Maturation decreases responsiveness of human bone marrow B lineage cells to stromal-derived factor 1 (SDF-1)

Eric R. Fedyk,* Daniel H. Ryan,† Ion Ritterman,† and Timothy A. Springer*

*Department of Pathology, The Center For Blood Research and Harvard Medical School, Boston, Massachusetts; and †Department of Pathology and Laboratory Medicine, University of Rochester Medical Center, New York

Abstract: We compared the chemotactic responsiveness of different subsets of human B lineage cells to stromal derived factor-1 (SDF-1). High percentages (30–40% of input) of purified bone marrow progenitors including non-B lineage progenitors, pro-B cells, and pre-B cells migrated to SDF-1α, demonstrating that SDF-1 is an efficacious chemoattractant of these cells. Pro-B cells responded optimally to a lower concentration of SDF-1 than other subsets, demonstrating that SDF-1 is a more potent chemoattractant of this subset. A lower percentage (10–15% of input) of mature B lymphocytes migrated to SDF-1α than pro-B cells, demonstrating that responsiveness of B lineage cells to SDF-1 decreases during differentiation. Inhibition by anti-CXCR4 mAb demonstrated that migration of B lineage cells to SDF-1 was completely dependent on CXC chemokine receptor-4 (CXCR4). Mature B cells expressed higher levels of CXCR4 receptors than uncommitted progenitors and pro-B cells, despite differences in responsiveness to SDF-1. CXCR4 receptors expressed by unresponsive and SDF-1-responsive B cells bound SDF-1α with similar affinities (K_D = 1.7–3.3 × 10^{-9} M). Therefore, elements downstream from CXCR4 appear to regulate responsiveness of B cells to SDF-1. We speculate that SDF-1 and CXCR4 direct migration of progenitor cells in microenvironments that promote B lymphopoiesis. *J. Leukoc. Biol.* 66: 667–673; 1999.

Key Words: *chemotaxis · CXCR4 · B lymphopoiesis · affinity · B lymphocyte · pro B cell*

INTRODUCTION

Stromal-derived factor-1 (SDF-1) is a structurally and functionally unique chemokine. Although categorized as a CXC chemokine, SDF-1 is almost equally related to both CXC- and CC-subfamilies. This unique intermediary relationship suggests that SDF-1 is a primordial chemokine [1–3]. SDF-1 is unusually conserved between species; human and mouse SDF-1α are 99% identical [2] and are the most highly conserved chemokines described to date. Two alternate splice variants of the *sdf1* gene give rise to SDF-1α and SDF-1β, which are identical except that SDF-1β contains an additional four amino acid residues in the carboxy terminus [2]. Targeted disruption of the mouse genes for SDF-1 or its receptor, CXC chemokine receptor-4 (CXCR4) [4, 5], is perinatally lethal [6–9], confirming that these proteins serve a vital function in development.

The supportive function of the bone marrow stromal microenvironment is a dominant factor in B lymphopoiesis and stromal cells express high levels of SDF-1 [1, 10]. The bone marrow is heterogeneous and contains different types of stromal cells [11] and cytokines [12] that are involved in B cell differentiation. This heterogeneity suggests that specialized microenvironments exist within the bone marrow which drive differentiation of B lineage cells [13]. Human B cell progenitors are actively motile within *in vitro* bone marrow stromal microenvironments [14], and migrate underneath stromal cells, a process that requires adhesion to stromal cells via β1 (VLA) integrins and appears to involve chemoattractants [15, 16]. SDF-1 is a chemoattractant for hematopoietic progenitor cells [17, 18] and promotes migration [19] and expansion [20] of mouse pro-B cells. Furthermore, SDF-1 is required for normal mouse B lymphopoiesis because SDF-1- and CXCR4-deficient fetuses exhibit greatly reduced numbers of pro-B cells [6–9]. SDF-1 could fulfill both migratory and proliferative activities. Herein we demonstrate that SDF-1 is a more efficacious chemoattractant of human B cell progenitors than mature B lymphocytes. We also demonstrate that chemotactic responsiveness does not correlate with the level of CXCR4 receptors expressed by B lineage cells, despite the finding that this receptor exclusively mediates chemotaxis to SDF-1.

MATERIALS AND METHODS

Chemokines and antibodies

Synthetic full-length, human SDF-1α (1–67) and an inactive truncated version SDF-1α (6–67), were gifts of Ian Clark Lewis (University of British Columbia, Vancouver, Canada). SDF-1α and SDF-1β were also obtained from R & D Systems (Minneapolis, MN). Interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1), and regulated upon activation, normal T cell expressed and secreted (RANTES) were purchased from Genzyme (Cambridge, MA), monokine induced by interferon-gamma (Mig) was from PharMingen (San Diego, CA), human interferon gamma inducible protein 10 (IP-10), macrophage

Correspondence: Timothy A. Springer, Ph.D., The Center for Blood Research, 200 Longwood Avenue, Room 251, Boston, MA 02115. E-mail: springer@sprsgi.med.harvard.edu

Received February 24, 1999; revised May 4, 1999; accepted May 6, 1999.

inflammatory protein-1α (MIP-1α), MIP-3α, MIP-3β, 6CKine, and HCC-1 were from R & D Systems, and neutrophil-activating protein-2 (NAP-2) and lymphotactin were from Peprotec (Rocky Hill, NJ). Recombinant complement component 5a (C5a) was obtained from Sigma (St. Louis, MO). A mAb to human CXCR4, 12G5, was kindly provided by J. A. Hoxie (University of Pennsylvania). Myeloma IgG1 was purified from supernatants of the X63 mouse myeloma and used as a control IgG. Phycoerythrin-conjugated 12G5 was purchased from PharMingen.

B cell progenitors and B lineage cell lines

Bone marrow aspirates (posterior iliac crest) were obtained from normal adult volunteer donors. Bone marrow aspirates from pediatric B cell precursor ALL patients were obtained from discarded biopsies. Light-density bone marrow cells were isolated by Ficoll/Hypaque (Ficoll-Histopaque 1077, Sigma) density-gradient centrifugation. $CD34^+$ cells were enriched from light density cells using the CEPRATE LC affinity column (CellPro Inc., Bothell, WA) as described [16]. The B lineage cell lines Nalm-6, Reh, Wil-2, Ramos, and IM-9 were maintained by Dr. Daniel Ryan, and SUP-B8, Daudi, Raji, ARH-77, RPMI-8226 and U266BL were obtained from the ATCC (Rockville, MD).

