Repopulating hematopoietic stem cells from steady-state blood before and after *ex vivo* culture are enriched in the CD34$^+$CD133$^+$CXCR4^{low} fraction

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ABSTRACT

The feasibility of *ex vivo* expansion allows us to consider the steady-state peripheral blood as an alternative source of hematopoietic stem progenitor cells for transplantation when growth factor-induced cell mobilization is contraindicated or inapplicable. *Ex vivo* expansion dramatically enhances the *in vivo* reconstituting cell population from steady-state blood. In order to investigate phenotype and the expression of homing molecules, the expression of CD34, CD133, CD90, CD45RA, CD26 and CD9 was determined on sorted CD34$^+$ cells according to CXCR4 ("neg", "low" "bright") and CD133 expression before and after *ex vivo* expansion. Hematopoietic stem cell activity was determined *in vivo* on the basis of hematopoietic repopulation of primary and secondary recipients - NSG immuno-deficient mice. *In vivo* reconstituting cells in the steady-state blood CD34$^+$ cell fraction before expansion belong to the CD133$^+$ population and are CXCR4^{low} or, to a lesser extent, CXCR4^{neg}, while after *ex vivo* expansion they are contained only in the CD133$^+$CXCR4^{low} cells. The failure of the CXCR4^{bright} population to engraft is probably due to the exclusive expression of CD26 by these cells. The limiting-dilution analysis showed that both repopulating cell number and individual proliferative capacity were enhanced by *ex vivo* expansion. Thus, steady-state peripheral blood cells exhibit a different phenotype compared to mobilized and cord blood cells, as well as to those issued from the bone marrow. These data represent the first phenotypic characterization of steady-state blood cells exhibiting short- and long-term hematopoietic reconstituting potential, which can be expanded *ex vivo*, a *sine qua non* for their subsequent use for transplantation.

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Introduction

The introduction into clinical practice of "mobilization" from the bone marrow to peripheral blood, was an approach that resulted in an impressive increase of the number of hematopoietic progenitor cells (HPCs) and hematopoietic stem cells (HSCs) available for collection by cytapheresis. As such, this approach represented a revolutionary event in hematopoietic transplantation[1] and, as a result, strategies involving steady-state peripheral blood (SS-PB)[2] were abandoned. However, the procedure of mobilization of HPCs and HSCs, as well as their collection from the bone marrow, are not without risks.[3] Such risks can also effectively pose a deterrent to the recruitment of voluntary donors. Besides, mobilization is contraindicated in some cases, leading to the exclusion of the potential donors. Thus, avoiding mobilization

or bone marrow collection would be of great interest, especially in the context of allogeneic transplantation.

Ex vivo expansion procedures have evolved over the last few years and it is now possible to amplify committed HPCs to a great extent without losing the long-term reconstituting HSCs.[4,5] Recently, we demonstrated the presence of both short- and long-term reconstituting HSCs in human SS-PB and also observed that the activity of these cells increases dramatically after *ex vivo* expansion.[6,7] In this manner, we can safely source substantial numbers of SS-PB HPCs and HSCs, thus overcoming major obstacles to subsequent transplantation. In the light of this, SS-PB HPCs and HSCs should be reconsidered in the context of hematopoietic transplantation.

Based on previous literature regarding HSC activity,[6] it was not possible to specify whether the increase in activity of HSCs capable of reconstituting *in vivo* hematopoiesis of severe combined immune-deficient mice (SCID) repopulating cells (SRCs) after *ex vivo* expansion is: (i) due to amplification of these cells during *ex vivo* culture; or (ii) corresponds to pre-existing SRCs before *ex vivo* expansion (at time 0), which during expansion (until day 7), gained the ability to engraft after transplantation; or (iii) a combination of the above.

In order to address this issue, we investigated both HSC functional capacity in *in vivo* assays and the expression of membrane markers known to be associated with cell adhesion and homing, such as CD9, CD26, CD49d, CD49e, CD49f and especially CXCR4, as well as markers enabling the enrichment of HSCs (CD133, CD90, CD45RA). The choice of the tetraspanin CD9 was based on the fact that it is regulated by the activity of stromal cell-derived factor-1 (SDF-1; the ligand of CXCR4 receptor)[8] and CD26, since it is known to be an inhibitor of activity of the SDF-1/CXCR4 couple,[9] which plays an essential role in HSC mobilization and homing.[10-12] CD49d (VLA4), CD49e (VLA5), and CD49f (VLA6) are adhesion molecules of the integrin family associated with the anchorage and adhesion of cells in different situations and are considered essential for HSC homing.[13] Furthermore CD49f, CD45RA and CD90 are used as markers of cord blood (CB) and/or bone marrow (BM) HSCs.[14,15] Despite the fact that it largely overlaps with CD34, CD133 was chosen since it is not expressed on some subpopulations of committed progenitors and, hence, is more likely to include the HSCs.[16-18]

We found that HSC activity increases due to both amplification in their number and to enhancement of their individual proliferative capacity. Furthermore, *in vivo* reconstituting cells (both short- and long-term reconstituting cells i.e. ST-HSCs and LT-HSCs, respectively) in the fresh SS-PB CD34+ cell population belong to the subpopulation of CD133+ cells which are either CXCR4low or CXCR4neg, while after *ex vivo* expansion they are present only in the CD133+CXCR4low population.

Methods

Human steady-state peripheral blood cells

Leukocytes were recovered from leukodepletion filters (T2975, Fresenius Kabi, Louviers, France) by counterflow elution as described elsewhere[6,19,20] with a slight modification, i.e. the cells were flushed directly into 50 mL tubes (Falcon, Dutscher, Brumath, France) (see *Online Supplementary Methods*).

Isolation and cryopreservation of CD34+ cells

CD34+ cells were isolated from the mononuclear cell fraction using Miltenyi's (Miltenyi Biotec, Paris, France) "indirect" immuno-magnetic technique[19] ("LS" columns; Vario Macs Device). The CD34+ cell purity was 85-90% and the yield was 3-5x10^5 CD34+ cells per leukodepletion filter.

For each sample, 20 to 24 leukodepletion filters were processed and CD34+ cells were pooled before cryopreservation (4% human serum albumin solution, 10% dimethylsulfoxide; Wak-Chemie, Steinbach, France).[21] Samples were thawed in cold 4% human serum albumin and washed in selection buffer. After thawing, the CD34+ cell purity was 90-95%.

Ex vivo expansion of CD34+ cells recovered from leukodepletion filters

All tests were performed on CD34+ cells after thawing, before expansion (day 0) and after expansion (day 7). Day-0 CD34+ cells were seeded at 2x10^4 cells/mL, and cultured in 75 cm^2 flasks (NUNC, Roskilde, Denmark) for 7 days in liquid (clinical-grade serum-free medium Macopharma HP01) cultures supplemented with granulocyte colony-stimulating factor 100 ng/mL (Neuropen, Amgen SAS, Neuilly-sur-Seine, France), stem cell factor 100 ng/mL, thrombopoietin 20 ng/mL and interleukin-3 0.5 ng/mL (all from Peproteck, Rocky Hill, NJ, USA) (see *Online Supplementary Methods*).

CD34+ cell detection, immunophenotypic analysis and selection of cell subfractions

The CD34+ cell concentrations/purities were determined as previously described.[19,22] Fluorescent monoclonal antibodies were used to analyze/isolate CXCR4neg, CXCR4low, CXCR4bright subfractions, and CXCR4negCD133-, CXCR4negCD133+, CXCR4lowCD133-, CXCR4lowCD133+, CXCR4brightCD133-, and CXCR4brightCD133+ subfractions. The details are provided in the *Online Supplementary Methods*.

