Bone marrow CD34+ progenitor cells may harbour HIV-DNA even in successfully treated patients

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Abstract

The issue about bone marrow hematopoietic progenitor cells harbouring HIV-DNA in infected patients is still under scrutiny. We studied nine HIV-infected individuals undergoing bone marrow aspiration for diagnostic purposes. In all patients, even in those receiving successful antiretroviral therapy for several years, HIV-DNA was detected in purified CD34+ lineage-bone marrow progenitor cells. This finding, although not conclusive due to the low number of patients examined, adds further evidence that current treatment strategies may be insufficient to resolve latent infection in bone marrow CD34+ hematopoietic progenitor cells.

Keywords: Bone marrow, hematopoietic progenitor cells, HIV infection, proviral DNA, viral load
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Introduction

The issue about HIV latent infection of CD34+ hematopoietic progenitor cells (HPCs) in HIV-positive individuals still needs clear answers [1,2]. All studies on this topic have considered small samples (on average, about 10 patients/paper, range 8–18) due to the difficulty in the enrolment of suitable patients. Nevertheless, it has been previously demonstrated that HPCs are infected in vivo [3–6], and more recently this has been shown even in successfully treated patients [7]. In contrast, others were unable to find detectable HIV-DNA, and no HIV replication was observed, after differentiation, in CD34+ bone marrow (BM)-HPCs from HIV-positive patients receiving successful antiretroviral therapy (ART) for more than 2 years [8,9]. Recently, in chronically infected subjects with undetectable plasma HIV-RNA, we have shown that BM-HPCs are apparently free of HIV replication, but are blunted in differentiation capability, leaving open the question about latent HIV infection [10]. Thus, the ability of CD34+ BM-HPC to provide a suitable reservoir during HIV infection is still under scrutiny. In the present study we addressed this issue by investigating whether, in untreated or ART-treated HIV-positive patients, CD34+ BM-HPCs may harbour HIV-DNA.

Peripheral blood (PB) and BM blood residual samples were obtained from 9 HIV-infected patients undergoing BM aspiration for diagnostic purposes. The present project has been approved by the local Ethical Committee (Approval n.4 dated January 22, 2013), and signed informed consent was obtained from all enrolled subjects. CD34+ HPCs were purified from BM mononuclear cells (MNC) obtained by density gradient centrifugation, by a two-step magnetic procedure (CD34 Diamond isolation kit, Miltenyi Biotec Inc., San Diego, CA, USA) involving a lineage (Lin) depletion of mature cells followed by positive CD34+ isolation (as shown in Fig. 1a). Phenotype analysis of CD34+ HPC from BM patients was performed by flow cytometry. Samples acquisition and data analysis were performed by a FACS Canto II flow cytometer (Becton Dickinson, San Jose, CA, USA) by using Diva software. In Fig. 1b and c, an example of the enriched Lin- cells and purity of the resulting CD34+Lin- cells is reported. The percentage of CD34+Lin- cells was 97.6% (interquartile range (IQR) 95.85–98.15), while the percentage of contaminating cells defined as CD34-CD4+ was 0.1% (IQR 0–0.15) (Fig. 1c, Fig. 2a). Proviral HIV-DNA load was measured by long terminal repeat (LTR)-specific quantitative real-time PCR, and normalized to cell number by human telomerase reverse transcriptase (hTERT) gene [11,12]. Statistical comparison (Mann-Whitney U test) and correlations (Spearman rank test) were assessed using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA).

Clinical and experimental data from enrolled patients are shown in Fig. 2a. HIV+ patients underwent BM aspiration because of clinical indication as follows: suspicion of opportunistic infection (all patients), pancytopenia ($n = 5$), fever of unknown origin ($n = 2$), and suspicion of lymphoma ($n = 1$). Typical HIV-related

