

Hematopoietic Stem Cells Are Uniquely Selective in Their Migratory Response to Chemokines

Douglas E. Wright,¹ Edward P. Bowman,^{2,3,4,5} Amy J. Wagers,¹
Eugene C. Butcher,^{2,3,4} and Irving L. Weissman¹

¹Departments of Pathology and Developmental Biology, ²Laboratory of Immunology and Vascular Biology, Department of Pathology, and the ³Digestive Disease Center, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305

⁴Center for Molecular Biology and Medicine, Veterans Affairs, Palo Alto Health Care System, Palo Alto, CA 94305

⁵Department of Immunology, DNAX Research Institute, Palo Alto, CA 94304

Abstract

Although hematopoietic stem cell (HSC) migration into and out of sites of active hematopoiesis is poorly understood, it is a critical process that underlies modern clinical stem cell transplantation and may be important for normal hematopoietic homeostasis. Given the established roles of chemotactic cytokine (chemokine)-directed migration of other leukocyte subsets, the migration of murine HSC to a large panel of CC and CXC chemokines was investigated. HSC migrated only in response to stromal derived factor-1 α , the ligand for the CXC chemokine receptor 4 (CXCR4). CXCR4 expression by HSC was confirmed by reverse transcription polymerase chain reaction analysis. Surprisingly, HSC also expressed mRNA for CCR3 and CCR9, although they failed to migrate to the ligands for these receptors. The sharply restricted chemotactic responsiveness of HSC is unique among leukocytes and may be necessary for the specific homing of circulating HSC to bone marrow, as well as for the maintenance of HSC in hematopoietic microenvironments.

Key words: chemokines • chemokine receptors • chemotaxis • mobilization • bone marrow

Introduction

Hematopoietic stem cells (HSC)* are rare, pluripotent, self-renewing cells that give rise to all hematopoietic lineages (1) and migrate among various hematopoietic tissues. The site of hematopoiesis changes several times during embryonic and fetal development in a process thought to be dependent on HSC migration (for review see reference 2). In normal adult animals, HSC continuously migrate from the

bone marrow (BM) to the blood and back to the BM, in such a way that circulating HSC are always available in peripheral blood to fill open BM HSC niches (3–5). Large-scale HSC mobilization from BM to blood is induced in adult animals by treatment with cytokines (e.g., G-CSF) and/or cytotoxic drugs (e.g., cyclophosphamide [Cy]) (1). Drug-induced HSC mobilization from BM to blood and the rehoming of transplanted HSC from blood to BM are critical for modern therapeutic transplants that include stem cells. Finally, HSC have recently been shown to be capable of giving rise to hepatocytes (6), and BM cells enriched for HSC have been shown to be capable of giving rise to epithelial cells (7) and cardiac muscle cells (8). It has been proposed that HSC migration into sites of injury may be a mechanism by which damaged tissues are repaired (6). These new discoveries lend urgency to efforts to understand the mechanisms of HSC migration.

Adhesion molecules, such as $\alpha 4\beta 1$ integrin, have been implicated in both HSC mobilization as well as in the rehoming of transplanted HSC placed in the circulation (9, 10). Roles for chemotactic cytokines (chemokines) in hematopoietic stem and progenitor cell biology have also

D.E. Wright and E.P. Bowman contributed equally to this work.

E.C. Butcher and I.L. Weissman served as co-principal investigators.

Address correspondence to Douglas E. Wright, Department of Pathology (5324), Beckman B-261, 279 Campus Drive, Stanford University School of Medicine, Stanford, CA 94305. Phone: 650-725-5808; Fax: 650-498-6255; E-mail: doug.wright@stanford.edu

*Abbreviations used in this paper: APC, allophycocyanin; BLC, B lymphocyte chemokine; BM, bone marrow; chemokine, chemotactic cytokine; Cy, cyclophosphamide; HSC, hematopoietic stem cell(s); LT-HSC, long-term repopulating hematopoietic stem cell(s); MPB, mobilized peripheral blood; MIG, monokine induced by IFN- γ ; MIP, macrophage inflammatory protein; RANTES, regulated upon activation, normal T expressed and secreted; RT, reverse transcriptase; SDF-1 α , stromal cell-derived factor-1 α ; SM, staining medium; ST-HSC, short-term repopulating hematopoietic stem cell(s); TECK, thymus-expressed chemokine; WBM, whole bone marrow.

been proposed. HSC are rapidly mobilized from BM to blood after the administration of the chemokine IL-8 to mice (11) or to monkeys (12), and serum IL-8 levels spike just before mobilization after the induction with G-CSF (13). However, the effect of IL-8 in inducing HSC mobilization appears to be indirect (14, 15). The possibility of a direct role for the chemokine stromal cell-derived factor-1 α (SDF-1 α) in progenitor mobilization was raised by a study of human BM and mobilized peripheral blood (MPB) CD34⁺ cells, in which MPB CD34⁺ cells did not respond as well to SDF-1 α as to CD34⁺ cells isolated from BM (16). The authors proposed that the alteration in SDF-1 α responsiveness might be part of the mechanism of progenitor mobilization, although only a fraction of human CD34⁺ cells are HSC (17, 18). Finally, SDF-1 α has been implicated in the strikingly specific homing of transplanted hematopoietic progenitors to BM. SDF-1 α is constitutively expressed on human BM endothelium and appears to cooperate with α 4 β 1 integrin (very late antigen-4) and α L β 2 integrin (LFA-1) in inducing the arrest of circulating progenitors on vascular endothelium (19, 20). As of this writing, however, large panel studies on the ability of chemokines to induce chemotaxis of normal and mobilized HSC have not been published. Therefore, we sought to examine chemotaxis of HSC in a mouse model using a panel of chemokines that bind most known chemokine receptors.