Detection of stem cells by their *in vivo* repopulating capacity

The only way to evaluate the activity of HSCs properly is to test their *in vivo* capacity of hematopoietic reconstitution.[23] Hence, we employed the most widely used assay based on the repopulation, by human cells, of hematopoietic tissues of immune-deficient mice, thereby evaluating the cells usually called SRCs (Figure 1). This approach enables the detection of two SRC populations:

i) Short-term HSCs (ST-HSCs). ST-HSC activity was evaluated *in vivo*, following transplantation of different phenotypically defined fractions of human SS-PB CD34+ cells in immunodeficient [NOD/SCID/gamma-null (NSG)] mice. As described previously,[24] the animal experiments were performed in compliance with French regulations (license n. 3306002) and with the approval of the Ethics Committee (n. 50120213-A). Either 1x10^5 CD34+ cells or 1x10^5 cells of sorted subfractions at day 0 were injected per mouse. After expansion, 2x10^5 of total day-7 cells or 2x10^5 cells of sorted subfractions were transplanted per mouse. In some experiments, the post-culture (day-7) equivalent of a defined number of day-0 cells i.e. the total day-7 progeny of a defined day-0 cell number, was injected per mouse (Figure 1). For all experiments, 10- to 12-week old female NSG mice (central animal-keeping facility of Bordeaux University) were conditioned by means of intra-peritoneal injections of 25 mg/kg busulfan (Busilvex, Pierre Fabre, Boulogne, France),[25,26] After 8 weeks, the animals were sacrificed and their femoral mononuclear BM cells isolated and analyzed for human CD45, CD19 and CD33 (with anti-human antibodies coupled with, respectively, fluorescein isothiocyanate, phycoerythrin and allophycocyanin; BD Biosciences, Le Pont de Claix, France) by flow-cytometry (FACS Canto II; BD Biosciences, Le Pont de Claix, France). To avoid false-

positive results due to control isotype, we used the non-injected mice to establish the "positivity threshold" for CD45, which was 0.1%.[24,27] Furthermore, to avoid inhibition of CXCR4 activity due to fixation of clone 12G5 antibody on the same external loop as SDF-1α (CXCL12, CXCR4 specific ligand),[28] we performed antibody elution by acid solution. For this, cells were incubated 20 min at 0°C in ACDA, pH 5 (Anticoagulant Citrate Dextrose solution formula A, Bioluz, Saint-Jean-de-Luz, France), then washed twice in RPMI medium before injection into mice. All cell suspensions were treated in an identical manner before injection.

To determine the SRC frequency, limiting dilution analysis[29,30] was performed for the $CD34^+CXCR4^{low}CD133^+$ subpopulation. The details are given in the *Online Supplementary Methods*.

ii) Long-term HSCs (LT-HSCs). For the detection of LT-HSCs, secondary recipient mice (Figure 1) were conditioned as primary recipients. The BM from both femora of primary recipients was flushed, resuspended and injected intrafemorally[6,25] into the secondary recipient NSG mice (Figure 1), as described in detail in the *Online Supplementary Methods*. The mice were sacrificed 7 or 8 weeks later and analyzed as described above.

Detection of colony-forming committed progenitors

Thawed $CD34^+$ cells were selected for their CXCR4 and CD133 expression and sorted as $CXCR4^{neg}CD133^-$, $CXCR4^{neg}CD133^+$, $CXCR4^{low}CD133^-$ and $CXCR4^{low}CD133^+$ subpopulations. Day-0 sorted subfractions were expanded separately *ex vivo* for 7 days. Day-0 and day-7 subpopulations were plated in methylcellulose cytokine-supplemented kits "Stemα-1D" (Saint Clement les Places, France) (1000 cells/mL for each cell population) and cultured for 14 days (37°C, 20% O_2, 5% CO_2) in 35 mm Petri dishes (NUNC, Roskilde, Denmark) in duplicate. The colonies (>50 cells) were scored[19] as burst-forming unit - erythroid (BFU-E), colony-forming unit - granulocyte and macrophage (CFU-GM) and multi-lineage colony-forming unit (CFU-mix).

Statistical analysis

The Mann-Whitney test for non-parametric values was applied. P values <0.05 were defined as statistically significant (*). P<0.01 (**) and P<0.001 (***) were highly significant values.

Results

Hematopoietic stem cells with short-term reconstituting capacity

To estimate ST-HSC activity directly before and after *ex vivo* expansion, the mice were injected with $2x10^5$ day-0 SS-PB $CD34^+$ cells or with their total day-7 progeny, hereafter referred to as "day-0 equivalent". These results from the NSG mice confirmed our previous findings obtained with NOD/SCID mice[6] demonstrating that 7 days of culture greatly enhanced SRC activity (P<0.05) while it also maintained the differentiation potential, as judged on the basis of the proportion of lympho (CD19)-myeloid (CD33) chimerism (Figure 2A).

Regarding the SS-PB $CD34^+$ population, the most prominent changes in culture were related to the expression of CXCR4 between day 0 (~16% cells expressing CXCR4)

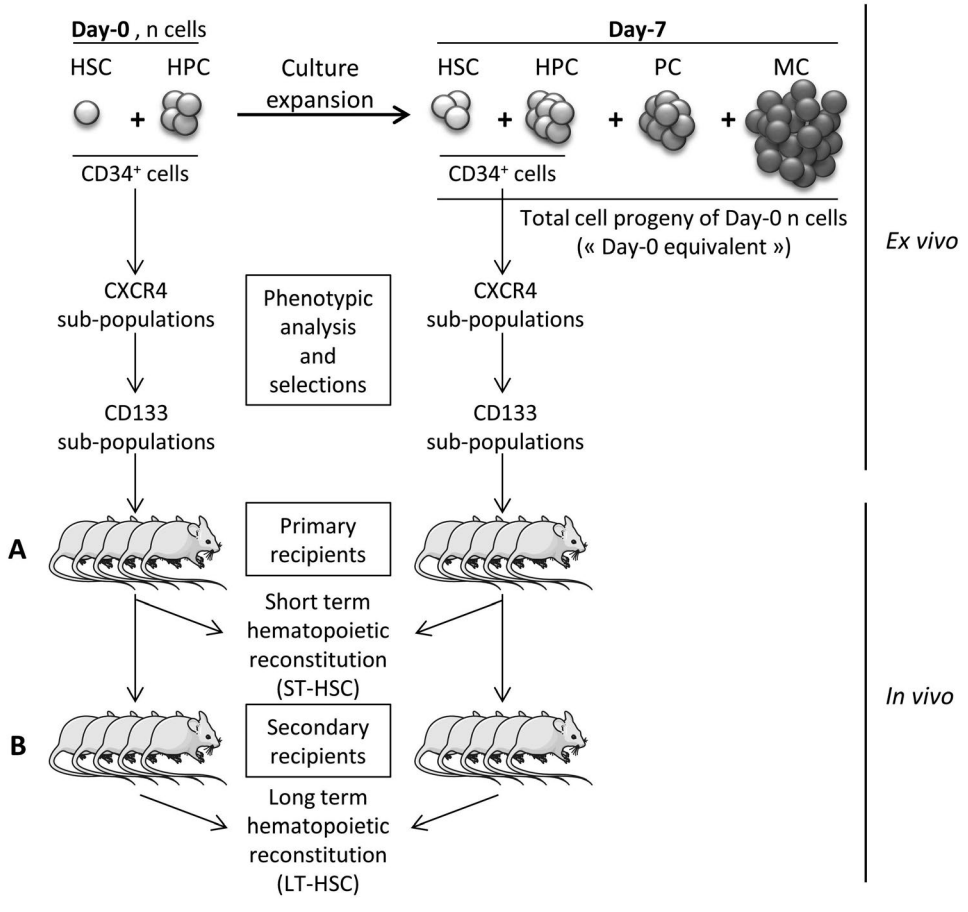


Figure 1. Experimental design. Evaluation of human stem cells by employing a severe combined immunodeficiency repopulating cell assay before (day 0) and after (day 7) *ex vivo* expansion culture. (A) Human cell chimerism in primary mice recipients reflects the activity of short-term hematopoietic stem cells (ST-HSC) while (B) human cell chimerism in secondary mice recipients reflects the activity of long-term hematopoietic stem cells (LT-HSC). HPC: hematopoietic progenitor cell; PC: precursor cell; MC: mature cell.

