Hematopoietic Precursor Cells Isolated From Patients on Long-term Suppressive HIV Therapy Did Not Contain HIV-1 DNA

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Background. We address the key emerging question of whether Lin−/CD34+ hematopoietic precursor cells (HPCs) represent an important latent reservoir of human immunodeficiency virus type 1 (HIV-1) during long-term suppressive therapy.

Methods. To estimate the frequency of HIV-1 infection in bone marrow, we sorted Lin−/CD34+ HPCs and 3 other cell types (Lin−/CD34−, Lin−/CD4+, and Lin+/CD4+) from 8 patients who had undetectable viral loads for 3–12 years. Using a single-proviral sequencing method, we extracted, amplified, and sequenced multiple single HIV-1 DNA molecules from these cells and memory CD4+ T cells from contemporaneous peripheral blood samples.

Results. We analyzed 100 000–870 000 bone marrow Lin−/CD34+ HPCs from the 8 patients and found no HIV-1 DNA. We did isolate HIV-1 DNA from their bone marrow Lin+/CD4+ cells that was genetically similar to HIV-1 DNA from lymphoid cells located in the peripheral blood, indicating an exchange of infected cells between these compartments.

Conclusions. The absence of infected HPCs provides strong evidence that the HIV-1 infection frequency of Lin−/CD34+ HPCs from bone marrow, if it occurred, was <.003% (highest upper 95% confidence interval) in all 8 patients. These results strongly suggest that Lin−/CD34+ HPCs in bone marrow are not a source of persistent HIV-1 in patients on long-term suppressive therapy.
A recent study by Carter et al reported that HIV-1 can infect CD34⁺ hematopoietic precursor cells (HPCs) from bone marrow in vitro. By isolating viral DNA from CD34⁺ HPCs isolated from bone marrow of 44% of the patients analyzed, they showed that these cells are viral reservoirs in patients on suppressive therapy [10]. The patients analyzed in this study had undetectable viral loads for at least 6 months at the time of sampling, but whether CD34⁺ HPCs act as an important HIV reservoir after several years of suppressive ART remains unknown.

To investigate whether HPCs are a persistent source of HIV-1 in persons on long-term suppressive therapy, we sorted and analyzed Lin⁻/CD34⁺ HPCs from the bone marrow of 8 patients enrolled in the Options Cohort at University of California, San Francisco, who had been on suppressive therapy with undetectable viral loads (<40–75 copies/mL) for 3–12 years. To further analyze the possibility of the bone marrow as a viral reservoir in these patients, we sorted and compared the HIV genetic populations in 3 additional cell types (Lin⁻/CD34⁺, Lin⁻/CD4⁺, and Lin⁻/CD4⁻) from bone marrow with HIV-1 populations isolated from contemporaneous memory CD4⁺ T cells sorted from peripheral blood.

**METHODS**

**Clinical Specimens**

Paired bone marrow and peripheral blood samples from 8 HIV-1 subtype B individuals were analyzed. The samples were obtained from individuals in the Options Cohort at University of California, San Francisco. The Options Cohort is an ongoing longitudinal observational cohort study of adults enrolled within 6 months of HIV-1 antibody seroconversion and followed throughout the course of HIV disease. In this study, we included 5 patients who had elected to begin therapy within 30 days of entering the cohort (acute/early infection, patients 1–5) and 3 who had chosen to begin therapy >1 year after infection (chronic infection, patients 6–8) (Supplementary Table 1). The criterion we used for selection of these patients was undetectable HIV-1 viremia (<40–75 copies/mL) for at least 3 years after the initiation of therapy. Informed consent was obtained from all eight patients. The study was approved by the institutional review boards at University of California, San Francisco and the Karolinska Institutet.

**Single-Proviral Sequencing**

To quantify and genetically characterize HIV-1 viral populations in the cells isolated from bone marrow and peripheral blood we used a modification of the newly developed single-cell sequencing assay [11] called single-proviral sequencing. The details for this assay are reported here.

**Isolation of Single Cells from Bone Marrow and Peripheral Blood**

Paired bone marrow and blood samples from the 8 patients were collected. The blood specimens were collected using tubes containing acid citrate dextrose as an anticoagulant, and the bone marrow aspirate was collected, following local administration of lidocaine, using 30-mL syringes containing 1 cc of heparin to collect up to 60 mL of bone marrow aspirate. Bone marrow cells and peripheral blood mononuclear cells were separated from plasma using Ficoll.

