HIV-1 gp120 impairs the differentiation and survival of cord blood CD34+ HPCs induced to the erythroid lineage

Silvia Morini[1], Giuseppina Musumeci[1], Isabella Bon[1], Anna Miserocchi[1], Francesco Alviano[2], Serena Longo[1], Alessia Bertoldi, Claudio V. Velati[3], Davide Gibellini[4], Maria Carla Re[1,4]

[1]Microbiology Section of the Department of Experimental, Diagnostic and Specialty Medicine, School of Medicine, University of Bologna, Italy;
[2]Histology Section of the Department of Experimental, Diagnostic and Specialty Medicine, School of Medicine, University of Bologna, Italy;
[3]Immunohematology and Transfusion Medicine Service, S. Orsola-Malpighi Hospital, University of Bologna, Italy;
[3]Microbiology Section, Department of Pathology and Diagnostics, University of Verona, Italy;
[4]Interuniversity Consortium, National Institute of Biostructures and Biosystems (INBB), Rome, Italy

SUMMARY

Anemia is the most common hematological abnormality in human immunodeficiency virus (HIV)-infected patients. Besides chronic disease, opportunistic infections, nutritional deficiencies and antiretroviral drug toxicity, the direct role of HIV in the development of anemia has not yet been fully investigated. To explore the HIV-related mechanisms involved in the genesis of anemia, we used two experimental designs. In the first, HPCs purified from cord blood were challenged with HIV-1IIIb or recombinant gp120 (rgp120) and then committed to erythrocyte differentiation (EPO-post-treated HPCs). In the second, HPCs were first committed to differentiate towards the erythroid lineage and only afterwards challenged with HIV-1_{IIIb} or rgp120 (EPO-pre-treated HPCs). Our results showed that HPCs and EPO-induced HPCs were not susceptible to HIV-1 infection. In addition, the two experimental designs (EPO post or pre-treated HPCs) independently showed that HIV-1_{IIIb} or rgp120 were able to induce the impairment of survival, proliferation, and differentiation albeit differing in kinetics and extent. Interestingly, the gp120 interaction with CD4 and CXCR4 played a pivotal role in the impairment of erythrocyte differentiation by inducing TGF-β1 expression. These observations reveal an important additional mechanism involved in the genesis of anemia suggesting a complex competition between EPO-positive regulation and HIV-negative priming regarding erythrocyte survival, proliferation and maturation.

KEY WORDS: HIV, CD34+ HPCs, erythropoiesis, apoptosis, glycophorin.

Received August 27, 2015 *Accepted* December 6, 2015

INTRODUCTION

Among the cytopenias commonly encountered in HIV-1-infected patients, anemia represents a major pathological manifestation during the course of infection [Zon *et al.,* 1987; Kreuzer and Rockstroh, 1997; Gibellini *et al.,* 2013).
The decreased erythrocyte number found in HIV-1-positive patients is frequently associated with alterations of erythrocyte form (Spivak *et al.,* 1984), and the severity of anemia seems to be strictly related to immunosuppression and disease stage (Volberding *et al.,* 2004). In particular, anemia, observed in 10% of asymptomatic HIV individuals and up to 92% of AIDS patients (Zon *et al.,* 1987; Kreuzer and Rockstroh, 1997; Gibellini *et al.,* 2013), is a negative prognostic factor correlated with a lower survival rate

Corresponding author
Maria Carla Re
Department of Experimental
Diagnostic and Specialty Medicine
School of Medicine
University of Bologna, Italy
Via Massarenti, 9 - 40138 Bologna, Italy
E-mail: mariacarla.re@unibo.it

(Kreuzer and Rockstroh 1997; Gibellini *et al.*, 2013; Spivak *et al.,* 1984; Volberding *et al.*, 2004; Forsyth *et al.,* 1996).

HIV infection per se and its indirect effects could play a crucial role in the genesis of anemia. Vitamin deficiencies, pharmacological treatments, iron metabolism derangement, neoplastic diseases, opportunistic infections and therapeutic protocols are indirect effects, but a direct viral effect on HPCs and erythroid lineage differentiation and proliferation must not be underestimated (Levine *et al.,* 2001; Semba *et al.,* 2002). HIV-1 may well have a central role in triggering apoptosis and impaired proliferation in HPCs through gp120 binding to the cellular membrane and HPCs. In addition, erythroid-committed cells like BFU-E (Zauli *et al.,* 1992; Zauli *et al.,* 1994; Lee *et al.,* 1999) might represent a target of HIV infection even though controversial results have been reported (Cleveland *et al.,* 1996; Carter *et al.,* 2011; McNamara *et al.,* 2012; Durand *et al.,* 2012; Josefsson *et al.,* 2012).

It is noteworthy that the HIV-1 deregulation of pro-inflammatory cytokines is related to impaired erythropoietin (EPO)-related feedback modulation. EPO is a cytokine produced by mesangial cells in the kidney and plays a central role in erythrocyte differentiation, proliferation and homeostasis regulation.

This study focused on the effects of early HIV-1 challenge on the survival/proliferation and maturation of the erythrocyte lineage derived from HPC differentiation by EPO. Two experimental designs were used. In the first, HPCs purified from cord blood before being committed to erythrocytes differentiation were challenged with HIV-1_{IIIb} or recombinant gp120 (rgp120) (*EPO-post-treated HPCs*). In the second, HPCs were first committed to erythrocyte differentiation and only afterwards challenged with HIV-1_{IIIb} or rgp120 (*EPO-pre-treated HPCs*).

Both experimental designs investigated susceptibility to HIV-1 infection, cell viability, cell survival/proliferation, apoptosis activation, glycophorin expression, colony formation units and the role of TGF-β1 to study the HIV-related mechanisms involved in impaired erythrocyte cell lineage survival and induction of EPO-driven differentiation.

MATERIALS AND METHODS

CD34+ HPC isolation and analysis of the cell surface (CD4, CXCR4 and glycophorin). $CD34^+$ cells were obtained from umbilical cord blood specimens. Collection and handling of samples were carried out according to the Helsinki declaration (Shephard DA, 1976). No approval from the Ethical Committee was requested because all the samples were anonymous and could not be related to any cord blood donors.

The efficiency of CD34+ HPCs purification was determined by flow cytometry. Briefly, cells were stained with phycoerythrin (PE)-conjugated anti-CD34 monoclonal antibody (Becton-Dickinson, San José, CA, USA) for 30 minutes in ice. After washing with PBS, stained cells were acquired on a FACSCalibur, and the data were analyzed with BD CellQuest™ Pro Software (Becton-Dickinson Biosciences). The percentage of CD34+ HPCs obtained ranged from 94 to 99% in all purified cord blood samples.

