Incorporation of CXCR4 into membrane lipid rafts primes homing-related responses of hematopoietic stem/progenitor cells to an SDF-1 gradient

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We found that supernatants of leukapheresis products (SLPs) of patients mobilized with granulocyte–colony-stimulating factor (G-CSF) or the various components of SLPs (fibrinogen, fibronectin, soluble vascular cell adhesion molecule-1 [VCAM-1], intercellular adhesion molecule-1 [ICAM-1], and urokinase plasminogen activator receptor [uPAR]) increase the chemotactic responses of hematopoietic stem/progenitor cells (HSPCs) to stromal-derived factor-1 (SDF-1). However, alone they do not chemoattract HSPCs, but they do increase or prime the cells' chemotactic responses to a low or threshold dose of SDF-1. We observed that SLPs increased calcium flux, phosphorylation of mitogen-activated protein kinase (MAPK) p42/44 and AKT, secretion of matrix metalloproteinases, and adhesion to endothelium in CD34+ cells. Furthermore, SLPs increased SDF-dependent actin polymerization and significantly enhanced the homing of human cord blood (CB)– and bone marrow (BM)–derived CD34+ cells in a NOD/SCID mouse transplantation model. Moreover, the sensitization or priming of cell chemotaxis to an SDF-1 gradient was dependent on cholesterol content in the cell membrane and on the incorporation of the SDF-1 binding receptor CXCR4 and the small GTPase Rac-1 into membrane lipid rafts. This colocalization of CXCR4 and Rac-1 in lipid rafts facilitated guanosine triphosphate (GTP) binding/activation of Rac-1. Hence, we postulate that CXCR4 could be primed by various factors related to leukapheresis and mobilization that increase its association with membrane lipid rafts, allowing the HSPCs to better sense the SDF-1 gradient. This may partially explain why HSPCs from mobilized peripheral blood leukapheresis products engraft more quickly in patients than do those from BM or CB. Based on our findings, we suggest that the homing of HSPCs is optimal when CXCR4 is incorporated in membrane lipid rafts and that ex vivo priming of HSPCs with some of the SLP-related molecules before transplantation could increase their engraftment. (Blood. 2005;105:40-48)

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Introduction

A better understanding of the mechanisms of hematopoietic stem/progenitor cell (HSPC) homing and engraftment could improve transplantation outcomes. Peripheral blood mobilized by cytokines and chemotherapy has become a major source of HSPCs for autologous transplantation and for an increasing number of allogeneic transplantations.[1-3] Hematopoietic engraftment after transplantation of granulocyte–colony-stimulating factor (G-CSF)–mobilized peripheral blood (mPB) HSPCs is significantly faster than after transplantation of bone marrow (BM) cells not primed by G-CSF, but the reasons for this are not fully understood.[4] Study findings on the expression of adhesion molecules that are essential for hematopoietic engraftment have been inconclusive[5]; however, the higher dose of progenitors in mPB responsible for short-term engraftment is one of the most likely explanations.[4] In addition, we have recently proposed that microvesicles derived from platelets (PMVs) activated during mPB HSPC collection (leukapheresis), as well as the higher expression of matrix metalloproteinases (MMPs) in mobilized cells, may facilitate the engraftment of mPB HSCs.[6,7]

The α-chemokine stromal-derived factor-1 (SDF-1) is secreted by the BM stroma under physiological conditions and during BM regeneration after myeloablative therapy given before transplantation.[8,9] SDF-1 strongly chemoattracts HSPCs, which express the CXCR4 receptor on their surfaces.[10-13] Recently, it has become evident that the SDF-1–CXCR4 signaling axis plays an essential role in the homing and engraftment of HSPCs.[14] SDF-1 also regulates tethering or adhesion to the endothelium and increases the expression of MMPs, other processes that are essential to HSPC homing and engraftment.[6,15,16] We have reported that the responsiveness of HSPCs to an SDF-1 gradient may be positively regulated by PMVs or small molecules, such as complement cleavage fragments (C3a anaphylatoxin), present in supernatants from leukapheresis products (SLPs).[7,17]

Hence, because inflammatory molecules are involved in regulating the responses of HSPCs to SDF-1, we hypothesized in this study that mPB HSPCs are primed for their responsiveness to SDF-1 by molecules present in leukapheresis products, allowing them to better sense an SDF-1 gradient and, therefore, to engraft

more quickly after transplantation. Supporting this contention is the fact that SLPs are enriched in several biologically active molecules, such as fibronectin, fibrinogen fragments, soluble vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1),[18] which, like PMVs,[7] C3a anaphylatoxin,[17] hyaluronic acid,[19] and sphingosine-1 phosphate,[20] may increase the responsiveness of HSPCs to an SDF-1 gradient. Given that the molecular explanation of how these molecules prime the SDF-1–CXCR4 axis has so far not been elucidated, we focused on this phenomenon in our current work.

CXCR4 is a 7-transmembrane, G-protein–coupled receptor, and recent evidence indicates that, for optimal signaling, it must be incorporated into membrane lipid rafts.[21-24] Lipid rafts have recently been shown to orchestrate the interaction of the small GTPases Rac and Rho with their downstream effectors (mDia and PAK, respectively).[25-28] It is believed that the spatially restricted activation of Rho-mDia and Rac-PAK pathways may control local microtubule stabilization and membrane ruffling, respectively; each event is essential to cell migration and adhesion.[22-28] Supporting this idea is the fact that the deletion of Rac1 and Rac2 murine alleles leads to a massive egress of HSPCs into the blood from the marrow, whereas Rac1$^{-/-}$ but not Rac2$^{-/-}$ HSPCs fail to engraft in the bone marrow of irradiated recipient mice.[26]

Thus, in this work, we further hypothesized that the molecular mechanisms of the priming effect we observed depend on increased CXCR4 incorporation into membrane lipid rafts and that cells with more CXCR4 in lipid rafts are better able to interact with small GTPases (eg, Rac-1) and thus better able to sense an SDF-1 gradient. Moreover, because SDF-1 regulates other steps of engraftment in addition to chemotaxis, such as adhesion to endothelium or migration across vascular basement membranes, we examined whether these responses of HSPCs to SDF-1 are also influenced by SLPs. To test the priming of CXCR4 responses, we evaluated the sensitivity of human BM and cord blood (CB) HSPCs to SDF-1 in the absence and presence of SLPs or their components (fibronectin and fibrinogen), and we attempted to determine whether the observed priming effect could be explained by the incorporation of CXCR4 into membrane lipid rafts. We also performed transplantation experiments in mice to evaluate the effects of priming murine and human HSPCs on their homing in vivo.

Patients, materials, and methods

Cells and supernatants from leukapheresis products

Light-density BM cells were obtained from healthy volunteers or unrelated bone marrow donors who had given informed consent; the protocols used were approved by the appropriate institutional ethics review boards. Light-density cells were depleted of adherent cells and T lymphocytes (A$^-$T$^-$ mononuclear cells [MNCs]) and were enriched for CD34$^+$ cells by immunoaffinity selection with MiniMACS paramagnetic beads (Miltenyi Biotec, Auburn, CA), as described previously.[17,29] The purity of isolated BM CD34$^+$ cells was greater than 95%, as determined by fluorescence-activated cell sorter (FACS) analysis using a FACScan (Becton Dickinson, San Jose, CA).

