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Pattern of expression of CXCR4 and adhesion molecules by human CD34+ cells from different sources: role in homing efficiency in NOD/SCID mice

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A B S T R A C T

Background and Objectives. The role of adhesion molecules (AM) and CXCR4 in the homing of CD34+ cells to NOD/SCID marrow and spleen is not completely elucidated. In this work, we study the differences in the expression of CXCR4 and AM by human CD34+ cells from different sources and their impact on homing ability in NOD/SCID mice.

Design and Methods. We used flow cytometry to analyze the expression of CXCR4 and AM (CD49d, CD49e, CD11a, CD58, CD54, CD31, CD62L, CD43 and CD44) on fresh CD34+ cells from bone marrow (BM), mobilized peripheral blood (MPB), positively selected CD34+ cells (PS) and after expansion cultures with two cytokine combinations. Secondly, we studied the homing efficiency of CD34+ cells from each source in 75 irradiated NOD/SCID mice, and finally the pattern of expression of CXCR4 and AMs by retrieved human CD34+ cells that had efficiently homed.

Results. The homing efficiency of PS CD34+ cells was significantly lower than that of BM and MPB CD34+ cells. Our results reveal that changes in the expression of CXCR4 and AM are induced by mobilization, PS and *in vitro* expansion. However none of these changes has definitive impact on the homing efficiency. Human CD34+ cells found in the marrow and spleen of NOD/SCID mice have the same adhesive profile as the injected cells: CXCR4, CD62L and CD11a mainly negative, and CD49d+, indicating that homing is not restricted to positive cells.

Interpretation and Conclusions. We conclude that changes induced in CXCR4 and AM expression after mobilization, selection and expansion of human CD34+ cells do not cause significant differences in the homing efficiency of these cells. The lower homing efficiency of PS CD34 cells could be explained by the absence of accessory cells.

Key words: CXCR4, adhesion molecules, homing efficiency, expansion cultures, NOD/SCID mice.

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The ability of hematopoietic stem cells (HSC) to reconstitute hematopoiesis in myeloablated recipients requires initially that these cells home within the bone marrow (BM) microenvironment. Homing is a complex multistep process that theoretically requires the presence of certain adhesion molecules (AM) expressed in the membrane of hematopoietic cells; these AM, through interactions with their receptors in the BM endothelium and extracellular matrix, should allow the HSC to reach and anchor appropriate bone marrow niches where active hematopoiesis takes place. Several studies suggested a role in homing for integrins (VLA4, VLA5, LFA-1), L-selectin, immunoglobulin-like adhesion molecules (ICAM-1, PECAM-1), and others such as LFA-3, CD43 and CD44.[1-4]

The CXC chemokine stromal cell-derived factor 1 (SDF-1) plays a major role in migration, proliferation, differentiation and survival of both murine and human hematopoietic progenitors.[5] CXCR4 is the 7-transmembrane receptor of SDF1, and is widely expressed by several hematopoietic cell types and different stromal cells.[6] Recently it has been proposed that SDF-1/CXCR4 has an essential role in both homing and multilineage hematopoietic repopulation of non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice transplanted with human cells derived from cord blood (CB), BM and mobilized peripheral blood (MPB).[7] In contrast, another study demonstrated that CXCR4+ and CXCR4- stem cells from cord blood have equivalent engrafting abilities, suggesting that human repopulating function may involve properties other than CXCR4 and SDF-1 interactions.[8] Other factors, such as the role of accessory cells present in the graft in promoting homing and

engraftment, have also been well documented. It is generally accepted that the mechanism by which $CD8^+$ T lymphocytes facilitate engraftment consists in the elimination of residual immune cells in the recipient, but stem cells transplanted into multi-immunodeficient recipients also benefit from accessory cells, suggesting the possibility of other roles for these so-called accessory cells in the graft.[9]

On the other hand, the exposure of stem cells to cytokines, both *in vivo* for mobilization purposes and *in vitro* in expansion cultures, has been described to induce several changes in the expression of AM that hypothetically could result in defects in the cells' homing and/or engraftment potential.[10] We have previously demonstrated that human positively selected $CD34^+$ cells from MPB expanded with stem cell factor (SCF), Flt3-ligand (FL) and thrombopoietin (TPO) engraft NOD/SCID mice at levels comparable to non-cultured cells, while the addition of interleukin-3 (IL-3) and interleukin-6 (IL-6) to the cultures completely abrogates the engraftment ability.[11] The engraftment deficiencies observed in expanded cells by us and others may be due to homing failure, differentiation with concomitant loss of pluripotentiality or both.