Chemotaxis assays

Chemotaxis assays employed the Transwell® culture insert (Costar, Cambridge, MA) or the ChemoTx System (Neuro Probe, Cabin John, MD). Transwell, 6.5-mm diameter, 5-μm pore polycarbonate membrane inserts were coated with human plasma fibronectin (15 μg/mL; Sigma, St. Louis, MO) and allowed to air dry. Membranes were then washed twice with phosphate-buffered saline (PBS) and the upper chambers were placed into the corresponding wells of a tissue culture dish that contained 600 μL of RPMI-1640 + 10% FBS + 50 μg/mL L-glutamine + 50 U/mL gentamicin plus chemokines at the indicated concentration. Next, 5×10^5 cells in 100 μL of RPMI-1640 + 10% FBS + 50 μg/mL L-glutamine + 50 U/mL gentamicin were added to this upper chamber and the plate was placed into a humidified, 37°C incubator for 3 h. Using the ChemoTx System, 3.25-mm diameter, 5-μm pore polycarbonate membranes were coated with human plasma fibronectin, as outlined above, and the membrane was placed over the wells of a corresponding 96-well tissue culture dish. Each well contained 28 μL of RPMI-1640 + 10% FBS + 50 μg/mL L-glutamine + 50 U/mL gentamicin plus chemokines at the indicated concentration. Next, 2.5×10^5 cells in 25 μL of RPMI-1640 + 10% FBS + 50 μg/mL L-glutamine + 50 U/mL gentamicin were added to the upper side of the membrane and the plate was placed into a humidified, 37°C incubator for 3 h. In both assays, cells in the lower chamber were vigorously resuspended and were then analyzed by forward versus side scatter using a FACScan® (Becton-Dickinson, San Jose, CA) and viable cells were counted for 30 s at 60 μL/min. To determine the percent of viable input cells that transmigrated to the lower chamber, 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128 dilutions of the input cell populations were counted by the same method.

Human umbilical vein endothelial cells (HUVECs) were freshly isolated as described [21] or obtained from the ATCC. Human fibronectin-coated (above), 6.5-mm-diameter, polycarbonate membrane (5-μm pore), Transwell inserts were placed into the corresponding wells of a tissue culture dish that contained 600 μL of HUVEC media (F-12K media (GIBCO-BRL, Grand Island, NY) + 10% FBS, 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 100 μg/mL gentamicin, and 50 μg/mL endothelial mitogen (Biomedical Technologies Inc., Stoughton, MA). Next, 1×10^5 HUVEC cells in 100 μL of media were added to the upper chamber and the plate was placed into a humidified, 37°C incubator. Every 48 h, media was exchanged and the monolayer was checked for confluency by removing one well, staining with Wright Giemsa and visually inspecting it under a microscope. Once confluent monolayers had been obtained, HUVEC transmigration assays were conducted as follows: 10 μL of a chemokine solution (60×) was added to the lower chamber and 5×10^5 cells in 10 μL were added to the upper chamber. The entire apparatus was then placed into a humidified 37°C incubator for 3 h. Transmigrated cells were harvested and analyzed as outlined for chemotaxis assays on fibronectin.

Identification of migrated cells

To phenotype transmigrated cells, aliquots of the input cells and transmigrated cell populations were stained with directly conjugated mAbs and analyzed by three-color flow cytometry. Transmigrated CD34-enriched populations were stained with anti-CD34-phycoerythrin (PE), anti-CD10-fluorescein isothiocyanate (FITC; Becton-Dickinson Immunocytometry Systems), and anti-CD19-ECD (Coulter Immunology, Marietta, GA) or the appropriate directly conjugated isotype controls. In contrast, transmigrated light density bone marrow cells were stained with anti-CD34-PE, anti-CD10-FITC, and anti-CD19-ECD, or anti-CD3-ECD (Coulter Immunology), anti-CD10-PE, and anti-CD20-FITC (Becton-Dickinson Immunocytometry Systems), or the appropriate directly conjugated isotype controls. Cells and mAbs were incubated at 4°C for 30 min. Cells were washed three times at 4°C with flow buffer and then analyzed with a Coulter EPICS Elite ESP (Coulter). Populations were analyzed until 25,000 events had been recorded or until no sample remained. Cell marker expression and the proportions of cells in the lymphocyte and blast scatter gates were used to determine the percentage of each subset in the migrated population. To determine the total number of migrated cells in each subset, the total number of migrated cells in the sample was then multiplied by the fraction of the migrated population that consisted of that subset.

Flow cytometry analysis of CXCR4 expression by B lineage cells

B lineage cell lines and progenitor cells were stained and analyzed by two different protocols. Normal B cell progenitors were analyzed by three-color flow cytometry. Cells were washed once in flow buffer [PBS + 1% bovine serum albumin (BSA) + 5 mM EDTA] and 2×10^5 cells were resuspended in a 10 μg/mL solution of directly conjugated anti-CXCR4-PE (PharMingen, San Diego, CA), anti-CD10-FITC (Becton-Dickinson Immunocytometry Systems), anti-CD19-ECD (Coulter Immunology), anti-CD34-Cy5 (Caltag Laboratories, South San Francisco, CA) or the appropriate directly conjugated isotype controls and incubated at 4°C for 30 min. Alternatively, B cell lines were analyzed by single-color flow cytometry using unconjugated anti-CXCR4 mAb 12G5 and FITC-conjugated, goat anti-mouse Ig $F(ab')_2$ (Caltag laboratories). After incubation with fluorescent mAbs, B cells were washed twice at 4°C with flow buffer and then analyzed with a FACScan (Becton-Dickinson Immunocytometry Systems) or a Coulter EPICS Elite ESP (Coulter).