After purification, HPCs ($1x10^5$) were analyzed for the presence of cell surface receptors and intracellular markers. Briefly, to determine the percentage of cells expressing CD4, CXCR4 and glycophorin A, cells were separately stained with FITC-conjugated-anti-CD4mAb (Becton-Dickinson), FITC-conjugated-anti-CXCR4mAb (R&D System, Minneapolis, MI, USA) and PE-conjugated-anti-glycophorin A mAb (R&D System) for 30 minutes in ice. The cells were extensively washed in PBS and then analyzed by flow cytometry.

HIV-1_{IIIb} and recombinant gp120 (rgp120) treatment. HIV-1_{IIIB} viral stock was produced as previously described (Gartner S and Popovic M, 1990) and titrated by ELISA HIV-1 p24 antigen kit (BioMerieux, Marcy L'Etoile, France). Isolated HPCs ($5x10^4$ cells/ml) were seeded in Iscove medium (Gibco, Paisley, UK) containing 2% FBS (Gibco), IL-3 (10 ng/ml; Roche, Mannheim, Germany), SCF (10 ng/ml; Roche), GM-CSF (250 ng/ml; Genzyme, Cambridge, UK).

Twenty-four hours later, HPCs were challenged with HIV-1_{IIIb} (5 ng/ml of HIV-1 p24) for 2 hours at 37°C. Cultures, seeded in 6-well plates, were extensively washed with PBS, kept in standard medium and supernatants and HPCs were harvested at days 3 and 7 post infection (pi).

Most experiments were performed treating HPCs with full-length recombinant gp120 (rgp120 (1 μg/ml, NIBSC, London, UK) for 2 hours at 37°C to verify the effect of envelope glycoprotein on progenitor cells on erythrocyte survival, proliferation and maturation.

HPC differentiation. Two different experimental designs concerning *in vitro* differentiation and HIV-1 pr gp120 challenge were performed, as shown in Table 1.

First: *EPO-post-treated HPCs*. HPCs ($5x10^4$ cells/ml) were challenged with HIV-1_{IIIB} or rgp120 and treated with EPO 24 hours later (4U/ml; Roche). Half the medium was changed every 4 days with fresh medium without SCF, with IL3 and EPO. In addition, HIV-1_{IIIB} or rgp120 were re-added every 4 days.

Second: *EPO-pre-treated HPCs*. Isolated HPCs ($5x10^4$ cells/ml) were pre-treated with EPO (4U/ml; Roche) and HIV-1_{IIIB} or rgp120 was added to cell cultures 24 hours later. Half the medium was changed every 4 days with fresh medium without SCF, with IL3 and EPO, including HIV-1_{IIIB} or rgp120 at the same concentration described forEPO-pre-treated HPCs.

As control, HPCs were kept in medium without EPO or in medium with EPO without HIV-1_{IIIb} and rgp120 challenges in both protocols.

HIV detection. The HIV-1 p24 content was assayed in supernatants by ELISA HIV-1 p24 antigen kit (BioMerieux Boxtel, The Netherlands). Cellular and proviral DNAs were extracted from samples by DNAeasy kit (Qiagen, Hilden, Germany). Purified DNA (0.5 μg) was amplified by PCR using SK431 and SK462 oligos, as previously described (Gibellini *et al.,* 2008). A specific amplicon of 142 bp was detectable by 2% agarose gel electrophoresis. As a control, parallel amplification of globin gene was carried out and the integrated HIV-1 proviral DNA was analyzed after gel purification of cell genomic DNA (Gibellini *et al.,* 2008) followed by nested Alu-PCR assay as assessed by O'Doherty and coworkers (O'Doherty *et al.,* 2002). The first nested PCR amplification was performed on cell genomic DNA (0.5 μg) with primers specific for *Alu* and *gag* sequences whereas the second amplification was carried out with the HIV-1 LTR oligonucleotide pair. A specific amplicon of 100 bp was detectable by 3% agarose gel electrophoresis.

Quantitative RT-PCR (qRT-PCR) for mRNA TGF-β detection. Total mRNA was extracted from HPCs by the High Pure RNA isolation kit (Roche) following the manufacturer's instructions. 50 ng of retro-transcribed total RNA was amplified using Quantitect SYBR Green RT-PCR kit (Qiagen) with 400 nM of each specific oligos (β-actin F: CATGTACGTTGCTATCCAGGC; β ac-

TABLE 1 - *Experimental design performed on CD34 HPCs purified from cord blood. On the left, HPCs firstly challenged with HIV-1IIIb or gp120 (rgp120) and then committed to erythrocytes differentiation (HPCs EPO post-treated). On the right HPCs firstly committed to erythrocytes differentiation and then challenged with HIV-1IIIb or rgp120 (HPCs EPO pre-treated).*

	HPCs EPO post-treated		*HPCs EPO pre-treated*
		HPCs isolation	
	HPCs plus HIV or gp120		HPCs plus EPO
	EPO addition		HIV or gp120 addition
Day 3	Cell count HIV DNA PCR Apoptosis Glycophorin expression TGF-β1 mRNA expression		Cell count HIV DNA PCR Apoptosis Glycophorin expression TGF-β1 mRNA expression
Day 4		*Fresh medium and HIV or gp120*	
Day 7	Cell count Apoptosis HIV DNA PCR Glycophorin expression TGF-β1 mRNA expression		Cell count Apoptosis Glycophorin expression TGF-β1 mRNA expression
Day 8		*Fresh medium and HIV or gp120*	
Day 12	Cell count Apoptosis TGF-β1 mRNA expression		Cell count Apoptosis TGF-β1 mRNA expression Glycophorin expression
Day 14		*CFU assay*	

tin R: GTCCTTAATGTCACGCACGAT, TGF- β1 F: 5'-CTTCAAGCTCCTGGGAAATGT-3',TGF- β1 R: 5'- GCAGAATAAAGCCTACCTTGAAAG-3' in a LightCycler instrument (Roche). The amplification was performed with RT step (1 cycle at 50°C for 20 min) followed by initial activation of HotStar Taq DNA polymerase at 94°C for 15 min and 40 cycles in 3 steps: 94°C for 10 s, 60°C for 30 s, 72°C for 60 s. The β-actin real time RT-PCR amplification was carried out with an annealing step at 60°C for 15 s and an extension time at 72°C for 25 s. The amplicons were also analyzed in 1.5% agarose gel electrophoresis.

Apoptosis analysis. HPCs were fixed in cold ethanol 70% for 15 minutes at 4°C and after 2 soft washings in PBS, the samples were treated with RNase (500 µg/ml, Sigma) and then stained with propidium iodide (50 µg/ml, Sigma). The samples were analyzed by flow cytometer (FACScan, Becton-Dickinson) at different experimental times.