Therefore, the mechanisms by which HSC home have been defined only to a limited extent and, at present, the *homing efficiency* seems to be related to several factors including the expression of AM, the presence of accessory cells in the graft, and previous exposure to cytokines *in vivo* or *in vitro*.

In the present work we study the kinetics of expression of CXCR4 and the major AM in HSC during mobilization, positive selection and *in vitro* expansion, as well as the role of these changes in the homing efficiency of HSC to the BM and spleen of NOD/SCID mice. For this purpose, we first compared the respective homing efficiencies of human $CD34^+$ cells from BM, MPB and expansion cultures according to their expression of CXCR4 and AMM, and secondly, we defined the phenotypic adhesive profile of human $CD34^+$ cells that had been able to home to the BM and spleen of 75 transplanted NOD/SCID mice.

Design and Methods

Human cells

Human BM cells and granulocyte colony-stimulating factor (G-CSF)-mobilized peripheral blood cells were obtained after informed consent from either healthy donors for allogeneic transplantation or patients undergoing autografting for non-hematologic malignancies. BM mononuclear cells were obtained by density-gradient centrifugation on Ficoll-Hypaque (Axis Shield, Oslo, Norway). In some experiments mononuclear cells from the apheresis products were used unmanipulated, and in others $CD34^+$ cells were isolated as previously described,[12] using the MACS cell isolation kit and the AutoMACS magnetic cell sorter (Miltenyi Biotec, Bergisch, Gladbach, Germany), according to the manufacturer's instructions.

Expansion cultures

$CD34^+$ selected cells were resuspended at 1×10^5 cells/mL in serum-free medium X-VIVO 15 (BioWhittaker, Walkersville, MD, USA) supplemented with 1% detoxified bovine serum albumin (BSA; Sigma, St. Louis, MO, USA), 2 mmol/L L-glutamine (Sigma), 10^{-4} mol/L 2-mercaptoethanol (Sigma), 100 U/mL penicillin and 100 U/mL streptomycin. Cells were cultured for six days in flat-bottomed 96-well plates with the following cytokine combinations: (i) 3-cytokine combination (3CK): thrombopoietin (TPO; 20 ng/mL; Genzyme, Cambridge, MA, USA), stem cell factor (SCF; 100 ng/mL; R&D Systems, Minneapolis, MN, USA), Flt-3 ligand (FL; 100 ng/mL; R&D Systems); (ii) 5-cytokine combination (5CK): TPO+SCF+FL (at the same doses) + interleukin 3 (IL-3; 20 ng/mL; Genzyme) and interleukin 6 (IL-6; 20 ng/mL; Genzyme). Plates were incubated at 37°C in 5% CO_2 in air.

Adhesion molecule profile of $CD34^+$ cells

Three-color immunofluorescence cytometry was used to quantify the expression of different AM on $CD34^+$ cells from steady-state BM, MPB, positive selection products and cytokine-mediated expansion cultures. Aliquots of $1\text{-}5\times10^5$ cells from mononuclear cells, were washed twice in phosphate-buffered saline + 0.5% w/v bovine serum albumin and 0.2% (w/v) sodium azide (PBA). The cells were then stained with phycoerythrin-cyanin-5 (PE-Cy5) or peridin chlorophyll protein (PerCP) labeled monoclonal antibodies (MoAb) against human CD34 (HPCA-2, Becton Dickinson, San Josè, CA, USA), and the following fluorescein isothiocyanate (FITC) or phycoerythrin (PE)-labeled mouse anti-human MoAbs: anti fusin (CXCR4 clone 12G5), anti-integrin VLA-4 (CD49d, clone 9F10), anti-integrin VLA-5 (CD49e clone IIA), anti-LFA-1 (CD11a, clone G43-25B), anti-LFA-3 (CD58 clone 1C3), anti-ICAM-1 (CD54 clone LB-2), anti PECAM-1 (CD31 clone WM-59), anti- L-selectin (CD62L clone Dreg56), anti-CD43 (clone 1G10) and anti-CD44 (clone L178) (all from Pharmingen, Becton-Dickinson, San Josè, CA, USA). After 10 minutes of incubation in the dark, cells were washed twice in PBA and analyzed immediately after in a dual-laser FACscalibur flow cytometer with Cell-Quest Software. Forward scatter versus side scatter signals were used to discriminate hematopoietic cell populations, erythrocytes and debris. The $CD34^+$ population was gated in a FL3 versus side scatter dot plot. The percentage of cells positive for

adhesion molecules was calculated using appropriate isotype controls. At least 2,000 $CD34^+$ events were collected for analysis.

