic diversity in the viral populations within an individual. Thus, it is probable that these lymphoid cells differentiated from a common HSPC that was initially infected.



### *Potential fates of the latent HIV reservoir in HSPCs*

Given the work that has been presented here, we can speculate on the potential outcomes of a latently infected HSPC *in vivo*, as depicted in the model in **Figure 4-2**. The first outcome to consider is that an HSPC infected with a latent provirus could persist through quiescence, which is a property attributed to true HSCs for their ability to survive in the bone marrow for the extent of a human's life. It is not yet known how non-stem cell progenitors are able to persist. Given the evidence discussed earlier that infected HSPCs can persist for years *in vivo* and that HSCs or persistent non-stem cell progenitors harbor provirus, this seems a likely way that the provirus is maintained. Another outcome could be homeostatic proliferation, which could occur with HSCs and, in some limited capacity, MPPs. In this case, the latently-infected HSPC would be activated to proliferate, without differentiation. Of the HSPC-derived amplicons presented in Chapter 3, no amplicons within a donor were identical to each other, which might support that this proliferation without loss of provirus is not possible. However, given the low frequency of detectable virus in HSPCs, further analysis of proviral sequence and integration sites may be required to identify identical provirus between multiple HSPCs.

A third outcome for the infected HSPC is that it could be stimulated to reactivate latent infection. Our first study of HSPCs showed *in vitro* that a latently infected HSPC when stimulated with GM-CSF and TNF- $\alpha$  could reactivate latent infection to release infectious virus, but would also succumb to cell death (Carter et al., 2010). However, it is not known if reactivated HSPCs can produce virus or if

reactivation leads to cell death *in vivo*. If the infection is reactivated and the HSPC began to release virus, it is possible that this virus could be released into circulation, as well as spread to other HSPCs or more differentiated targets, including CD4<sup>+</sup> T cells. If therapy is stopped in an HIV<sup>+</sup> person, this could allow for the infection in HSPCs to lead to the resurgence of viremia. In preliminary evidence presented in the Appendix, sequences from residual plasma virus were identical to HSPC-derived sequences in 5 donors, which could be due to virion release from infected HSPCs.

A final outcome to be considered is that a latently-infected HSPC could potentially differentiate with maintenance of provirus. Differentiation of HSPCs is thought to reactivate the virus, as shown previously with stimulation towards myeloid-differentiation by GM-CSF and TNF- $\alpha$  *in vitro* (Carter et al., 2010), and so it did not seem possible that provirus could be maintained through the differentiation process. However, as evidenced by the analysis of CD4-negative lymphoid cells in Chapter 3 discussed earlier, this could likely occur *in vivo*. Indeed, it seems that HSPCs targeted by both CCR5- and CXCR4-tropic virus can undergo this differentiation, at least into lymphoid cells, with preservation of the provirus in the differentiated cells. This could be explained perhaps by the provirus not being reactivated during differentiation in human bone marrow. This could be due to preserved blocks to active infection which allowed the infection to be latent in the first place or the provirus in HSPCs is defective thus preventing the production of viral proteins that lead to cytotoxicity. Another possibility is that HSPCs do reactivate latent infection during differentiation, while possibly releasing virus into circulation, but simply do not experience cytotoxic effects, and the virus eventually

reverts to a latent state in the differentiated cell. Reactivation without cell death could be due to some signaling factor or cell interaction in the niche, which would not have been re-capitulated in our laboratory's initial *in vitro* infections of HSPCs. Macrophages are another cell type which has less cytotoxicity during active infection, and thus can continue to release virus without dying.

#### *Additional questions and future directions*

Although the studies presented in Chapters 2 and 3 further our understanding of the HIV reservoir in HSPCs and have implications for our understanding of hematopoiesis, important questions remain to be addressed by future research. Further studies are needed to understand whether HSPCs are truly a reservoir of HIV *in vivo*. Additional studies of the bone marrow in HIV+ patients on therapy would hopefully provide these answers. Chapters 2 and 3 have provided additional information on which cells to focus on within the HSPC population, likely HSCs, MPPs, and some myeloid progenitors (CMPs and GMPs), although the study of provirus in differentiated cells also implicates lymphoid progenitors, such as an MLPs. Within these subsets, the progenitors with relatively high CD4 expression would be of most interest. In order to confirm the subsets infected *in vivo*, bone marrow from HIV+ donors on therapy could be separated into the different subsets using a panel of flow markers, in addition to CD4 staining to find populations that are enriched for HIV. This is challenging though, given the limited availability of BM MNCs from HIV-infected donors and the cell loss that occurs with flow cytometric sorting to get pure populations. It is also possible that latently-infected cells could also have downregulated CD4 through some residual Nef expression, despite the