Colony-forming unit (CFU) assay. The CFU assay was performed using MethoCult™ assay (StemCell Technologies, Milan, Italy) following the manufacturer's instructions. In the first protocol HPCs were gp120-treated and then seeded in MethoCult™ medium. In the second protocol purified HPCs were seeded in MethoCult™ medium and gp120-challenged only 24 hours later. At day 14, CFUs were enumerated following the manufacturer's instructions.

Recombinant proteins and polyclonal antibody. Briefly, recombinant TGF-β1 (r TGF-β1; 900 pg/ml; Peprotech London, UK), p5p (pyridoxal-phosphate), as antagonist of gp120-CD4 binding (5 ng/ml, Sigma, St Louis, MO, USA) and AMD3100, as antagonist of gp120- CXCR4 binding (0.5 µM, Sigma, St Louis, MO, USA) were added to purified CD34+ daily in some experimental steps to verify the individual role of different molecules in survival/proliferation and maturation of the HPC-derived erythrocyte lineage. TGF-β1 neutralization experiments were performed using anti-TGF-β1 polyclonal antibody (pAb; 1 µg/ml; R&D Systems, Minneapolis, MI, USA) to study the potential role of TGF-β1.

Statistical analysis. The data are expressed as means ± standard deviation (±SD) of 3 separate experiments performed in duplicate. Statistical analysis was performed using Student's two-tailed t-test.

RESULTS

Cord blood HPCs and EPO-induced HPCs are not susceptible to HIV-1 infection. The percentage of purified HPCs (CD34 ranging from 94 to 99%) expressing CD4 and CXCR4 was 5±1.5 and 32 ± 3% respectively.

HPCs and HPCs differentiated towards the erythroid lineage (48 hours after EPO treatment) exposed to HIV-1_{IIIb} (5ng p24/ml) were analyzed at days 3 and 7 p.i. for the presence of HIV proviral DNA by PCR and HIV p24 by ELFA technique (Vidas IV p24, BioMerieux, Marcy-l'Etoile, France) in cell culture supernatants. Neither HIV sequences (Figure 1) nor HIV-1 p24 protein were detected at any experimental time point, indicating that HPCs and EPO-induced HPCs were not susceptible to HIV-1 infection.

HIV-1 or rgp120-impaired cell survival/ proliferation of cord blood CD34+ HPCs through apoptosis activation. We performed 2 sets of experiments treating HPCs with HIV-1or rgp120 before or after EPO stimulation. In highly purified CD34+ HPCs challenged with virus and then triggered to differentiation *(EPO-post-treated HPCs)*, cell viability (by trypan blue exclusion technique) showed a significant cell reduction at different times post-infection (days 3, 7, 12), evident early on (day 3) both in the presence of HIV-1_{IIIb} and rgp120 (data not shown). Moreover, a significant in-

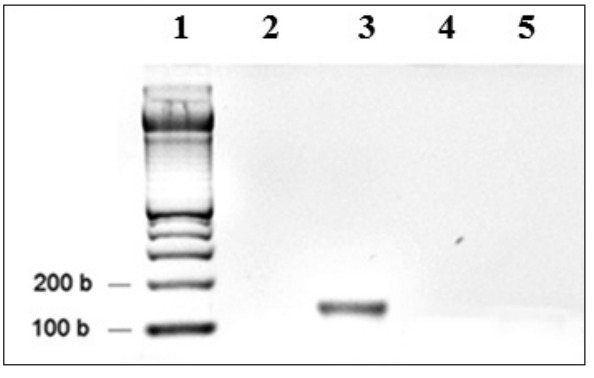


FIGURE 1 - *HIV DNA PCR in HPCs and HPCs differentiated towards the erythroid lineage (48 hours after EPO treatment) exposed to HIV-1IIIb. Lane 1: Molecular weight markers. Lane 2: Negative control (PBMCs). Lane 3: Positive control (HIV-1-infected PBMCs cells at day 3). Lane 4: HPCs challenged with HIV-1 at day 3. Lane 5: EPO-treated HPCs challenged with HIV-1 at day 3.*

crease in apoptotic cells from day 3 onwards (12±2%, 23.1±2.6% and 40±6, at days 3, 7 and 12 respectively) was also observed both in HIV-1 $_{IIIb}$, (data not shown) and rgp120-treated HPCs (Figure 2A) in comparison with control cells (4±1.2%, 6.8±1.3% and 8.5±1.8, at days 3, 7 and 12 respectively).

In the second set of experiments, cells were first triggered to erythroid differentiation and only 24 hours later challenged with viral stocks or rgp120 *(EPO-pre-treated HPCs)*. In this instance, results showed a later reduction of cell viability and apoptosis. In particular, after a slight and not significant increase in cell number at days 3 and 7 p.i., HPCs showed a drastic reduction in viable cells coinciding with apoptosis activation from 12 days onwards. However, the high apoptosis levels recorded in the first set of experiments (*EPO-post-treated HPCs)* were never reached, thereby allowing more experimental points to be checked. In particular, we observed a higher apoptosis percentage (up to