Transplantation of human cells into NOD/SCID mice

NOD/LtSz-scid/scid mice (NOD/SCID) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed in specific pathogen-free conditions in an isolator and supplied with sterile food and drinking water *ad libitum*. Before transplantation, 8 to 12-week old NOD/SCID mice received a sublethal dose of 310 cGy total-body irradiation (TBI) at 5 cGy/minute using a Gammacell (Nordion International Inc, Ontario, Canada) equipped with a ^{137}cesium source. Mononuclear cells containing 1 - 2×10^6 $CD34^+$ cells from BM, MPB, positive selection products or expansion cultures were injected into the tail vein of 75 mice approximately 2 hours after irradiation.

Analysis of homing in NOD/SCID mice

Mice were killed 24 hours after injection of the human cells. Murine BM cells were flushed from both femora of each mouse using a syringe and a 26-gauge needle, and cell suspensions were obtained from the mice spleen. The percentage and phenotypic profile of human cells were measured in BM and spleen using multiparameter flow cytometry. After counting the total number of cells obtained from both femora and spleen, aliquots were washed twice in PBA and stained for 10 min at room temperature with allophycocyanin-conjugated anti-human CD45 (Clone HIe-1) in combination with the following FITC or PE conjugated anti-human MoAbs: CD34 (clone HPCA-2), anti fusin (CXCR4 clone 12G5), anti-LFA-1 (CD11a, clone G43-25B), anti- L-selectin (CD62L clone Dreg56), and anti-integrin VLA-4 (CD49d, clone 9F10). After two washes with PBA, red blood cells were lysed by adding 2.5 mL of lysis solution (0.155 mol/L NH_4Cl+0.01 mol/L $KHCO_3$+10^{-4} mol/L EDTA). Then, cells were resuspended in PBA + 1μg/mL propidium iodide (PI). At least 50,000 events were acquired and analyzed in a dual-laser FACscalibur flow cytometer with Cell-Quest Software. Non-viable cells were excluded after gating on PI low cells.

All MoAbs were tested in non-transplanted NOD/SCID showing non-cross reactivity. We had previously performed dilution experiments to establish a sensitivity level of 0.02% of human cells (*data not shown*).

Assessment of the homing efficiency of the transplanted cells

The percentages of human $CD34^+$ cells were used to calculate total numbers of human progenitor cells recovered by using direct cell counts from the spleen and adjusted cell counts from the marrow, assuming that 1 femur contains approximately 7% of the total murine BM cellularity.[13] Homing efficiency is expressed as the percentage of $CD34^+$ infused cells that are recovered from the total murine BM or the spleen.

Statistics

All results are reported as mean ± standard error of the mean (SE). Differences between populations of cells were analyzed using a two-tailed Student´s t test. Correlations between pairs of values were established using Pearson's correlation coefficient.

Results

Changes in the expression of AM and CXCR4 during mobilization, selection and expansion

We analyzed the percentage of $CD34^+$ cells expressing AM and CXCR4 in BM, unmanipulated MPB, positively selected cells and expanded cells. Figure 1 shows that the percentages of cells positive for CD11a, CD54, CD58 and CD62L were lower in MPB than in steady-state BM, while the percentages of cells expressing CD31, CD43, CD44, CD49d and CD49e were not significantly different. Positive selection induced an increased percentage of cells expressing CD11a, CD49d and CD58 while no changes were observed in the proportion of cells positive for the remaining AM. After G-CSF mobilization, the percentage of $CD34^+$ cells expressing CXCR4 decreased significantly from 40.6±5.6% in BM to 9.7±2.5% in unmanipulated MPB (p=0.01), and was further reduced after immunomagnetic positive selection to 2.8±0.9% (p=0.021) (Figure 1). Figure 2 shows the changes in the expression of CD11a, CD62-L and CXCR4 in a representative case.

Six days of incubation with TPO+SCF+FL and TPO+SCF+FL+IL3+IL6 produced a significant and similar increase in the percentage of cells expressing CD11a, CD54, and CXCR4 with respect to the preincubation level. The proportion of cells expressing CD62L and CD31 decreased significantly only after culture with the 5CK combination, while the remaining AM were expressed by similar percentages of cells before and after culture regardless of the cocktail of cytokines added.