We have previously shown that virtually all of the reconstituting activity of HSC isolated from the BM of untreated C57BL/Ka-Thy-1.1 mice and of mobilized HSC isolated from Cy/G-CSF-treated C57BL/Ka-Thy-1.1 mice is contained in two phenotypically defined populations: Thy-1.1^{lo}Sca-1⁺Lin⁻c-Kit⁺Mac-1⁻ cells that are mostly long-term repopulating HSC (LT-HSC), and Thy-1.1^{lo}Sca-1⁺Lin⁻c-Kit⁺Mac-1^{lo} cells that are mostly short-term repopulating HSC (ST-HSC) (21–23). Here, the *in vitro* chemotaxis of LT-HSC and ST-HSC, obtained from the BM of untreated mice, in response to a panel of chemokines binding most currently described chemokine receptors from the CC and CXC families, was investigated. Chemotaxis of HSC obtained from mice treated with a mobilizing regimen of Cy/G-CSF in response to SDF-1 α was also studied. In parallel, the expression of mRNA of chemokine receptors on both LT-HSC and ST-HSC derived from the BM of untreated mice was examined. These studies provide a large-scale survey of HSC chemotactic responses and provide evidence that mouse HSC possess mRNA for several chemokine receptors, yet only migrate *in vitro* in response to one chemokine, SDF-1 α .

Materials and Methods

Mice

6–12-wk-old C57BL/Ka-Thy-1.1 mice were bred and maintained at the Stanford University Laboratory Animal Facility, and given acidified water (pH 2.5) and rodent chow *ad libitum*.

HSC Mobilization

Cy and G-CSF were administered as previously described (23). HSC were analyzed on day 4 of the regimen, due to the expansion of HSC in the BM and the large numbers of HSC in the blood and spleen on that day (23).

Tissue Processing

Single cell suspensions of BM and spleen cells were prepared as previously described (23). Blood was collected from animals after heparin injection and induction of anesthesia with methoxyflurane (Mallinckrodt Veterinary, Inc.). After the incision of the right atrium, blood was removed from the thoracic cavity with a Pasteur pipette. The left ventricle was then perfused via a 25-gauge needle with 10 mM EDTA in HBSS (GIBCO BRL) and blood/EDTA from the thoracic cavity was removed. Blood was subjected to dextran sedimentation and ammonium chloride erythrocyte lysis as previously described (23). Splenic erythrocytes were lysed in all experiments and BM erythrocytes were lysed in most experiments.

Antibodies

mAbs used in immunofluorescence staining were prepared from hybridomas and included 19XE5 (anti-Thy-1.1), 2B8 (anti-c-Kit), and E13 (anti-Sca-1, Ly6A/E). Lineage marker mAbs included KT31.1 (rat anti-mouse CD3), GK1.5 (rat anti-mouse CD4), 53-7.3 (rat anti-mouse CD5), 53-6.7 (rat anti-mouse CD8), Ter119 (rat anti-mouse erythrocyte-specific antigen), 6B2 (rat anti-mouse B220), 8C5 (rat anti-mouse Gr-1), and M1/70 (rat anti-mouse Mac-1). Fc receptors were blocked with mAb 2.4G2 (anti-FcR) at appropriate points in staining procedures.

Depletion of Lineage⁺ Cells for Chemotaxis Assays

Cells were stained in HBSS/2% calf serum with the lineage marker antibodies listed above at 10⁸ cells/ml of previously determined optimal concentrations of antibody solution on ice for 25 min, rinsed twice in staining medium (SM), and enriched by incubation on a rocking platform at 4°C for 20 min with SM-washed sheep anti-rat IgG magnetic beads (Dynabeads; Dynal) at a 1:1 ratio of beads/cells in SM. After the removal of beads and attached cells, the remaining cells were incubated on a rocking platform at 4°C for 30 min with fresh washed beads at a 4:1 ratio of beads/cells, and a second round of bead/cell removal was accomplished. Typical lineage staining profiles of whole BM and lineage-depleted BM are shown in Fig. 1 A.

Chemotaxis Assay

Lineage-depleted tissues were suspended in RPMI medium (GIBCO BRL) with 10% serum and incubated in polystyrene tissue culture flasks at 37°C for 1 h to remove adherent cells and allow time for resensitization of potentially desensitized chemokine responses (24). Cells were then placed into a transwell chemotaxis assay as previously described (25) at 5 × 10⁵ – 2 × 10⁶ cells per top well. The following chemokines were added to the bottom well, and cells were allowed to migrate for 2 h at 37°C: mouse JE, mouse eotaxin, human thymus- and activation-regulated chemokine, mouse regulated upon activation, normal T expressed and secreted (RANTES), mouse macrophage inflammatory protein (MIP)-1 α , human MIP-3 α , human I-309, mouse KC, and human SDF-1 α (PeproTech); mouse MIP-1 β , human MIP-3 β , mouse thymus-expressed chemokine (TECK), mouse monokine induced by IFN- γ (MIG), and mouse B lymphocyte

chemokine (BLC)/BCA-1 (R&D Systems); and human IL-8 (a gift from K. Matsushima, University of Tokyo, School of Medicine, Tokyo, Japan). Optimal chemokine concentrations were determined in preliminary chemotaxis assays using BM cells or splenocytes. Two chemokines (I-309 and eotaxin) did not elicit responses from any BM or splenic subset, and therefore were used at published concentrations (24). Biological activity of I-309 and eotaxin was confirmed using cell lines expressing CCR8 and CCR3, respectively. Chemokines were used at final concentrations of 100 nM, with the following exceptions: 1 nM JE; 3 nM MIP-1 β ; 5 nM G-CSF; 50 nM SDF-1 α ; 300 nM KC and TECK; and 500 nM BLC.