Heterogeneous competition binding assays

Cell lines were washed twice in L15 medium + 2.5% BSA (4°C). Cells were resuspended at 4×10^6 cells/mL and 25-μL aliquots were added to wells of a 96-well microtiter plate. Twice the final indicated concentrations of SDF-1α were prepared in L15 media + 2.5% BSA (4°C) and added to the cell suspensions in 25-μL aliquots. A saturating concentration of PE-conjugated anti-CXCR4 mAb 12G5 (PharMingen) or an equal concentration of PE-conjugated isotype control Ig were immediately added (final concentration of 6.4×10^{-7} M) and the cells, mAb and SDF-1α were incubated at 4°C for 60 min. Cells were washed twice with L15 media + 2.5% BSA (4°C), and then analyzed with a FACScan (Becton-Dickinson). In parallel, homologous competition binding assays between unconjugated and PE-conjugated anti-CXCR4 mAb 12G5 (PharMingen) were conducted using the same protocol. Values for IC_{50} were calculated using equations from two nonlinear regression programs (GraphPad Prism 2.0, GraphPad Software Inc., San Diego, CA). Receptor affinities were calculated using the equations of Cheng and Prusoff [22]. The fluorescently labeled antibody was used at a saturating concentration, so it was assumed that no (minimal) cooperativity of binding existed. In parallel with receptor binding assays, Quantum Simply Cellular Microbeads (Flow Cytometry Standards Corp., San Juan, PR) were used as internal standards to measure the amount of anti-CXCR4 mAb binding. Beads were incubated with saturating amounts of PE-conjugated anti-CXCR4 mAb, washed twice with L15 media + 2.5% BSA, and then analyzed with a FACScan (Becton-Dickinson). Using fluorescence values corresponding to predetermined numbers of binding sites per bead, a standard curve was constructed. Values for the total number of binding sites per B cell were calculated using linear regression (GraphPad Prism 2.0), assuming one binding site per mAb.

Calcium mobilization assays

Human B cell lines were incubated at 1×10^7 cells/mL in loading buffer containing Hanks' balanced salt solution (HBSS), 25 mM HEPES, 0.1% BSA, pH 7.2, and 10 μg/mL Fura-2/AM (Molecular Probes, Eugene, OR) for 30 min at 37°C. The loaded cells were centrifuged, resuspended in fresh loading buffer,

added to a stirred cuvette (4×10^6 cells/mL), and inserted into a Hitachi F2000 spectrophotometer. Chemokine was added in 10 μL such that the final concentration was 1 μg/mL.

RESULTS

SDF-1α is a more efficacious chemoattractant of human progenitor B cells than mature B lymphocytes

To examine whether B cell progenitors migrate to SDF-1, hematopoietic progenitor cells were enriched from human bone marrow aspirates by Ficoll-Hypaque centrifugation and affinity to anti-CD34 columns. Both $CD34^+$ cells (**Fig. 1A**) and light-density cells (Fig. 1B) were tested for migration across fibronectin-coated membranes to human SDF-1α. High percentages of pro-B cells ($CD34^+$, $CD10^+$, $C19^+$) migrated to SDF-1α at the optimal dose of 100–1000 ng/mL (Fig. 1A). High percentages of total $CD34^+$ progenitor cells and $CD34^+$ non-B progenitor cells migrated at an optimal dose of 1,000 ng/mL (Fig. 1A). Within this fraction, residual mature B lymphocytes ($CD34^-$, $CD19^+$) also migrated to SDF-1α with a dose-response similar to pro-B cells; however, significantly ($P < 0.05$) lower percentages of these cells responded (Fig. 1A). In contrast, pre-B cells ($CD34^-$, $CD10^+$, $CD20^-$) and mature B cells ($CD34^-$, $CD10^-$, $CD20^+$) responded optimally to at least 1000 ng/mL of SDF-1α (Fig. 1B). At this concentration, a significantly lower percentage ($P < 0.05$) of input mature B lymphocytes migrated than pre-B cells (Fig. 1B). Finally, T lymphocytes ($CD34^-$, $CD3^+$) migrated optimally to at least 1000 ng/mL of SDF-1α (data not shown).

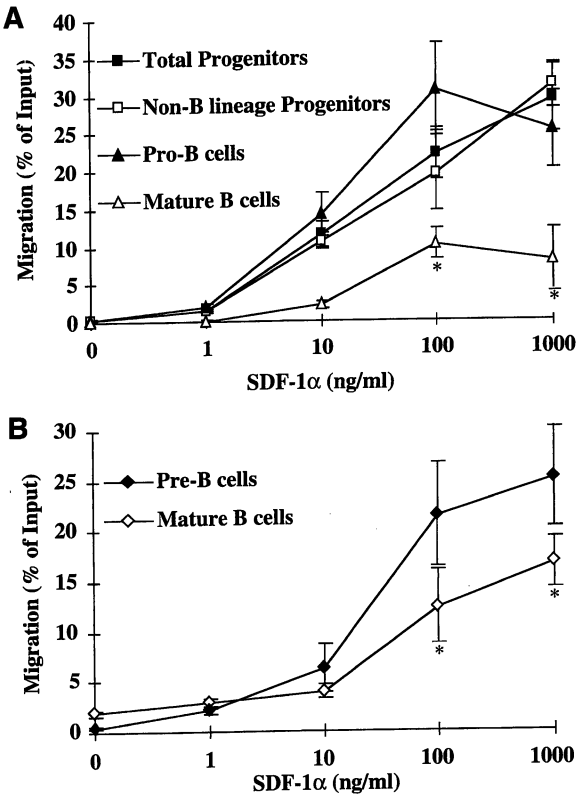

Fig. 1. SDF-1α is a more efficacious chemoattractant of human progenitor B cells than mature B lymphocytes. Human $CD34^+$ progenitor cells (A) and light-density bone marrow cells (B) were purified from fresh bone marrow aspirates of healthy volunteers. Human $CD34^+$ progenitor cells and light-density bone marrow cells were added to the upper compartment of a fibronectin-coated chemotaxis chamber while SDF-1α was added to the lower chamber at the indicated concentrations. Aliquots of the transmigrated populations were removed and the number of cells that migrated was determined by manual counting or by flow cytometry. The remaining cells were stained and analyzed by three-color flow cytometry. The number of transmigrated cells for each B cell subset was determined by multiplying the total number of transmigrated cells by the fraction of that population that contained the B cell subset of interest. Transmigration for each subset was determined as the percent of the number of input cells in each subpopulation. Cell subsets were defined as follows: total progenitors ($CD34^+$), non-B lineage progenitors ($CD34^+CD10^-CD19^-$), pro-B cells ($CD34^+CD10^+CD19^+$), pre-B cells ($CD34^-CD10^+CD20^-$), mature B cells (panel A, $CD34^-CD19^+$; panel B, $CD34^-CD10^-CD20^+$). The data are the mean of three independent experiments employing three different donors. $^*P \leq 0.05$, $^{**}P \leq 0.01$.