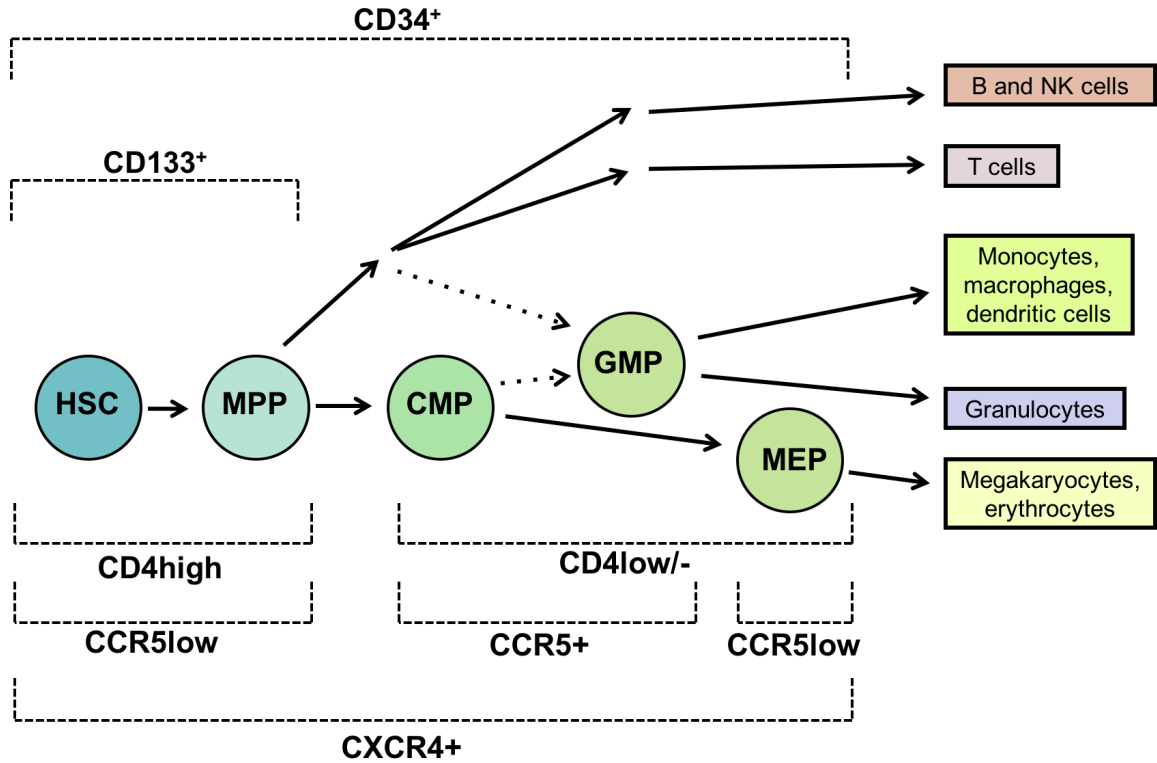
major transcriptional blocks that keep the provirus latent, and thus CD4 could not be used to enrich for infected HSPCs *in vivo*. A new technology called PrimeFlowRNA (Hanley et al., 2013) adapted for flow analysis of HIV-infected cells based on probes for HIV transcripts could aid in simultaneously assessing the expression of flow cytometric markers that delineate HSCs and other progenitor subsets and detecting infection of HSPCs. This was recently reported to be able to detect and purify a latent HIV-infected cell, as rare in the starting population as 1 in  $10^4$  or  $10^5$  cells, that was spontaneously expressing RNA transcripts or had been induced by a latency-reversing agent (Romerio and Zapata, 2015).

In addition to confirming which HSPCs are infected *in vivo*, further analysis of HIV<sup>+</sup> donor bone marrow could allow for an increased understanding of the establishment of infection in HSPCs and its effects on hematopoiesis. Assessing the HSPC reservoir in donors who began treatment during acute or primary HIV infection would be important to see if the HSPC reservoir is consistently detected in these individuals after viral suppression. There have been limited studies on the effects of HIV on hematopoiesis (Redig and Berliner, 2013), but infection and immune activation within the bone marrow, even within components of the cellular niche, could play a role on when the reservoir is established and which HSPCs are prone to active and latent infection during early infection. Although cART has been shown to improve the anemia commonly seen in HIV-infected individuals, anemia is still often seen in these individuals on treatment (Redig and Berliner, 2013) and so further studies could elucidate the effect of HIV infection on the survival and lineage potential of bone marrow HSPCs.

HIV reservoirs have currently been defined as a cell population that can harbor replication-competent virus for an extended time period (Martin and Siliciano, 2016). Thus, further studies need to build on the work presented in Chapters 2 and 3 to confirm that the latently infected HSPCs do persist in HIV-infected people and also assess if the provirus persisting in these cells can produce infectious virus that could lead to rebound viremia after treatment cessation. In respect to persistence of the reservoir, longitudinal sampling in donors who have suppressed viremia for even longer periods of time would point towards how long these cells which are presumed to be infected early in infection could remain infected *in vivo*. Sequence analysis over time, ideally of the entire proviral genome which has been difficult thus far, could provide information about viral evolution in the bone marrow. If virus is mainly latent, I would expect to see limited to no divergence in viral sequences in HSPCs over time, and would also expect a homogenous population of HSPC sequences that is genetically similar to circulating virus present before the initiation of therapy. Minimal evolution could be seen if there is ongoing replication in the bone marrow despite the presence of cART or stochastic reactivation which leads to new infections. Cell-to-cell spread has been shown to occur even in the presence of anti-retrovirals (Sigal et al., 2011), which could explain this possibility.