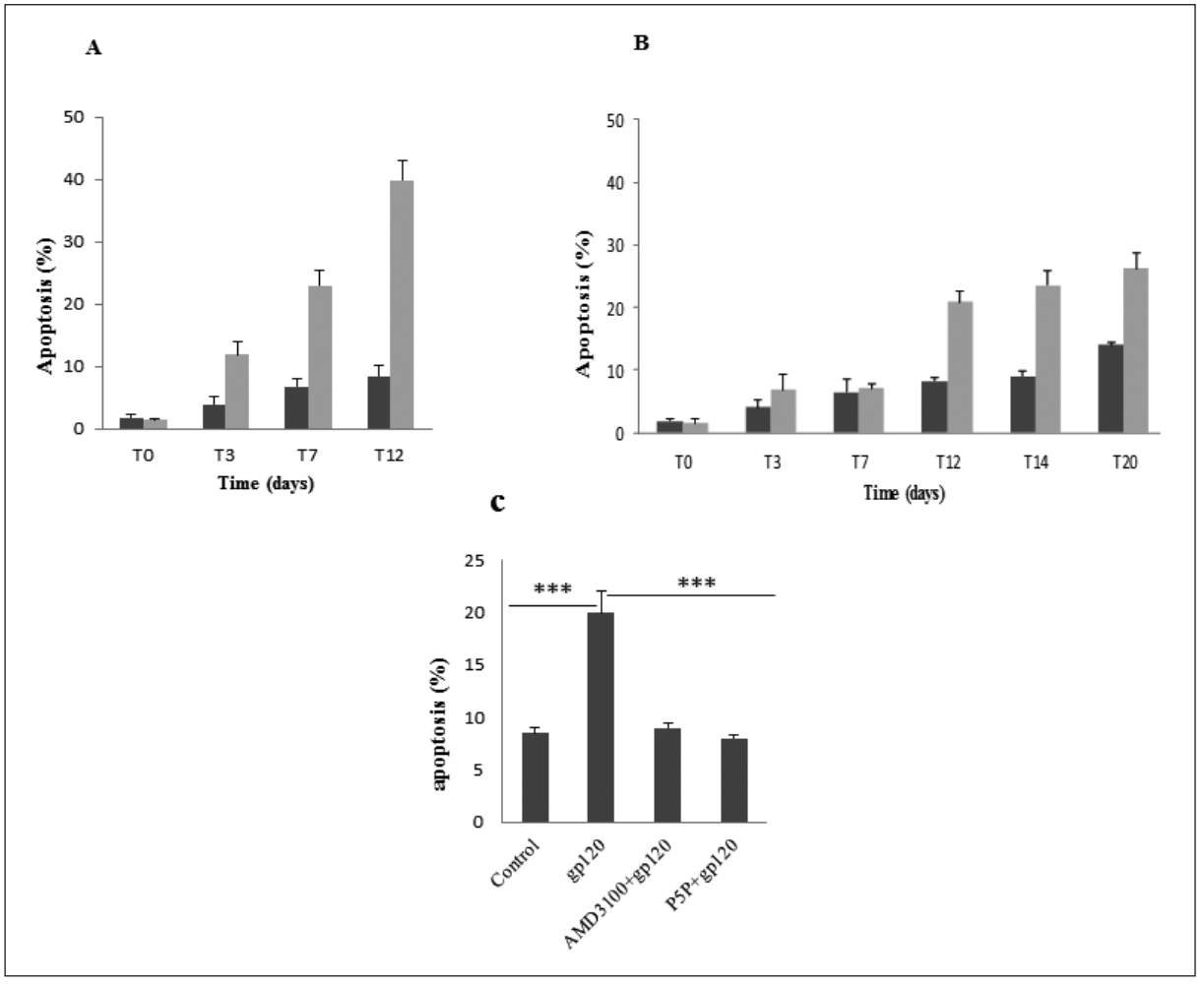


FIGURE 2 - *Panel A. Percentage of apoptosis (y axis) detectable by flow cytometry in purified HPCs challenged with recombinant gp120 and subsequently committed to erythrocyte differentiation (EPO-post-treated HPCs). Black columns: control CD34+ HPCs; gray columns: rgp120-treated CD34 HPCs. Panel B. Percentage of apoptosis (y axis) detectable by flow cytometry in purified HPCs first committed to erythrocyte differentiation and then challenged with rgp120 (EPO-pre-treated HPCs). Black columns: control HPCs CD34+; gray columns: rgp120-treated CD34 HPCs. Panel C. Percentage of apoptosis (at day 12) in purified EPO-pre-treated HPCs separately challenged with HIV rgp120, HIV rgp120 plus p5p (as inhibitor of gp120/CD4 binding) and HIV rgp120 plus AMD3100 (as inhibitor of gp120/CXCR4 binding).*

40±6%) in *EPO-post-treated HPCs* at day 12 in comparison to *EPO-pre-treated HPCs* where the level of apoptosis did not exceed 20% and was maintained within the 30% throughout our experimental times and up to day 20 (Figure 2B).
In a parallel experiment, $5x10^4$ cells/ml HPCs committed to differentiation by EPO, exposed to HIV gp120 (1 ng/ml) 24 hours later (*EPO-pre-treated HPCs*) were then separately treated with inhibitors of gp120/CD4 binding (p5p (5ng/ml)) or gp120/CXCR4 binding (AMD3100 (0.5 μM)). HIV gp120 was re-added every 4 days while P5P and AMD 3100 were added every day and the percentage of apoptosis was checked at days 7 and 12. Even if no difference was observed at day 7 (data not shown), later on (at day 12), as expected, HPCs treated only with rgp120 showed a clear increase ($p<0.05\%$) in apoptosis levels (20±2%) in comparison with HPCs treated also with p5p (9±0.3)% or AMD3100 (11±0.6%) showing apoptosis values similar to controls (Figure 2C).

Glycophorin expression in EPO-post and pre-treated HPCs was downregulated by challenge with rgp120. Glycophorin A erythroid antigen expression was studied in CD34+ HPCs to evaluate whether the pre- or post-challenge with rgp120 might affect the subsequent EPO-driven differentiation. Results demonstrated a strong percentage of cells expressing glycophorin A with clear differences in the two protocols. In highly purified CD34+ HPCs challenged with gp120 and then triggered to differentiation (*EPO-post-treated HPCs*), glycophorin expression was very low compared to control cells both at day 3 and day 7 (1.8±0.3% versus 4.2±1.3% and 2.2±0.6% versus 8.1±1.8%) (Figure 3A).
In *EPO-pre-treated HPCs*, despite an early higher and constant glycophorin expression with respect to *EPO-post-treated HPCs,* a sharp and significant reduction in comparison to control cells was observed from day 12 up to day 20 (11.9+3 versus 22.26±5 (day12), 12.3+2.4 versus 24.2+4.2 (day 14), 12.5+3 versus 31.4+5(day 20)) (Figure 3B).
Glycophorin expression was evaluated by flow cytometry in control HPC cells (purified HPCs maintained in the presence of IL3 and EPO (as described in Material and Methods) and in HPCs challenged with p5p and AMD3100 - separately - to verify the role of CD4 and or CXCR4 and evaluated at day 12.
HPCstreatment with P5P resulted in an inhibition of glycophorin expression, comparable to gp120-treated HPCs (8±2.3 and 9.52±3.4