and the end stage of the *ex vivo* culture (day 7) (~67% cells expressing CXCR4) (*Online Supplementary Table S1*). In all experiments, three distinct subpopulations of cells with respect to CXCR4 expression level were evidenced: CXCR4^{neg}, CXCR4^{low} and CXCR4^{bright} (*Online Supplementary Figure S1*). Flow cytometry analysis after sorting showed that the cells belonged to only one of the subpopulations, categorized according to CXCR4 expression (*Online Supplementary Figure S2*). Most cells with *in vivo* repopulating capacity in the day-0 population were predominantly concentrated in the CXCR4^{low} fraction, although some minor activity was found in the CXCR4^{neg} and CXCR4^{bright} populations (Figure 2B); these HSCs exhibit a lower lymphoid differentiation potential compared to CXCR4^{neg} and, especially, CXCR4^{low} repopulating HSCs.

The engraftment of CXCR4^{neg} cells prompted us to explore the hypothesis that at least some of the CXCR4^{neg} cells can express CXCR4 once in an *in vivo* microenvironment of 37°C (i.e. after injection and transplantation). Thus, after an overnight incubation, 30% of the CD34^{+} cells that were initially CXCR4^{neg}, became CXCR4^{low} (*Online Supplementary Figure S3*). These data from the bulk CD34^{+} cultures were confirmed in the cultures initiated with the sorted CXCR4^{neg} cells (*Online Supplementary Figure S4*). After *ex vivo* expansion, almost all cells with engraftment capacity (SRCs) were concentrated in the CXCR4^{low} fraction (Figure 2C) and fully maintained their day-0 differentiation potential, although from these results it appears that SRC activity after *ex vivo* expansion (day-7) is lower than that of non-expanded cells at day 0. However,

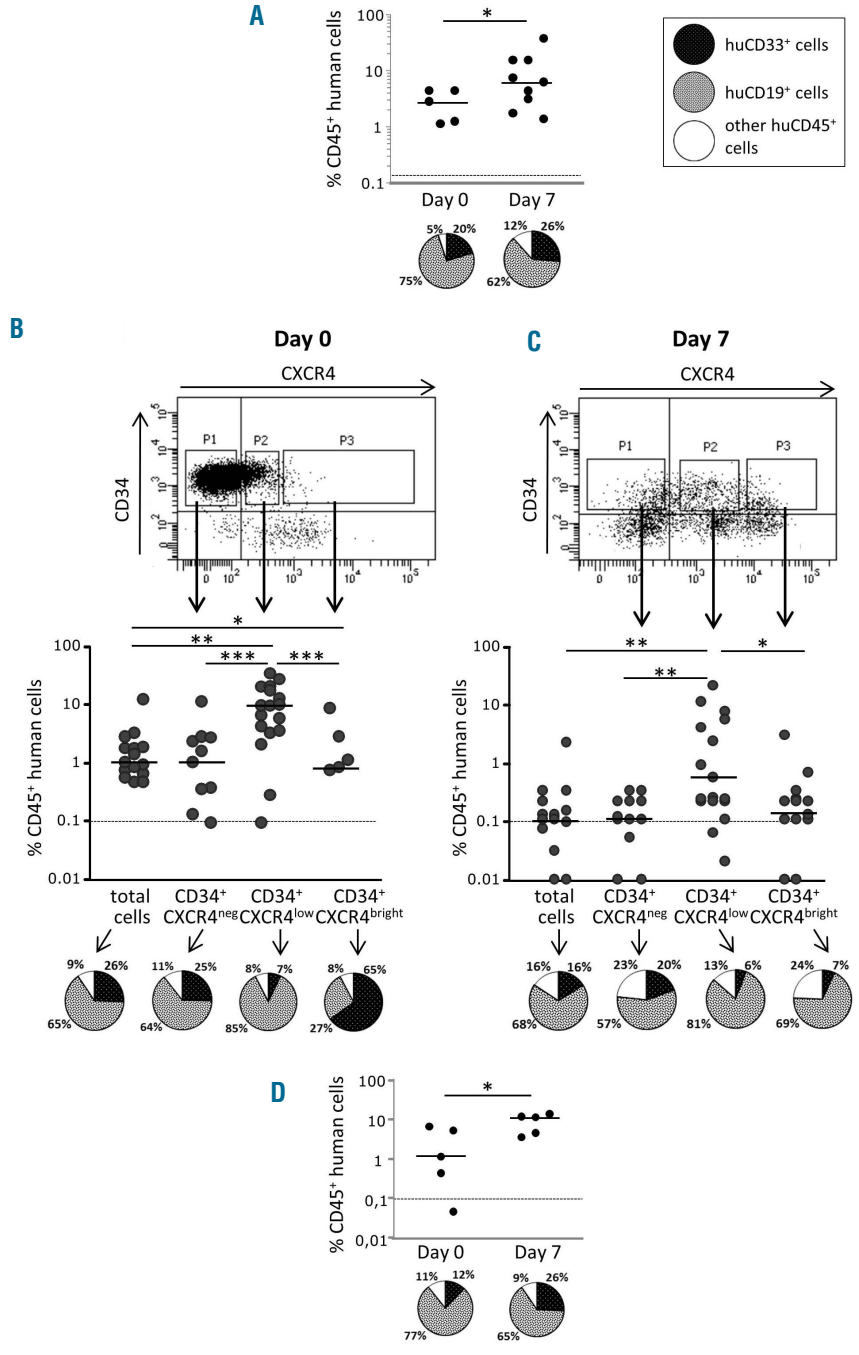


Figure 2. CXCR4 expression is related to the engraftment capacity of hematopoietic stem cells contained in the steady-state peripheral blood CD34^{+} cell population before and after *ex vivo* expansion. (A) The activity of severe combined immunodeficiency repopulating cells (SRCs) in CD34^{+} steady-state peripheral blood (SS-PB) cells is enhanced by 7 days of culture. Day 0: 2x10^{5} SS-PB CD34^{+} cells were injected per mouse. Day 7: the total progeny of 2x10^{5} day-0 SS-PB CD34^{+} cells was injected per mouse. (B-C) After culture day-0 (B) and day-7 (C) culture, three cell subpopulations were selected for injection into the recipient mice: P1, cells defined and sorted as the CD34^{+}CXCR4^{neg} subpopulation; P2, cells defined and sorted as the CD34^{+}CXCR4^{low} subpopulation; P3, cells defined and sorted as the CD34^{+}CXCR4^{bright} subpopulation. Day 0: 1x10^{5} SS-PB total CD34^{+} cells or 1x10^{5} cells from each sorted cell subpopulation were injected per mouse. Day 7: 2x10^{5} of the total expanded cell population or 2x10^{5} cells from each sorted cell subpopulation were injected per mouse. (D) Effect of ex vivo expansion on SRC activity in SS-PB CD34^{+}CXCR4^{low} cells: Day 0: 1x10^{5} SS-PB CD34^{+}CXCR4^{low} cells were injected per mouse; day 7: the total progeny of 1x10^{5} day-0 SS-PB CD34^{+}CXCR4^{low} cells were injected per mouse. (A-D) SRC activity was evaluated by short-term reconstitution (8 weeks) in NSG mice; each point represents the percentage of CD45^{+} human cells in one mouse bone marrow. For each condition (A-D) the "pie" graphs show the relative proportion of CD19^{+} and CD33^{+} cells of human origin within the huCD45^{+} population. Statistical significance: *P<0.05; **P<0.01; ***P<0.001.