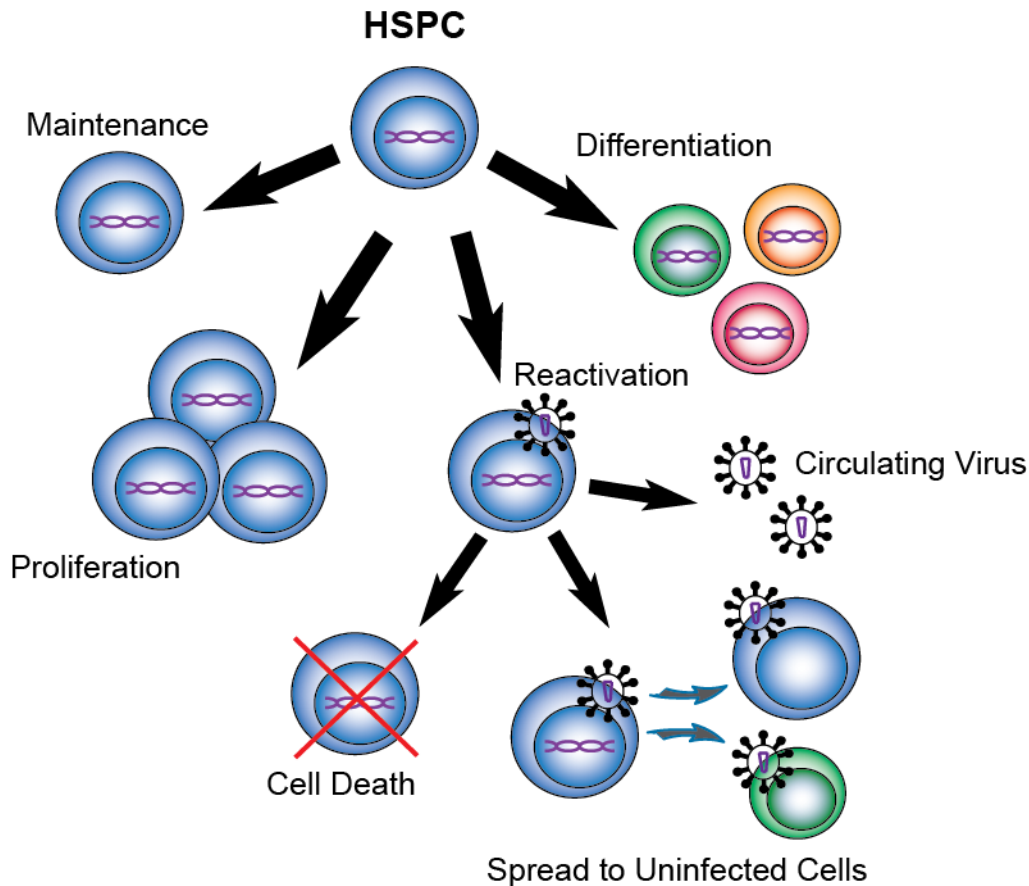
As for whether HSPCs can produce infectious virus, the preliminary work presented in the Appendix indicates that some circulating virus may come from HSPCs. However, additional studies are ongoing to perform a viral outgrowth assay from donor-derived HSPCs by stimulating the HSPCs with agents such as TNF- $\alpha$  that

have been shown to reactivate latent HIV in these cells and culturing with an infectable cell line. These assays have been used to detect even a single resting CD4<sup>+</sup> T cell with replication-competent virus in peripheral blood from HIV-infected donors on treatment (Siliciano and Siliciano, 2005). This may prove to be very difficult in HSPCs given the low frequency of provirus, limited cell numbers from donor samples, and high rates of defective provirus in latent reservoirs, as recently found by an assessment of the resting CD4<sup>+</sup> T cell reservoir in peripheral blood (Ho et al., 2013). A more direct way to assess if the HSPC reservoir can produce replication-competent virus that is relevant to HIV-infected people on therapy is through sequence analysis of the virus in rebounding viremia during treatment interruption in comparison to proviral sequences in bone marrow HSPCs from the same individual. Although these types of studies have been limited due to potential clinical consequences, carefully monitored treatment interruptions in patients may be done more often in the future after a recent study where this was safely performed (Martin and Siliciano, 2016; Rothenberger et al., 2015). Such studies would provide the greatest evidence of the contribution of the reservoir in HSPCs to viral persistence in HIV-infected patients. In sum, this dissertation presents work that provides insights into HSPCs as a reservoir of HIV, and also raises important questions regarding normal human hematopoiesis and the relevance of this reservoir to HIV-infected people.



**Figure 4-1. Overview of HIV receptor expression on HSPCs.**

CD4, CCR5, and CXCR4 receptor expression in human bone marrow stem and progenitor cells based on data presented in Chapter 3. HSC, hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; MEP, megakaryocyte/erythrocyte progenitor; GMP, granulocyte/monocyte progenitor.