SDF-1α and SDF-1β are chemoattractants for B acute lymphoblastic leukemia cell lines

We compared cell lines representing different stages of B cell development for migration to SDF-1. Nalm-6, Reh, JM-1, and SUP-B8 were derived from acute B lymphocytic leukemias (B-ALL) and are arrested at the pro- and pre-B cell stages of development. Each of these cell lines migrated to SDF-1α and SDF-1β with kinetics and dose-responses resembling normal pro-B cells (**Fig. 2** and data not shown). Ramos, Daudi, Raji, and Wil-2 are B cell lines that are arrested at the mature B cell stage. They did not migrate through fibronectin or HUVEC monolayers to SDF-1α (Fig. 2A and data not shown). The human myeloma cell lines ARH-77, IM-9, RPMI-8226, and U266BL did not migrate to SDF-1α in fibronectin chemotaxis assays (data not shown).

Migration of B-ALL lines was characterized further. Nalm-6 cells migrated across fibronectin and HUVEC-coated membranes only toward higher concentrations of SDF-1α (**Table 1** and data not shown) demonstrating that SDF-1 stimulates chemotaxis and not random migration. Similar results were obtained with Reh cells (data not shown). Nalm-6 cells did not migrate to the CXC chemokines IL-8, NAP-2, MIG, and IP-10; the CC-chemokines HCC-1, MCP-1 MIP-1α, MIP-3α (Exodus-1), MIP-3β (ELC), RANTES, and 6Ckine (SLC); the C-chemokine, lymphotactin; C5a, or a truncated version of SDF-1α that lacks six amino acids from the amino terminus (SDF-1α 6–67; data not shown).

Expression levels of CXCR4 receptors do not correlate with chemotactic responsiveness of B lineage cells

Human bone marrow light-density cells and $CD34^+$ cells (>90% purity) were analyzed for CXCR4 expression by three-color flow cytometry. Pre-B cells ($CD10^{low}$, $CD19^+$) and mature B cells ($CD10^-$, $C19^+$) expressed the highest levels of CXCR4 (**Fig. 3A**). Lower levels of expression were detected on pro-B cells ($CD34^+$, $CD19^+$ or $CD10^{2+}$, $CD19^+$) and some non-B progenitors ($CD34^+$, $CD19^-$; **Fig. 3, A** and **B**). We also examined CXCR4 receptor expression by B cell lines and calculated the number of receptors per cell by linear regression, using fluorescent beads as standards (**Table 2**). The mature B

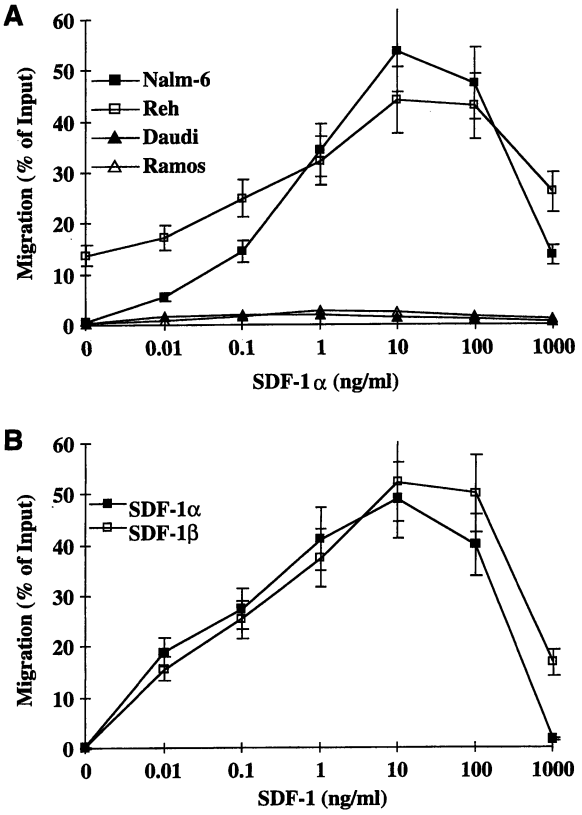

Fig. 2. Human acute B lymphocytic leukemia cell lines migrate on fibronectin to SDF-1α and SDF-1β. Human B cell lines were added to the upper compartment of a chemotaxis chamber containing a fibronectin-coated membrane, while SDF-1α (A and B) or SDF-1β (B) was added to the lower chamber at the indicated concentrations. Transmigration is shown as percent of input cells. The data are the mean of nine wells gathered from three independent experiments.

cell lines Ramos and Daudi expressed the highest number of CXCR4 receptors, the pro-/pre-B lines Nalm-6 and Reh expressed intermediate levels, and the myelomas ARH-77, IM-9, RPMI-8226, and U266BL expressed undetectable levels of this receptor (Table 2 and data not shown).

CXCR4 receptors exclusively mediate migration of SDF-1-responsive B lineage cells

To examine whether CXCR4 receptors mediate chemotaxis of B-ALL cell lines or freshly isolated B-ALL cells to SDF-1α

TABLE 1. Checkerboard Chemotaxis Assay of the Nalm-6 Cell Line Responding to SDF-1α[a]

SDF-1α in lower chamber (ng/mL)	SDF-1α in upper chamber (ng/mL)			
	0.0	0.1	1.0	10.0
	Nalm-6 cell migration (%)			
0.0	1.00	0.63	0.56	0.63
0.1	7.60[c]	3.43	0.80	0.60
1.0	59.80[c]	20.36[b]	3.93	0.91
10.0	85.93[c]	44.46[c]	16.50[b]	1.27

[a] Nalm-6 cells migrated through fibronectin-coated membranes. Data are the mean of three independent experiments. *P* values represent comparisons between samples receiving SDF-1α and media alone in the lower chamber.—[b] $P \leq 0.05$, [c] $P \leq 0.01$.