Enumeration of Migrated HSC

After the addition of a fixed number of 15 μ m polystyrene beads (Polysciences) for normalization of cell numbers among wells as previously described (25), migrated cells were carefully collected from bottom wells, centrifuged, restained with lineage marker antibodies, and then stained with PE-conjugated goat anti-rat polyclonal antibody (Jackson ImmunoResearch Laboratories) to visualize lineage markers. Cells were then washed twice and stained with a cocktail containing FITC-conjugated (Molecular Probes) anti-Thy-1.1 (19XE5), allophycocyanin-conjugated (APC; Cytotech) anti-c-Kit (2B8), and TxR-conjugated (Molecular Probes) anti-Sca-1 (E13). In some experiments, PE-conjugated lineage antibodies were also included with the anti-Thy-1.1, anti-c-Kit, and anti-Sca-1 antibodies. Stained cells were resuspended in SM containing propidium iodide in preparation for flow cytometry.

Flow Cytometry

Cells were analyzed and sorted by multiparameter flow cytometry on either a modified 2-laser FACS Vantage[®] (Becton Dickinson), or a modified 3-laser cytometer (Cytomation, Inc. and Becton Dickinson), made available through the flow cytometry shared user group at Stanford University. Flow cytometry data were analyzed using FloJo[®] software (Treestar, Inc.). Representative gating for LT-HSC and ST-HSC is shown in Fig. 1 A.

Isolation of HSC for Reverse Transcription (RT)-PCR

HSC were enriched by positive selection for the c-Kit antigen with magnetic beads (Miltenyi Biotec) as previously described (26), and stained for flow cytometry with PE-conjugated lineage mAbs as previously described, FITC-conjugated mAb against Thy1.1, TxR-conjugated mAb against Sca-1, and APC-conjugated mAb against c-Kit. Lin⁻¹⁰Thy-1.1^{lo}Sca-1⁺c-Kit⁺ HSC were double sorted for RT-PCR analysis.

RT-PCR

RNA Isolation. RNA from FACS[®]-sorted HSC (~20,000 cells) was extracted with Trizol (GIBCO BRL) according to the manufacturer's instructions. Isolated RNA was resuspended in diethylpyrocarbonate (DEPC)-treated dH₂O and incubated with DNaseI (RNase-free; Boehringer) for 20 min at 37°C to remove contaminating genomic DNA. Before RT, DNaseI was inactivated and RNA was denatured by incubation at 70°C for 10 min.

RT. After chilling on ice, RNA samples were split equally into two 0.5-ml Eppendorf tubes (3.5 μ l per tube), and used for either the RT reaction (+RT), or for a control reaction in which the RT was omitted (-RT control). 16.5 μ l of the appropriate (+ or -RT) RT master mix was added to each tube containing 3.5 μ l RNA, for a final volume of 20 μ l.

RT Reaction Per Tube. 4 μ l 5 \times RT buffer; 2 μ l 0.1 M dithiothreitol; 1 μ l random hexamer (137 pmoles/ μ l); 8 μ l dNTPs (2.5 mM each dNTP); 0.5 μ l RNAsin (Promega); plus either 1 μ l Superscript II RT (+RT; GIBCO BRL) or 1 μ l DEPC-dH₂O (-RT control). RT reactions were performed by incubation at 42°C for 1 h.

PCR. Specific cDNA were amplified from the RT reactions by hot-start PCR using 2 μ l per tube of RT reaction as template and a total volume of 50 μ l per reaction. PCR master mix per tube: 5 μ l 10 \times PCR buffer; 5 μ l 25 mM MgCl₂; 4 μ l dNTPs (2.5 mM each); 1.5 μ l primer 1 (16.7 μ M stock concentration); 1.5 μ l primer 2 (16.7 μ M stock concentration); 0.25 μ l Taq polymerase; 30.75 μ l dH₂O; and 2 μ l template (RT reaction). PCR parameters were optimized for each primer set. PCR amplification was performed for 40 cycles. B. Fife (Northwestern University, Chicago, IL) provided some of the chemokine receptor PCR primer sequences.

PCR Primer Sequences. Oligonucleotide sequences specific for chemokine receptors are listed in Table I.

Statistics

The RxC test of independence (using the G test) with the Williams' correction was used to analyze the data in Table III. The Student's *t* test was used to analyze data in Figs. 1 and 4 A. *P* value of 0.05 was chosen as the limit of statistical significance.

Results

LT-HSC and ST-HSC Migrate Exclusively to SDF-1 α . We assayed the migration of LT-HSC and ST-HSC to a panel of chemokines that bind the receptors listed in Table II. In addition, G-CSF, a nonchemokine cytokine widely used for HSC mobilization in experimental animals and in humans, was tested. BM enriched for HSC was placed in the top well of a transwell chamber, and chemokine-containing medium was placed in the bottom well. Responding cells were collected from the bottom well and stained with antibodies to identify and enumerate HSC by flow cytometry (Fig. 1 A). Migration data were expressed as the percentage of input HSC contained in the population placed in the top well that migrated to the bottom well. Strikingly, both LT-HSC (Fig. 1 B) and ST-HSC (Fig. 1 C) migrated only in response to SDF-1 α , and did not respond significantly to any of the other chemokines or to G-CSF. Although the magnitude of SDF-1 α -induced migration varied among experiments (5.8–49% for LT-HSC and 7.3–57% for ST-HSC), there was no significant difference in the degree of migration to SDF-1 α of LT-HSC and ST-HSC. Furthermore, the pattern of nonresponsiveness to other chemokines did not vary between LT-HSC and ST-HSC.