With their informed consent and the approval of the appropriate ethics review boards, leukapheresis products (LPs) were obtained from patients with non-Hodgkin lymphoma who had been mobilized with a standard protocol using G-CSF (Filgrastim; Amgen, Thousand Oaks, CA; 10 μg/kg per day subcutaneously) by means of the Cobe Spectra Apheresis System (COBE, Lakewood, CO). Supernatants were then obtained by centrifugation of the LPs.

Mononuclear CB cells were obtained and for some experiments were enriched for CD34$^+$ cells using immunoaffinity MiniMACS paramagnetic

beads as above.[30] Human hematopoietic cell lines used in this study included MOLT-4, Nalm-6, and THP-1, obtained from the American Type Culture Collection (Manassas, VA) and cultured as described.[15]

In some experiments BM MNCs were obtained from BALB/c mice, and their Sca-1$^+$ cells were isolated as described.[7]

Calcium flux assay

Briefly, cells were incubated for 30 minutes at 30°C with 1 to 2 μM Fura-2/AM (Molecular Probes, Eugene, OR). After incubation, the cells were washed once, resuspended in loading buffer without fetal bovine serum (FBS), stimulated with 3% vol/vol SLPs (combined sample derived from 3 donors) or SDF-1β (500 ng/mL), and analyzed within 1 hour as previously described.[15]

Chemotaxis assay

Cells were resuspended in RPMI with 0.5% bovine serum albumin (BSA) and were equilibrated for 10 minutes at 37°C. Prewarmed serum-free medium containing SDF-1 at low or high doses and SLP or one of the priming molecules to be tested—such as fibrinogen (4 μg/mL), fibronectin (2 μg/mL), urokinase plasminogen activator receptor (uPAR; 1 μg/mL), ICAM (1 μg/mL), VCAM (1 μg/mL) (R&D Systems, Minneapolis, MN), and complement C3a (1 μg/mL) (Calbiochem, San Diego, CA)—were added to the lower chambers of a Costar Transwell 24-well plate (Costar Corning, Cambridge, MA). In some experiments, SLP and priming agents were added instead to the upper chambers 15 minutes before the chemotaxis assay. Aliquots of the cell suspension (1 × 10^5 cells/100 μL) were loaded onto the upper chambers, which were incubated for 3 hours (37°C, 95% humidity, 5% CO$_2$), and cells from the lower chambers were scored using FACS analysis. The results are presented as a chemotactic index (the ratio of the number of cells migrating toward the medium with SDF-1 to the number of cells migrating toward the medium alone). In some experiments, CD34$^+$ cells recovered from the lower chambers were plated in semisolid clonogenic assays supplemented with artificial serum and were stimulated to grow mixed colony-forming unit (CFU-Mix), granulocyte macrophage CFU (CFU-GM), erythroid blast-forming unit (BFU-E), and megakaryocyte CFU (CFU-Meg) colonies, as described elsewhere.[17]

To evaluate the influence of lipid raft formation on chemotaxis, in some experiments cells were preincubated before the chemotaxis assay with molecules that disturb lipid raft formation: 2.5 mM methyl-β-cyclodextran (MβCD), 25 μg/mL nystatin, or 5 μg/mL amphotericin B (Sigma, St Louis, MO), as described.[31-34]

Phosphorylation of intracellular pathway proteins

Western blot analysis was performed on extracts prepared from cell lines and CD34$^+$ cells that had been kept in RPMI medium containing low levels (0.5%) of BSA to render the cells quiescent. Cells were stimulated with SDF-1 (300 ng/mL) in the presence or absence of SLP for 10 minutes at 37°C and then lysed for 10 minutes on ice in M-Per lysing buffer (Pierce, Rockford, IL) containing protease and phosphatase inhibitors (Sigma). Extracted proteins were separated on a 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel and were transferred to a nitrocellulose membrane (Hybond ECL; Amersham Life Sciences, Little Chalfont, United Kingdom). Phosphorylation of 44/42 MAPK and AKT proteins was detected by protein immunoblotting using mouse monoclonal 44/42 phospho-specific MAPK antibody and rabbit phosphospecific polyclonal antibodies (all from New England Biolabs, Beverly, MA), with horseradish peroxidase–conjugated goat antimouse immunoglobulin G (IgG) or goat antirabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) as secondary antibodies, as described.[15,16] Equal loading in the lanes was evaluated by stripping the blots and reprobing them with the appropriate monoclonal or polyclonal antibodies: p42/44 anti MAPK antibody clone 9102 and anti AKT antibody clone 9272 (New England Biolabs). The membranes were developed with an ECL reagent (Amersham Life Sciences), dried, and exposed to film (HyperFilm; Amersham Life Sciences).

Isolation of lipid rafts

Hematopoietic THP-1 cells (5 × 10^7) were lysed in 300 μL MEB buffer (150 mM NaCl, 20 mM MES, pH 6.5) containing 1% Triton X-100 and

protease inhibitors (500 μM phenylmethylsulfonyl fluoride [PMSF] and 5 mM iodoacetamide) for 1 hour on ice. The cells were mixed with an equal volume of 80% sucrose in MEB and were placed at the bottom of a centrifuge tube. Samples were overlain with 30% and 5% sucrose in MEB and were centrifuged at 100 000g for 17 hours. Fractions were gently removed from the top of the gradient, and n-octylglucoside was added to each fraction (60 μM final) to solubilize rafts. To detect ganglioside M1 (GM1), Western blot analysis was carried out using standard techniques with a CXCR4 antibody (Serotec, Oxford, United Kingdom) and cholera toxin subunit B conjugated with horseradish peroxidase (HRP) (Sigma).[32-34]

Confocal analysis

For visualization of lipid rafts, cells were fixed in 3.7% paraformaldehyde/Ca- and Mg-free phosphate-buffered saline (PBS) for 15 minutes and were permeabilized by Triton X-100 in PBS for 5 minutes at room temperature (RT). The primary antibodies used for raft analysis were cholera toxin B–subunit conjugated with fluorescein isothiocyanate (FITC) (Sigma Aldrich, St Louis, MO) and mouse monoclonal anti-hCXCR4 IgG (R&D Systems). After they were rinsed in PBS, the sections were incubated with Alexa Fluor 568 goat antimouse IgG (Molecular Probes) for 45 minutes. Stained cells were examined using a BX51 fluorescence microscope (Olympus America, Melville, NY) equipped with a charge-coupled device camera (Olympus America). Separate pictures were merged using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). Each staining was repeated 3 times on separate samples.