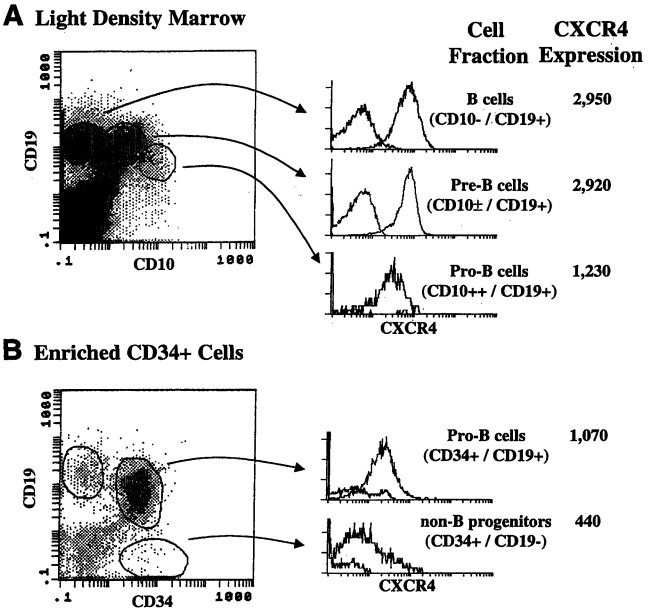

Fig. 3. Pre-B and mature B cells express higher levels of CXCR4 receptors than pro-B cells. Light-density marrow (A) and enriched $CD34^+$ cells (B) were isolated from fresh bone marrow aspirates of healthy volunteers and incubated with directly conjugated mAbs against CD34, CD10, CD19, and CXCR4. Solid lines are subsets stained with anti-CXCR4 mAb and lighter lines are subsets stained with isotype control Ig. The values reported for CXCR4 expression are the mean fluorescence values of the populations stained with anti-CXCR4. The data are representative three independent experiments employing three different donors.

and/or SDF-1β, cells were pretreated with the anti-CXCR4 mAb 12G5 or control mouse IgG and then subjected to chemotaxis assays. The anti-CXCR4 mAb 12G5 completely inhibited migration of Nalm-6 and Reh cell lines to SDF-1α or SDF-1β, whereas control mouse IgG had no effect (**Fig. 4A** and data not shown). Anti-CXCR4 mAb also inhibited migration to SDF-1α of $CD19^+$ blasts from three primary pediatric B-ALL cases (Fig. 4B). The G-protein inhibitor pertussis toxin also completely inhibited migration of Nalm-6 and Reh cells to SDF-1α whereas the b-oligomer subunit was 1000-fold less effective (Fig. 4C and data not shown).

Unresponsive and SDF-1-responsive cell lines bind SDF-1α with similar affinities but differ in mobilization of intracellular calcium

We investigated whether responsiveness to SDF-1 is determined by differences in binding of this chemokine to CXCR4

TABLE 2. Expression of CXCR4 Receptors by Human B Cell Lines

B cell line	Phenotype	CXCR4 site per cell[a]	K_D SDF-1α[b]	K_D SDF-1β[b]
Nalm-6	Pro/Pre-B cell	121,000	1.7×10^{-9} M	6.6×10^{-9} M
Reh	Pro/Pre-B cell	25,000	3.3×10^{-9} M	n.d.[c]
Daudi	Mature B cell	242,000	1.7×10^{-9} M	n.d.[c]
Ramos	Mature B cell	355,000	3.0×10^{-9} M	6.2×10^{-9} M

[a] CXCR4 sites per cell was determined by linear regression, using fluorescent beads as standards.—[b] K_D was determined by heterogeneous competition binding assays between SDF-1 and a directly-conjugated anti-CXCR4 mAb (12G5-PE).—[c] n.d., not determined.