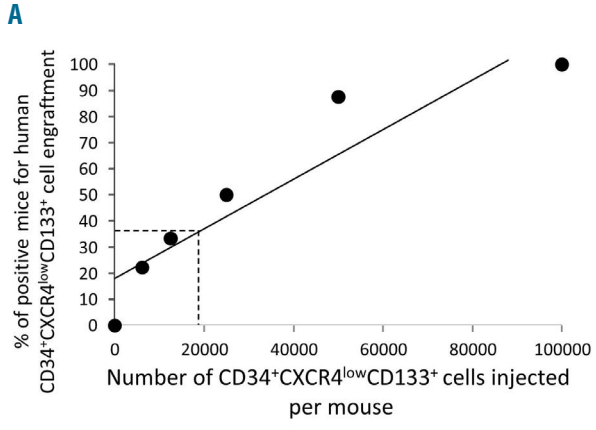
although injecting the same number of cells from each fraction into mice can show in which fraction the SRCs are concentrated, it cannot provide insight into changes of specific SRC activity during expansion culture. To obtain this information, we injected each mouse with the full day-7 progeny (equivalent) of $2x10^5$ day-0 $CD34^+CXCR4^{low}$ cells (Figure 2D) (the $CD34^+CXCR4^{low}$ fraction was chosen since effectively all SRC activity is concentrated in this fraction). In this way, we obtained unequivocal proof that SRC activity was enhanced after the *ex vivo* expansion culture. To quantify these data, we performed a limiting dilution assay[31] on the $CD34^+CXCR^{low}CD133^+$ population at the beginning and after culture (see further text for the CD133 issue), the results of which showed an ~4.2-fold expansion of SRCs after 7 days with respect to day 0 (Figure 3A-C). Furthermore, the mean individual SRC proliferative capacity was ~4-fold higher after 7 days of expansion culture compared to the capacity at day 0 (Figure 3D). In the same time period, a 14.2-fold expansion of the $CD34^+CXCR4^{low}CD133^+$ fraction was found (Figure 3C; *Online Supplementary Table S2*).

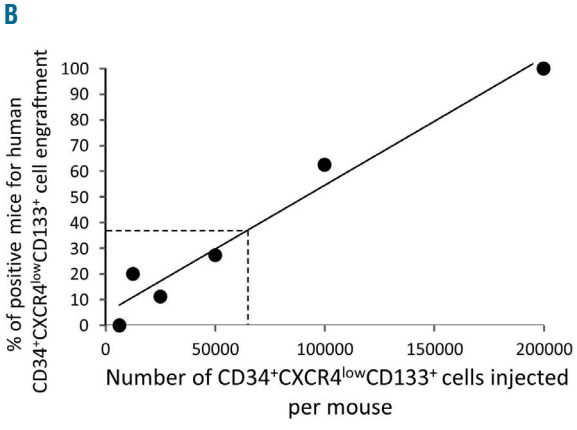
All (100%) SS-PB $CD34^+$ cells, whatever their CXCR4 expression pattern, expressed all adhesion molecules analyzed (LFA-1, VLA-4, VLA-5, VLA-6) before and after *ex vivo* expansion (*data not shown*). Neither CD90 nor CD45RA was expressed by $CD34^+$ SS-PB cells: all the sorted subpopulations were $CD90^-$ and $CD45RA^-$ (*data not shown*).

Before and after culture expansion, expression of the tetraspanin CD9 correlated closely with the expression of CXCR4 (Figure 4A). CD26 was not expressed on $CXCR4^{neg}$ or $CXCR4^{low}$ fractions of $CD34^+$ cells at either day 0 or day 7, however 22% and 38% of $CXCR4^{bright}$ cells expressed CD26 on day 0 and day 7, respectively (Figure 4B). It is noteworthy that the expression of CD26 by CXCR4-expressing cells coincides with their loss of engraftment capacity (Figure 2).

On day-0 $CD34^+$ cells, CD133 was primarily expressed on $CXCR4^{neg}$ and $CXCR4^{low}$ $CD34^+$ cell fractions (Figure 5A). In contrast, after expansion culture (day 7), the $CD133^+$ cells were exclusively concentrated in the $CXCR4^{low}$ fraction of the cells remaining $CD34^+$ (Figure 5B).

When day-0 cells from these fractions defined on the basis of CXCR4 and CD133 expression were injected into NSG mice, SRCs were evidenced only in $CD133^+$ fractions, i.e. $CXCR4^{neg}CD133^+$ and $CXCR4^{low}CD133^+$ (Figure 6A). Furthermore, after expansion at day 7, the main SRC activity remained in the $CXCR4^{low}CD133^+$ fraction: with the cell dose employed, all mice were "positive" and with high chimerism (Figure 6B). At day 0, we observed that SRCs were much more frequent in the $CXCR4^{low}$ fraction than in the $CXCR4^{neg}$ fraction ($P<0.01$) (Figure 4A). With regard to differentiation potential, a predominant "lymphoid" profile characterized the repopulating HSCs of $CD133^+$ fractions, while the rare repopulating HSCs detected in $CD133^{neg}$ fractions showed much higher proportion of, or predominantly exhibited, a myeloid differentiation potential (Figure 6A). At day 7, only the $CD34^+CXCR4^{low}CD133^+$ fraction yielded HSCs capable of





C

Cell population	Number per culture		Fold amplification
	Day 0	Day 7	
SRC	4,43 ±2,63	18,63 ±5,46	4,20 ±1,58
$CD34^+CXCR4^{low}CD133^+$	80834 ±32930	1148066 ±467781	14,20 ±5,77

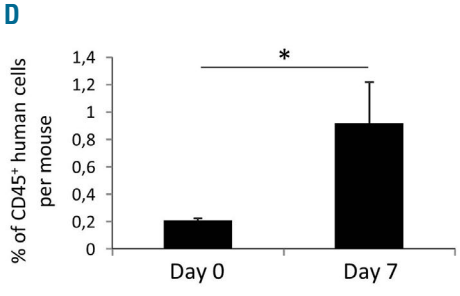


Figure 3. Frequencies and individual proliferative capacity of severe combined immunodeficiency repopulating cells within the $CD34^+CD133^+CXCR4^{low}$ cell population before and after *ex vivo* expansion. (A,B) Percentage ofmice "positive" for human CD45, 8 weeks after injection of $CD34^+CXCR4^{low}CD133^+$ cells, with respect to the cell dose, before expansion (A) and after expansion (B). (C) Absolute number of severe combined immunodeficiency repopulating cells (SRCs) estimated on the basis of the extreme limiting dilution assay (ELDA). (D) Mean chimerisms of the individual SRCs (only the doses giving less than 37% of positive mice were taken into consideration and only positive mice from these conditions were analyzed). The results presented were generated from the individual data given in *Online Supplementary Table S2*. Statistical significance: *$P<0.05$.

in vivo reconstitution. In this case, they displayed a predominant lymphoid differentiation potential (Figure 6B).