Rac activation assay

THP-1 cells were grown in complete media containing 10% FBS. Before assay (Rac activation assay kit 17-283; Upstate Biotechnology, Lake Placid, NY) cells were kept in RPMI 1640 medium containing low levels (0.5%) of BSA to render the cells quiescent. Cells were then divided and stimulated by optimal doses of SDF-1 (200 ng/mL), fibrinogen (4 μg/mL), or fibronectin (2 μg/mL) for 3 minutes. Treated cells were lysed with Mg^{2+} lysis buffer (MLB) containing 10% glycerol, 10 μg/mL each of leupeptin and aprotinin, and phosphatase inhibitors NaF (25 mM) and Na_2VO_5 (1 mM). Cell lysates were precleared at 14 000 rpm for 5 minutes at 4°C, and protein concentration was determined by Bradford assay. Lysate from each of the treatments was incubated with PAK binding domain–tagged agarose (10 μg) by gentle rocking at 4°C for 1 hour. Agarose beads were then washed 3 times with MLB and suspended in LDS sample buffer. The GTP-bound form of Rac was detected through Western blot analysis using monoclonal Rac antibody.

Actin polymerization assay

Cells were washed 3 times and were resuspended in assay medium (RPMI with 0.5% BSA) at a concentration of 10^6 cells/mL. Before the experiment, cells and ligands were incubated separately at 37°C for 10 minutes. Ligands were added to the cell suspension, and, at indicated time points, 100 μL cell solution was transferred to the fixation solution (Cytofix/Cytoperm; Phar-Mingen, San Diego, CA). Subsequently, cells were incubated in the fixation solution for 20 minutes. Thereafter, cells were centrifuged and resuspended in 100 μL permeabilization reagent (Perm/wash buffer; PharMingen). After 5 minutes of incubation in this solution, 1 U/mL Alexa 488 phalloidin (Molecular Probes) was added to visualize the F-actin. After 20 minutes, the cells were centrifuged and resuspended in PBS with 0.5% BSA, and MFI was measured by FACScan (Becton Dickinson).

trans-Matrigel migration assay and zymography

Migration of CB and BM CD34$^+$ cells was evaluated after their incubation with SLP using the *trans*-Matrigel assay as described previously.[6,17,35] Briefly, polycarbonate filters (13-mm diameter, 8-μm pore size) coated with 25 μg Matrigel were placed between the upper and lower compartments of modified Boyden chambers. CB and BM CD34$^+$ cells that had been preincubated with or without SLP (obtained from 2 patients) were loaded into the upper compartments (2 $\times$ 10^5 cells/chamber) and were incubated for 3 hours at 37°C and 5% CO$_2$. Cells that invaded the Matrigel barrier

toward media (control, Iscove modified Dulbecco medium [IMDM] with 0.1% BSA) or toward SDF-1 gradients (20 or 300 ng/mL) were recovered from the lower compartments and were counted using a Neubauer hemocytometer (Carl Zeiss, Jena, Germany). The *trans*-Matrigel migration index was calculated based on the ratio of the number of cells crossing the Matrigel toward SDF-1 gradients to the number of cells migrating toward media (control). Each experiment was performed at least twice using 4 chambers for each condition.

MMP-2 and MMP-9 activities were evaluated using zymography, as previously described.[6,15-17,35] Briefly, CB and BM CD34$^+$ cells (2 $\times$ 10^6/mL) were incubated for 48 hours at 37°C and 5% CO$_2$ in serum-free media in the absence (control) or presence of SLPs (from 2 patients). Cell-conditioned media were collected and analyzed, and the intensity of the bands in the zymograms was quantitated using Kodak Digital Science ID Image Analysis software (Eastman Kodak, Rochester, NY). Fold-stimulation of MMP secretion was calculated relative to the control.

Adhesion to HUVECs

Before the adhesion assay, CB CD34$^+$ cells were pretreated with SLPs (5% vol/vol), SDF-1β (1 μg/mL) or SDF-1 + SLPs. Cells were labeled with the fluorescence dye calcein-AM and was subsequently added (for 5 minutes) to 96-well plates covered by human umbilical vein endothelial cells (HUVECs). After the nonadherent cells had been discarded, the cells that adhered to the HUVECs were counted under a microscope, as described.[36]

Homing assay

For transplantation experiments, BALB/c or NOD/SCID mice (Jackson Laboratory, Bar Harbor, ME) were irradiated with a lethal dose (1000 cGy) of γ-irradiation. After 24 hours, the BALB/c or NOD/SCID mice underwent transplantation (by tail-vein injection) with 10^5 murine BM MNC Sca-1$^+$ cells or 10^6 human BM or CB CD34$^+$ cells, respectively. Recipient mice were killed 16 hours after cell injection, and BM MNCs were isolated from the femurs as described.[17] Day-12 spleen cell CFU (CFU-S) in BM cavities of mice that underwent transplantation with murine Sca-1$^+$ cells was evaluated through the secondary transplantation of cells recovered from the femurs of primary recipients into lethally irradiated syngeneic mice. Numbers of CFU-GMs (murine and human) in the femurs of mice after transplantation were evaluated by performing clonogenic assays in serum-free methylcellulose cultures with cells recovered from the femurs of mice that underwent transplantation, as described.[7,17]

Statistical analysis

Arithmetic means and standard deviations were calculated on a Macintosh Powerbase 180 computer, using Instat 1.14 (GraphPad, San Diego, CA) software. Statistical significance was defined as $P < .01$. Data were analyzed using the Student t test for unpaired samples.

Results

SLPs and their selected components prime/enhance HSPC chemotactic responses to an SDF-1 gradient