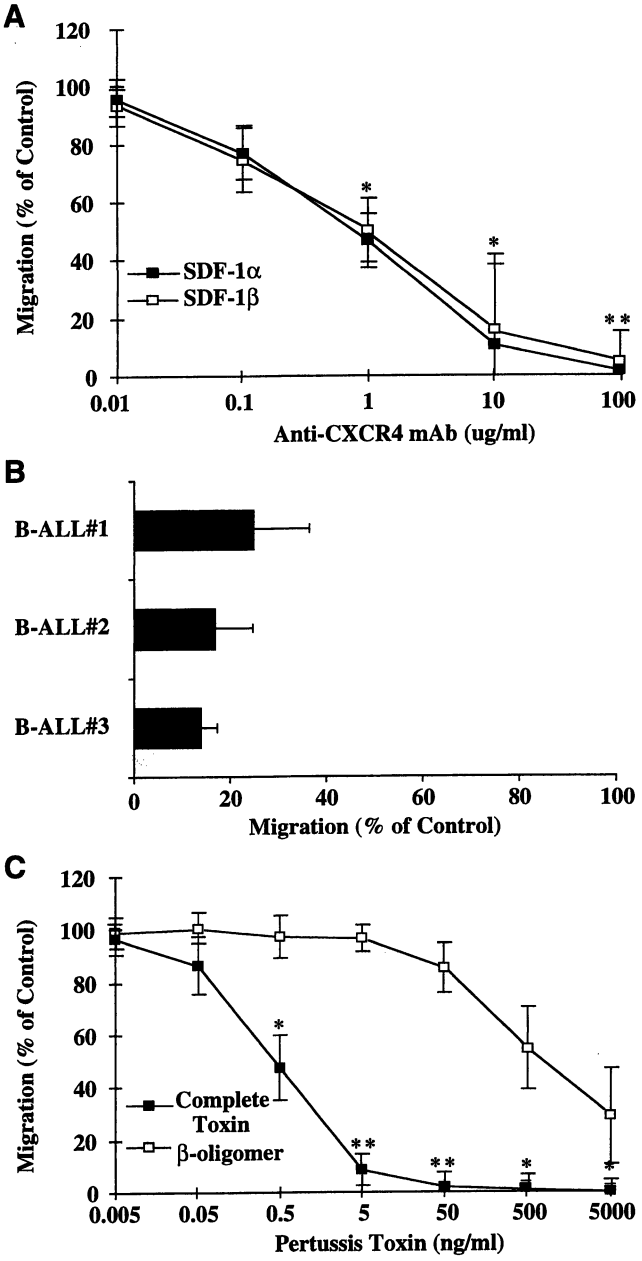

Fig. 4. The CXCR4 receptor and a pertussis toxin-sensitive pathway exclusively mediate chemotaxis of human B lineage cells to SDF-1. (A) Nalm-6 cells were preincubated with the indicated concentration of the anti-CXCR4 mAb 12G5 or control Ig and subjected to chemotaxis to 10 ng/mL of SDF-1α. Data are from nine wells assayed in three independent experiments. (B) Mononuclear cells from bone marrow of three cases of pediatric B-ALL were isolated and preincubated with the anti-CXCR4 mAb 12G5 or control Ig (100 μg/mL) and were subjected to chemotaxis to SDF-1α (1000 ng/mL). Transmigrated B-ALL cells were identified by a blast phenotype and expression of CD19. Data are the mean of triplicate wells. (C) Nalm-6 cells were incubated with the indicated concentrations of intact pertussis toxin or the b-oligomer (control) at the indicated concentrations. Cell suspensions were washed and subjected to chemotaxis to 100 ng/mL of SDF-1α. Data points are from triplicate wells and the histogram is representative of three independent experiments. In panels A and B, migration is presented as percent of migration of the Ig control, and in panel C, of the media control. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$.

receptors expressed by the pro-/pre-B cell lines Nalm-6 and Reh and the mature B cell lines Daudi and Ramos. Previous attempts to study SDF-1 binding with radioligands were unsuccessful so we set up a heterogeneous competition binding assay between SDF-1 and a directly conjugated anti-CXCR4 mAb (12G5-PE). Cells were first stimulated with various concentrations of SDF-1α or media alone at 4°C (to prevent receptor internalization) and then a saturating concentration of 12G5-PE was added. Once binding reached equilibrium, cells were washed and the amount of bound antibody was measured via flow cytometry analysis. In the absence of SDF-1, virtually 100% of each cell population bound 12G5-PE. SDF-1α could completely inhibit this binding (**Fig. 5**). Using equations derived by nonlinear regression analysis, we calculated the IC_{50} for SDF-1α binding to each cell line: 0.021 μg/mL for Nalm-6, 0.041 μg/mL for Reh, 0.021 μg/mL for Daudi, and 0.037 μg/mL

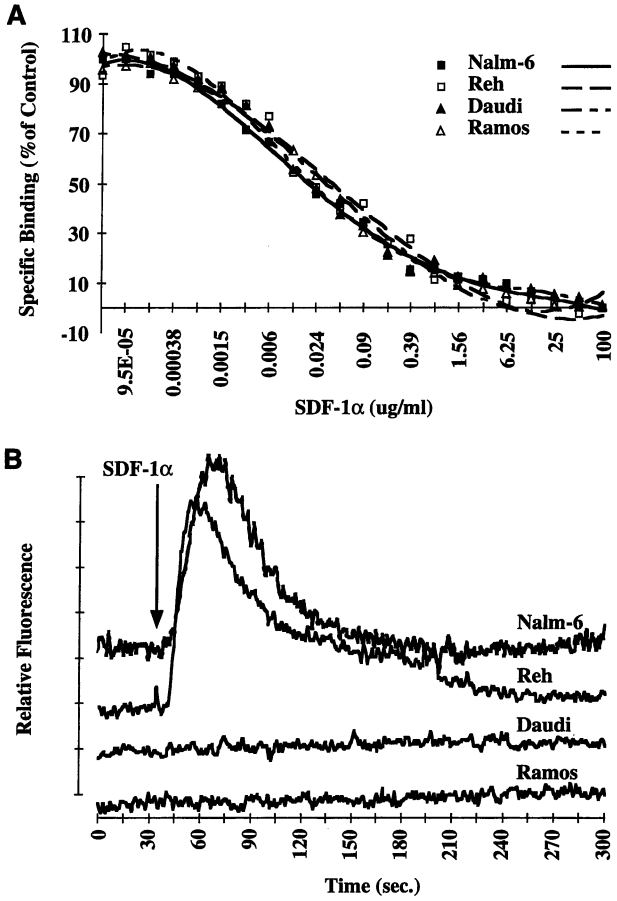

Fig. 5. Unresponsive and SDF-1-responsive B cell lines bind SDF-1α with similar affinities but differ in mobilization of intracellular calcium. (A) Competition by SDF-1 of PE-conjugated mAb 12G5 binding to CXCR4 receptors expressed by Nalm-6, Reh, Daudi, and Ramos cells. Cells were incubated with a mixture of SDF-1 (or media control) and PE-conjugated 12G5 mAb or control Ig for 1 h at 4°C. Cells were washed and subjected to flow cytometry. Specific binding was calculated by subtracting fluorescence values for the isotype control Ig-PE from all values for 12G5-PE mAb. Binding of 12G5 mAb was then calculated as percent binding in the absence of SDF-1. The data points represented are the mean percent of specific binding of three individual experiments. (B) Mobilization of calcium in human B cell lines stimulated with SDF-1α. B cell lines were loaded with Fura-2/AM fluorescent dye, stimulated with SDF-1α (arrow) and fluctuations in intracellular calcium (fluorescence) were monitored for 5 min.

for Ramos. Stimulation of B cell lines with SDF-1β could also completely inhibit binding of 12G5-PE and yielded similar IC_{50}s: 0.08 μg/mL for Nalm-6 and 0.076 μg/mL for Ramos (data not shown). Using the dissociation constant (K_D) of 12G5-PE (calculated from homologous competition binding assays) and the IC_{50} value for SDF-1α binding to each cell line, we calculated the K_Ds of SDF-1α and SDF-1β binding to these cell lines (Table 2). SDF-1α and SDF-1β bound to CXCR4 receptors on each B cell line with nearly identical affinities (Table 2).

We investigated whether intracellular signaling correlates with chemotactic responsiveness to SDF-1 in B cell lines. The human B cell lines Nalm-6, Reh, Daudi, and Ramos were loaded with Fura-2/AM, added to a spectrophotometer, and subsequently stimulated with SDF-1α. Nalm-6 and Reh cell lines mobilized calcium in response to stimulation with SDF-1α, whereas Daudi and Ramos cells were unresponsive (Fig. 5B).