**Figure 4-2. Potential outcomes of latent infection in a hematopoietic stem and progenitor cell (HSPC).**

Diagram representing conceivable fates of a hematopoietic progenitor with an integrated viral genome (purple). An infected HSPC can maintain or expand the pool of latently infected cells through remaining quiescent or proliferating without differentiation. With stimulation by cytokines or reactivation agents, the HSPC could go from a latent to an actively infected state, where cell death could be induced, virus could be produced to infect other cells and new virions could contribute to plasma virus. An HSPC could theoretically differentiate into a mature hematopoietic cell such as a T cell and retain viral DNA.<sup>7</sup>

<sup>7</sup> Reproduced with permission from Sebastian, N.T., and Collins, K.L. (2014). Targeting HIV latency: resting memory T cells, hematopoietic progenitor cells and future directions. *Expert Rev Anti Infect Ther* 12, 1187-1201..



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# **Appendix A**

## **Provirus in Bone Marrow Hematopoietic Progenitor Cells Matches Residual Plasma Virus in HIV+ People with Optimal Viral Suppression**

### **Introduction**

Life-long combination anti-retroviral therapy (cART) is required for the majority of HIV-1-infected individuals to maintain clinically undetectable plasma viral loads (<50 copies per ml) and uphold CD4+ T cell levels for sufficient immune function. Despite years of optimal cART that suppresses HIV replication, treatment interruptions lead to a rebound of circulating virus (Coffin, 1995). The rebounding virus is thought to be due to persistence of the virus through either ongoing replication via cell-to-cell spread or latent infection (Martin and Siliciano, 2016). Therapy intensification does not reduce levels of residual circulating virus which makes it unlikely that ongoing replication is occurring (Dinosa et al., 2009; McMahon et al., 2010) although recent studies do suggest that lymphoid tissue may harbor continued replication due to decreased drug penetration (Fletcher et al., 2014; Lorenzo-Redondo et al., 2016). A recent study characterizing plasma viral sequences before and during cART in 14 patients compared with virus after treatment interruption in five of them, showed that residual virus during therapy

evolves minimally and viral sequences post-treatment interruption represent the viral populations present before initiation of therapy (Kearney et al., 2014). Thus, it is likely that rebounding viremia comes from the activation of longer-lived clones that were infected before initiation of therapy and suppression of plasma viremia, instead of just an increase in cART-resistant on-going replication (Kearney et al., 2014; Martin and Siliciano, 2016; Trono et al., 2010).

Although the best studied reservoir of latent infection is in resting memory CD4+ T cells which are long-lived, it has been demonstrated that the sequences of genomes in circulating viral genomes do not exactly match those detected in circulating latently-infected T cells pre-interruption (Bailey et al., 2006; Brennan et al., 2009; Sahu et al., 2009). Viral load decay kinetics indicate that long-lived clones are potentially from a variety of reservoirs within a patient – with at least one of them decaying minimally after 7 to 12 years of treatment (Dahl et al., 2010; Riddler et al., 2016). Thus, a stem or progenitor cell could serve as this long-lived reservoir, and studies do implicate hematopoietic progenitors in bone marrow as that important reservoir (Bordoni et al., 2015; Carter et al., 2011; Carter et al., 2010; Redd et al., 2007).

In the preliminary analysis presented here, we amplified HIV sequences from purified HSPCs, PBMCs, and plasma virus from an ongoing cohort of HIV+ patients with viral suppression for at least 6 months to examine the relationship between residual plasma virus and provirus in HSPCs and PBMCs.

## Results and Discussion

*A subset of donors with optimal viral suppression have detectable provirus in HSPCs*

To get a more complete look at how often HIV can be detected in bone marrow HSPCs in HIV-infected individuals, we recruited a cohort of HIV+ donors with suppressed viremia on optimal treatment for at least 6 months from which we received bone marrow aspirates and peripheral blood. Here, I describe the overall cohort from which a subset of donors was presented in Chapter 3. As described in Chapter 3, we isolated two highly pure populations of HSPCs from the bone marrow aspirates, Sort 1 (CD133-sorted) and Sort (CD34-sorted, CD133-depleted). We also isolated PBMCs from the peripheral blood.

We used a single-copy sensitive multiplex nested PCR assay with donor-specific primers sets optimized with donor PBMC DNA. This allowed us to amplify sequence from two regions of HIV, an 800-bp Gag amplicon from the 5' region of *gag* and a 450-bp V3 amplicon including the V3 loop of *env*. Gag and V3 amplicons were amplifiable in PBMCs from the majority of donors (43 out of 44), but we were only able to detect Gag and/or V3 in Sort 1 or Sort 2 for 27 out of the 44 donors assessed (**Table A-1**). For the 17 negative donors, at least 100,000 cells were tested in all donor HSPC samples for the majority of donors (only 5 donors had less than 100,000 cells tested in either Sort 1 or Sort 2). This is comparable to the rate of positivity in CD133-sorted HSPCs in our previous 11 donor-cohort analysis described in Chapter 2 (6 out of 11 donors with HIV detected by qPCR).