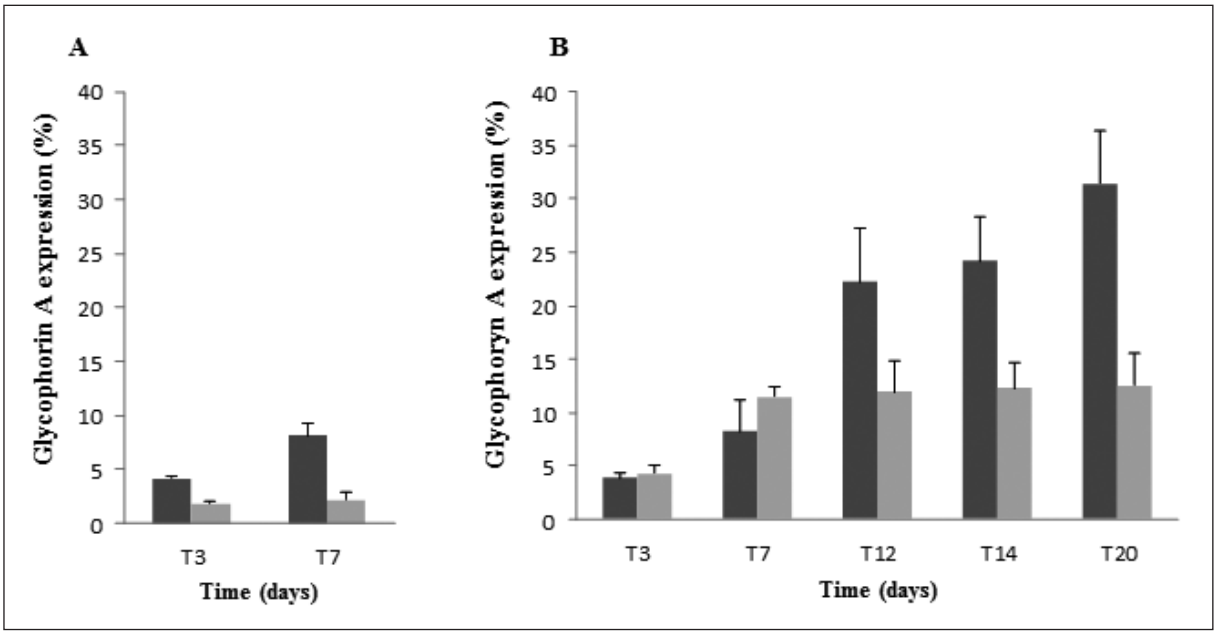
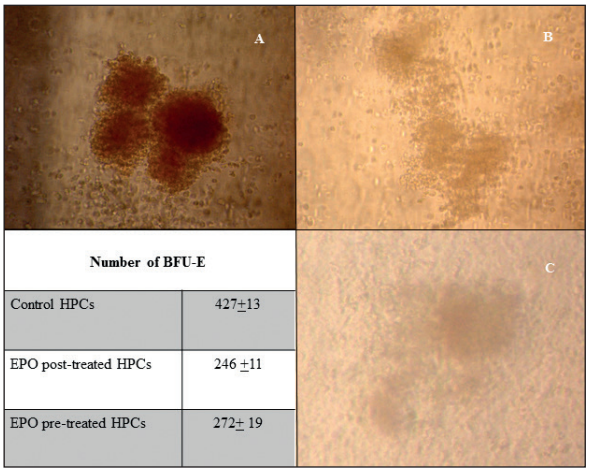


FIGURE 3 - *Glycophorin A expression in EPO-post-treated HPCs (Panel A) at days 3 and 7 and EPO-pre-treated HPCs (Panel B) from day 3 to day 20. Black columns: control CD34+ HPCs; gray columns: rgp120-treated CD34 HPCs.*



Number of BFU-E	
Control HPCs	427±13
EPO post-treated HPCs	246 ±11
EPO pre-treated HPCs	272± 19

FIGURE 4 - *Colony formation assay in HPCs (A: control), in EPO-pre-treated (B) and EPO-post-treated HPCs challenged with rgp120 (C). Table reports the number of colonies obtained by triplicate experiments in EPO-post and pre-treated HPCs.*

respectively) in comparison with control cells (22.26±3.2). On the other hand, pre-treatment with AMD3100 partially restored glycophorin expression (14±2.1) probably suggesting a role of gp120 binding and CXCR4 in the differentiation process.

Colony formation is hampered in both post and pre-treated HPCs. In parallel, we evaluated the impact of rgp120 on EPO-driven colony formation in semisolid methylcellulose agar. The number of BFU-E was clearly less in rgp120-challenged EPO post (BFU-E number: 246±11) and pre-treated HPCs (BFU-E number: 272±19) at day 14 in comparison to control cells (BFU-E number: 427±13) with a clear downregulation of hemoglobin in the colonies determined by a pale red color with respect to the brilliant red detectable in the HPC control cells (Figure 4).

mRNA expression of TGF-β1 is elevated in both EPO post and pre–treated CD34+ HPCs. Since TGF-β and related factors exert autocrine and/or paracrine effects on hematopoiesis by regulating cell proliferation, differentiation, and survival (Gibellini *et al*. 2013; Xie *et al*. 2015), to further investigate the role of TGF-β in the development of erythroid progenitors we focused on TGF-β1 induction in EPO post (Figure 5A**)** and pre-treated CD34+ HPCs (Figure 5B**)** from day 3 onwards.

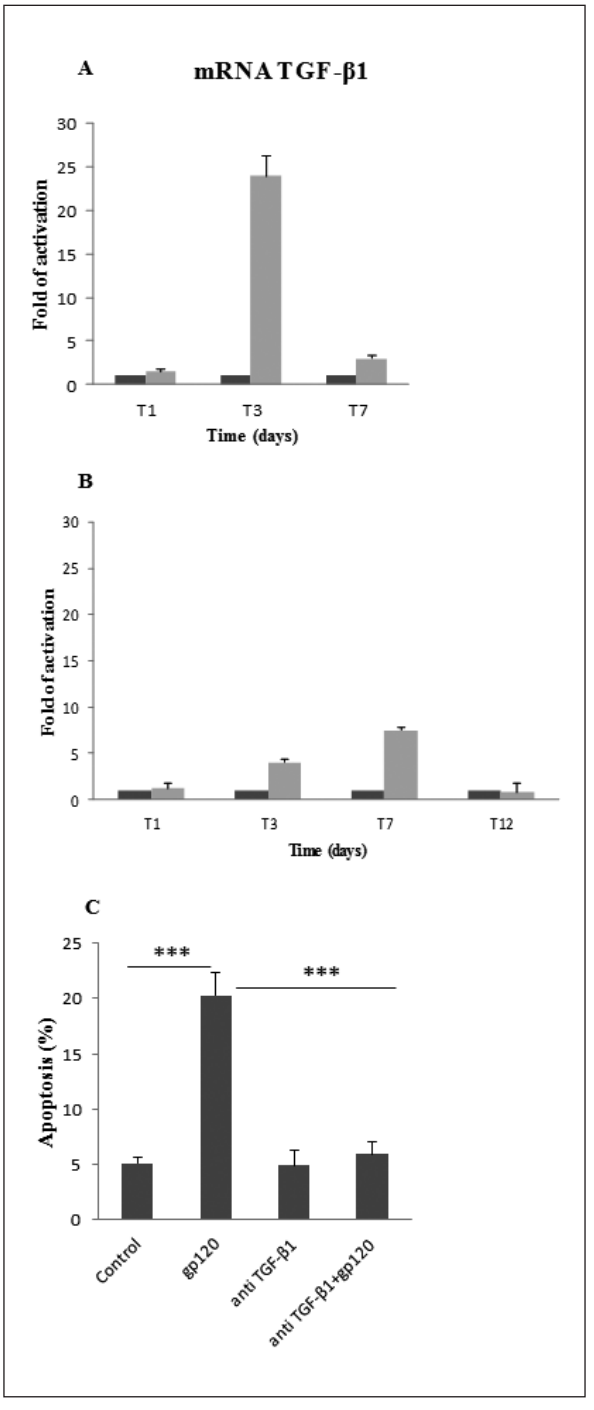


FIGURE 5 - *Panel A. mRNA TGF-β1 expression in EPO-post treated HPCs. Black columns: control HPCs CD34+; gray columns: rgp120-treated HPCs. Panel B: mRNA TGF-β1 expression in EPO-pre-treated HPCs. Black columns: control CD34+ HPCs; grey columns: rgp120 treated HPCs. Panel C: percentage of apoptosis at day 12 in HPCs (control cells, HPCs treated with gp120 or TGF-β1 only or gp120 plus anti-TGF-β1.*