To test the hypothesis that HSPCs are primed in their responses to SDF-1 by molecules present in LP, we pooled SLPs from 3 patients and exposed BM CD34$^+$ cells to the sample during chemotaxis to a low (10 ng/mL) dose of SDF-1. We found that CD34$^+$ cells placed in the upper chambers with SLPs loaded in the upper or the lower chambers responded significantly better to the low dose of SDF-1 than CD34$^+$ cells without SLPs, reaching approximately 45% of their maximal chemotactic response achieved with a high (optimal) dose of SDF-1 (300 ng/mL) (Figure 1A). SLPs on their own only slightly chemoattracted CD34$^+$ cells and did not increase chemotaxis of CD34$^+$ cells to a high dose of SDF-1 (Figure 1A). Because a population of CD34$^+$ cells is heterogeneous and clonogenic
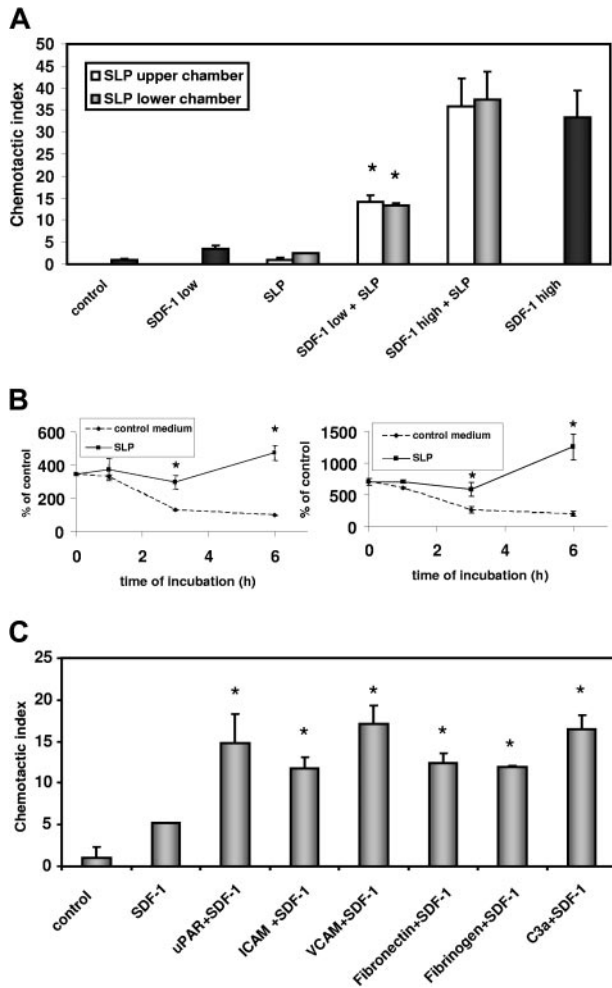
Figure 1. Effect of SLPs and components on chemotaxis of CD34+ cells.
(A) Chemotaxis of BM CD34+ cells toward medium alone (control), SDF-1 low (10 ng/mL) alone, SLPs (combined SLPs from 3 patients), SDF-1 low + SLP, and SDF-1 high (300 ng/mL). SDF-1 was always added to the lower chamber. Data are pooled from quadruplicate samples from 3 independent experiments (*$P < .00001$). (B) mPB CD34+ cells were kept in SLPs (solid line) or were washed out of SLPs and resuspended in control medium (dashed line). At set time points, cells were isolated from the samples and assayed for their responsiveness to a low dose of SDF-1 (30 g/mL) in chemotaxis assays. After chemotaxis, cells were collected from the lower chambers, cells were counted (left panel), and clonogenic CFU-GMs collected from the lower chambers were assayed in secondary methylcellulose cultures (right panel). Data are pooled from quadruplicate samples from 3 independent experiments (*$P < .00001$). (C) Chemotaxis of CD34+ cells to medium alone (control), SDF-1 low (10 ng/mL) alone, and SDF-1 low (10 ng/mL) + uPAR (1 μg/mL) or + ICAM (1 μg/mL) or + VCAM (1 μg/mL) or + fibronectin (2 μg/mL) or + fibrinogen (4 μg/mL) or + C3a (1 μg/mL). Data are pooled from quadruplicate samples from 3 independent experiments (*$P < .00001$).

HSPCs account for only approximately 3% to 5% of it,[37] we investigated whether SLPs could also prime the chemotactic responses of clonogenic progenitors to SDF-1. After chemotaxis, CD34+ cells were collected from the lower chambers and were plated to grow CFU-GM, BFU-E, and CFU-Meg colonies. We found that SLPs efficiently increased the chemotactic responses of clonogenic CD34+ progenitors and that heat inactivation of SLPs significantly decreased (by approximately 50%) the priming effect with respect to a low dose of SDF-1 (not shown).

To evaluate whether mPB CD34+ cells remain in a primed status when they are resuspended in SLPs, in the next set of experiments mPB CD34+ cells were maintained in SLPs or were washed out of the SLPs and resuspended in fresh leukapheresis buffer (Figure 1B). We observed that the priming responses of mPB

CD34+ cells and of CD34+ clonogenic CFU-GMs to the low threshold dose of SDF-1 decreased to control values after cells were washed from the SLPs.

Moreover, because SLPs contain several components that may prime the responses of CD34+ cells—the most important of which are PMV,[7] activated complement proteins such as anaphylatoxin C3a,[17] soluble VCAM-1, soluble ICAM-1, uPAR, fibrinogen, and fibronectin[18]—we evaluated whether these factors, when used alone, could have induced the chemotaxis of HSPCs and found no evidence that they did (not shown). Given that we previously showed that PMV[7] and C3a[17] prime the response of HSPCs to SDF-1, here we examined whether fibrinogen, fibronectin, soluble VCAM-1, soluble ICAM-1, and uPAR also prime HSPCs in their responses to SDF-1 using C3a as the positive control. We found that when these molecules were added to the lower chambers together with SDF-1, they increased the chemotactic response of CD34+ cells to SDF-1 (Figure 1C). To determine whether this also affects CD34+ clonogenic progenitors, CD34+ cells were collected from the lower chambers after the chemotaxis assay and were plated in methylcellulose cultures. We found that, like SLP, all these compounds increased the SDF-1–directed chemotaxis of clonogenic myeloid (CFU-GM), erythroid (BFU-E), and megakaryocytic (CFU-Meg) progenitors. Moreover, a similar effect for the established hematopoietic cell lines Molt 4, Nalm-6, and THP-1 was also found (data not shown).

SLP induces calcium flux, phosphorylation of AKT and MAPK p42/44, and actin polymerization

In another set of experiments, we attempted to elucidate the molecular mechanisms by which SLPs enhance the responsiveness of hematopoietic cells to an SDF-1 gradient. BM CD34+ cells were cultured in SLP or control medium (mock buffer used for leukapheresis), and the influence of this incubation on the expression of CXCR4 was evaluated after 0, 12, and 24 hours using FACS analysis, with negative results (not shown). Then we examined whether the priming molecules affect the internalization of CXCR4 in cells stimulated by SDF-1 but found no effect (not shown). Next we performed signaling studies that revealed that SLPs alone (3% vol/vol) induced calcium flux (Figure 2A) and phosphorylation of AKT and MAPK p42/44 in CD34+ cells (Figure 2B). The effect on phosphorylation was slightly greater when SLPs were combined with SDF-1 (Figure 2B). Similarly, we observed that actin polymerization of CD34+ cells to a low dose of SDF-1 was enhanced in the presence of SLPs (Figure 2C).

Incorporation of CXCR4 into lipid rafts is responsible for the priming effect in normal human BM CD34+ cells

Because it has been demonstrated that optimal signaling from CXCR4 correlates with its presence in lipid rafts,[21-24] we asked whether the priming molecules we were investigating modulated the incorporation of CXCR4 into lipid rafts. Given that lipid raft formation is perturbed by polyene antibiotics (eg, nystatin, amphotericin B), we first examined whether these compounds inhibited the chemotaxis of CD34+ cells to an SDF-1 gradient. We found that both compounds, used at doses not toxic to the cells, significantly inhibited SDF-1–mediated migration of CD34+ cells and CD34+ clonogenic progenitors, confirming that the presence of CXCR4 in lipid rafts is crucial for the chemotactic response of these cells (Figure 3A).