**Fluorescence-Activated Cell Sorting**

Fluorescence-activated cell sorting was carried out on a FACSAria (BD). Four different cell types were sorted from the patient bone marrow samples: Lin⁻/CD34⁺ and Lin⁻/CD34⁻ HPCs, and Lin⁻/CD4⁺ and Lin⁻/CD4⁻ lymphoid cells. The monoclonal antibodies used for phenotypic isolation of these 4 different cell types were: CD34-PE, Lin-FITC (combination of CD3, CD14, CD16, CD19, CD20, and CD56), CD4-PE-TxRED, and CD45-A700. To isolate memory CD4⁺ T cells from peripheral blood we used CD4-PE-TxRED, CD3-PE, CD45-PE, CD8 Qdot605, CD45RO FITC, and CD27 AeF780. Dead cells were excluded by staining with aqua-fluorescent amine reactive dye. (These monoclonal antibodies are from Invitrogen and BD/Pharmingen.) Detailed information about the gates used during the sorts is presented in Figure 1. For each cell type, 10⁵–11 × 10⁶ cells were sorted into facs tubes. The cells were then divided into 1.5-mL Eppendorf tubes and spun down. The supernatant was removed, and the pellet was stored at −70°C before being sent on dry ice to Sweden for further analysis.

**DNA Amplification and Detection**

Cells were lysed by adding 100 µL of lysis buffer, and DNA was released by incubating the samples at 55°C for 1 hour and subsequently at 85°C for 15 minutes [11, 12]. After cell lysis, all samples were stored at −20°C. To obtain polymerase chain reaction (PCR) products derived from single HIV-1 DNA molecules, the cellular lysate with HIV-1 DNA was serially diluted 1:3 in 5-mM Tris-HCl (pH 8.0) to a maximum dilution of 1:243. According to Poisson’s distribution, the cellular dilution yielding PCR product in 3 of 10 PCR reactions contains 1 copy of HIV-1 DNA per positive PCR about 80% of the time. Thus, PCR plates were set up to identify the optimal cellular lysate dilution yielding ≤30% positive PCR reactions for each cell type and patient by the following procedure.

The PCR plates were set up with 8 µL in each of 86 wells containing the following components at the indicated final amounts or concentrations in sterile molecular grade water: 2 × PCR Buffer (Invitrogen), 4 mM MgSO₄, 400 nM of specific outer primers, 0.08 U/µL Taq platinum polymerase (Invitrogen), and 400 µM nucleotide mix (Promega). Next, 2 µL from
each of the different cellular dilutions was added to each well of a PCR row for a total of 10 wells per cellular dilution and a total of 5 rows with cellular dilutions spanning from undiluted to 1:243. Six control wells were included on each PCR plate. A plasmid containing DNA from a laboratory strain (pNL4-3) of HIV-1 was added to 2 wells as a positive control (1 μL of 10 copies/μL), and molecular-grade water was added to 4 wells as negative controls. The p6-RT region of the HIV-1 DNA in the wells from the plate was then amplified using a nested PCR protocol (outer primers 1894+ and 3500−; inner primers 1870+ and 3410−) as previously described [11, 13]. Positive HIV-1 DNA amplicons were identified on a 96-well 1% agarose gel with ethidium bromide (Invitrogen). None of the negative control wells were positive. From the number of HIV-1 DNA molecules amplified by PCR, we then determined the cellular dilution yielding ≤3 HIV-1 amplicons per 10 wells. Using this cellular dilution, up to 8 additional HIV-1 DNA amplification PCR plates were set up for each cell type from each of the 8 patients. The amplified HIV-1 products were sequenced by direct dideoxyterminator sequencing (Applied Biosystems) in both directions using overlapping internal primers [13].

Our ability to detect single HIV-1 DNA molecules has been shown in an earlier study using 293T cells infected with defined numbers of HIV-based vectors [11]. To further validate the ability of the single-proviral sequencing assay to detect all the HIV-1 DNA molecules in the sorted cell samples, we sorted and analyzed patient samples with known HIV-1 infection frequencies. The frequency of infection of these patient samples was determined previously by single-cell sequencing [11]. The single-proviral sequencing analysis resulted in the amplification of the expected number of HIV-1 DNA molecules for the infection frequencies of these patient samples.

**Statistical Methods**

Conducting single-proviral sequencing on specific numbers of patient cells, we determined the HIV-1 infection frequency in each cell type using a maximum likelihood statistical analysis. For detailed statistical methods and calculations, please see the Supplementary Material.