In *EPO-post-treated HPCs*, we observed a peak of mRNA TGF-β1 at day 3 (24±2.3 fold of activation) followed by a clear decrease five days later (3±0.4 fold of activation). As expected, no further times should be analyzed since number of cells available was not more sufficient for additional experimental points
On the contrary in *EPO-pre-treated HPCs* only a slight increase in TGF-β1 levels, never reaching the activation fold registered in *EPO-post-treated HPCs*, was observed only at days 3 (4±0.4 fold of activation versus 24±2.3 fold) and 7 (7.9±0.7 fold of activation versus 3.1±0.3 fold), with a clear decrease at later times. In particular, comparing the level at day 3 in *EPO-post and pre-treated HPCs I,* a significant difference was observed. On the basis of these results and to verify the role of TGF-β1 in apoptosis induction, HPC cell cultures ($1x10^4$ cells) were treated with gp120 plus anti-TGF-β1 pAb (1 microgram/ml) added daily up the end of our experimental times (day 12). Results obtained showed a consistent apoptosis inhibition in *EPO-pre-treated HPCs* suggesting that a specific treatment with anti-TGF-β1 pAb completely re-established the apoptosis levels as in control cells (Figure 5C).

DISCUSSION

Anemia is detectable in many HIV patients and is related to increased morbidity with a lower survival rate with respect to HIV patients without anemia (Opie J., 2012; *Gibellini et al.,* 2013; Redig and Berliner 2013). cART therapy is able to raise erythrocyte and hemoglobin levels in a good percentage of patients (up to 55%) even if some antiretroviral agents induce bone marrow suppression with a derangement of erythrocyte cell lineage differentiation (Alexaki and Wigdahl 2008).
The pathogenesis of HIV-related anemia is multifactorial and involves both the loss of differentiated erythrocytes and impairment of the erythrocyte differentiation process. Generation and maturation on erythrocytes takes place in the bone marrow, which represents one of the main replication sites during HIV infection. HIV infection affects bone marrow function via several mechanisms, including deregulation of cytokine networks, stromal cell damage, and direct infection of bone marrow macrophages. HIV infection has also been linked to decreased erythropoietin (EPO) synthesis resulting from HIV-related kidney damage, and to anti-EPO autoantibodies in several HIV-positive patients (Sipsas *et al*., 1999; Tsiakalos *et al.,* 2010).
EPO is a 30 kDa protein that plays an essential role in the production and differentiation of red blood cells (Jelkmann, 2013). The molecule is mainly expressed after birth by peritubular fibroblasts in the renal cortex even though EPO mRNA is detectable in other tissues such as liver, brain, lung, spleen and testis. EPO plays a crucial role in the differentiation of erythrocytes, modulating the replication and differentiation of CFU-E to generate large amounts of pro-erythroblasts and normoblasts. Importantly, EPO is an anti-apoptotic molecule for CFU-E and some studies (Spivak *et al.,* 1984; Wang *et al.,* 1993; Kreuzer *et al.,* 1997) have demonstrated that the same erythroid progenitors contained EPO mRNA and protein. This suggests a twofold regulation of CFU-E biology where autocrine stimulation by CFU-E-produced EPO may modulate basal erythropoiesis whereas circulating renal EPO induces a strong response to hypoxic stimulation (Gibellini *et al.,* 2013).
In this complex scenario where anemia may represent a consistent negative prognostic factor during the disease course and the role of human progenitor cells in the pathogenesis of HIV infection remains controversial, we investigated the HIV-related mechanisms involved in the impairment of erythrocyte cell lineage survival and differentiation. We used two experimental - in vitro - protocols to evaluate the effect before and after the induction of EPO-driven differentiation of cord blood CD34+ HPCs.
Since the HPC/HIV interaction is still a controversial issue, our first aim was to verify HPC susceptibility to HIV-1. We and others (Sakaguchi *et al.,* 1990; Re *et al.,* 1993; Re *et al.,*1994; Molina *et al.,* 1999; Zauli *et al.,* 1994) previously demonstrated the lack of HPC infection after *in vivo* or *in vitro* exposure to HIV despite detectable amounts of CD4 and CXCR4 receptor on their surface (Deichmann *et al.,* 1997; Aiuti *et al.,* 1999; Shen *et al.,* 1999). By contrast, recent papers have shown that a low percentage of HPCs could be infected and HIV proviral