Hematopoietic stem cells with long-term reconstituting ability

While the results presented above concern ST-HSCs, we also employed the primary/secondary recipient transplantation approach to detect the LT-HSC subpopulation in SS-PB CD34+ cells depending on their CD133 and CXCR4 expression pattern before and after *ex vivo* expansion.[6,24,32] In fact, we tested the presence of LT-HSCs at day 0 and after expansion culture (day 7) in the total cell population, in the CD34+CXCR4lowCD133+ population (which, as described above and shown in Figure 7A,B, contains most of the ST-HSCs), and in the fraction containing all remaining cells after removal of the CD34+CXCR4lowCD133+ population (Figure 7). With the number of cells injected in our experiments, the LT-HSCs were practically undetectable both at day 0 (before expansion) and at day 7 (after expansion) (see CD45 chimerism in secondary recipients, Figure 7C,D), indicating that their frequency in the total CD34+ cell population is extremely low. However, once concentrated in the CD34+CXCR4lowCD133+ population, LT-HSCs become clearly detectable both before and after expansion (Figure 7C,D). Since we did not find "positive" secondary recipient mice after injection of BM from the primary recipient mice which had received the cell population composed of all other cells except CD34+CXCR4lowCD133+ ones, it can be concluded that the LT-HSCs are limited to the CD34+CXCR4lowCD133+ phenotype. In view of the fold expansion of the total cells (25.1 ± 9.9) (*Online Supplementary Table S1*) and the fact that the injected cell dose after expansion was only eight times higher than before expansion, it can be estimated that LT-HSCs were at minimum maintained during the culture. It is very interesting to note that the day-0 LT-HSCs (Figure 7C) showed a relatively lower lymphoid differentiation capacity compared to cultured (day-7) LT-HSCs (Figure 7D).

Committed hematopoietic progenitors

The content of committed progenitors in CD34+ cells belonging to the fractions defined by CXCR4 and CD133 expression is presented in Figure 8. Interestingly, the

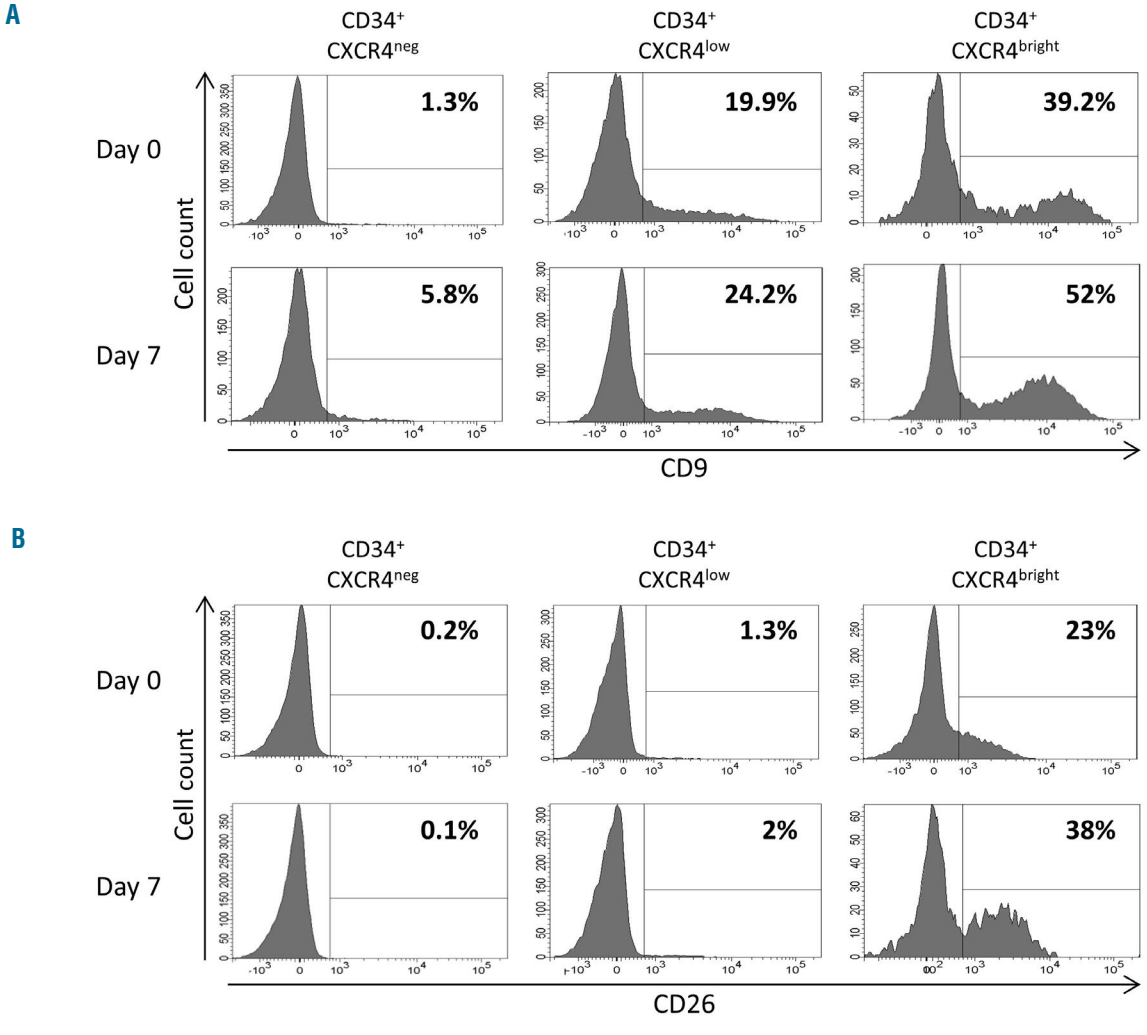


Figure 4. CD9 and CD26 cell expression among CXCR4 cell subpopulations. (A) CD9 expression at day 0 and after 7 days of culture. (B) CD26 expression at day 0 and after 7 days of culture. The same CD34+CXCR4neg, CD34+CXCR4low and CD34+CXCR4bright subpopulations were selected as those for the in vivo reconstitution experiments. Percentages of CD9+ or CD26+ cells are indicated for each subpopulation. Day 7: cell subpopulations were defined among the progeny of total day-0 CD34+ cells.