Besides the changes in the percentage of $CD34^+$ cells expressing CXCR4 and AM, the respective mean fluorescence intensity (MFI), which is related to the number of antigenic determinants on the cell surface, showed exactly the same behavior for CXCR4 and each one of the AM studied: it was significantly lower for MPB than for BM, remain unchanged after the positive selection and showed a significant increase

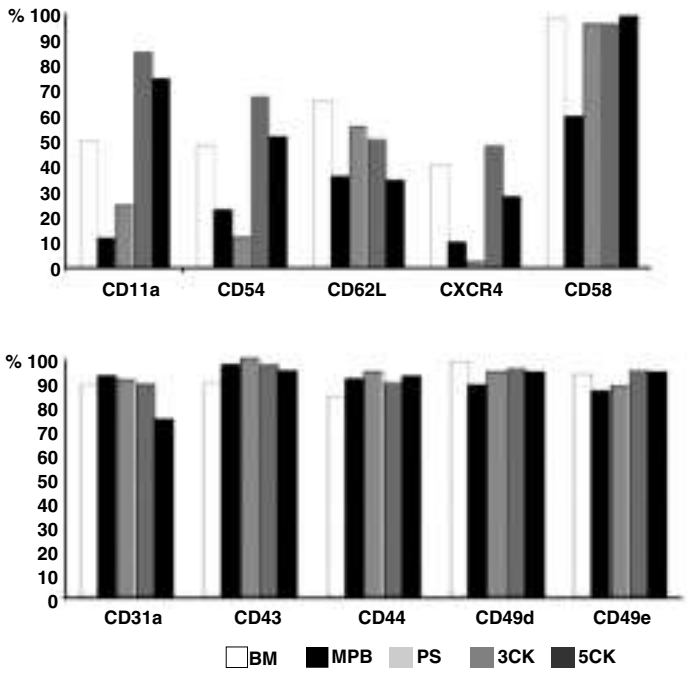

Figure 1. Percentages of CD34⁺ cells from BM, MPB, and after positive selection and expansion cultures, expressing CXCR4 and AM. Compared to steady-state BM, a significantly lower percentage of CD34⁺ cells in MPB express CXCR4 (p=0.01), CD11a (p<0.05), CD54 (p<0.05), CD58 (p<0.001) and CD62L (p<0.05); percentages of cells expressing CD31, CD43, CD44, CD49d and CD49e did not change (p=NS) compared to unmanipulated CD34⁺ cells from MPB. Positive selection induced a significant increase in the percentage of cells expressing CD11a (p<0.001), CD49d (p=0.01) and CD58 (p<0.001) along with a decrease in cells positive for CXCR4 (p<0.05). After culture with TPO+SCF+FL+IL3+IL6 the percentage of CD34⁺ cells expressing CD62L and CD31 decreased (p<0.05 for both markers), while those of cells positive for CD11a, CD54 and CXCR4 were significantly higher (p<0.001); similar increse in the expression of these three AM was induced by culture with TPO+SCF+FL (p<0.001). (n=16).

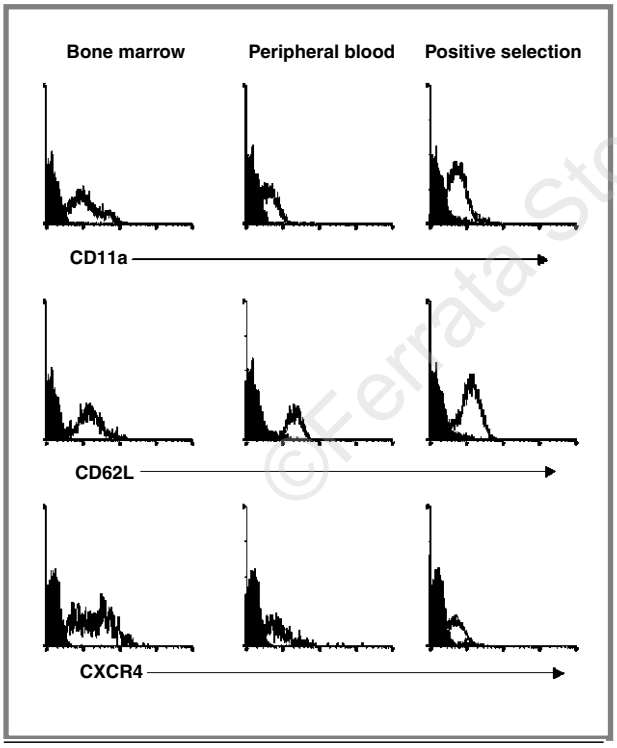

Figure 2. Flow cytometric analysis of CD11a, CD 62L and CXCR4 expression of CD34⁺ cells from BM, MPB, positive selection (PS). The plots show fluorescence intensity (log) (x-axis) vs relative cell number (y-axis) in a representative case.