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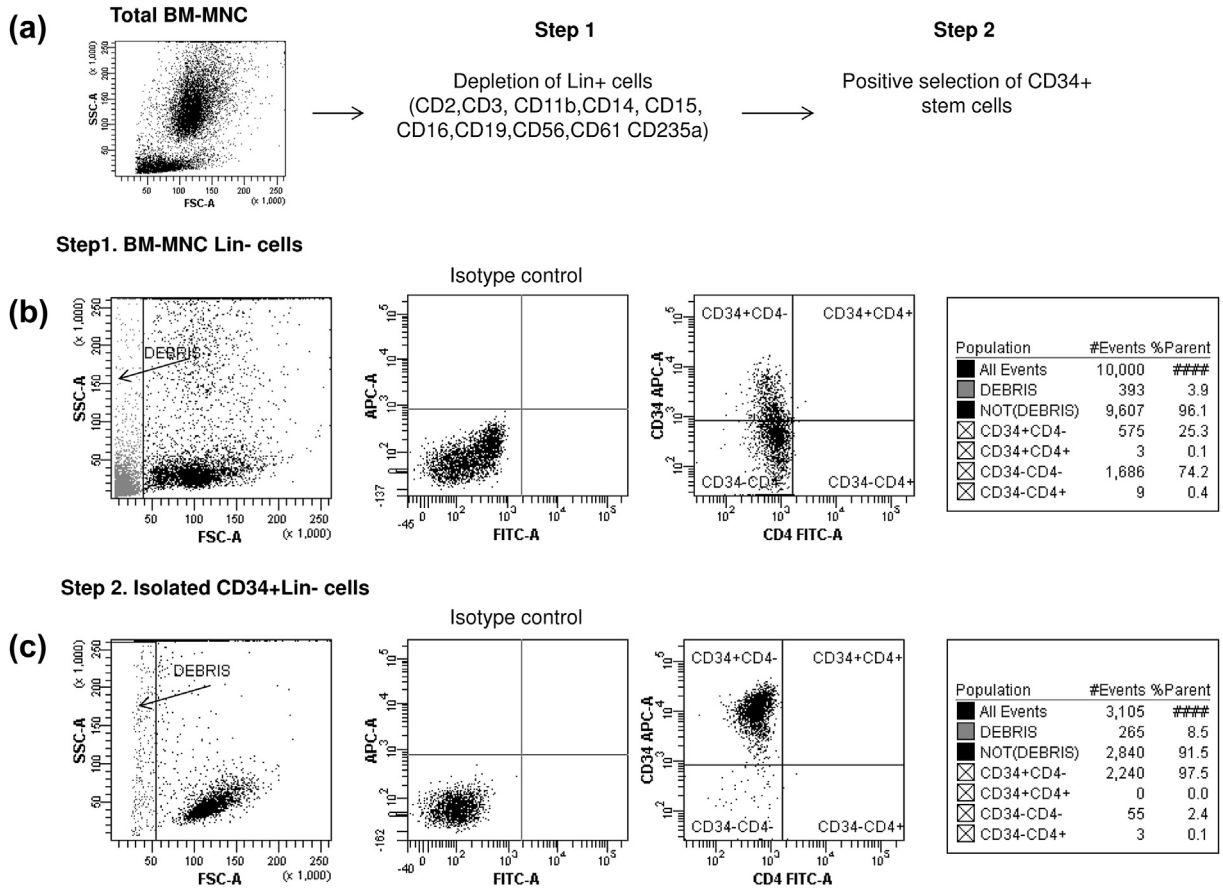

FIG. 1. Flow cytometry analysis of purification method used to analyse HIV-DNA in CD34+ hematopoietic progenitor cells (HPC). (a) Method used to isolate CD34+ HPC. CD34+ cells were first enriched from bone marrow mononuclear cells (BM-MNC) using indirect magnetic labelling of Lineage (Lin) positive cells. Highly pure CD34+Lin- populations were then obtained by a positive selection of CD34+ HPC using a direct magnetic labelling of lineage negative populations. (b) One representative flow cytometry analysis showing the percentage of CD34+ and CD4+ cells on Lineage-depleted populations of BM-MNC and (c) the corresponding purity of the CD34+Lin- cells obtained.

bone marrow morphology (from normocellular ($n = 5$) to hypocellular ($n = 4$) marrow) was observed with dysplastic changes most commonly seen in the granulocytic series. Plasma cells were often increased. Two opportunistic infections (leishmaniasis) were found in the bone marrow. Five patients were ART-untreated (HIV-RNA, median 5.92×10^5 cp/mL, IQR 2.6×10^4–2.6×10^6), while four patients were on successful ART (HIV-RNA <50 cp/mL). Patients treated with ART received antiretrovirals for a median of 78 months (IQR 10–168 months).

Haematological features were not significantly different between the ART-treated and untreated patients. As expected, the proportion of peripheral blood CD4+ cells was higher in ART-treated patients (24.6%; IQR 20.45–33.20) in comparison to untreated patients (median 11.9%; IQR 9.15–22.3; p = 0.05) (Fig. 2a). Since enrolled patients presented clinical indication for BM aspiration, they showed hematologic abnormalities, including deep lymphopenia. Thus, the absolute peripheral blood CD4+ T cell counts did not significantly differ among ART-treated and untreated patients (median 127.5/mm^3, IQR 73–1121 vs. 245/mm^3, IQR 125–361, p = 0.36). Total BM-MNCs in untreated and ART-treated patients were not significantly different (median 5×10^7, IQR 1.6×10^7–1×10^8/10 mL, vs. median 8×10^7, IQR 2×10^7–2.5×10^8/10 mL, respectively, p = 0.36). On the contrary, the yield of CD34+Lin- cells was higher from treated patients with respect to untreated patients (median 1.1×10^5, IQR 9.0×10^4–3.96×10^5, vs. median 7.5×10^4, IQR 3.0×10^4–2.84×10^4, p = 0.05).