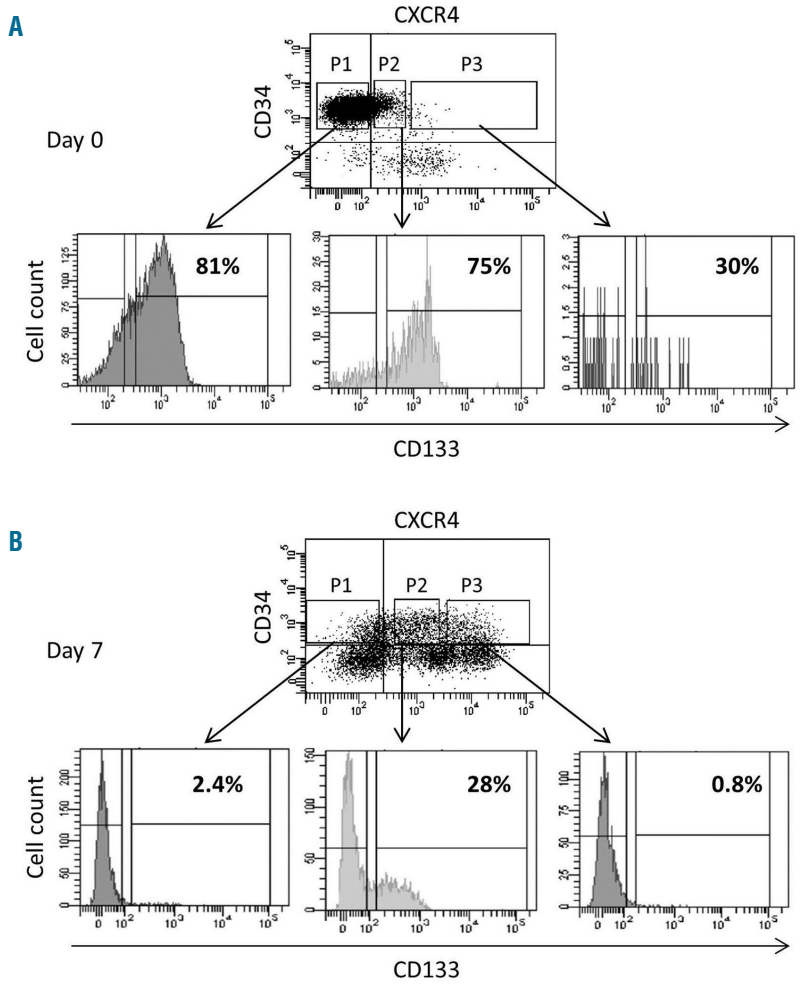


Figure 5. CD133 expression by CD34⁺ cells selected on the basis of CXCR4 expression. Three gates were delimited: P1, CD34⁺CXCR4^{neg} cell subpopulation; P2, CD34⁺CXCR4^{low} cell subpopulation; P3, CD34⁺CXCR4^{bright} cell subpopulation. Among these cell gates, CD133⁺ and CD133⁻ fractions were defined and six cell subpopulations were sorted as CXCR4^{neg}CD133⁻, CXCR4^{neg}CD133⁺, CXCR4^{low}CD133⁻, CXCR4^{low}CD133⁺, CXCR4^{bright}CD133⁻, and CXCR4^{bright}CD133⁺ subfractions. For each of these subpopulations, the percent of CD133-expressing cells is indicated.

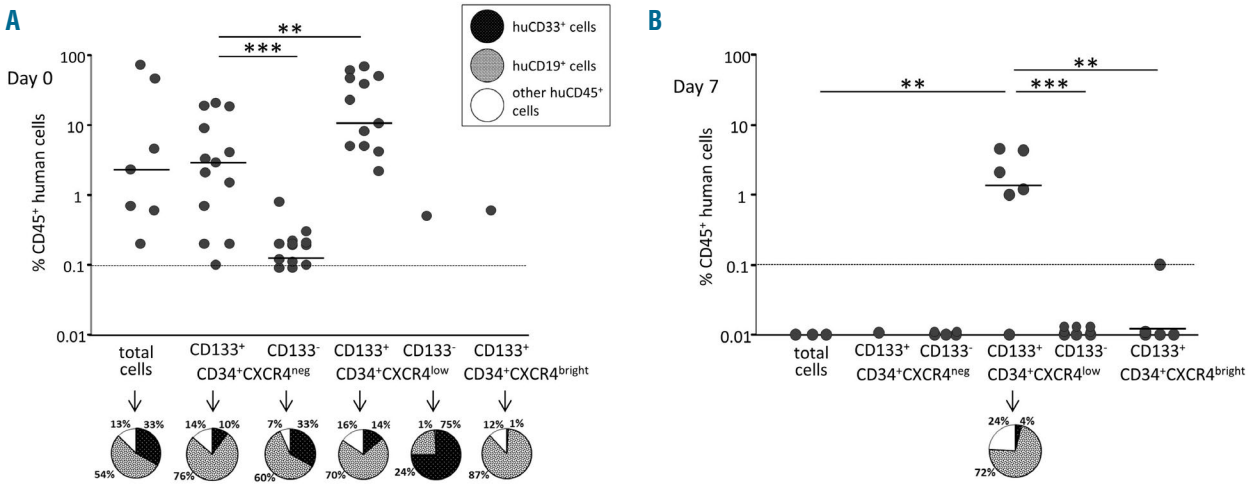


Figure 6. CD133 determines hematopoietic severe combined immunodeficiency repopulating cell capacity of CXCR4-expressing CD34⁺ steady-state peripheral blood cells, before and after *ex vivo* expansion. Severe combined immunodeficiency repopulating cell (SRC) activity was evaluated by short-term engraftment (8 weeks) in NSG mice. Each point of the graphs represents the percentage of CD45⁺ human cells in one mouse bone marrow. (A) Day 0: 1x10⁵ SS-PB total CD34⁺ cells or 1x10⁵ cells from each sorted cell subpopulation were injected per mouse. (B) Day 7: 2x10⁵ of the total expanded cell population or 2x10⁵ cells from each sorted cell subpopulation were injected per mouse. The "pie" graphs in (A) and (B) show the relative proportions of CD19⁺ and CD33⁺ cells pf human origin within the huCD45⁺ population. Statistical significance: ***P*<0.01 and ****P*<0.001.

CD133- fractions before and after *ex vivo* expansion contained exclusively erythroid progenitors (BFU-E) irrespective of CXCR4 expression, while CD133+ cells always contained three classes of progenitors (CFU-GM, BFU-E and CFU-Mix). Furthermore, the committed progenitors were five times less concentrated in the CXCR4lowCD133- fraction than in the CXCR4lowCD133+ one.

Discussion

The findings presented in this article clearly show that ST-HSCs and LT-HSCs present in SS-PB have particular phenotypic properties, which are different from those of HSCs in CB, BM or mobilized peripheral blood (M-PB). It is evident that the pattern of CXCR4 expression is related to the functional abilities of SS-PB ST-HSCs and LT-HSCs. This is not surprising since CXCR4 and its ligand SDF-1 have been demonstrated to have a major role in homing/mobilization of HPCs and HSCs.[33,34]

The presence of a small fraction (8%) of CXCR4-expressing CD34+ cells in SS-PB was first observed by Lataillade *et al.*[35] Here, we found 16% of CXCR4+CD34+ cells in the mononuclear SS-PB fraction issued from leukodepletion filters. Only a very small fraction of M-PB CD34+ cells express CXCR4; these cells exhibit an *in vitro*