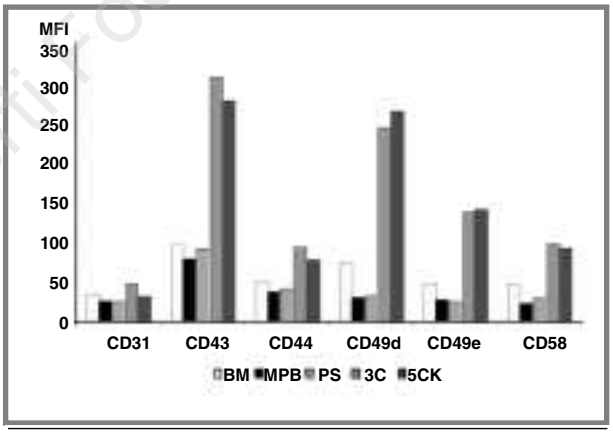

Figure 3. Mean fluorescence intensity (MFI) for AM expression on CD34⁺ cells from BM, MPB, and after positive selection (PS) and expansion cultures with TPO+SCF+FL (3CK) and TPO+SCF+FL+IL3+IL6 (5CK). MFI (arbitrary units) was significantly lower for CD31 (p<0.05), CD43 (p<0.05), CD44 (p<0.05), CD49d (p<0.001), CD49e (p<0.001) and CD58 (p<0.001) in cells from MPB than in BM cells. After culture with 3CK and 5CK, the MFI for all AM was increased (p< 0.05 for CD31 and CD44, and p<0.0001 for CD43, CD49d, CD49e and CD58) (n=16).

after cytokine-induced expansion. Figure 3 shows the changes in MFI of CD31, CD43, CD44, CD49d, CD49e and CD58, indicating that although the percentages of cells expressing these AM were unchanged, their staining intensity was significantly reduced after mobilization, unchanged by positive selection and increased by expansion cultures.

Homing efficiency of CD34⁺ cells according to their source

The homing efficiencies of human BM and MPB CD34⁺ cells to NOD/SCID marrow were respectively 0.5±0.2% and 1.26±0.5% (mean±SE, *p*=NS). The seeding efficiency decreased significantly after positive selection (0.14±0.03%; *p*=0.03). The homing efficiency of 6-day cultured cells seemed to be higher for cells cultured with the 5CK combination (0.43±0.1%) and lower for those cultured with 3CK (0.05±0.05%), in comparison to positively selected uncultured CD34⁺ cells. However, these differences were not statistically significant (*p*=0.19, and *p*=0.27, respectively)

We did not observe any significant differences between the homing efficiencies of the three progenitor sources to the murine spleen (0.07±0.03% for BM, 0.34±0.15 for MPB and 0.11±0.03 for CD34⁺ selected cells).

Homing efficiency of CD34⁺ cells according to the expression of CXCR4 and AM

We investigated whether the homing efficiency of human CD34⁺ cells to murine BM and spleen is related to the percentage of CD34⁺ cells in the graft that co-express individual AM and CXCR4. Regardless of the source of progenitors, we did not find any correlation between the homing efficiency to murine bone marrow and the percentage of CD34⁺ cells in the graft expressing CXCR4 and AM (R=0.19, *p*=0.2 for CXCR4; R=0.07, *p*=0.6 for CD11a; R=0.02, *p*=0.8 for CD31; R=0.2, *p*=0.2 for CD43; R=0.05, *p*=0.7 for CD44; R=0.05, *p*=0.7 for CD49d; R=0.09, *p*=0.9 for CD49e; R=0.2, *p*=0.2 for CD54 and R=0.05, *p*=0.7 for CD62L). Similar results were obtained for the homing efficiency to murine spleen.

To identify the role of the expression of CXCR4 and selected AM on the homing of CD34⁺ cells to NOD/SCID marrow more accurately, we analyzed separately the homing efficiencies of CD34⁺ cells positive for CXCR4 and the major AM and their negative counterparts, based on the total number of positive and negative cells in the inoculum and the total number of positive and negative cells retrieved from murine bone marrow. Figure 4 shows that the homing efficiency of CD34⁺ human cells to murine bone marrow seems to be slightly superior for cells expressing CXCR4 (0.44±0.2%), CD49d (0.04±0.02%), CD62L (0.05±0.03%) and CD11a (0.47±0.3%) than for their negative counterparts (0.23±0.1%, 0.02±0.01%, 0.04±0.02%, and 0.2±0.1%, respectively), but these differences were not statistically significant. Therefore, our data indicate that CD34⁺ cells positive and negative for CXCR4, CD62L, CD11a, and CD49d exhibit identical homing efficiency to NOD/SCID marrow.