DNA has been detected in bone marrow HPCs purified from cART-treated patients, suggesting HPCs are a reservoir of HIV infection (McNamara and Collins 2011; Carter *et al.,* 2011).
In our experimental conditions, the results obtained showed a lack of HPC infection both in HPCs treated with HIV and HPCs after EPO-driven differentiation induction, confirming previous results and also suggesting that EPO treatment does not modify the lack of HIV-1 susceptibility.
Although we did not observe any proviral DNA in our experimental conditions, this cannot rule out a possible HIV infection in other experimental procedures where different HIV strains, HPC purity, time of HIV exposure, cell-to-cell infection, or deregulation of cytokines at bone marrow level can promote HPC infection by HIV (Zhang and Crumpacker, 2010).
Despite the lack of infection, HPC cultures challenged with HIV showed a reduced cell viability and a higher apoptosis rate than untreated cultures. Increased levels of apoptosis were observed in HPC cells challenged with either virus or gp120 before and after inducing erythrocyte differentiation by EPO treatment. Challenging EPO post-treated HPCs with HIV or rgp120 resulted in a dramatic reduction of viable cells mainly by apoptosis activation.
The significant increase in apoptotic cells, already detectable at day 3 pi with a peak at days 7 and 12 pi suggested that subsequent EPO treatment is not able to tackle the negative effect induced by HIV or rgp120 treatment. These data confirm previous studies where HIV induced apoptosis in CD34+HPCs (Zauli *et al.,* 1992, Gibellini *et al.,* 2014; MacEneaney *et al.,* 2011), and stress the importance of apoptosis in the pathogenesis of different cytopenias during HIV infection.
Interestingly, the results obtained by the second protocol (*EPO-pre-treated HPCs*) showed a decreased viability, but only at later times and never reaching the high levels shown in EPO-post-treated HPCs, suggesting that EPO efficiently reduces the dramatic negative HIV effects on viability and proliferation.
In addition, results obtained treating purified HPCs with an antagonist of gp120-CD4 binding or an antagonist of gp120-CXCR4 binding showed apoptosis levels very similar to HPCs treated with only gp120, suggesting a mechanism related to CD4 and CXCR4 interaction with gp120 in triggering apoptosis.
Data obtained by the analysis of glycophorin expression in HPCs treated with rgp120 are consistent with the detrimental effect demonstrated by the gp120/HPC interaction. In our experimental conditions, HIV glycoprotein downregulated glycophorin A erythroid antigen expression, even if with clearly different results in the two protocols. While the expression of glycophorin was very low at early experimental times in *EPO-post-treated HPCs*, the reduction of glycophorin expression was observed only later (form day 12) in *EPO-pre-treated HPCs* confirming that EPO is able to attenuate the detrimental effect of gp120 also in this case.
Colony formation was also drastically hampered in both post and pre-treated HPCs and the impact of rgp120 was associated with a clear downregulation of hemoglobin. The reduction of BFU-E and downregulation of hemoglobin in the methylcellulose-based cultures suggests that HIV-1 could perturb HPC differentiation towards the erythrocytic lineage resulting in decreased differentiation and an inhibition of erythroid specific markers.
Finally, to try to understand the mechanism of the detrimental effects of HIV or its envelope protein we studied the expression of TGF-β1, a cytokine required for both HPC self-renewal and replication/differentiation control and involved in the HIV-1-related depression of bone marrow and differentiation of cell lineages such as the megakaryocyte lineage.
However, we verified whether an increase in TGF-β1could be detected after EPO treatment. TGF-β1 mRNA appeared significantly increased already at day 3 pi and this upregulation was found at days 6 and 10 when high levels of apoptotic cells were detected. Interestingly, TGF-β1 is, at least in part, involved in apoptosis induction.
Apoptosis induction was mediated by the increased expression of TGF-β1. TGF-β1 has been implicated in the control of erythrocyte generation by determining the levels of apoptosis in progenitor cells (Gibellini *et al.,* 2013; Xie *et al.,* 2015). Moreover, anti-TGF-β1pAb treatment in the supernatant in our experimental system inhibited TGF-β1-paracrine/autocrine activity,

consistently reducing the number of apoptotic cells in HIV-1 and rgp120-treated cell cultures. In addition, CD4 and CXCR4 are present even on BFU-E and CFU-E (Kitano *et al.,* 1991; Lee *et al.,* 1999) suggesting that the HIV-1 or rgp120-related negative effect involves not only HPC apoptosis but also the first steps of differentiation. EPO did not seem able to tackle the negative effect, since the amount of CFU-E was dramatically decreased in gp120-treated cultures.

In conclusion, anemia and the progressive decline of erythrocytes and hemoglobin play an important role in both HIV disease progression and the clinical and therapeutic management of HIV-positive patients. By certifying the lack of HPC infection, a dramatic reduction of viable cells mainly by apoptosis activation, and a downregulation of glycophorin A erythroid antigen expression, our data add important information to the pathogenesis of HIV infection underlying the central role of TGF-β1 in HPC replication and differentiation.

ACKNOWLEDGEMENTS

The authors thank Dr Elisa De Crignis for her helpful discussion of results.

Transparency Declaration: all the authors declare that they have no relationship (commercial or otherwise) that may constitute a dual or conflicting interest.

Grant sponsor: Fondazione Cassa di Risparmio Bologna, Italy (n° 2006.0035, June 2006); Fondazione del Monte di Bologna e Ravenna, Italy (n° 376 bis/2009); AIDS projects of the Italian Ministry of Health (n° 40H5); University of Bologna funds for selected research topics and MURST 60.

REFERENCES

Aiuti A., Turchetto L., Cota M., Cipponi A., Brambilla A., Arcelloni C., et al. (1999). Human CD34(+) cells express CXCR4 and its ligand stromal cell-derived factor-1. Implications for infection by T-cell tropic human immunodeficiency virus. *Blood.* **94**, 62-73.

Alexaki A., Wigdahl B. (2008). HIV-1 infection of bone marrow hematopoietic progenitor cells and their role in trafficking and viral dissemination. *PLoS Pathog.* **4**, e1000215.

Carter C.C., McNamara L.A., Onafuwa-Nuga A., Shackleton M., Riddell J. 4th, Bixby D., et al. (2011). HIV-1 utilizes the CXCR4 chemokine receptor to infect multipotent hematopoietic stem and progenitor cells. *Cell Host Microbe.* **17**, 223-234.

Cleveland R.P., Liu Y.C. (1996). CD4 Expression by erythroid precursor precursor cells in human bone marrow. *Blood.* **87**, 2275-2282.

Deichmann M., Kronenwett R., Haas R. (1997). Expression of the human immunodeficiency virus type-1 coreceptors CXCR-4 (fusin, LESTR) and CKR-5 in CD34+ hematopoietic progenitor cells. *Blood.* **89**, 3522-3528.

Durand C.M., Ghiaur G., Siliciano J.D., Rabi S.A., Eisele E.E., Salgado M., et al. (2012). HIV-1 DNA is detected in bone marrow populations containing CD4+ T cells but is not found in purified CD34+ hematopoietic progenitor cells in most patients on antiretroviral therapy. *J. Infect. Dis.* **205**, 1014-1018.

Forsyth B.W., Andiman W.A., O'Connor T. (1996). Development of a prognosis-based clinical staging system for infants infected with human immunodeficiency virus. *J. Pediatr.* **129**, 648-655.

Gartner S., Popovic M. (1990). Virus Isolation and production. In Aldovini A., Walker B.D. Editors. HIV techniques, Stockton Press NY. 53-69.

Gibellini D., Vitone F., Buzzi M., Schiavone P., De Crignis E., Cicola R., et al. (2007). HIV-1 negatively affects the survival/maturation of cord blood CD34(+) hematopoietic progenitor cells differentiated towards megakaryocytic lineage by HIV-1 gp120/CD4 membrane interaction. *J. Cell. Physiol.* **210**, 315-324.

Gibellini D., De Crignis E., Ponti C., Cimatti L., Borderi M., Tschon M., et al. (2008). HIV-1 triggers apoptosis in primary osteoblasts and HOBIT cells through TNFalpha activation. *J. Med. Virol.* **80**, 1507-1514.

Gibellini D., Clò A., Morini S., Miserocchi A., Ponti C., Re M.C. (2013). Effects of human immunodeficiency virus on the erythrocyte and megakaryocyte lineages. *World J. Virol.* **2**, 91-101.

Jelkmann W. (2013). Physiology and pharmacology of erythropoietin. *Transfus. Med. Hemother.* **40**, 302-309.