To address this issue further, the formation of rafts in the cells was perturbed by MβCD, which depletes cholesterol from the cell
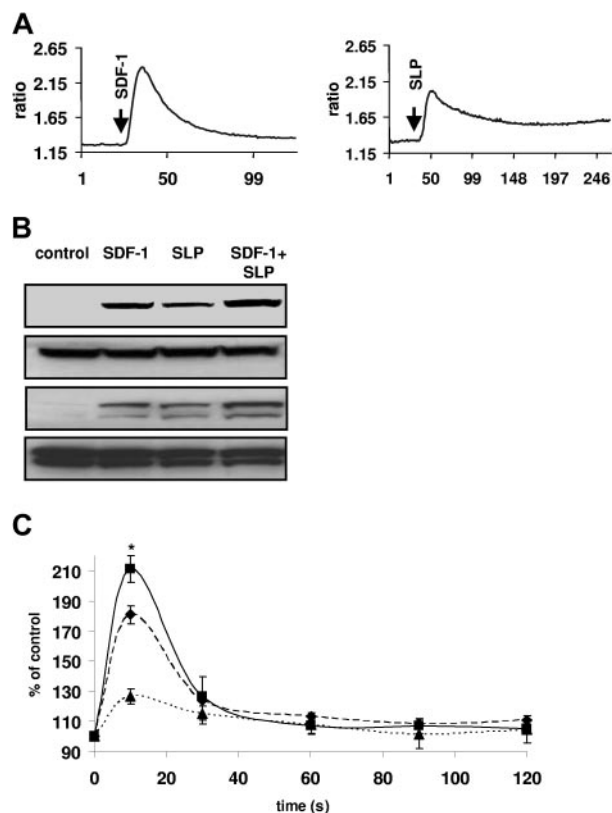
Figure 2. SLPs induce calcium flux, phosphorylation of AKT and MAPK p42/44, and actin polymerization. (A) Calcium flux studies of Fura-2–loaded normal human CD34^{+} cells. Cells were stimulated with 3% vol/vol SLP or SDF-1β (500 ng/mL), and subsequently calcium flux was evaluated using a spectrophotofluorimeter. Data presented are of a representative experiment that was repeated 3 times and yielded similar results. (B) SLP-induced phosphorylation of AKT and MAPK p42/44. BM CD34^{+} cells were stimulated by SDF-1 (200 ng/mL, lane 2), by SLPs (3% vol/vol), or by SDF-1 (200 ng/mL) + SLP (3% vol/vol). (Lane 1) Nonstimulated cells. The experiment was repeated twice and yielded similar results. (C) Effect of SLPs on SDF-1–induced formation of filamentous actin. Purified CB CD34^{+} cells were stimulated with SDF-1 (100 ng/mL), 5% vol/vol SLPs, or both, and the formation of filamentous actin was analyzed by flow cytometry. Data are pooled from 3 independent experiments. *$P < .001$.

membrane.[21-24] The CD34^{+} cells were preincubated with MβCD for 1 hour, and chemotaxis to a low dose of SDF-1 in the presence of fibronectin and fibrinogen (in the upper chambers) was evaluated. We found that preincubating CD34^{+} cells with MβCD inhibited by approximately 60% the priming effect of fibrinogen or fibronectin (Figure 3B). Consistent with this observation, confocal-like microscopy analysis revealed that priming of hematopoietic THP-1 cells with fibrinogen or fibronectin increased the incorporation of CXCR4 into ganglioside M1 (GM1)–enriched lipid rafts (not shown). More important, by using a similar strategy, we observed that CXCR4 is present in membrane lipid rafts in mPB CD34^{+} cells, and its association with membrane lipid rafts ceased when mPB CD34^{+} cells were washed out of the SLPs and were resuspended in a control buffer (Figure 3C).

CXCR4 in lipid rafts interacts with and activates Rac-1

Direct evidence of the effect of priming agents on CXCR4 incorporation into membrane lipid rafts was obtained from Western blot analysis of CXCR4 expression in various fractions of cell membranes isolated according to their lipid raft content (Figure 4). We found that stimulation of hematopoietic THP-1 cells by fibronectin or fibrinogen stimulates the incorporation of CXCR4
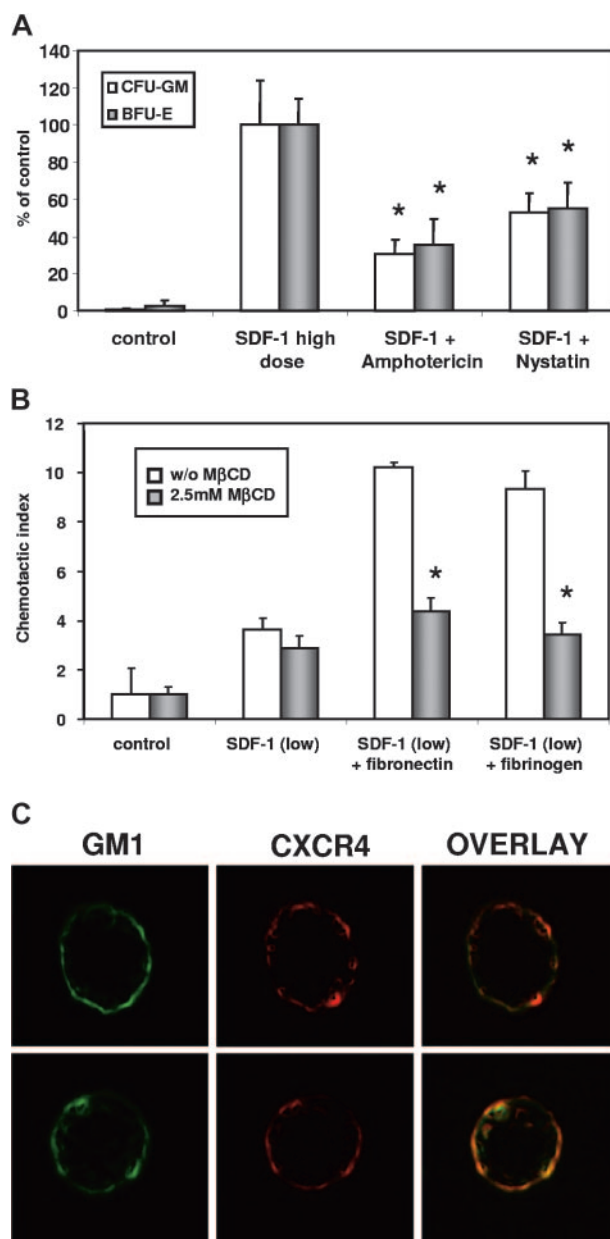