**Phylogenetic Analyses**

Analyses of the intracellular HIV-1 populations were performed using an in-house computer program written in Perl.
scripting language (available upon request). For phylogenetic analysis of the HIV-1 populations, maximum likelihood trees were constructed using Mega5 (http://www.megasoftware.net/). The model of evolution was selected using the find model tool from the Los Alamos HIV Sequence Database (http://www.hiv.lanl.gov/content/index). Statistical support of the tree structures was obtained by 100 bootstrap replicates. Measurements of the HIV-1 genetic diversity (average pairwise distance) within each cell type was calculated using MEGA5.0.

RESULTS

HPCs Isolated From Bone Marrow Do Not Contain HIV-1 DNA
To assess the HIV-1 infection frequency of HPCs in patients on long-term suppressive therapy, we isolated HPCs from bone marrow of 8 patients on suppressive antiretroviral therapy for at least 3 years. The HPCs were sorted from the bone marrow aspirate of the 8 patients based on their specific phenotype (Lin−/CD34+) (Figure 1). "Lin" represents markers of specific hematopoietic lineages, and CD34 is a cell surface marker on many HPCs. The number of Lin−/CD34+ HPCs sorted from the bone marrow was 1.0–8.7 × 10^5. Using single-proviral sequencing, the amount of HIV-1 DNA was assessed in these HPCs for each patient.

None of the HPCs sorted from the 8 patients were found to contain HIV-1 proviral DNA. The lack of any infected Lin−/CD34+ HPCs in the 8 patients that we studied provides strong evidence that the true infection frequency of these cells in any individual, if it was present, was .0003%–.003% (upper 95% confidence bounds) (Table 1).

Differing Levels of HIV-1 DNA in Lin−/CD4+ and Lin+/CD4+ Cells Isolated From Bone Marrow
To further analyze the viral content in cells from bone marrow, we isolated Lin−/CD34−, Lin−/CD4+, and Lin+/CD4+ cells in addition to Lin−/CD34+ HPCs. The number of Lin−/CD34+ HPCs sorted from the bone marrow was 5.8–59 × 10^5 cells, and none of these cells were found to contain HIV-1 DNA. This equates to upper 95% confidence bounds on infection frequency of .0001%–.0005% in Lin−/CD34− cells. When analyzing the Lin−/CD4+ cellular population, we found no HIV-1 DNA in any of the samples from patients who initiated therapy during acute/early infection (370 000–1 400 000 cells analyzed). Conducting single-proviral sequencing, we did, however, find HIV-1 proviral DNA in this cellular population in all of the patients who initiated therapy during chronic HIV-1 infection (patients 6–8). The level of HIV-1 infection was low, ranging 0.0003%–0.0008% in these 3 patients (for individual values and 95% confidence intervals [CIs], see Table 1). When analyzing the Lin+/CD4+ cells, we found HIV-1 DNA in this cellular population in all 8 patients. The

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Abbreviations: CI, confidence interval; HPCs, hematopoietic precursor cells.

aOne cannot rule out the possibility of low but nonzero infection rates in cases where estimates were zero, as shown by the upper confidence bounds.
estimated level of HIV-1 infection for these cells ranged 0.0003%–0.01% in the patients who initiated therapy during acute/early infection. The infection frequency of these cells was found to be higher in patients who initiated therapy during chronic infection (ranging 0.02%–0.1%). The geometric mean HIV-1 infection frequency of these Lin+/CD4+ cells was 21-fold higher in the chronic-infection patients (95% CI, 1.8-fold–235-fold; $P = .02$, unpaired $t$ test).

**Similar HIV-1 Populations Found in Cells From Bone Marrow and Paired Peripheral Blood Memory T Cells**

To investigate whether the HIV-1 populations isolated from the bone marrow cells were unique, we compared the HIV-1 genetic populations in Lin−/CD4+ and Lin+/CD4+ from bone marrow to HIV-1 populations isolated from contemporaneous memory CD4+ T cells sorted from peripheral blood. Using single-proviral sequencing, we found the peripheral blood–derived memory CD4+ T cells from all the patients contained HIV-1 DNA (Figure 1). We found a 0.0006%–0.2% infection frequency in the memory T cells isolated from patients who initiated therapy during acute/early infection and a 0.02%–0.4% infection frequency in memory T cells from patients who initiated therapy during chronic infection (for individual values and 95% CI, see Table 1). The geometric mean infection frequency of these cells was 13-fold higher in patients who initiated therapy during chronic infection (95% CI, 5-fold–363-fold; $P = .08$).