SDF-1 α Induces HSC Chemotaxis That Does Not Require Non-HSC BM Cells. To further characterize SDF-1 α -induced HSC migration, we tested the requirement for an SDF-1 α gradient to induce HSC migration, or for non-HSC BM cells to stimulate HSC migration to SDF-1 α in vitro. HSC migration to SDF-1 α was chemotactic (polarized migration toward a chemokine source) not chemokinetic (randomly induced migration), because HSC migration only occurred in a gradient of SDF-1 α (bottom well

Table I. PCR Primers Used for Analysis of Chemokine Receptor mRNA Expression by HSC. DHFR, Dihydrofolate Reductase

Receptor	Sequence	Size
		bp
XCR1	Sense 5'-CAT CCC TGA TGC TGT CTT CCA C-3' Anti-sense 5'-AAT GAG AGA AGG CCA AAT GGC G-3'	346
CCR1	Sense 5'-AGC CTA CCC CAC AAC TAC AGA A-3' Anti-sense 5'-CTT GTA GGG GAA ATG AGG GCT A-3'	546
CCR2	Sense 5'-GGT CAT GAT CCC TAT GTG G-3' Anti-sense 5'-CTG GGC ACC TGA TTT AAA GG-3'	253
CCR3	Sense 5'-TGG GCA ACA TGA TGG TTG TG-3' Anti-sense 5'-GCT GTC TTG AGA CTC ATG GA-3'	383
CCR4	Sense 5'-CCA AAG ATG AAT GCC ACA GAG-3' Anti-sense 5'-CCT TAC AAA GCG TCA CGG AAG-3'	1,090
CCR5	Sense 5'-GCT GAA GAG CGT GAC TGA TA-3' Anti-sense 5'-GAG GAC TGC ATG TAT AAT GA-3'	362
CCR6	Sense 5'-GGG CAA CAT TAT GGT GGT GAT GAC-3' Anti-sense 5'-ACC GCA GTC ACG AGG AGG ACC ATG-3'	650
CCR7	Sense 5'-ACA GCG GCC TCC AGA AGA ACA GCG G-3' Anti-sense 5'-TGA CGT CAT AGG CAA TGT TGA GCT G-3'	344
CCR8	Sense 5'-CGA TGG AGC CCA ACG TCA CG-3' Anti-sense 5'-GGC CGT CCT CAC CTT GAT GGC-3'	424
CCR9	Sense 5'-TGC TGA TCT GCT CTT TCT TG-3' Anti-sense 5'-GTG CTT GGA TGA CTT CTT GG-3'	477
CXCR2	Sense 5'-AAC AGT TAT GCT GTG GTT GTA-3' Anti-sense 5'-CAA ACG GGA TGT ATT GTT ACC-3'	483
CXCR3	Sense 5'-GAA CGT CAA GTG CTA GAT GCC TCG-3' Anti-sense 5'-GTA CAC GCA GAG CAG TGC G-3'	631
CXCR4	Sense 5'-GGC TGT AGA GCG AGT GTT GC-3' Anti-sense 5'-GTA GAG GTT GAC AGT GTA GAT-3'	390
CXCR5	Sense 5'-AAA CGA AGC GGA AAC TAG AGC C-3' Anti-sense 5'-GCC CAG CTT GGT CAG AAG CC-3'	402
CX3CR1	Sense 5'-GCC ATC GTC CTG GCC GCC AAC TCC-3' Anti-sense 5'-GAC CGC ACA GGA CGG CCA GGC AC-3'	564
DHFR	Sense 5'-CCA CAA CCT CTT CAG TGG AAG GTA AAC AGA-3' Anti-sense 5'-TTG GCA AGA AAA TGA GCT CCT CGT GG-3'	159

only) and not when SDF-1 α was presented in a uniform manner (top and bottom wells) (Fig. 2 A). In addition, HSC chemotaxis did not require the presence of non-HSC BM cells. When HSC alone were sorted directly into the top well of the transwell chamber, these individual sorted HSC migrated in response to SDF-1 α , with a migration index in the same range as that determined using a heterogeneous mixture of lineage-depleted BM cells (Fig. 2 B).

Exposure to SDF-1 α Does Not Alter the In Vivo Engraftment Potential of HSC. We addressed the possibility that the functional reconstituting ability of phenotypically identified migrated HSC was altered by exposure to SDF-1 α over the time course of our assay. We tested whether ex

vivo incubation or exposure to SDF-1 α altered the engraftment potential at near limit dilution doses of LT-HSC. As shown in Table III, neither ex vivo incubation nor exposure to SDF-1 α significantly altered the engraftment potential of LT-HSC. 31 wk after the injection of 40 LT-HSC that had been incubated alone for 1 h, followed by incubation with SDF-1 α for 2 h, all recipient mice were engrafted by donor cells and four out of eight mice displayed long-term multilineage reconstitution. In a second experiment, 17 wk after the injection of 30 LT-HSC that had been incubated alone for 1 h, followed by incubation with SDF-1 α for 2 h, four out of seven mice were reconstituted with donor cells, and three out of seven mice displayed long-term multilineage reconstitution.