The frequency of provirus in Sort 1 and Sort 2 HSPCs was assessed by the number of 1<sup>st</sup> round PCR reactions, set up at limiting dilution, which produced a gag

and/or V3 amplicon, out of the total number of cells assayed for each population (**Table A-2**). For the donors where provirus was detected, the proviral frequency ranged from 1.0 to 23 provirus per  $10^6$  cells in Sort 1 and 1.4 to 29 provirus per  $10^6$  cells in Sort 2, with similar mean frequencies between positive Sort 1 samples and positive Sort 2 samples (Sort 1 had 11 per  $10^6$  cells and Sort 2 had 9.1 per  $10^6$  cells; **Table A-2**). The majority of amplicons were unlikely to be due to CD3<sup>+</sup> T cell contamination, as assessed in Chapters 2 and 3. This is similar to our previous study in Chapter 2, where a frequency of 12 to 37 provirus per  $10^6$  cells was reported by qPCR analysis of CD133-sorted HSPCs in 5 of the positive donors (27,000 to 84,000 cells assessed), although the sixth donor had a much higher frequency of 420 provirus per  $10^6$  cells in only 4,800 cells tested. The analysis presented here uses a PCR assay for larger regions of the HIV genome, which may decrease sensitivity, but did assess higher cell numbers than our analysis in Chapter 2. In comparison, the reported frequency by viral outgrowth of replication-competent provirus in resting CD4<sup>+</sup> T cells is approximately 1 in  $10^6$  cells, although the total number of provirus (defective and replication-competent) may be closer to 300 in  $10^6$  resting CD4<sup>+</sup> T cells as indicated by a recent study (Eriksson et al., 2013). Thus, HSPCs have a much lower frequency of provirus than the CD4<sup>+</sup> T cell reservoir, but may still persist with replication competent virus that is important *in vivo*.



*Provirus in HSPCs has sequence identity with plasma virus*

To compare the circulating virus in the plasma to bone marrow HSPCs, the plasma for each donor was pelleted to concentrate virions and the pellet was used to isolate viral RNA for synthesis of cDNA. Given that plasma virus levels were below detection by clinical assays (less than 20-48 copies/mL), an optimized protocol was developed using Raji RNA as a carrier and internal control to maximize yield for RNA isolation and cDNA synthesis, allowing amplification of viral sequences from a very low number of virions. Using the same nested multiplex PCR assay on the viral cDNA for each donor, plasma virus was analyzed in 26 donors thus far and sequence was amplifiable in 18 of those donors (**Table A-1**). However, a few donors of these donors tested initially in the cohort and negative for amplifiable virus were not processed with the optimized RNA isolation and cDNA synthesis protocol. Phylogenetic analysis of plasma virus in patients on therapy with low viremia saw multiple identical viral sequences, often with a predominant plasma clone (Bailey et al., 2006). As expected, in our study, clonal plasma viral sequences were detected in several donors.

Interestingly, in a sequence alignment and pairwise genetic distance analysis of nucleotide sequences from each donor, we found identical sequence in Gag or V3 amplicons isolated from HSPCs and plasma virus in five donors thus far (**Table A-3, Figure A-1**). In all cases, there was at least one sequence detected in PBMCs that had the same sequence, although these sequences were derived from bulk PBMCs which contains a variety of cell types. It is assumed that the majority of these sequences come from resting CD4<sup>+</sup> T cells in HIV-infected people on cART, but other

cell types including macrophages could also be infected. In the analysis presented in Chapter 3, we were able to detect provirus in CD4-negative lymphoid subsets that are not thought to be infected and the provirus was statistically unlikely to be due to CD4<sup>+</sup> T cells. Therefore, it is possible that the plasma viral sequences could still indicate virions that are released from infected HSPCs, and potentially infected a PBMC or the HSPC differentiated into a cell type found in PBMCs. However, further analysis and comparison is needed.

#### *Conclusion and Future Directions*

The ongoing analysis of this cohort presents further evidence that HIV provirus can be detected in a subset of HIV<sup>+</sup> donors with viral suppression on therapy and that HIV in HSPCs contributes to residual viremia in these donors.

As additional sequences are amplified from bone marrow, peripheral blood, and plasma virus samples, we hope to have a more complete picture of viral dynamics between these three viral populations. Compartmentalization analysis would compare the relationship between sequences within each population and between the three different populations to assess if there are significant differences in the population structures. Indeed, Slatkin-Maddison analysis, which looks at the minimum inter-population migration events to explain the phylogenetic structure of populations, was applied in a study to V3 sequences from plasma virus and PBMCs in 30 chronically-infected HIV<sup>+</sup> donors with suppressed viremia on treatment (Pou et al., 2013). The authors found that in some cases there was compartmentalization between plasma virus and PBMCs, which has implications on whether PBMC

provirus can be used as a true representation of residual virus in the presence of treatment. Compartmentalization of bone marrow HSPCs from PBMC or plasma virus could indicate different selective pressures by immune activation or anti-retroviral therapy in the bone marrow, in addition to differences in the phenotype of these viruses such as tropism or pathogenicity (Zarate et al., 2007).