Using single-proviral sequencing, we sequenced approximately 20 single-proviral HIV-1 genomes from the cells sorted from bone marrow and memory CD4+ T cells sorted from peripheral blood from each of the 8 patients. To evaluate the genetic relationship between the HIV-1 populations in the cells from these 2 different compartments, we conducted phylogenetic analyses (Figure 2). Sequences from all 8 patient cell samples as well as standard laboratory viruses formed independent populations that were at least 5% different from one another on a phylogenetic tree with no intermingling (data not shown), demonstrating that the viruses found in these patients were genetically distinct. The intrapatient comparisons showed that all 5 patients who initiated therapy during acute/early infection had nearly monomorphic viral populations derived from the cells located in both compartments (an example is shown in Figure 2A). The homogeneity of viral sequences in patients with early infection contrasted with the heterogeneity of sequences found in patients with chronic infection (examples shown in Figure 2B).

![Figure 2. Phylogenetic analysis of viral sequences from cells isolated from bone marrow and peripheral blood. Maximum likelihood trees of bone marrow Lin+/CD4+–derived (open circles), Lin−/CD4+–derived (open squares), and peripheral blood memory CD4+ T-cell–derived (filled circles) sequences from patients initiating antiviral therapy during acute/early HIV-infection (patient 3) (A) and during chronic HIV infection (patients 6 and 7; B and C, respectively). Patient 7 initiated therapy in chronic infection and had identical clonal human immunodeficiency virus type 1 (HIV-1) sequences containing a large 380–base pair deletion (C). All trees are rooted to pNL43 (filled square).](image-url)
For all patients analyzed, the phylogenetic distribution of DNA sequences of cells from bone marrow was similar to memory CD4+ T cells derived from peripheral blood taken at the same time (Figure 2A–C). The genetic similarity between HIV-1 populations in cells from bone marrow and peripheral blood implies that cells from peripheral blood were present in bone marrow specimens or that there is ongoing cellular exchange between these 2 compartments. In samples from patient 7, who initiated therapy in chronic infection, we found identical clonal HIV-1 sequences in cells from both the bone marrow and peripheral blood compartments, which supports this hypothesis. This clonal sequence contains a large 380-base pair deletion, essentially eliminating the protease from this HIV-1 population, indicating this viral population is not replication competent and must result from the expansion cells containing this replication-incompetent HIV-1 population (Figure 2C).

HIV-1 Genetic Diversity Is Similar in Cells From Bone Marrow and Peripheral Blood Compartments

To further assess the genetic relationship between HIV-1 populations found in cells from bone marrow and memory CD4+ T cells from peripheral blood, the diversity of sequences from the cells located in these compartments for each patient was calculated using average pairwise distance. In patients who initiated therapy during acute/early infection (1–5), the average pairwise distance of the HIV populations from Lin+/CD4+ cells located in the bone marrow ranged 0.06%–0.17%, and in memory CD4+ T cells from peripheral blood the average pairwise distance ranged 0.08%–0.14%; the corresponding ranges were 0.69%–1.44% and 0.60%–1.37% in patients who initiated therapy in chronic infection (patients 6–8). The average pairwise distance of the HIV-1 populations located in cells from bone marrow versus peripheral blood were similar, with the within-patient ratios ranging 0.65–1.44 (geometric mean, 1.05; 95% CI, .85–1.31; P = .58), which is consistent with the tree analysis (Figure 2).