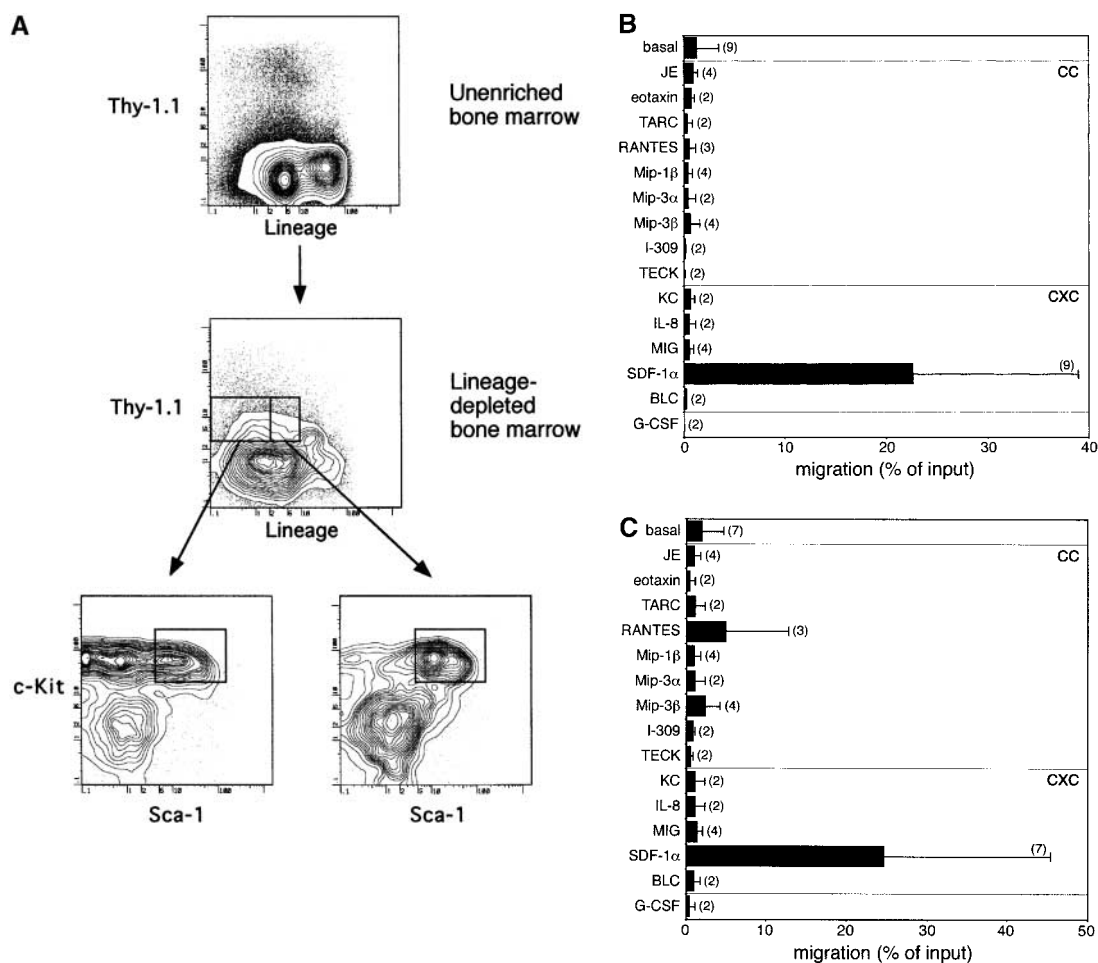


Figure 1. LT-HSC and ST-HSC migrate in response to SDF-1 α , but are refractory to other chemokines and to G-CSF. (A) Flow cytometry contour plots showing partial enrichment of BM cells for HSC by lineage depletion (upper two panels), and gating of lineage⁻ LT-HSC (boxed cells, lower left) and lineage^{lo} ST-HSC (boxed cells, lower right). See the Materials and Methods section for details. (B and C) Lineage-depleted BM cells were prepared and added to inserts placed in wells containing medium alone, the listed chemokines, or G-CSF (see the Materials and Methods section for concentrations). Responding cells were harvested, stained for HSC markers, and analyzed for the presence and number of (B) LT-HSC and (C) ST-HSC by flow cytometry. The data presented are the means \pm SD of two to nine independent experiments and represent the percentage of input HSC that migrated to each chemokine or to G-CSF. Numbers in parentheses indicate the number of experiments performed for each agent. CC and CXC refer to the receptor families to which the listed chemokines belong. Compared with basal migration, only migration to SDF-1 α was statistically significant ($P < 0.05$).

HSC Express mRNA for CCR3, CCR9, and CXCR4. In parallel with the chemotaxis studies, we tested for expression of mRNAs encoding the chemokine receptors for the chemokines addressed in this study, plus the chemokine receptors XCR1 (lymphotactin [XCL1] receptor) and CX3CR1 (fractalkine [CX3CL1] receptor). Chemokine receptor expression was determined by RT-PCR from samples representing 1,000 double-sorted HSC (this sorted population contained both LT-HSC and ST-HSC) or 1,000 unfractionated whole bone marrow (WBM) cells as a positive control. CXCR4 (SDF-1 α receptor) mRNA was easily detected in HSC at the 1,000 cell level. CCR3 (eotaxin and RANTES receptor) and CCR9 (TECK receptor) messages were also detected (Fig. 3 A and Table IV), and XCR1, CXCR2 (KC/IL-8 receptor), and CXCR5 (BLC receptor) mRNA were observed inconsistently. Other chemokine receptors were not detectably expressed

in HSC, although they were clearly present in RNA isolated from an equivalent number of WBM cells. Because it is conceivable that the *in vivo* migratory behavior of LT-HSC and ST-HSC differ, and that these differences could be reflected in chemokine receptor expression, we further examined CCR3, CCR9, and CXCR4 mRNA expression by LT-HSC and ST-HSC individually. CCR3, CCR9, and CXCR4 mRNA expression was detected in both LT-HSC and ST-HSC (Fig. 3 B).