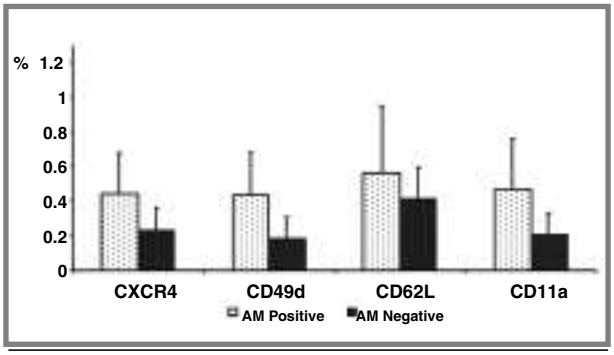

Figure 4. Homing efficiency of human CD34⁺ cells to murine bone marrow according to the expresssion of AM and CXCR4. Results are expressed as mean±SE of the homing efficiency of human CD34⁺ cells positive and negative for CXCR4 and selected AM to the bone marrow of 75 NOD/SCID mice. Data are pooled from all the investigated sources. Comparisons between the homing efficiency of positive and negative cells for the expression of each marker were made using the Student´s t test for paired samples. No statistically significant differences were found.

Expression of AM and CXCR4 by human 'homed' cells

In an attempt to establish the profile of expression of AM on the human cells that had been able to home, we analyzed the co-expression of CXCR4 and the major AM by the human CD34⁺ cells found in murine bone marrow and spleen. For this purpose, human cells retrieved from murine marrow and spleen were analyzed as described in Design and Methods. Figure 5 shows the expression of CXCR4, CD49d and CD62L by human CD34⁺ cells obtained from the BM of a representative mouse. Interestingly, human CD34⁺ cells were mainly CXCR4 negative, both in murine marrow and spleen, regardless of the source of progenitors infused. The mean percentages of human CD34⁺ cells in murine BM and spleen that co-expressed CXCR4, CD49d, CD62L and CD11a are shown in Figure 6. As can be seen, most CD34⁺ cells were CXCR4, CD62L and CD11a negative (only 9.08±2.5%, 9.5±3.1% and 16.6±4% respectively co-expressed these molecules on their surface) while 96.7±1.5% were CD49d positive. This profile of expression is almost identical to that of the human cells which had homed in murine spleen: CXCR4 negative, CD49d positive, CD62L negative and CD11a negative (Figure 6).

Discussion

The results we present here demonstrate several changes in the expression of AM by CD34⁺ cells from BM and MPB before and after positive selection as

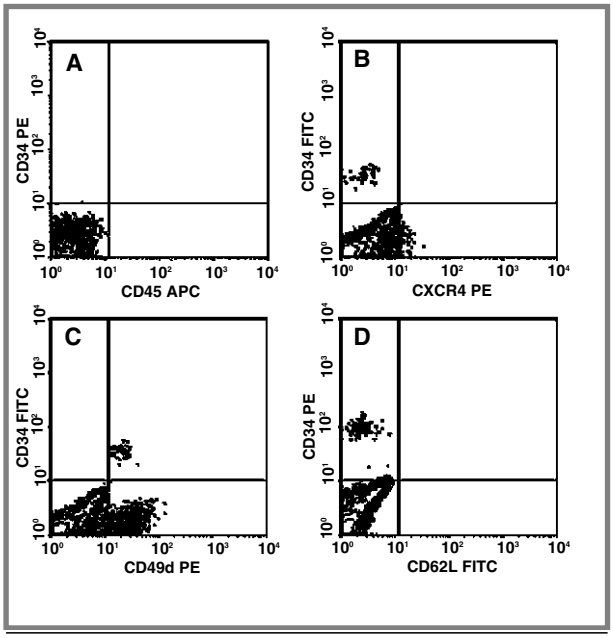

Figure 5. Flow cytometric analysis of the expression of CXCR4 (B), CD49d (C) and CD62L(D) by human CD34 positive cells obtained from the BM of a representative mouse. Human CD34⁺ cells present in both murine marrow and spleen were mainly CD49d positive, CD62L negative and CXCR4 negative, regardless of the source of progenitors infused. Density plot A represents a control mouse that was sacrified after irradiation without transplantation of human cells.

well as after expansion cultures with two cytokine combinations. However, none of these changes seems to be of capital importance in the homing efficiency of CD34⁺ cells to NOD/SCID marrow and spleen, because the respective homing efficiencies of cells positive and negative for the expression of CXCR4 and the major AM were not significantly different. Although there are several reports on the expression of AM by unmanipulated and purified CD34⁺ cells, as well as by *in vitro* expanded cells, these studies did not assess the impact of the observed changes on the *in vivo* homing efficiency.