Thus far, there have been sequences that share identity between all three compartments, but it is difficult to resolve the direction of viral flow between bone marrow HSPCs with plasma or peripheral blood. Analysis of the number of HSPC sequences with mutations that predict defective provirus, in addition to experiments to stimulate HSPCs to measure virion production could help explain if plasma virus sequences are potentially coming from HSPC provirus. Longitudinal plasma samples from repeat donors within our cohort could give insight into how often HSPCs reactivate infection and whether infected HSPCs continue to contribute to plasma viremia over time. Overall, the analysis presented here does have important implications for whether an HIV reservoir in HSPCs could contribute to viral persistence by causing rebound viremia in HIV-infected individuals who cease treatment.

## **Methods**

### **Clinical Samples**

We recruited HIV-positive donors currently receiving antiretroviral therapy from the University of Michigan HIV-AIDS Treatment Program and the Henry Ford Health System. Informed consent was obtained according to a protocol approved by

the University of Michigan Institutional Review Board and Henry Ford Institutional Review Board. At the time of sample acquisition, all donors were >18 years old, had normal white blood cell counts, and had plasma viral loads of <48 copies/mL for at least 6 months. 20 ml of bone marrow was aspirated from the posterior iliac crest, collected in preservative-free heparin, and processed immediately. 100 mL of peripheral blood was collected in K2-EDTA vacutainer tubes and also processed the same day. Bone marrow mononuclear cells (BMMCs) and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque density separation (GE Healthcare). Sort 1 and Sort 2 HSPCs were then isolated from BMMCs as indicated below. Diluted plasma from the Ficoll-Paque separation of the peripheral blood was pelleted by ultracentrifugation at 112,398xg for 1.5-3 hours at 4°C and resuspended in 1 mL Trizol Reagent (ThermoFisher) and stored at -80°C.

A fraction of each clinical sample was stained with R-phycoerythrin-conjugated anti-CD133 (Miltenyi Biotec), fluorescein isothiocyanate-conjugated anti-CD34 (eBioscience), allophycocyanin-conjugated anti-CD3 (eBioscience), and 7-aminoactinomycin D (7-AAD). Samples were analyzed on a BD FACSCanto. The donors who provided samples analyzed in this study are a subset of a larger cohort and four of the donors included provided multiple donations with 3-9 months between donations. Only samples that were at least 80% CD133<sup>+</sup> for Sort 1 or 80% CD34<sup>+</sup> for Sort 2, with < 2.0 % CD3<sup>+</sup> contamination were considered in our analysis (**Table 3-2**).

### **Cell Isolation**

Adherent cells were depleted from CMBCs or BMMCs by incubation in serum-free StemSpan media (StemCell Technologies) for 1-2 hours at 37°C, and then Sort 1 (CD133<sup>+</sup> cells) was isolated by magnetic separation with a CD133 MicroBead Kit (Miltenyi Biotec) according to the manufacturer's protocol with the following modifications. To achieve higher yields of CD133<sup>+</sup> cells, 1.5x the recommended ratio of CD133 MicroBeads to cells was used for donations 451000, 453000, and 454304. CBMCs or BMMCs that flowed through the first column (CD133-depleted) were used for isolating the Sort 2 (CD133<sup>low/-</sup>CD34<sup>+</sup>) fraction by magnetic sorting with the EasySep Human CD34 Positive Selection Kit (StemCell Technologies). For both CD133 and CD34 magnetic isolations, cells were sequentially sorted on 2 columns to increase purity.

### **RNA Isolation and cDNA Synthesis**

Plasma viral RNA was isolated either using a Trizol-chloroform extraction protocol or RNeasy micro column purification kit (Qiagen). Samples were all DNase treated before cDNA synthesis. cDNA synthesis was performed using SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). RNA isolation and cDNA synthesis efficiency was verified by spiking Trizol samples with Burkitts Lymphoma (Raji) Total RNA and yields were monitored by Human ACTB (Beta Actin) TaqMan Gene Expression Assay (ThermoFisher). cDNA samples were diluted 1:10 or greater when used as a template in the PCR reactions described below.

### **Polymerase Chain Reaction (PCR)**

Cells were lysed in MagNA Pure DNA Lysis/Binding Buffer (Roche), and DNA extracted using a MagNA Pure Compact System (Roche). A 2-step PCR assay was validated for single copy sensitivity on ACH-2 cell DNA. For each donor, first and second round primers used for HSPC DNA analysis were first verified by amplification of proviral sequences from PBMC DNA. Primer sequences are listed in **Table 3-5**. First round primer pairs for Env included 5036d plus LTR-pA-R, 5956d-f plus LTR-pA-R, or envC2F2 plus envC4R1, along with primers to amplify a region of Gag (U5-577.9662-f plus tagD4.6b-p24R1d plus or minus long1316-D4.6b depending on the patient sequence). Second round primer pairs included 5956d-f plus LTR-pA-R, envC2F2 plus envC4R1, or env1in5 [Brennan et al., 2009] plus env1in3 [Brennan et al., 2009] for *env* amplification. For *gag* amplification, second round primers were 626s [Hasegawa et al., 1985] plus D4.6b [Buszczak et al., 2014].