SDF-1 α Responses of HSC from BM of Untreated Mice Are Indistinguishable from Responses of HSC Derived from BM, Blood, and Splens of Cy/G-CSF-treated Mice. It has been suggested that decreased responsiveness to SDF-1 α may play a role in the mobilization of human hematopoietic progenitors from BM to blood (16). We tested this hypothesis in the mouse by examining the chemotaxis of HSC derived from BM, blood, and splens of Cy/G-CSF-treated

Table II. Chemokines Used and Their Known Receptors. Standardized Chemokine Names Are Given in Parentheses

Receptors	Chemokines
CCR1	RANTES (CCL5)
CCR2	JE (CCL2)
CCR3	Eotaxin (CCL11), RANTES (CCL5)
CCR4	TARC (CCL17)
CCR5	RANTES (CCL5), MIP-1 β (CCL4)
CCR6	MIP-3 α (CCL20)
CCR7	MIP-3 β (CCL19)
CCR8	I-309 (CCL1)
CCR9	TECK (CCL25)
CXCR2	KC (CXCL1-3), IL-8 (CXCL8)
CXCR3	MIG (CXCL9)
CXCR4	SDF-1 α (CXCL12)
CXCR5	BLC (CXCL13)

animals. When compared with HSC from the BM of untreated mice, PB-, BM-, and spleen-derived HSC from Cy/G-CSF-treated animals showed indistinguishable responsiveness to SDF-1 α (Fig. 4). In addition, both MPB

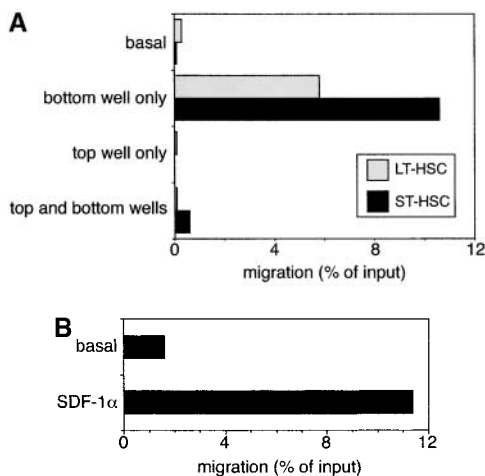


Figure 2. (A) HSC migration to SDF-1 α is chemotactic, not chemokinetic. Lineage-depleted BM cells were prepared and added to inserts placed in wells. SDF-1 α was present in either the bottom well, the top well (the insert), or in both the top and bottom wells. Responding cells were harvested, stained for HSC markers, and analyzed for LT-HSC and ST-HSC by flow cytometry. The data represent the percentage of input HSC that migrated to the bottom well. HSC migrated only in the presence of a gradient of increasing SDF-1 α concentration. (B) HSC chemotaxis to SDF-1 α does not require cells other than HSC. 15,000 Thy-1.1^{lo}Sca-1⁺Lin^{-/lo}c-Kit⁺ cells (this population contains both LT-HSC and ST-HSC) from BM of untreated mice were sorted directly into inserts placed in wells containing SDF-1 α . 30,000 of the same cells were sorted into inserts placed in wells only containing medium to establish basal migration. Responding cells were collected and analyzed by flow cytometry. The data represent the percentage of input HSC that migrated to the bottom well.

Table III. Exposure to SDF-1 α Does Not Alter the Long-Term Engraftment Potential of LT-HSC Injected at Near Limit Dilution Doses

n	Hours of exposure		Reconstitution pattern				
	In culture	SDF-1 α	LTMR	Percent			
			LTMR	B, T	B, M	B	None
A							
6	0	0	4	67	0	0	2
6	3	0	4	67	2	0	0
5	3	0.5	3	60	2	0	0
6	3	1	4	67	1	0	1
8	3	2	4	50	3	1	0
B							
8	3	0	4	50	1	0	2
7	3	2	3	43	1	0	3

(A) Experiment 1. The long term competitive repopulation assay was performed (Materials and Methods) with 40 double-sorted LT-HSC injected per recipient mouse. LT-HSC were incubated in vitro without (in culture) or with SDF-1 α as indicated. Peripheral blood of recipient mice was analyzed by flow cytometry for the presence of donor-derived B, T, and myeloid cells 31 wk after transplant. Data shown are the number or percentage of mice with the indicated reconstitution patterns. An *RxC* test of independence using the G test with the Williams' correction revealed that there are no significant differences among the groups ($0.9 < P < 0.975$). (B) Experiment 2. Same as experiment 1, except that 30 LT-HSC per recipient were injected and the mice were analyzed 17 wk after transplant. An *RxC* test of independence using the G test with the Williams' correction revealed that there are no significant differences between the groups ($0.5 < P < 0.9$). Abbreviations: B, B cells; LTMR, long-term multilineage reconstitution; M, myeloid cells; T, T cells.

and mobilized spleen HSC expressed easily detectable CXCR4 mRNA by RT-PCR (unpublished data).

Discussion

HSC migrate in several circumstances: (a) HSC are thought to migrate through the blood to seed new sites of hematopoiesis during embryonic and fetal development (for review see reference 2); (b) HSC migrate continuously between BM and blood in normal adult animals (3-5); (c) HSC migrate in large numbers from BM to blood after the treatment of animals with cytotoxic agents such as Cy and/or cytokines such as G-CSF (23, 27); and (d) injected HSC migrate efficiently to hematopoietic tissues after transplantation in experimental animals, or after clinical transplantation in humans (28). Because HSC have now been shown to also have the capacity to give rise to nonhematopoietic tissues such as liver, migrating HSC may represent a source of pluripotent cells that are constantly available for the repair of damaged organs. Here, we report the first comprehensive study to address the chemotactic responsiveness of HSC to chemokines.