DISCUSSION

It is widely believed that B lymphopoiesis requires interaction of progenitor cells with stromal elements within discrete microenvironments of the bone marrow. Human B cell progenitors migrate to discrete locations within *in vitro* bone marrow microenvironments and adhere to stromal cells [14]. SDF-1 is chemotactic for uncommitted human hematopoietic progenitor cells [17] and mouse bone marrow stroma abundantly produces this chemokine [1, 10]. SDF-1 is also required for fetal B lymphopoiesis in mice [6] and is a chemoattractant of mouse B cell progenitors and human pro- and pre-B cell lines [19]. We demonstrate that SDF-1 is an efficacious attractant of human pro-B and pre-B bone marrow cells, pro-B and pre-B cell lines, and fresh B-ALL cells. Concentrations of SDF-1α that were optimal for pro-B cell migration were lower than concentrations that were optimal for uncommitted progenitors, non-B progenitors and $CD3^+$ T lymphocytes. Finally, we demonstrate that B lineage cells exhibit directed migration to SDF-1α rather than chemokinesis. Hence, we conclude that SDF-1α is a potent and efficacious chemoattractant of human B cell progenitors. We speculate that the ability of pro-B cells to respond to lower concentrations of SDF-1 may preferentially direct these cells into microenvironments promoting B lymphopoiesis.

In bone marrow, a significantly ($P < 0.05$) smaller percentage of bone marrow B lymphocytes (10–15%) migrate to SDF-1α than B cell progenitors (30–35%). This percentage of responsive mature B lymphocytes is similar to the percentage of unfractionated tonsillar B lymphocytes that migrated to SDF-1α [23]. A similar phenomenon has been observed for mouse B lineage cells. B lymphocytes derived from mouse bone marrow are significantly less responsive to SDF-1α than pro- and pre-B cells [19]. We also report that high percentages of human pro- and pre-B cell lines migrated to SDF-1α or SDF-1β, whereas mature B cell lines and the myelomas were unresponsive. These data demonstrate that responsiveness to SDF-1 decreases as B cells mature and differentiate. It is noteworthy that mature B lymphocytes do respond to SDF-1, although less efficaciously than pre-B or pro-B cells. Mature B lymphocytes normally emigrate from the bone marrow into the vasculature and then immigrate into secondary lymphoid organs. A decrease in responsiveness to SDF-1 by mature B lymphocytes may allow these cells to leave the bone marrow.

We report that CXCR4 receptors and pertussis toxin-sensitive G proteins exclusively mediate chemotaxis of human B lineage cells to SDF-1. Furthermore, B lineage cells that do not express CXCR4 receptors (Wil-2, ARH-77, and IM-9), do not migrate to SDF-1 (data not shown). These data demonstrate that CXCR4 is the only receptor on human B lineage cells that mediates chemotaxis to SDF-1α or SDF-1β. These data are consistent with the finding that targeted disruption of either the mouse SDF-1 or CXCR4 genes results in identical phenotypes [7–9].

It is interesting that the level of CXCR4 receptors expressed by human B lineage cells did not correlate with the chemotactic responsiveness of these cells. Most strikingly, Daudi and Ramos cell lines expressed high levels of CXCR4 but failed to chemotax to SDF-1. Among normal B lineage cells, human B lymphocytes expressed the highest levels of CXCR4 but are the least responsive subset to SDF-1. A similar phenomenon is found with human germinal center B lymphocytes, which retain expression of CXCR4 but lose responsiveness to SDF-1 [23]. We did not detect SDF-1 expression by any B cell line through the use of highly sensitive ELISA and RNase protection assays (data not shown), therefore endogenous production of SDF-1 by Daudi and Ramos cell lines does not explain their failure to chemotax. Using a heterogeneous competition binding assay between SDF-1 and a directly conjugated anti-CXCR4 mAb (12G5-PE), we demonstrated that both unresponsive and responsive B cell lines bind SDF-1 with nearly identical affinities. These K_Ds agree with those from the lone investigation reporting the binding of radiolabeled SDF-1 to a human T cell line [24] and are in general agreement with K_Ds for other chemokine receptors and their ligands. In sum, expression of CXCR4 receptors is required for migration of B lineage cells to SDF-1 but expression levels and affinities of CXCR4 receptors for SDF-1 do not correlate with chemotactic responsiveness. Therefore, the factors that regulate chemotactic responsiveness to SDF-1 must exist downstream of CXCR4 receptors. We demonstrate that Nalm-6 and Reh cells mobilize calcium when stimulated with SDF-1α, whereas Daudi and Ramos cell lines do not. Furthermore, stimulation by exogenous SDF-1α increases the percentage of Nalm-6 and Reh cells adhering to human bone marrow stroma by a VLA-4-dependent mechanism (data not shown). In contrast, stimulation by SDF-1α does not induce adhesion of Daudi and Ramos to human bone marrow stroma (data not shown) despite expression of VLA-4 by these cells [25]. These data demonstrate that CXCR4 receptors on unresponsive B cell lines are uncoupled from intracellular signaling mediators and integrins. Further investigation is required to identify the intracellular mediators that cause the chemotactic responsiveness of B lineage cells to SDF-1.

Disruption of the mouse SDF-1 or CXCR4 genes greatly reduces fetal B lymphopoiesis [6–9]. An early investigation described SDF-1 as a proliferative factor that augmented IL-7-stimulated growth of mouse pro-B cells [20]. The absence of this proliferative stimulus could explain the lack of B

lymphopoiesis in SDF-1- or CXCR4-deficient mice [6]. Another explanation is that the chemotactic activity of SDF-1 is required for normal B lymphopoiesis. We hypothesize that B lymphopoiesis requires both the chemotactic and proliferative activities of SDF-1. Specifically, we propose that SDF-1 elicits chemotaxis of progenitors to the source of SDF-1, stromal cells [1, 10]. Once within these regions, high concentrations of SDF-1 cause progenitors to firmly adhere to stromal cells, which in turn stimulates proliferation and B cell maturation. Once differentiated into mature B lymphocytes, responsiveness to SDF-1 decreases, and this may allow B cells to detach from stromal cells and immigrate into the vasculature.