In the first round, 5  $\mu$ L of template DNA at limiting dilution (maximum 0.1 copies/ $\mu$ L with  $\leq 25\%$  of reactions expected positive) was amplified in 50- $\mu$ L reactions containing 10  $\mu$ l of 5X Phusion HF Buffer (ThermoFisher), 1U of Phusion Hot Start II High Fidelity DNA Polymerase (ThermoFisher), 500nM of each primer, and 200  $\mu$ M deoxyribonucleotide triphosphates (dNTPs). ACH-2 [Clouse et al., 1989] cell DNA was diluted in DNA from uninfected PBMCs to serve as a positive control (10 HIV genomes per  $\mu$ l) or control for sensitivity (0.2 HIV genomes per  $\mu$ l). DNA from uninfected PBMCs was used as a negative control. Thermocycling was conducted using a BioRad C1000 thermocycler with conditions indicated in **Table 3-5**.

In the second round, 1  $\mu$ l of the first round reaction was amplified in 50- $\mu$ l

reactions containing 10 µl of 5X Phusion HF Buffer, 1U of Phusion Hot Start II High Fidelity DNA Polymerase (ThermoFisher), 500nM of each primer and 200 µM dNTPs. Thermocycling was conducted using a BioRad C1000 thermocycler with cycling conditions as in **Table 3-5**.

### **DNA Sequencing Analysis and Cloning**

PCR reactions were run on 1.5% agarose Tris-acetate-EDTA gels with 1X GelRed (Biotium), the amplicons excised, extracted using QIAquick Gel Extraction Kit (Qiagen), and then sequenced by Sanger dideoxy sequencing. Consensus sequences were generated using SeqMan (DNASTar) and contaminants were excluded after comparison to all previously generated donor sequences and lab strains in MEGA6 [Tamura et al., 2013].

Molecular phylogenetic analysis was performed by maximum likelihood method using MEGA7 [Kumar et al., 2016]. The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model [Hasegawa et al., 1985]. The tree with the highest log likelihood (-2805.4022) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.8699)). Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were

eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position.

### **Statistical Analysis**

T cell contamination analysis was performed for HSPC sequences as described as in McNamara *et al* [\[McNamara et al., 2013\]](#). All other statistical tests were performed using Excel or GraphPad Prism 5.0a.



**Table A-1. HIV Detection in HIV+ Donors on Therapy with Optimal Viral Suppression**

<b>Source of HIV</b>	<b>Donors Positive (n)</b>	<b>Rate of HIV Detection</b>
HSPC (Sort 1 and 2) DNA	27 (44)	61%
Flowthrough 1 and 2 DNA	41 (44)	93%
PBMC DNA	43 (44)	98%
Plasma Virus RNA	18 (26)	69%
n = total donors tested		

**Table A-2. Proviral Frequency in Donors Positive for HIV in HSPCs**

Donation ID <sup>a</sup>	Sort 1		Sort 2	
	Proviral Frequency <sup>b</sup> (per 10 <sup>6</sup> cells)	Cells Analyzed (x10 <sup>4</sup> )	Proviral Frequency <sup>b</sup> (per 10 <sup>6</sup> cells)	Cells Analyzed (x10 <sup>4</sup> )
405000	12	8.6	NA	NA
406000	17	12	<4.9	20
412406	<4.9	20	5.9	34
435412406	8.4	24	<1.8	56
407000	NA	NA	4.4*	23
433407	<11	9.4	<8.6	12
408000	<49	2.0	<33	3.0
428408	23	13	18	5.5
409000	21	9.5	<9.3	11
411000	<12	8.5	4.5	22
413402	20	5.0	<5.4	19
414000	7.1	14	3.7	27
415000	3.5	28	NA	NA
419000	3.2	31	NA	NA
420000	12	26	4.9*	41
421000	17	17	8.2	37
423000	1.7	58	1.4	70
434423	<1.5	67	1.9	53
424000	6.9*	14	NA	NA
426000	10	20	29	38
431000	14	21	<3.9	26
432000	7.6	40	9.5	32
436000	<17	6.1	29	10
437000	5.9	17	2.4	43
439000	10	9.9	<7.9	13
445000	5.6*	54	<7.7	13
446000	4.4	23	<1.8	56
449000	3.4	29	6.7	60
450000	1.0*	103	NA	NA
451000	NA	NA	5.4	19
453000	<2.5	41	2.2	45
454304	<8.3	12	14*	14

<sup>a</sup>First 3 digits is donation number; subsequent groups of 3 digits are ID of previous donation(s) from the same individual, if any.

<sup>b</sup>Proviral frequency calculated by number of 1<sup>st</sup> round PCR replicates at limiting dilution that amplified either gag and/or V3 in the 2<sup>nd</sup> round.