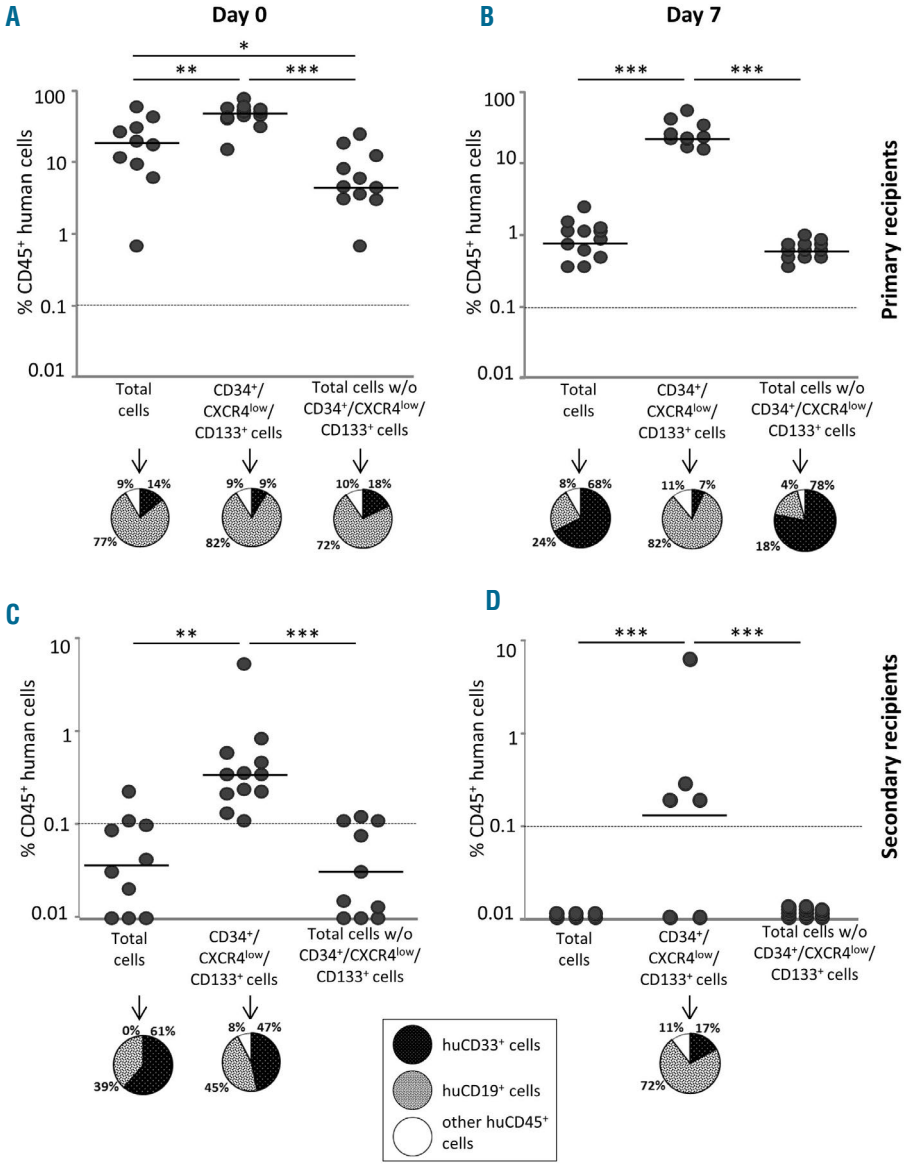


Figure 7. Capacity of long-term hematopoietic reconstitution of NSG mice is restricted to the CD34+CXCR4lowCD133+ steady-state peripheral blood cell fraction. Before (day 0) (A, C) and after ex vivo expansion (day 7) (B, D), three cell populations were selected and sorted: total CD34+ steady-state peripheral blood (SS-PB) cells, the CD34+CXCR4lowCD133+ selected subpopulation, and total CD34+ cells without the CD34+CXCR4lowCD133+ subpopulation. (A) Day-0, short-term reconstitution in primary recipients, 2x10^5 cells of each subpopulation were injected intravenously per mouse. (B) Day-7, short-term reconstitution in primary recipients, 1.6x10^6 cells of each subpopulation were injected intravenously per mouse. Day-7 subpopulations were defined among the progeny of total day-0 CD34+ cells. (C) Day-0, long-term reconstitution in secondary recipients. Bone marrow cells from both femora of each primary recipient were injected into the bone marrow of the secondary recipient NSG mouse. (D) Day-7, long-term reconstitution in secondary recipients. Bone marrow cells from both femora of each primary recipient were injected into the bone marrow of the secondary recipient NSG mouse. For each condition (A-D) the "pie" graphs show the relative proportions of CD19+ and CD33+ cells of human origin within the huCD45+ population. Statistical significance: *P<0.05; **P<0.01; ***P<0.001.

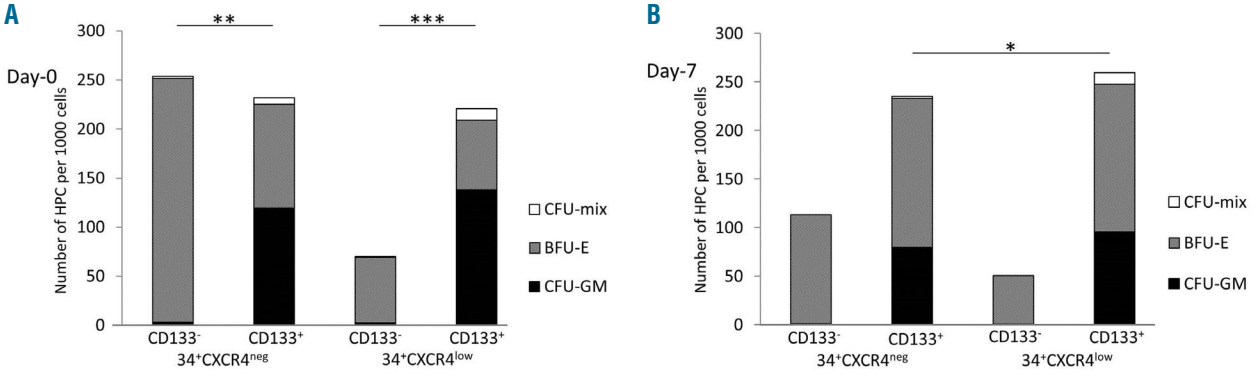


Figure 8. Hematopoietic committed progenitors in the CD34⁺ cell populations selected on day 0 on the basis of CXCR4 and CD133 expression. Day-0 (A) sorted subpopulations were expanded separately *ex vivo* and day-7 (B) clonogenic capacities of these subpopulations were analyzed. CFU-GM: colony-forming unit - granulocyte/monocyte; BFU-E: burst-forming unit – erythroid; CFU-mix: colony-forming unit - mixed. Statistical significance: *P<0.05; **P<0.01; ***P<0.001.

capacity to migrate towards a SDF-1α gradient and result in high levels of multilineage engraftment upon injection into NOD/SCID mice.[10] In CB, both CXCR4⁺ and CXCR4^{neg} subsets were shown to be capable of engrafting NOD/SCID mice with similar frequencies[36] suggesting that CXCR4 is not a suitable marker for purification of human CB HSCs before transplantation.

In addition, CXCR4 expression is rapidly regulated by environmental factors or induced *ex vivo* by cytokines such as granulocyte colony-stimulating factor (also used in our cultures) in CD34⁺ cells from all sources, including SS-PB CD34⁺ cells with primitive features.[35] Furthermore, induction of CXCR4 expression on CB and M-PB CD34⁺ cells increased their capacity for *in vivo* engraftment in NOD/SCID mice.[37] Apparently contradictory conclusions were reached in a study that found that the homing efficiency of CD34⁺ cells selected from BM or M-PB was not related to expression of either CXCR4 or adhesion molecules.[38] However, blocking CXCR4 signaling on transplanted CB CD34⁺ cells prevented homing, whereas pretreatment of cells with cytokines led to up-regulation of CXCR4 expression and increased mice engraftment,[39] which clearly highlights the crucial importance of CXCR4 expression for HSC engraftment.

This point is important because it helps to avoid erroneous conclusions due to artifacts induced by the technical procedure related to CXCR4 expression-based cell sorting; a low repopulating capacity of CD34⁺CXCR4⁺ cells (the authors did not discriminate between low and bright populations) from CB and BM could result from the neutralizing activity of the anti-CXCR4 monoclonal antibody that was used for cell sorting. This antibody (clone 12G5) binds the site that serves for the binding and signaling of the CXCR4 specific ligand SDF-1α[28,39] and, depending on its concentration, can have either inhibitory or stimulatory effects.[11] To avoid any artifacts in assaying CXCR4 activity and grafting capacity of CD34⁺ cells, we used antibody elution after cell subfraction sorting and before any engraftment assays.