Figure 3. Chemotaxis of cells to an SDF-1 gradient is lipid raft dependent. (A) Chemotaxis of CD34^{+} cells to medium alone (control), SDF-1 (300 ng/mL), and SDF-1 (300 ng/mL) after preincubation for 1 hour with amphotericin (10 μg/mL) or nystatin (50 μg/mL). Cells were collected after chemotaxis from the lower chambers and were plated in clonogenic assay. The number of CFU-GM and BFU-E colonies formed by harvested cells is shown as a percentage of control values. Data are pooled from quadruplicate samples from 3 independent experiments. *$P < .0001$. (B) Chemotaxis of CD34^{+} cells toward medium alone (control), SDF-1 low (10 ng/mL) alone, or SDF-1 low with cells exposed to fibronectin (2 μg/mL) or fibrinogen (4 μg/mL). Cells pretreated before chemotaxis for 1 hour with MβCD (2.5 mM) are shown as red bars. Data are pooled from quadruplicate samples from 3 independent experiments. *$P < .00001$. (C) Lipid raft formation on CD34^{+} cells. mPB CD34^{+} cells were kept in SLPs (lower panel) or were washed out of SLPs (upper panel) and resuspended in control medium. Primary antibodies used for raft analysis were cholera toxin β-subunit conjugated with FITC and mouse monoclonal anti-hCXCR4 IgG. Stained cells were examined using a BX51 fluorescence microscope (Olympus America, Melville, NY) equipped with a charge-coupled device camera (Olympus America). Separate pictures are merged using Image-Pro Plus software (Media Cybernetics Inc., Silver Spring, MD). Lipid raft formation was analyzed on samples from 3 patients who underwent mobilization with G-CSF. Results from a representative study are shown. Colocalization of GM1 and CXCR4 is shown as yellow patchy staining. Magnification × 60.
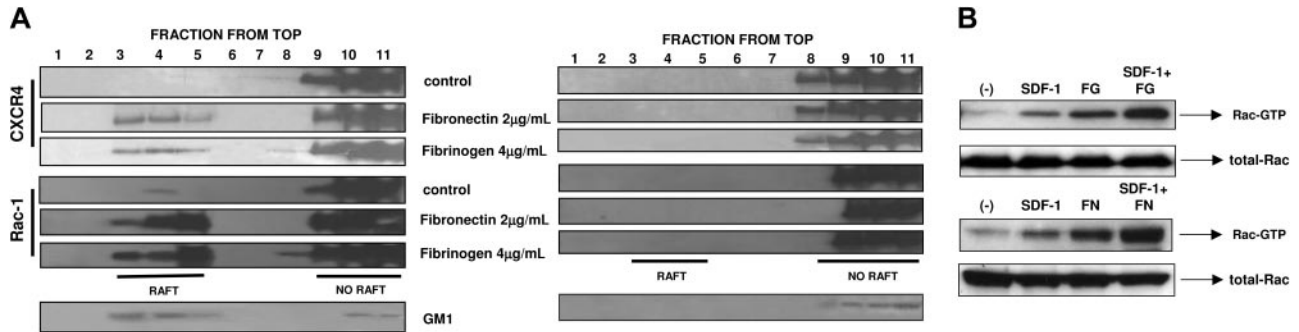
Figure 4. Western blot analysis of the colocalization of CXCR4 with Rac-1 in various fractions of cell membranes. Membranes enriched in lipid rafts (fractions 3-5) and depleted of lipid rafts (fractions 9-11). (A) Hematopoietic THP-1 cells were stimulated with fibronectin (2 μg/mL) or fibrinogen (4 μg/mL) or were not stimulated (control). CXCR4 and Rac-1 were detected in these membrane fractions by Western blot along with ganglioside GM1, a marker of lipid rafts. Experiments were performed 3 times with similar results. (B) In control experiments, cells were pretreated for 1 hour with 10 mM MβCD. A representative study is shown. (C). Detection of GTP-bound (active) form of Rac in THP-1 cells nonstimulated (−) or stimulated with SDF-1, fibrinogen (FG), or SDF-1 + FG (left panel) or with SDF-1, fibronection (FN), or SDF-1 + FN (right panel). Activated Rac was detected in lysates of stimulated cells by using Western blot, as described in "Patients, materials, and methods." Experiments were performed 3 times with similar results. Representative results are shown.

into GM1-enriched membrane lipid rafts (fractions 3-5) (Figure 4A). At the same time, we observed that priming of THP-1 cells with fibronectin or fibrinogen increased the incorporation of Rac-1, a small GTPase that is crucial for cell migration and adhesion,[22-28] into membrane lipid rafts (Figure 4B). The interaction between CXCR4 and Rac-1 resulted in enhanced activation of Rac-1 in the presence of a low dose of SDF-1 (Figure 4C). Thus, certain SLP-related molecules increase the incorporation of CXCR4 and Rac-1 into membrane lipid rafts, thereby promoting Rac activation and resulting in enhanced sensitivity and responsiveness of hematopoietic cells to an SDF-1 gradient.

SLPs increase the adhesion of CD34+ cells to HUVECs

Next we examined the effects of priming agents on other processes essential for the engraftment of HSPCs. HSPCs must adhere to the endothelium of BM sinuses before they can migrate and reach niches in the hematopoietic microenvironment. To mimic this process in vitro, we evaluated the influence of SLPs (5% vol/vol) on the adhesion of CB CD34+ cells to HUVECs. Figure 5 shows that SLP increased the adhesion of CB CD34+ cells to HUVECs. When SLPs were used together with SDF-1 (1 μg/mL), we observed a slight, though not statistically significant, increase in the adhesion of CD34+ cells.

SLPs increase trans-Matrigel migration and MMP secretion

Subsequently, we evaluated whether SLPs could promote the migration of CB and BM CD34+ cells across the subendothelial basement membrane, a process in which MMPs play a crucial role.[6,35] To address this question, we performed a trans-Matrigel migration assay and found that SLP obtained from 2 patients significantly increased the trans-Matrigel migration of CB and BM CD34+ cells to a lower dose (20 ng/mL) of SDF-1 (Figure 6A). We found that SLP-primed BM cells responded comparably to unprimed cells that were exposed to SDF-1 at a high dose (300 ng/mL). Furthermore, we found that SLPs obtained from 2 patients[1,2] increased MMP-9 and MMP-2 secretion by CB and BM CD34+ cells (Figure 6B).

SLPs enhance homing of HSPCs to bone marrow

As a final proof of our hypothesis and to test whether the primed cells home better to BM, we primed murine BM-derived Sca-1+

and human BM- and CB-derived CD34+ cells with SLPs before their transplantation into lethally irradiated mice. Sixteen hours after transplantation, recipient mice were killed, and cells were recovered from their marrow cavities. These cells were subsequently assayed in in vitro clonogenic assays for the number of clonogenic murine and human CFU-GMs that homed to the BM of BALB/c and NOD/SCID mice, respectively, and they were assayed for the number of murine CFU-Ss that formed day 12 colonies in the spleens of lethally irradiated secondary recipients. Figure 7 shows that murine CFU-GMs and CFU-Ss (Figure 6A) and human CFU-GMs (Figure 6B) homed better to the BM of lethally irradiated recipient mice when they were preincubated in SLPs for 30 minutes before transplantation. Of note, no CFU-GMs or CFU-Ss were recovered from the femurs of lethally irradiated control animals that did not receive hematopoietic cells.
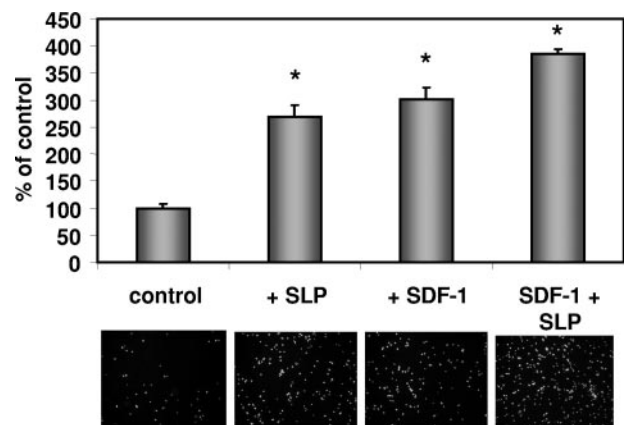