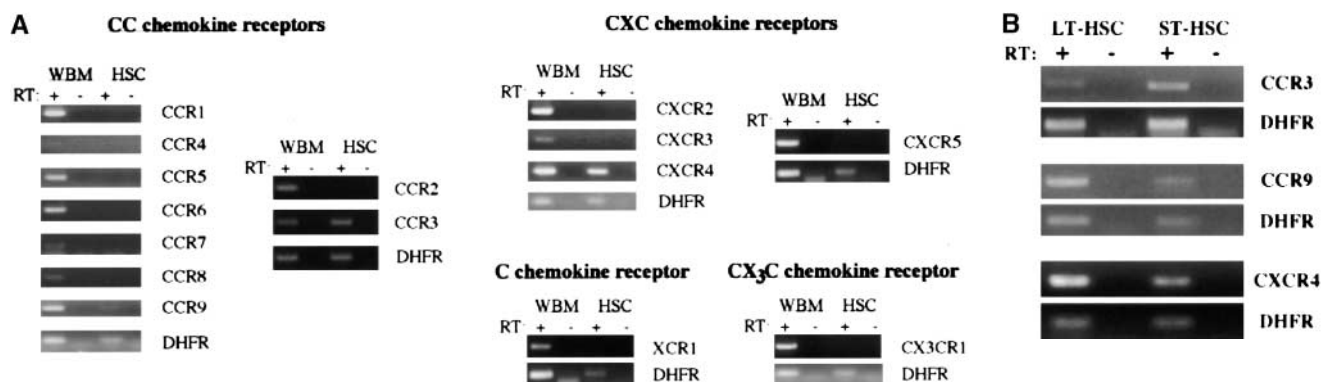


Figure 3. LT-HSC and ST-HSC contain mRNA for CCR3, CCR9, and CXCR4. (A) RT-PCR analysis of mRNA for chemokine receptors of combined LT-HSC and ST-HSC (Thy-1.1^{lo}Sca-1⁺Lin^{-/lo}c-Kit⁺ cells). RT-PCR was performed on RNA isolated from the equivalent of 1,000 double-sorted HSC, or from 1,000 unfractionated WBM cells. Representative data are shown (see Table IV for data summary). (B) Additional RT-PCR was performed on mRNA isolated from the equivalent of 1,000 LT-HSC or 1,000 ST-HSC for the receptors found to be positive in the first screen. Both LT-HSC and ST-HSC contained mRNA for CXCR4, CCR3, and CCR9. See the Materials and Methods section for RT-PCR protocol. DHFR, dihydrofolate reductase; WBM, whole bone marrow.

The role of chemokines in hematopoiesis includes guiding progenitor cells to microanatomical sites in BM or thymus for proper maturation (29). However, the roles of chemokines in the migrations of HSC during embryonic and fetal development, the maintenance of adult HSC niches, and the physiological flux of HSC between BM and blood in adults are unclear. Here we showed that in normal adult animals, BM-derived LT-HSC and ST-HSC displayed a sharply restricted responsiveness pattern to chemokines; they only migrated appreciably in response to SDF-1 α . Consistent with this, HSC express mRNA for CXCR4, the only known receptor for SDF-1 α . The SDF-

1 α -CXCR4 interaction is important in hematopoietic development, and may have a role in engraftment of BM by HSC. Among other defects, mice lacking either SDF-1 α or CXCR4 lack lymphomyeloid hematopoiesis in fetal BM (30, 31). The antibody blockade of CXCR4 prevented engraftment of adult SCID mouse BM by CD34⁺-enriched human cord blood cells (32).

Our data suggest that HSC, in contrast to other leukocyte subsets, only respond to SDF-1 α . However, the possibility exists that HSC may respond to other chemokines or chemoattractants that we have not tested in this study. Although HSC express mRNA for CCR3 and CCR9 (lack of antibodies against murine chemokine receptors precluded determination of whether CCR3 or CCR9 proteins were present on the cell surface of HSC), they did not migrate in response to eotaxin or RANTES, ligands for CCR3, or in

Table IV. Summary of RT-PCR Analysis of Chemokine Receptor mRNA Expression by HSC

Receptor	Expression ^a
XCR1	± (1/3)
CCR1	- (0/2)
CCR2	- (0/4)
CCR3	+ (4/6)
CCR4	- (0/2)
CCR5	- (0/2)
CCR6	- (0/2)
CCR7	- (0/2)
CCR8	- (0/2)
CCR9	+ (2/2)
CXCR2	± (2/5)
CXCR3	- (0/2)
CXCR4	+ (6/6)
CXCR5	± (1/5)
CX3CR1	- (0/3)

^aNumbers in parentheses indicate the fraction of independent experiments in which a PCR product was detectable.

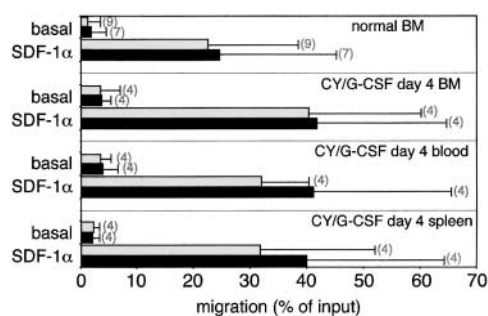


Figure 4. The magnitude of the chemotactic response to SDF-1 α by HSC from BM of untreated mice is indistinguishable from the magnitude of the chemotactic responses of HSC derived from BM, blood, or spleens of Cy/G-CSF-treated mice. Lineage-depleted BM cells from untreated animals, or BM, blood, or spleen cells from day 4 Cy/G-CSF-treated animals were prepared and added to inserts placed in wells containing medium alone or SDF-1 α . Responding cells were harvested, stained for HSC markers, and analyzed for the presence and number of LT-HSC and ST-HSC by flow cytometry. The data presented are the means \pm SD of four to nine independent experiments and represent the percentage of input HSC that migrated. Numbers in parentheses indicate the number of experiments performed. Shaded bar, LT-HSC; filled bar, ST-HSC.