The analysis of HIV proviral DNA, reported in Fig. 2a, indicates that the levels found in total BM were not significantly different from those in PB (Fig. 2b, left and middle panels, p = 0.34). Interestingly, while virtually all patients harboured HIV-DNA in CD34+ BM-HPCs, irrespective of being treated or not, the amount of HIV-DNA was significantly lower in patients on effective ART as compared to untreated patients (median 7 cp/10^4 cells, IQR 3.5.5–8.5 vs. 72 cp/10^4 cells, IQR 34.5–433, p = 0.05, Fig. 2b, right panel). Finally, a significant positive

(a)

Patient ID (age in y, gender)	Months on ART (years)	lymphs (mm^3)[a]	CD4 cell/μλ	CD4 (%)[b]	PB plasma HIV-RNA (cp/mL)	PB HIV-DNA (cp/10^5 cells)	Total BM-MNCs/10 mL	BM plasma HIV-RNA (cp/mL)	BM HIV-DNA (cp/10^5 cells)	yield of CD34+cells	CD34+ cells (%)	CD34+ HIV-DNA (cp/10^4 cells)	CD34- CD4+ (%)
1 (M, 42)		3.8	452	11.9	437005	1719	2.2E+07	8830	nd	8.80E+04	97.7	754	0
2 (M, 45)		2.1	208	9.9	746051	538	1.0E+07	63224	39	5.00E+04	95.2	65	0.4
3 (F,24)		0.5	42	8.4	2357630	7554	6.0E+07	116918	2398	7.00E+04	92.4	112	0.2
4 (M,62)		1	271	27.1	317	1150	5.0E+07	272	1232	8.00E+04	97.5	72	0.1
5 (F,42)		1.4	245	17.5	51674	1602	1.4E+08	29608	578	48.0E+04	99.6	4	0
6(F,39)	10	0.4	162	40.4	<50	3235.3	3.8E+08	<80	176	67.3E+04	96.5	9	0
7 (M,52)	60 (5)	0.4	93	23.2	<50	888	1.3E+08	79	3587	10.0E+04	98.4	6	0.1
8 (F,51)	96 (8)	8	2080	26	<50	nd	1.0E+07	<80	164.1	12.0E+04	97.6	1	0.1
9 (M,47)	168 (14)	0.3	53	17.7	<50	84.5	3.0E+07	<80	26.9	8.00E+04	97.9	8	0.1

Abbreviations: PB, peripheral blood; BM, bone marrow; MNC, mononuclear cells; nd, not determined

[a] Normal range: 1.2-4.8 mm^3 ; [b] Normal range: 36-49%;