ACKNOWLEDGMENTS

We thank Dan Jones for supplying pediatric B-ALL biopsies. This work was supported by National Institutes of Health Grants HL 48675 and NCI CA69502.

REFERENCES

1. Bleul, C. C., Fuhlbrigge, R. C., Casasnovas, J. M., Aiuti, A., Springer, T. A. (1996) A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). J. Exp. Med. 184, 1101–1110.
2. Shirozu, M., Nakano, T., Inazawa, J., Tashiro, K., Tada, H., Shinohara, T., Honjo, T. (1995) Structure and chromosomal localization of the human stromal cell-derived factor 1 (SDF1) gene. Genomics 28, 495–500.
3. Dealwis, C., Fernandez, E. J., Thompson, D. A., Simon, R. J., Siani, M. A., Lolis, E. (1998) Crystal structure of chemically synthesized [N33A] SDF-1α, a potent ligand for the HIV-1 "fusin" co-receptor. Proc. Natl. Acad. Sci. USA 95, 6941–6946.
4. Bleul, C. C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., Springer, T. A. (1996) The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. Nature 382, 829–833.
5. Oberlin, E., Amara, A., Bachelerie, F., Bessia, C., Virelizier, J.-L., Arenzana-Seisdedos, A., Schwartz, O., Heard, J.-M., Clark-Lewis, I., Legler, D. F., Loetscher, M., Baggiolini, M., Moser, B. (1996) The CXC chemokine, stromal cell derived factor 1 (SDF-1), is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. Nature 382, 833–835.
6. Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S.-I., Kitamura, Y., Yoshida, N., Kikutani, H., Kishimoto, T. (1996) Defects of B-cell lymphopoiesis and bone marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature 382, 635–638.
7. Zou, Y-R., Kottmann, A. H., Kuroda, M., Taniuchi, I., Littman, D. R. (1998) Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 393, 595—599.
8. Tachibana, K., Hirota, S., Lizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, K., Yoshida, N., Nishikawa, S-I., Kishimoto, T., Nagasawa, T. (1998) The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. Nature 393, 591–594.
9. Ma, Q., Jones, D., Borghesan, P. R., Segal, R. A., Nagasawa, T., Kishimoto, T., Bronson, R. T., Springer, T. A. (1998) Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. Proc. Natl. Acad. Sci. USA 95, 9448–9453.
10. Tashiro, K., Tada, H., Heilker, R., Shirozu, M., Nakano, T., Honjo, T. (1993) Signal sequence trap: A cloning strategy for secreted proteins and type I membrane proteins. Science 261, 600–603.
11. Witte, P. L., Robinson, M., Henley, A., Low, M. G., Stiers, D. L., Perkins, S., Fleischman, R. A., Kincade, P. W. (1987) Relationships between B-lineage lymphocytes and stromal cells in long-term bone marrow cultures. Eur. J. Immunol. 17, 1473–1484.
12. Witte, P. L., Frantsve, L. M., Hergott, M., Rahbe, S. M. (1993) Cytokine production and heterogeneity of primary stromal cells that support B lymphopoiesis. Eur. J. Immunol. 23, 1809–1817.
13. Funk, P. E., Kincade, P. W., Witte, P. L. (1994) Native associations of early hematopoietic stem cells and stromal cells isolated in bone marrow cell aggregates. Blood 83, 361–369.
14. Ryan, D. H., Tang, J. (1995) Regulation of human B cell lymphopoiesis by adhesion molecules and cytokines. Leuk. Lymphoma 17, 375–389.
15. Miyake, K., Hasunuma, Y., Yagita, H., Kimoto, M. (1992) Requirement for VLA-4 and VLA-5 integrins in lymphoma cells binding to and migration beneath stromal cells in culture. J. Cell Biol. 119, 653–662.
16. Tang, J., Scott, G., Ryan, D. H. (1993) Subpopulations of bone marrow fibroblasts support VLA-4-mediated migration of B-cell precursors. Blood 82, 3415–3423.
17. Aiuti, A., Webb, I. J., Bleul, C., Springer, T. A., Gutierrez-Ramos, J. C. (1997) The chemokine SDF-1 is a chemoattractant for human $CD34^+$ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of $CD34^+$ progenitors to peripheral blood. J. Exp. Med. 185, 111–120.
18. Kim, C. H., Broxmeyer, H. E. (1998) In vitro behavior of hematopoietic progenitor cells under the influence of chemoattractants: stromal cell-derived factor-1, steel factor, and the bone marrow environment. Blood 91, 100–110.
19. D'Apuzzo, M., Rolink, A., Loetscher, M., Hoxie, J. A., Clark-Lewis, I., Melchers, F., Baggiolini, M., Moser, B. (1997) The chemokine SDF-1, stromal cell-derived factor 1, attracts early stage B cell precursors via the chemokine receptor. Eur. J. Immunol. 27, 1788–1793.
20. Nagasawa, T., Kikutani, H., Kishimoto, T. (1994) Molecular cloning and structure of a pre-B-cell growth-stimulating factor. Proc. Natl. Acad. Sci. USA 91, 2305–2309.
21. Gimbrone, M. A., Jr. (1976) Culture of vascular endothelium. Prog. Hemost. Thromb. 3, 1—28.
22. Cheng, Y., Prusoff, W. H. (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 23, 3099–3108.
23. Bleul, C. C., Schultze, J. L., Springer, T. A. (1998) B lymphocyte chemotaxis regulated in association with microanatomic localization, differentiation state, and B cell receptor engagement. J. Exp. Med. 187, 753–762.
24. Crump, M. P., Gong, J-H., Loetscher, P., Rajarathnam, K., Amara, A., Arenzana-Seisdedos, F., Virelizier, J-L., Baggiolini, M., Sykes, B. D., Clark-Lewis, I. (1997) Solution structure and basis for functional activity of stromal cell-derived factor-1; dissociation of CXCR4 activation from binding and inhibition of HIV-1. EMBO J. 16, 6996–7007.
25. Sanchez-Aparicio, P., Dominguez-Jimenez, C., Garcia-Pardo, A. (1994) Activation of the alpha 4 beta 1 integrin through the beta 1 subunit induces recognition of the RGDS sequence in fibronectin. J. Cell Biol. 126, 271–279.