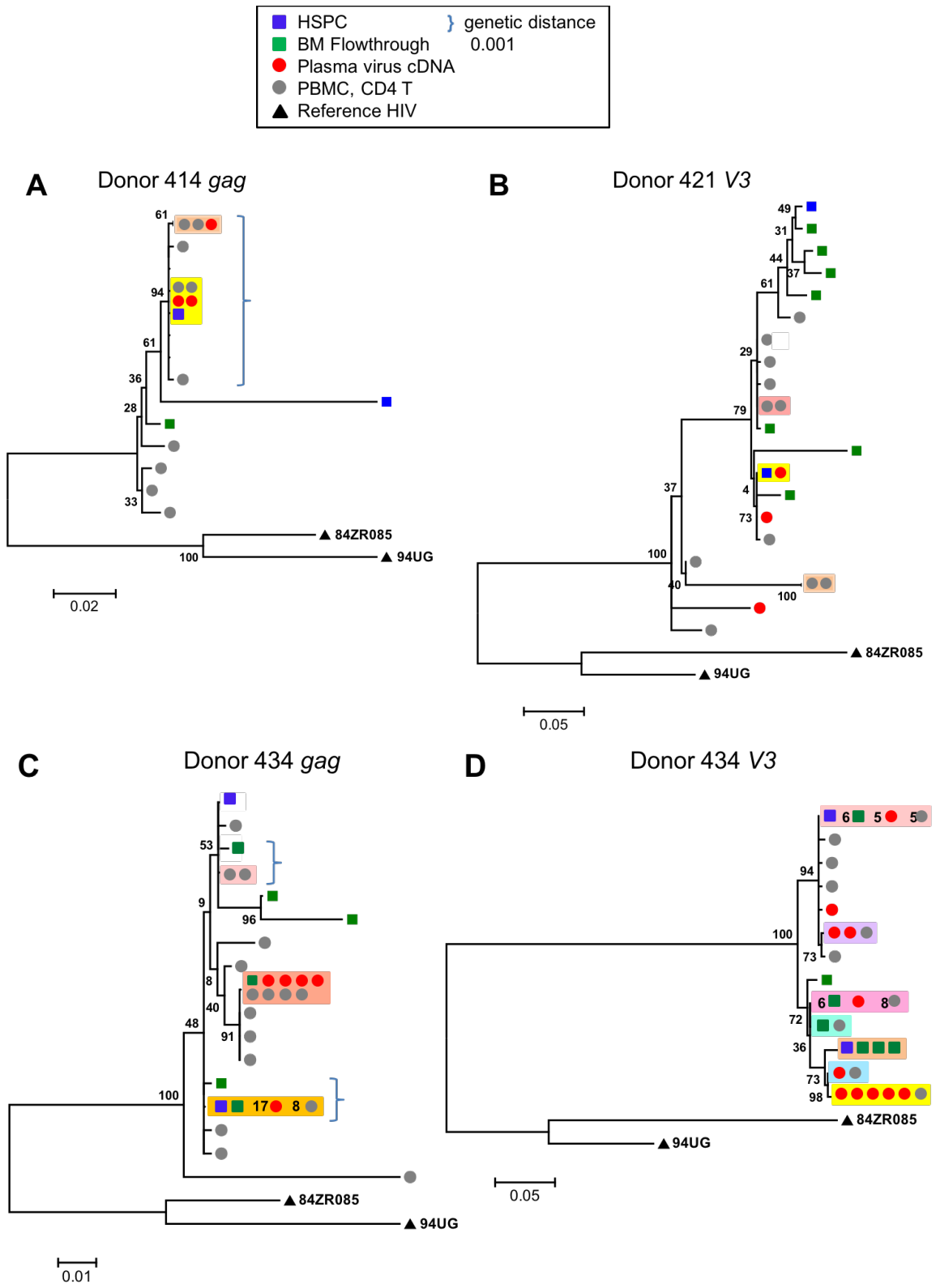
\*Indicates that gag or V3 amplicons could have originated from contaminating T cell DNA, so true HSPC proviral frequency may be lower than reported. Analysis to exclude T cell contamination was performed as previously published (McNamara et al., 2013). Data for T cell analysis was not available for donor 405000.

Bold borders indicate multiple donations from the same individual. Gray boxes indicate samples that did not meet criteria for purity. Abbreviations: NA, not analyzed

**Table A-3. Several Plasma Virus and PBMC *gag* and V3 *env* Sequences are Identical to HSPC Amplicons**

Donor ID*	Gag			V3		
	HSPC Amplicons	Amplicons Identical to HSPC		HSPC Amplicons	Amplicons Identical to HSPC	
		Plasma	PBMC		Plasma	PBMC
414000	1	2	2	1	0	7
421000	0	0	0	1	1	4
434423	1	17	8	1	5	5
435412406	0	0	0	1	0	3
				1	3	18
				1	0	2
436000	0	0	0	1	22	2

\*First 3 digits is donation number; subsequent groups of 3 digits are ID of previous donation(s) from the same individual, if any.



**Figure A-1. Plasma virus sequences are often clonal and are identical to HSPC amplicons in several donors.**

Maximum likelihood phylogenetic trees from three representative donors show genetic relationships of *gag* and *env* sequences from bone marrow, peripheral blood, and plasma virus. Highlighted areas indicate groups of identical amplicons. Scale indicates nucleotide substitutions per site.<sup>8</sup>

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<sup>8</sup> Figure created by Val Terry.

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