Josefsson L., Eriksson S., Sinclair E., Ho T., Killian M., Epling L., et al. (2012). Hematopoietic precursor cells isolated from patients on long-term suppressive HIV therapy did not contain HIV-1 DNA. *J. Infect. Dis.* **206**, 28-34.

Kitano K., Abboud C.N., Ryan D.H., Quan S.G., Baldwin G.C., Golde D.W. (1991). Macrophage-active colony-stimulating factors enhance human immunodeficiency virus type 1 infection in bone marrow stem cells. *Blood.* **77**, 1699-1705.

Kreuzer K.A., Rockstroh J.K. (1997). Pathogenesis and pathophysiology of anemia in HIV infection. *Ann Hematol.* **75**, 179-187.

Lee B., Ratajczak J., Doms R.W., Gewirtz A.M., Ratajczak M.Z. (1999). Coreceptor/chemokine receptor expression on human hematopoietic cells: biological implications for human immunodeficiency virus-type 1 infection. *Blood.* **93**, 1145-1156.

Levine A.M., Berhane K., Masri-Lavine L., Sanchez M., Young M., Augenbraun M., et al. (2001). Prevalence and correlates of anemia in a large cohort of HIV-infected women: Women's Interagency HIV Study. *J. Acquir. Immune Defic. Syndr.* **26**, 28-35.

MacEneaney O.J., Connick E., DeSouza C.A. (2011). Effects of HIV-1 gp120 and protease inhibitors on apoptotic susceptibility of CD34+ hematopoietic progenitor cells. *J. Acquir. Immune Defic. Syndr.* **56**, e49-50.

McNamara L.A., Collins K.L. (2011). Hematopoietic stem/precursor cells as HIV reservoirs. *Curr. Opin. HIV AIDS.* 643-648.

McNamara L.A., Ganesh J.A., Collins K.L. (2012). Latent HIV-1 infection occurs in multiple subsets of hematopoietic progenitor cells and is reversed by NF-κB activation. *J. Virol.* **86**, 9337-9350.

Molina J.M., Sakaguchi M., Groopman J.E. (1990). Effect of recombinant soluble CD4 on the in vitro growth of hematopoietic progenitor cells from AIDS patients. *Blood.* **75**, 1379-1380.

Opie J. (2012). Haematological complications of HIV infection. *S. Afr. Med. J.* **102**, 465-468.

Re M.C., Zauli G., Gibellini D., Furlini G., Ramazzotti E., et al. (1993). Uninfected haematopoietic progenitor (CD34+) cells purified from the bone marrow of AIDS patients are committed to apoptotic cell death in culture. *AIDS.* **7**, 1049-1055.

Re M.C., Furlini G., Zauli G., La Placa M. (1994). Human immunodeficiency virus type 1 (HIV-1) and human hematopoietic progenitor cells. *Arch. Virol.* **137**, 1-23.

Redig A.J., Berliner N. (2013). Pathogenesis and clinical implications of HIV-related anemia in 2013. *Hematology Am. Soc. Hematol. Educ. Program.* 377-381.

Semba R.D., Shah N., Klein R.S., Mayer K.H,, Schuman P,, Vlahov D. (2002). Prevalence and cumulative incidence of and risk factors for anemia in a multicenter cohort study of human immunodeficiency virus-infected and -uninfected women. *Clin. Infect. Dis.* **34**, 260-266.

Shen H., Cheng T., Preffer F.I., Dombkowski D., Tomasson M.H., Golan D.E., et al. (1999). Intrinsic human immunodeficiency virus type 1 resistance of hematopoietic stem cells despite coreceptor expression. *J. Virol.* **73**, 728-737.

Shephard D.A. (1976). The 1975 Declaration of Helsinki and consent. *Can. Med. Assoc. J.* **115**, 1191-1192.

Sipsas N.V., Kokori S.I., Ioannidis J.P., Kyriaki D., Tzioufas A.G., Kordossis T. (1999). Circulating autoantibodies to erythropoietin are associated with human immunodeficiency virus type 1-related anemia. *J. Infect. Dis.* **180**, 2044-2047.

Spivak J.L., Bender B.S., Quinn T.C. (1984). Hematologic abnormalities in the acquired immune deficiency syndrome. *Am. J. Med.* **77**, 224-228.

Tsiakalos A., Kordossis T., Ziakas P.D., Kontos A.N., Kyriaki D., Sipsas N.V. (2010). Circulating antibodies to endogenous erythropoietin and risk for HIV-1-related anemia. *J. Infect.* **60**, 238-243.

Volberding P.A., Levine A.M., Dieterich D., Mildvan D., Mitsuyasu R., Saag M. (2004). Anemia in HIV infection: clinical impact and evidence-based management strategies. *Clin. Infect. Dis.* **38**, 1454-1463.

Wang L., Mondal D., La Russa V.F., Agrawal K.C. (2002). Suppression of clonogenic potential of human bone marrow mesenchymal stem cells by HIV type 1: putative role of HIV type 1 tat protein and inflammatory cytokines. *AIDS Res. Hum. Retroviruses.***18**, 917-931.

Wang Z., Goldberg M.A., Scadden D.T. (1993). HIV-1 suppresses erythropoietin production *in vitro*. *Exp. Hematol*. **21**, 683-688.

Xie Y.L., Bai H., Liu Y., Hoyle D.L., Cheng T., Wang Z.Z. (2015). Cooperative effect of erythropoietin and TGF-β inhibition on erythroid development in human pluripotent stem cells. *J. Cell. Biochem*. Epub ahead of print.

Zauli G., Re M.C., Visani G., Furlini G., Mazza P., Vignoli M., La Placa M. (1992). Evidence for a human immunodeficiency virus type 1-mediated suppression of uninfected hematopoietic (CD34+) cells in AIDS patients. *J. Infect. Dis*. **166**, 710-716.

Zauli G., Vitale M., Re M.C., Furlini G., Zamai L., Falcieri E., et al. (1994). In vitro exposure to human immunodeficiency virus type 1 induces apoptotic cell death of the factor-dependent TF-1 hematopoietic cell line. *Blood.* **83**, 167 -175.

Zauli G., Furlini G., Vitale M., Re M.C., Gibellini D., Zamai L., et al. (1994). A subset of human CD34+ hematopoietic progenitors express low levels of CD4, the high-affinity receptor for human immunodeficiency virus-type 1. *Blood.* **84**, 1896-1905.

Zhang J., Crumpacker C.S. (2010). Hematopoietic stem and progenitor cells in HIV/AIDS and immune reconstitution. *Cell. Res.* **20**, 745-747.

Zon L.I., Arkin C., Groopman J.E. (1987). Haematologic manifestations of the human immune deficiency virus (HIV). *Br. J. Haematol.* **66**, 251-256.