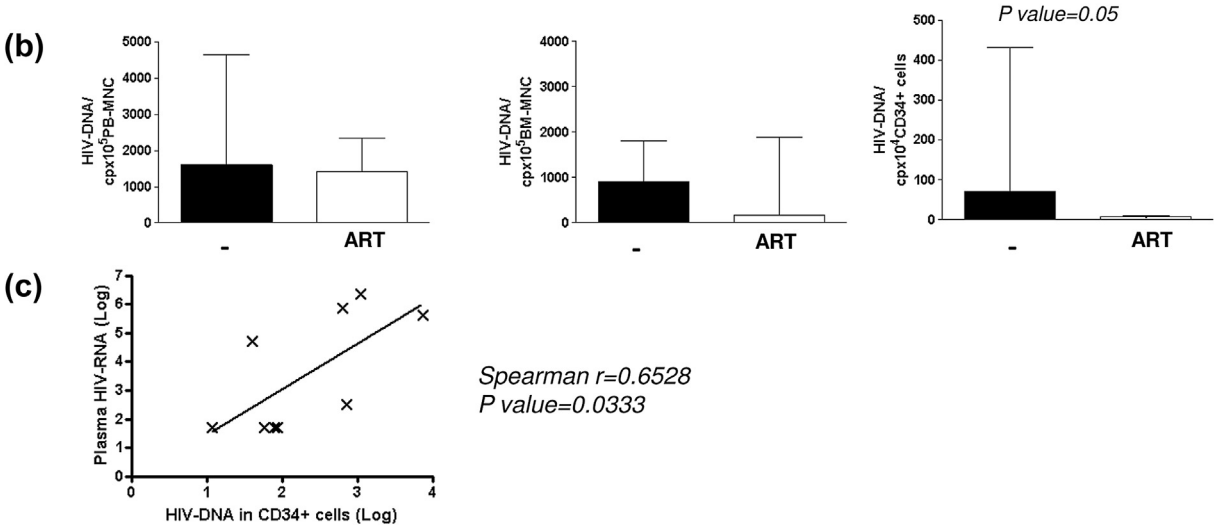

FIG. 2. Analysis of HIV-DNA in untreated and antiretroviral therapy (ART)-treated HIV patients. (a) Clinical and experimental data from enrolled subjects. (b) HIV-DNA levels in bone marrow, peripheral blood, and CD34+Lin- cells from untreated and ART-treated patients (left, middle, and right panel). Bars show medians with range. (c) Linear regression analysis between HIV-DNA levels in CD34+Lin- BM-HPCs and plasma HIV-RNA levels; r = 0.6528, p = 0.0333. HIV-DNA levels were measured by PCR and then normalized to cell equivalents by using hTERT as reference.

correlation between plasma HIV-RNA and CD34+ BM-HPCs HIV-DNA was observed (r = 0.6528, p = 0.03, Fig. 2c); in contrast, no correlation was found with PB-MNC HIV-DNA. Interestingly, HIV+ naive subjects display significantly higher HIV-DNA in CD34+ BM-HPS, but not in PB-MNC and BM-MNC. Many different mechanisms may cause this discrepancy, included wide dispersions due to the small sample size. This issue will be investigated in the ongoing work.

The ambitious aim of HIV eradication or functional cure critically depends on the full understanding of viral reservoirs establishment mechanisms [13]. We observe that HIV-DNA may be detected in CD34+Lin- BM-HPCs regardless of ART, and even after many years of treatment. The difference between our findings and those from other groups, who were unable to detect HIV-DNA in isolated CD34+ BM-HPCs [8,9,14,15], may be due to differences in study populations. In particular, our patients selectively included patients with HIV-associated myelodysplasia or with clinical indication for BM aspiration, and this may represent a major confounder. Indeed, it is possible that HPC in these patients are impaired and more susceptible to HIV infection than HPC of HIV-infected individuals without major hematologic abnormalities.

Moreover, technical issues, such as the method used for CD34+ HPCs isolation leading to a non-homogenous cell population with the enrichment of a specific subset [16], may influence the results. Durand *et al.* have hypothesized that contamination by HIV-infected CD4 T cells could produce false-positive results in beads-enriched HPCs [8], such as those in the Carter *et al.* work [7]. In our study we used a double-bead method to isolate very pure CD34+ cells (purity always >97.6 %). It is interesting to note that the sorter method used in the Durand *et al.* study did not consider as "contaminating" the population of CD3−CD4+ cells present in the bone marrow and in the enriched fraction [8]. Moreover, the sorter method used by Josefsson to isolate CD34+Lin- cells gave a purity of 76.7% [9]. In our view, our data may exclude the possibility that a contamination of <0.1% CD34-CD4+ cells may totally account for the proviral load found in the CD34+Lin- population.

Our work has limitations and cannot be considered conclusive due to the low number of patients recruited; however, work is in progress aimed at both increasing sample size and widening the representation of HIV-associated clinical scenarios.

In conclusion, our results suggest that CD34+Lin- BM-HPCs may harbour HIV genomes, and that HIV-DNA levels in CD34+Lin- BM-HPCs may correlate with the extent of plasma viremia. Bone marrow infection seems to be persistent even in the context of plasma HIV-RNA suppression after several years of ART. Further critical issues need to be evaluated, such as whether the infection of progenitor BM cells is productive, and/or how this may impact the proliferation and differentiation functional capabilities of BM-HPCs.

Transparency declaration

The authors have no conflicts of interest to declare.

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Author contributions: V.B. and F.M. conceived the study; M.B. performed the bone marrow procedures. M.B. and A.A. were in charge of patients and provided comments to the manuscript; V.B. and D.V. performed BM-MNC isolation and CD34 purification/analysis; I.A., G.R., A.A. performed extractions and assays for HIV and hTERT gene; A.R. processed peripheral blood samples; V.B., C.A., F.M., and M.R.C. analyzed the data and prepared the manuscript. All authors have read and approved the article. This work was supported by the Italian Health Ministry Ricerca Corrente grant for IRCCS.

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