Figure 5. Stimulation of CB CD34+ cell adhesion to HUVECs by SLPs. Calcein-AM–labeled CD34+ cells were not preincubated (left panel) or were preincubated with SLPs (3% vol/vol) (right panel) or with SDF-1 and subsequently were layered over HUVECs. After 30 minutes, nonadherent cells were removed, and adherent cells were counted using a spectrophotofluorimeter. (Upper panel) Relative fluorescence intensity. (Lower panel) Inverted microscope analysis of fluorochrome-labeled adhered cells. Cells were examined using Nikon Eclipse TS100 inverted microscope equipped with ELWD epifluorescence attachment (Nikon Instruments Inc., NY, USA), and digital images were captured using an Olympus DP10 camera (Olympus America). Original magnification 100 × (objective 20 ×, N.A 0.45). Data from 3 separate experiments were pooled together. Values shown are mean ± SD. *As compared with SDF-1 low dose.
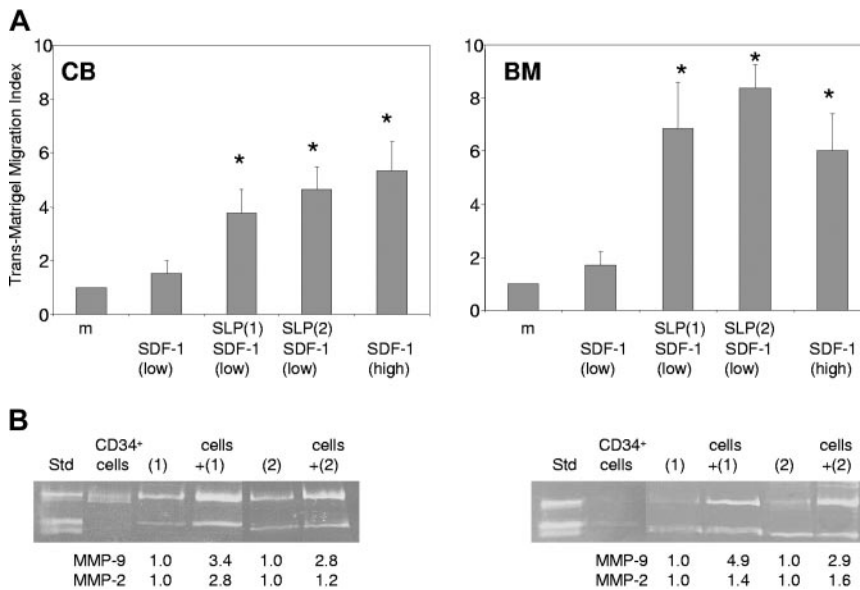
Figure 6. Stimulation of SDF-1–dependent *trans*-Matrigel migration of CB and BM CD34+ cells and MMP secretion by SLPs. (A) CB and BM CD34+ cells preincubated with SLPs from 2 patients[1,2] were loaded into Boyden chambers and allowed to migrate toward a low (20 ng/mL) SDF-1 gradient in a *trans*-Matrigel assay. Unstimulated cells migrated across Matrigel toward media alone (control) or toward low or high (300 ng/mL) SDF-1 gradients. Each experiment was performed in triplicate using 4 chambers for each condition (*P < .0003). (B) Media conditioned by CB and BM CD34+ cells preincubated with SLP from patients 1 and 2 were analyzed by zymography. Bands indicating MMP-9 and MMP-2 activity were quantitated by densitometric analysis, and fold-increase in the presence of SLP was calculated relative to the control (SLP alone). Each experiment was performed 3 times, and representative data are shown.
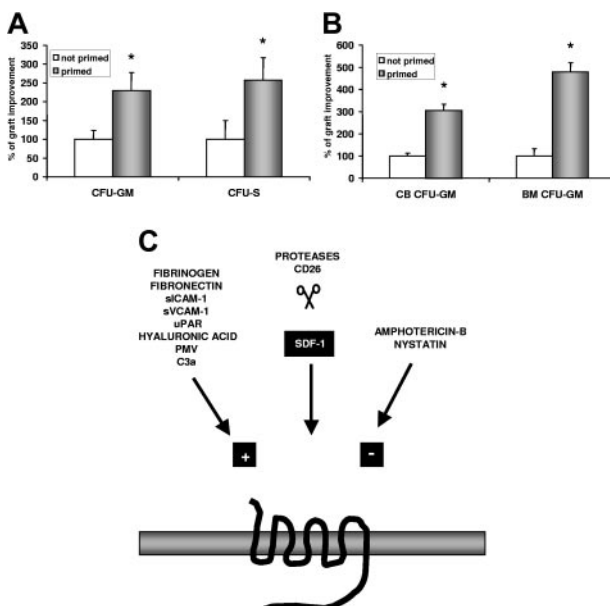

Figure 7. Priming of HSPC as a new strategy to enhance the efficiency of transplantation. (A) SLPs primed homing of murine CFU-GM and CFU-S into bone marrow of lethally irradiated mice. Murine BM-derived Sca-1+ cells (10^5/mouse) were transplanted into lethally irradiated syngeneic mice. Sixteen hours after transplantation, cells were recovered from the femurs and were assayed in secondary cultures for the number of clonogenic CFU-GMs (left panel) and for transplantation into secondarily irradiated animals for the number of day-12 CFU-Ss (right panel). Data are pooled from 3 independent experiments using 10 mice each per tested cells (n = 30) (*P < .0001). No HSPCs were recovered from the marrow cavities of control animals (irradiation; no transplantation). (B) SLPs primed homing of human CB and BM CD34+ cells into the bone marrow of lethally irradiated NOD/SCID mice 16 hours after transplantation of human CD34+ cells (10^6)/animal. Mice were killed, and CFU-GM colonies were assayed in methylcellulose cultures. Data are pooled from independent experiments performed on 3 different CB and 3 different BM samples (2-4 mice/tested cell sample) (P < .0001). (C) Schema of SDF-1–CXCR4 axis modulation by various factors. The SDF-1–CXCR4 axis is modulated by various external factors that target, on the one hand, SDF-1 or the N-terminus of CXCR4, which are cleaved by leukocyte-derived proteases, MMPs, or cell surface–expressed CD26/dipeptidylpeptidase IV, and, on the other hand, the SDF-1–CXCR4 axis, which can be primed positively (eg, by PMV, C3a, des-Arg C3a, thrombin, uPAR, fibrinogen, fibronectin, hyaluronic acid, soluble ICAM-1, soluble VCAM-1) or negatively (eg, by polyene antibiotics). Several of these molecules can also be found in tissues affected by inflammation and can modulate the responsiveness of CXCR4+ cells to an SDF-1 gradient.