response to TECK, the ligand for CCR9. These data indicate that receptor mRNA expression alone does not adequately predict chemotactic responsiveness, as has been observed in other studies (24, 33, 34). Knockout mice that do not express functional CCR3 have been prepared and no hematopoietic defects have been observed (Gerard, C., personal communication). Similarly, preliminary analyses of CCR9 knockout mice do not reveal notable defects in the development of most lymphoid compartments (Wurbel, M.A., and Malissen, B., personal communication [35]). Lack of migration by HSC to eotaxin, RANTES, and TECK is also consistent with the absence of essential roles for CCR3 and CCR9 in hematopoiesis.

Current models of leukocyte trafficking hold that chemotactic responsiveness plays a critical role in the homing of cells to particular microenvironments and in positioning cells within these microenvironments (36). Most leukocyte subsets express multiple chemokine and chemoattractant receptors, and migrate in response to several chemokines (37, 38). The possession of multiple chemokine and chemoattractant receptors has been shown to allow cells to maneuver in a stepwise fashion through spatial arrays of chemokine and chemoattractant gradients (39). This raises the question of the significance of the extremely selective responsiveness pattern to chemokines exhibited by HSC.

BM has the challenging task of promoting the growth of several cell lineages. The presumption that various lineages have unique requirements for maturation has led to the notion that BM is partitioned functionally into specialized microenvironments, or “niches.” Although HSC niches have not been characterized, evidence of their existence comes from the observation that cloned stromal lines are heterogeneous in their ability to maintain hematopoiesis *in vitro* (40–43). A second observation supporting the niche hypothesis is that transplanted HSC, when injected intravenously as discrete boluses, do not engraft BM of syngeneic recipient animals unless the dose of HSC is extremely large (44–48), or the recipient’s BM is injured by radiation/cytotoxic drugs.

Given the unique requirement of HSC niches in supporting both the maintenance of multipotency and differentiation, it is tempting to speculate that the restricted chemotactic responsiveness of HSC we observed might be important in localizing HSC within their niches, at least when they are actively participating in hematopoiesis. Possession by HSC of multiple functional chemokine receptors in a complex environment such as BM would be undesirable as it could lead to the inappropriate migration of HSC out of the HSC niche. Ma et al. (49) recently proposed that CXCR4 is required for the retention of B lineage and granulocytic precursors in the fetal liver and BM. The possibility that CXCR4 might similarly promote the retention of HSC in their BM niches remains to be fully tested, but is supported by the observation that the administration to mice of an SDF analogue that downmodulates CXCR4 resulted in a greater than 30-fold increase in the number of circulating Thy-1.1^{lo}Lin⁻Sca-1⁺c-Kit⁺ cells (50).

In addition to promoting the maintenance of HSC in specific microanatomical sites within BM, CXCR4 might allow efficient homing back to BM by those HSC that are released into the bloodstream. Using a cross-circulation model, we have found that HSC continuously flux between BM and blood under physiological conditions. HSC released from the BM into the circulation recolonize BM in other locations (5). Therefore, the circulating pool of HSC must have a mechanism for the efficient rehoming to BM. The SDF-1 α -CXCR4 interaction appears to play a key role in the homing of transplanted human hematopoietic progenitors to BM of NOD/SCID mice (32), and SDF-1 α promotes integrin-mediated arrest on vascular endothelium of circulating CD34⁺ cells (19). The SDF-1 α -CXCR4 interaction may have a similar role in the rehoming to BM of murine HSC released physiologically into the circulation or injected intravenously for transplantation.

Aiuti et al. (16) raised the possibility of a direct role for SDF-1 α in hematopoietic progenitor mobilization. In a study of human BM and MPB CD34⁺ cells, fewer MPB CD34⁺ cells responded to SDF-1 α than CD34⁺ cells isolated from BM, and it was suggested that the reduction in SDF-1 α responsiveness might be part of the mechanism of progenitor mobilization. But only a fraction of CD34⁺ cells are HSC (17, 18). We found that mouse HSC from MPB, or from BM or spleens of Cy/G-CSF-treated animals, were equally responsive to SDF-1 α as HSC from BM of untreated control animals. These data do not support a role for changes in SDF-1 α responsiveness in cytokine-induced HSC mobilization. The disparity between the results of the current study and those of Aiuti et al. (16) might be due to the differences between progenitors and HSC, or it may reflect species- or mobilization protocol-specific effects. In preliminary experiments, the migration of mobilized HSC to the following chemokine panel was also tested: JE, eotaxin, thymus- and activation-regulated chemokine, RANTES, MIP-1 β , MIP-3 α , MIP-3 β , I-309, KC, IL-8, MIG, and SDF-1 α . Similar to untreated BM, mobilized HSC migrated only to SDF-1 α and did not respond to the other chemokines or to G-CSF (unpublished data).

In conclusion, we report that HSC migrate to SDF-1 α but not to chemokines signaling through other known chemokine receptors. To our knowledge, this is the first report of a leukocyte subset that responds to a single chemokine, which makes HSC highly specialized in this regard. It remains to be determined whether SDF-1 α -CXCR4 interactions play a role in HSC localization within BM, or in the rehoming to BM by HSC that are released physiologically into the circulation. SDF-1 α responsiveness is preserved in HSC isolated from BM, blood, and spleens of Cy/G-CSF-mobilized mice.

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