DISCUSSION

HIV-1 infection of HPCs in patients on long-term suppressive therapy, if it occurs, will greatly impede the possibility of a cure for HIV-1 infection. To evaluate if HPCs serve as an HIV-1 reservoir in patients on long-term suppressive therapy, we isolated and analyzed Lin+/CD34+ HPCs from bone marrow from 8 patients with undetectable viral loads for at least 3 years. In this study, we did not find a single HIV-infected Lin+/CD34+ HPC in any of the 8 patients. In total, we analyzed 100 000–870 000 Lin+/CD34+ HPCs per patient. The lack of any infected HPCs provides strong evidence that if these cells are infected in patients on long-term suppressive therapy, their infection frequency is very low. Our results differ from a recent study by Carter et al, who identified HIV-1 DNA in total CD34+ cells isolated from bone marrow from 4 of 9 patients on suppressive antiretroviral therapy [10]. These patients had undetectable viral loads for at least 6 months and had an infection frequency of 1 infected cell per 10 000 CD34+ cells (0.01%). We analyzed 100 000–870 000 Lin+/CD34+ precursor cells from each patient; at an infection rate of 0.01%, this should have yielded about 10–80 infected cells or HIV-1 amplicons using our single-proviral sequencing method. However, there were several differences between our studies. The Carter study used an immunomagnetic bead procedure to isolate total CD34+ cells from bone marrow, whereas we employed multiparameter cell sorting to isolate the Lin− fraction of CD34+ bone marrow cells and eliminate dead cells and cells expressing CD4. In addition, there were differences in treatment periods: 3–12 years in our study versus 6 months for the majority of patients in the Carter et al study. This suggests that HPCs are not a source of persistent HIV in patients on long-term suppressive therapy. However, the HIV-1 infection frequency of HPCs in our patients could have been very low, but based on the number of cells analyzed by our very sensitive assay in conducting this study, our upper confidence bounds on infection provide strong evidence that the infection rates were <0.0009% in 7 of the 8 patients and <0.003% in one patient with fewer cells analyzed. An additional limitation is that some patients on long-term suppressive therapy could differ from the 8 examined here. Earlier studies have shown that HPCs are more susceptible to infection with subtype C HIV strains; however, it has recently been reported that CD34+ cells are infected in 40% of patients with subtype B HIV-1 on suppressive therapy [10, 14]. If 40% of patients on suppressive therapy have HIV-infected CD34+ cells, 3 of the 8 HIV-1-infected patients included in this study would be expected to have had HIV−1-infected Lin−/CD34+ cells, and there would only be a 2% chance that all 8 would be negative.

In addition to isolation and analysis of Lin−/CD34+ HPCs, we also analyzed Lin−/CD34− cells. The HIV-1 infection frequency of Lin−/CD34− bone marrow cells is reported to be lower than that of Lin−/CD34+ cells [10], but we sorted 900 000–5 800 000 Lin−/CD34− cells, which provides strong evidence that, if these cells are infected with HIV-1 in vivo, then the infection frequency for this phenotype is <.0005% (highest upper 95% CI) in all 8 patients.

We did find infected Lin−/CD4+ cells but only in the patients who initiated therapy during chronic infection. The frequency of infection of these cells was, however, very low (<0.0003%–0.0008%). The purity of this sort was 72%–85%, hence we cannot rule out the possibility that these Lin−/CD4+ HIV−1–infected cells also contained a low number of Lin−/CD4+ cells. The fact that we only found these cells to be infected in samples from patients initiating therapy during chronic infection may be due to an overall higher frequency of
infection of their Lin+/CD4+ lymphoid population in the bone marrow. We sorted bone marrow Lin+/CD4+ cells with lymphoid side scatter characteristics that are most likely to be CD4+ T cells and found these cells to be infected in all patients (infection frequency ranging 0.0003%–0.1%). The infection frequency of these cells was significantly higher in patients who initiated therapy during chronic infection ($P = .02$) than in patients who initiated therapy during acute/early infection, indicating that a larger pool of HIV-1–infected CD4+ T cells has been established in patients during chronic infection.

To investigate whether the HIV-1 populations isolated from cells in the bone marrow are unique or whether they are similar to HIV-1 populations in peripheral blood, we analyzed the genetic relationship between HIV-1 DNA populations in Lin+/CD4+ cells from bone marrow and memory CD4+ T cells from peripheral blood for each patient. We found that intracellular viral DNA sequences in the Lin+/CD4+ cells from bone marrow were phylogenetically similar to sequences derived from contemporaneous memory CD4+ T cells from peripheral blood. We also found that the genetic diversity of the HIV-1 populations in these 2 compartments was similar. Interestingly, in patient 7 we found a distinctive viral sequence in both cells isolated from bone marrow and peripheral blood that appears to be replication incompetent. These findings suggest that there is active cellular exchange between the bone marrow and peripheral blood compartments. However, it is also possible that the HIV-1–infected Lin+/CD4+ cells were derived from peripheral blood within the bone marrow.

Determining the HIV-1 cellular reservoir during long-term suppressive therapy is crucial so that these cells can be targeted for HIV-1 eradication. Our results provide evidence that Lin+/CD34+ HPCs are not HIV-1 infected. These findings strongly suggest that HPCs are not a major viral reservoir in patients on long-term suppressive therapy. The HIV-1–infected Lin+/CD4+ cells can be isolated from bone marrow. However, the genetic similarity of the virus in these cells to virus isolated from memory CD4+ T-cells in peripheral blood indicates an exchange of infected cells between bone marrow and peripheral blood.

**Supplementary Data**

Supplementary materials are available at the Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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