In our hands, the engraftment capacity of the CD34⁺CXCR4^{neg} subfraction in SS-PB was much lower than that of the CD34⁺CXCR4^{low} population, as found for CB CD34⁺CXCR4^{neg} *versus* CD34⁺CXCR4^{pos} cells (including both "low" and "high" CXCR4 expression).[12] However, a substantial number of CD34⁺CXCR4^{neg} cells expresses CXCR4 (i.e. they become "low expressing") after at least overnight *ex vivo* cytokine treatment, an effect which is even more pronounced after 4 days in culture (*Online Supplementary Figure S3*; confirmed also in the cultures initiated with the CD34⁺ cells sorted on the basis of CXCR4 expression: *Online Supplementary Figure S4*). This phenomenon could be proposed as the explanation for the *ex vivo* culture enhancing effect on the engrafting capacity of ST-HSCs and LT-HSCs. However, this is not the case, since practically all CXCR4^{neg} cells which became CXCR4⁺ in culture lost their CD133 expression by day 4 of culture (only CD133⁺ cells exhibited engrafting capacity, see below) (*Online Supplementary Figure S3*). However, the CD133 expression was maintained for at least for 24 h in culture mimicking the *in vivo* situation after cell injection. This could explain some minor engraftment capacity of the CXCR^{neg} cell population before expansion. In fact, these cells could become CXCR4^{low} *in vivo*, during the first hours after injection. It should be emphasized that the highest engraftment capacity before (at day 0) or after (at day 7) cell culture is concentrated in the CXCR4^{low} fraction and not in the CXCR4^{bright} one, which seems to be surprising. Actually, the expression of CD26 (that, in our protocol, could be induced by granulocyte colony-stimulating factor, as shown for CD34⁺CD38⁻ CB cells[40]), which is related only to CXCR4^{bright} cells, can explain the decrease in CXCR4^{bright} engrafting efficiency. It has been shown that CD26/dipeptidyl peptidase IV is a membrane-bound extracellular peptidase that cleaves polypeptides such as SDF-1, thus reducing CXCR4 activity. Furthermore, CD26 expression might be part of a mechanism regulating CXCR4 activity. The inhibition of CD26 expression on CB CD34⁺ cells enhances the *in vitro* migratory effect against the SDF-1 gradient[9] and improves *in vivo* long-term engraftment in NOD/SCID mice.[41,42] Furthermore, pretreatment of mice with a specific CD26 inhibitor (diprotin A) enhances engraftment of mouse BM cells in primary and secondary recipients.[43] This is being considered among emerging strategies to improve homing and engraftment of HSCs in clinical transplants.[44] A similar approach, allowing the CXCR4^{bright} HSCs to engraft, might

still enhance the engraftment efficiency of SS-PB after *ex vivo* expansion, although this remains to be confirmed. On the other hand, recent studies have shown that CD34+ cells also home to the BM in an SDF1-CXCR4 axis-independent manner and that "priming factors"[45] as well as "mild heat treatment" facilitate incorporation of CXCR4 into functional lipid rafts.[46] This might constitute another strategy to enhance engraftment of SS-PB cells.

Concerning our observation of a close relationship between the expression of CXCR4 and CD9, CD9 has been implicated in the regulation of various physiological processes, including cell motility and adhesion. Trafficking and homing is a multistep process, as demonstrated for lymphocytes and myeloma cells, in which CD9 has been proven essential for transendothelial invasion.[47] In human CB, CD9 is expressed by CD34+ cells and is regulated by SDF-1. Anti-CD9 antibody alters migratory and adhesive functions of CB CD34+ cells *in vitro* and CD9 neutralization impairs homing of transplanted CD34+ cells in NOD/SCID mice.[8] The functional relationship between CD9 and CD26 on CDCXCR4bright cells remains to be elucidated.

In our hands, all the sorted SS-PB CD34+ subpopulations were CD90- and CD45RA-. This phenotype is associated with committed progenitor cells in BM and CB CD34+ cells since HSCs seem to be CD90+[48] and/or CD45RA+.[14] However, our CD34+CXCR4lowCD133+CD90-CD45RA- SS-PB cells are enriched in true HSCs, as proven by efficient secondary recipient hematopoietic engraftment. CD49f, claimed to be a specific marker of CB repopulating HSCs,[15] is expressed on all CD34+CD133+ SS-PB cells whatever their CXCR4 expression.

Perhaps the most interesting information emerging from our study is the fact that all SS-PB HSCs exhibiting *in vivo* repopulating capacity (both ST- and LT-HSCs) are found to be exclusively a CD133+ population of CD34+ cells, highly concentrated in the CXCR4low population. This particular phenotypic determinant does not change after *ex vivo* expansion. With respect to the committed progenitors in the CD34+ population, our results (Figure 8) clearly show that before and after *ex vivo* expansion, CFU-GM and CFU-Mix reside exclusively in the CD133+ population, whereas BFU-E are present in both the CD133+ and CD133- populations. This is in line with recent findings obtained with CB CD34+ cells.[49-51] CD133 has long been considered a marker of stemness for CB, BM and M-PB cells although also expressed by most committed progenitor cells.[16,18,49] Here, we show that CD133 could also be used for the enrichment of SS-PB HSCs. In CB, BM and M-PB cytokine-activated CD34+ cells, CD133 is concentrated in the uropod of the polarized migrating cells.[52] A functional relationship has been observed between CD133/prominin-1 and CXCR4 in specific membrane micro-domains of magnupodia,[17] suggesting a favored cell migration towards the *in vivo* hematopoietic niche and, hence, engraftment. Since LT-HSCs are present only in the CD133+ fraction of CD34+CXCR4low SS-PB cells before and after expansion, the loss of this particular phenotypically-defined population in the course of *ex-vivo* manipulation could be indicative of a loss of the long-term repopulating capacity of the graft. Clinical scale CD133+ selection is also considered among emerging strategies and alternative methods in clinical transplantation.[53]

BM mesenchymal stromal cell proliferation, but also fluctuation of the number of HSCs in peripheral blood are related to circadian oscillations.[54] Since similar oscillations exist in humans,[55] the circadian rhythm must be taken into consideration to optimize collection of SS-PB HSCs and HPCs.

Large, phenotypic and HPC analysis was performed on CD34+ cells isolated from SS-PB.[56] *Ex vivo* culture of CD34+ SS-PB cells enhanced the total number of HSCs exhibiting *in vivo* repopulating capacity as well as their individual proliferative capacities, as shown by our limiting-dilution experiments. The maintenance of the lymphoid differentiation potential of repopulating HSCs after *ex vivo* culture is an additional important argument, since a shift towards predominant myeloid potential, as we detected in the rare CXCR4low and CD133- repopulating HSCs, has been found to occur during aging.[57] In fact, our results suggest that reducing HSC differentiation capacity to the myeloid lineage represents a degree of HSC commitment. In this respect, aging is characterized by a higher proportion of more committed HSCs[58] in a context of general "consumption" and is the first sign of imminent exhaustion of the system. This suggests that *ex vivo* expansion can provide an adequate tool to produce enough hematopoietic stem and progenitor cells to constitute a single hematopoietic graft from the contents of only one or two steady-state leukapheresis collections. The efficiency of the expansion procedure could, most likely, be further improved by using new approaches, for example the TAT-protein transduction peptide fused to regulatory factors or inhibition of HOXB4 degradation,[59-62] which is the object of our ongoing work. Furthermore, the CD34neg fraction containing immuno-competent cells (T and B lymphocytes) can be preserved and an appropriate dose of these cells injected either during transplantation or later, depending on the need for an allogeneic immuno-effect. Furthermore, lymphocyte efficiency can be enhanced and specified by *ex vivo* engineering.

Taken together, the results presented here might help in the design of novel, advanced graft generation, which could simultaneously provide efficient immuno-hematopoietic reconstitution and a graft-*versus*-tumor/leukemia effect. Future work in our laboratory aims to explore this strategy.

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