Discussion

Only a few options are available to accelerate hematopoietic reconstitution after transplantation, and these are used to harvest greater numbers of HSPCs, their ex vivo expansion before transplantation, or possibly their intramedullary injection.[1-3] Unfortunately, the number of HSPCs available for allogeneic or autologous transplantation is often limited (eg, from CB or the blood of poor mobilizers), and the same is true of HSPCs expanded ex vivo using current strategies.[38] Techniques to improve engraftment by direct injection of HSPCs into BM are still at the experimental stage in rodents and have not yet proven effective in a large animal model.[39,40]

Based on the principle that HSPCs can be primed with certain recombinant molecules we have identified here, we now propose a new approach to enhancing hematopoietic recovery in patients after transplantation. We present evidence that HSPCs primed by SLPs or some of their components (eg, soluble fibrinogen or fibronectin, uPAR, ICAM, and VCAM) better sense and respond to an SDF-1 gradient, a chemoattractant that is crucial for their homing. This finding is consistent with our previous observations that the chemotactic responses of HSPCs to SDF-1 could be primed or enhanced by certain factors, such as PMV or C3a anaphylatoxin.[7,29] We also reported that murine HSPCs that had been primed before transplantation by PMV[7] or C3a anaphylatoxin[17,29] engrafted more quickly in lethally irradiated mice, though we could not suggest a molecular explanation for this phenomenon at the time. Similarly, enhancement of cell responsiveness to SDF-1 in vitro and in vivo was also recently described for hyaluronic acid,[19,41] L-selectin,[42] and sphingosine-1 phosphate.[20] Again, no molecular explanations for these phenomena were given except the suggestion that L-selectin may inhibit the internalization of CXCR4 from the cell surface.[42] However, in our current study, molecules present in SLP did not affect the level of CXCR4 expression or its internalization after stimulation with SDF-1.

The most important finding of our current study is the observation that the priming effect of the SDF-1 response occurs through increased incorporation of CXCR4 into membrane lipid rafts. Several reports have suggested that for the most effective signaling,

CXCR4 must be incorporated into lipid rafts.[21-24] The 2 molecules (fibrinogen and fibronectin) we investigated here bind to various receptors and adhesion molecules present on the cell surface,[43] and we suggest that this interaction is crucial for increasing the incorporation of CXCR4 into membrane lipid rafts. Moreover, we found that this interaction was also crucial for the inclusion in lipid rafts and for subsequent activation of Rac-1, a small GTPase involved in the migration of HSPCs.[25-28] In support of this notion, it has been demonstrated that Rac-1$^{-/-}$ mice have an increased number of circulating HSPCs in peripheral blood[26] and that their HSPCs do not engraft in lethally irradiated littermates.[26] Hence, our data provide the first evidence that, during priming, CXCR4 and Rac-1 assemble together in membrane lipid rafts on the cell surface, an apparently critical step for the optimal chemotactic response of HSPCs to an SDF-1 gradient. The elucidation of other molecular events that regulate formation of the chemotactic synapse in hematopoietic cells requires further study. It has been suggested that the activation of cell-surface integrins may be crucial for this phenomeon.[25-28] Supporting this concept is our finding that all the molecules investigated in our (fibrinogen, fibronectin, and PMV) and others' (hyaluronic acid) studies interact with various integrin receptors expressed on the surface of the HSPC.

We also provide evidence that although CXCR4 is present in membrane lipid rafts in CD34$^+$ cells freshly isolated from leukapheresis products, its association with lipid rafts decreases after the cells have been washed out of the SLPs. This reversibility of the association of CXCR4 with lipid rafts, and the fact that CXCR4 expression tends to be lower on mPB CD34$^+$ cells than on BM- or CB-derived cells, may explain why mPB CD34$^+$ cells do not respond as well to an SDF-1 chemotactic gradient[44] and engraft less efficiently in NOD/SCID mice.[45]

Thus, a new and more complex picture of the mechanisms that regulate the activity of the SDF-1–CXCR4 axis is emerging (Figure 7C). The functionality of the CXCR4 receptor on HSPCs is modulated by several factors: (1) the level of receptor expression on the cell surface,[46-48] (2) the sulfation status of its N-terminus,[49] (3) the availability of SDF-1,[50] (4) the cleavage of the CXCR4 N-terminus on the cells and SDF-1 in extracellular space by serine proteases and MMPs,[8,50,51] (5) the heterologous desensitization by the CCR5 receptor,[52] and, our finding in this study, (6) the modulation of CXCR4 incorporation into membrane lipid rafts (Figure 7C).

Implications of this last phenomenon may be important from a clinical point of view. We have identified several factors here that increase the incorporation of CXCR4 into lipid rafts, resulting in better responsiveness of HSPC to an SDF-1 gradient, which could lead to better engraftment. However, at the same time, we have identified potential inhibitors, such as the antifungal polyene antibiotics (eg, nystatin, amphotericin B), that, like MβCD, disrupt lipid raft formation and are strong inhibitors of the chemotaxis of CD34$^+$ cells to an SDF-1 gradient. This implies that the administration of some antifungal antibiotics to patients at the time of stem cell transplantation must be critically re-examined in clinical trials because these drugs may cause impaired stem cell homing or engraftment.

Moreover, we present evidence that SLPs regulate several mechanisms relevant to stem cell homing besides increasing the responses of HSPCs to an SDF-1 gradient, such as enhancing the adhesion of HSPCs to endothelium and increasing MMP-9 and MMP-2 secretion. To parallel the mobilized PB HSPCs collected by leukapheresis that are already primed during this procedure, ex vivo priming of BM- or CB-derived HSPCs before transplantation could be particularly useful. Especially with respect to the latter, given that the number of HSPCs present in CB samples is often too low to ensure successful transplantation in adult patients, ex vivo priming of CB cells could enhance their homing and seeding efficiency, allowing for optimal hematopoietic engraftment after transplantation. Because molecules such as PMV, C3a, hyaluronic acid, fibrinogen, fibronectin, soluble VCAM-1, soluble ICAM-1, and uPAR do not negatively affect the clonogenic potential of CD34$^+$ cells[7,17,19,29] and are available as recombinant proteins, the proposed strategy to prime HSPCs ex vivo before transplantation should be safe and without unwanted side effects. We have already demonstrated the efficiency of this strategy for PMV and C3a anaphylatoxin in an animal model.[7,17]

The effects of CXCR4 receptor priming by several small molecules observed here may also have an important implication for other biologic processes in which the SDF-1–CXCR4 axis plays an important role. In light of our observations, all these small molecules present in inflammatory areas may modulate the responses of CXCR4$^+$ immunocompetent cells or CXCR4$^+$ tumor cells to SDF-1 and affect cell trafficking/metastasis.[36,53] The biologic consequences of this phenomenon require further study in appropriate animal models.

Based on our observations, we conclude that the responsiveness of HSPCs to SDF-1 can be modulated positively (SLPs and their small molecular components) and negatively (polyene antibiotics). These observations have important clinical implications for improving the engraftment of HSPCs by developing efficient clinical protocols for ex vivo HSPC priming before transplantation and by using treatment regimens that avoid the use of polyene antibiotics at the time of stem cell infusion. We have already demonstrated the efficacy of this priming strategy in an animal model,[7,17,29] and clinical trials with patients undergoing CB or BM transplantation are now indicated.

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