The observed loss of expression of CD11a, CD54, CD62L, and CXCR4 in CD34⁺ cells from MPB in comparison to BM CD34⁺ cells is not surprising; in fact, the mobilization of HPS from their bone marrow niches into the peripheral circualtion is nowadays understood as a process that involves sequential loss or alteration in the adhesive interactions between progenitors and marrow stromal cells.[5,14-18] This loss of expression of important AM could theoretically decrease the homing ability of MPB, but this fact has never been observed either experimentally or clinically. Indeed, our results reveal that cells from MPB have a homing efficiency slightly higher than that of their BM counterparts although the difference is not statistically significant. The percentage of CD34⁺ cells expressing CXCR4 decreases significantly during the immuno-

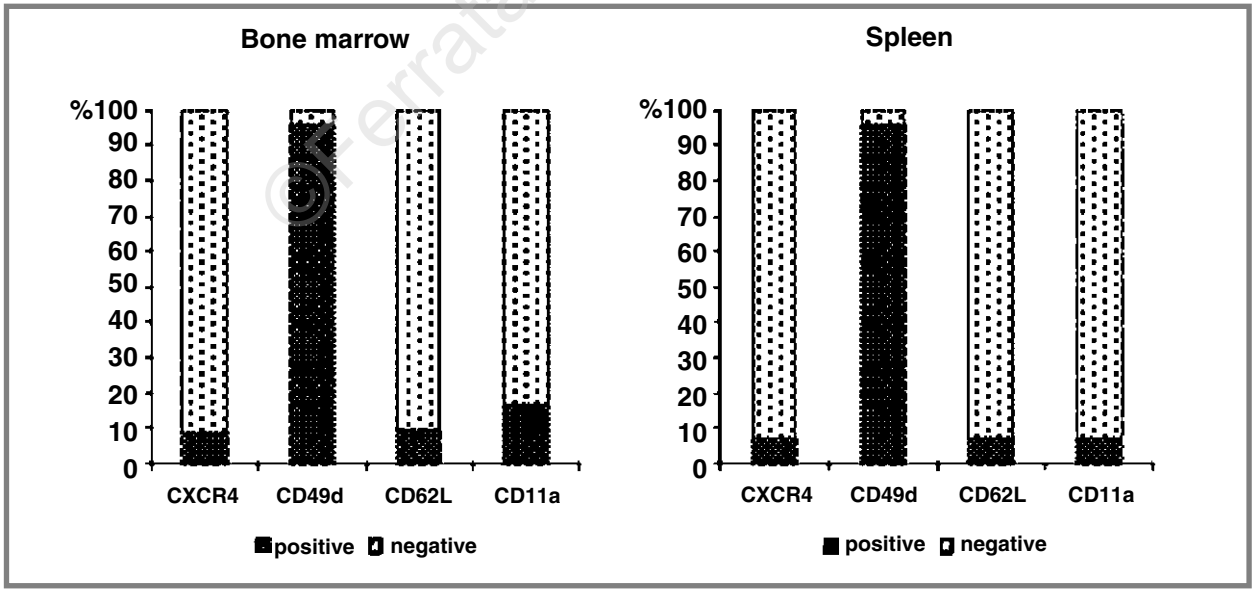

Figure 6. Expression of CXCR4 and AM by human CD34⁺ cells present in marrow and spleen of transplanted NOD/SCID mice. Data are the mean percentage of human CD34⁺ cells in murine BM and spleen that coexpress CXCR4, CD49d, CD62L and CD11a. Human CD34⁺ cells were mainly CXCR4, CD62L and CD11a negative (only 9.08±2.5%, 9.5±3.1% and 16.6±4%, respectively, co-expressed these molecules on their surface) while 96.7±1.5% were CD49d positive. (n=75 mice).

magnetic positive selection, and this phenomenon is obviously due exclusively to the manipulation procedure. Although we did not investigate whether this loss of expression is transient or not, the observation could prompt us to interpret the lower homing efficiency of positively selected CD34+ cells as a consequence of the lower percentage of cells expressing CXCR4. However, our results comparing the homing efficiencies of CXCR4 positive and negative CD34+ cells, as well as those comparing AM positive and negative cells did not demonstrate any significant differences. Therefore, we should conclude that the lower homing efficiency of CD34 selected cells in comparison to that of non-selected cells is not related to the loss of expression of CXCR4 or AM, and that it should probably be explained by the presence of accessory cells in non-selected grafts, as has been suggested by others.[9]

Several studies have examined changes in the expression of AM and CXCR4 after expansion cultures, particularly for HPS from cord blood or BM.[19-23] There are two reports suggesting that the expression of AM on CD34+ cells from CB during expansion cultures is unchanged.[20,21] Contrary data by Chute *et al.*[19] reported a significant increase in the expression of VLA-4, CD58 and L-selectin but not of LFA-1 or CD44 after culturing CD34+ cells from BM with cytokines for 7 days on porcine microvascular endothelial cells. Yet others have found a consistent increase in the expression, measured as the mean staining intensity, of VLA-4, VLA-5, L-selectin, LFA-1 and CD44, as well as a 4.1-fold decrease of CXCR4 expression on CD34+ cells from cord blood cultured with a growth factor combination that sustains repopulating cells.[12] With a different source of hemopoietic progenitors, MPB, and different cytokine combinations, we found a significant increase in the expression of CXCR4 and AM, as reflected by mean fluorescence intensity. The fact that the percentage of CD62L and CD31 positive cells decreased after culture with the 5CK combination could be explained by the greater increase in total cell number observed in these cultures than in those cultured with the 3CK combination, resulting in a higher proportion of immature cells that still do not present CD62L and CD31 on their surface. Alternatively, these AM could be down-regulated directly by IL3, IL6 or both. In any case, this lower percentage of CD34+ cells expressing CD31 and CD62L had no impact on their homing ability, and therefore, is not related to the lack of engraftment of cells cultured with the 5CK combination that we reported previously.[11] The lack of ability of CD34+ cells cultured with determined combinations of cytokines to repopulate NOD/SCID mice has also been reported previously,[24-26] but the causes for this phenomenon should be sought in other factors, such as changes in cellular cycle or cell differentiation into more mature cells.

The expression of CXCR4 on CD34+ cells was shown to be critical for engraftment in mice in a study by Peled *et al.*[7] in which treating human cells with antibodies to CXCR4 prevented engraftment. In contrast the results from Rosu-Myles[8] demonstrated that CXCR4+ and CXCR4− cells are distinct populations of primitive hematopoietic progenitors and, when transplanted into immune-deficient mice, both populations possess comparable engraftment ability *in vivo*. These two studies are obviously contradictory, and data in favor of each of them have been published: genetic evidence of the role of SDF-1 and its receptor CXCR4 is provided by studies in knockout mice,[27-29] in which null mutations of either ligand or receptor are lethal during embryonic development; however, transplantation of CXCR4 -/- fetal liver stem cells into normal recipients permits bone marrow engraftment with eventual defects in myelopoiesis and B-lymphopoiesis.[30,31] In spite of this accumulated evidence, the role played by the interactions SDF-1/CXCR4 in bone marrow homing and engraftment is unclear. In this sense, the results we present here clearly demonstrate that the homing efficiency of CD34+ cells from MPB is not related to the presence of CXCR4 on their surface: the homing efficiencies of CXCR4 positive and negative cells are not significantly different; moreover, approximately 90% of CD34+ cells that have homed to murine marrow and spleen are CXCR4 negative. It could be argued that these human CD34+ cells were initially CXCR4 positive and they became apparently negative after *in vivo* blocking of CXCR4 by its ligand SDF-1; however, this explanation can be excluded, because most human CD34− cells retrieved from murine bone marrow, and obviously also in contact with murine SDF-1, are CXCR4+. To the best of our knowledge, similar results have not been previously published, because the methods used by other authors were different. Several works have employed highly purified CXCR4 positive and negative cells transplanted into NOD/SCID mice to study the engraftment and/or the homing abilities of each subpopulation;[8,32] unfortunately the methods of purification that use monoclonal antibodies for fluorescence activated cell sorting of CXCR4 positive and negative cells uniformly have a problem: the neutralizing activity of anti-CXCR4 could theoretically result in a modified repopulating capacity of the sorted cells.[33-35] To circumvent this problem, we transplanted unmanipulated CD34+ cells, and the homing efficiencies of the cells positive and negative for CXCR4 and AM were calculated from the total number of positive and negative cells infused. Using this method, which is more likely to resemble the process of homing in clinical transplantation, we did

not find any significant differences between the homing efficiencies of progenitor cells positive and negative for the expression of CXCR4 and the studied AM. Therefore, our results clearly demonstrate that human CD34+ cells from MPB are able to home despite the absence of CXCR4 on their cell surface, in agreement with previous studies demonstrating the heterogeneity of CXCR4 expression on human repopulating stem cells.

In summary, our results provide further evidence for the role of both manipulation and exposure to cytokines in inducing changes in the expression of CXCR4 and the major adhesion molecules on the surface of CD34+ cells from peripheral blood. However, none of these changes, individually considered, is of particular value in predicting the homing efficiency of hematopoietic progenitors, which is similar for cells that do or do not express AM and CXCR4.

All persons designated as authors qualified for authorship by contributing to the design and development of the study as well as the interpretation of data. All of them approved the final version of the manuscript. We are indebted to Manuel del Castillo and Mercedes García for their excellent technical assistance.

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