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CXCR4 Regulates Migration and Development of Human Acute Myelogenous Leukemia Stem Cells in Transplanted NOD/SCID Mice

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ABSTRACT

The chemokine stromal cell-derived factor-1 (SDF-1) and its receptor CXCR4 participate in the retention of normal hematopoietic stem cells within the bone marrow (BM) and their release into the circulation. Homing and engraftment of human stem cells in immunodeficient mice are dependent on cell surface CXCR4 expression and the production of BM SDF-1, which acts also as a survival factor for both human and murine stem cells. However, the role of SDF-1/CXCR4 interactions in the control of human acute myelogenous leukemia (AML) cell trafficking and disease progression is poorly understood. In this study, we report that although some AML cells do not express surface CXCR4, all AML cells tested express internal CXCR4 and SDF-1. Culture of AML cells with SDF-1 promoted their survival, whereas addition of neutralizing CXCR4 antibodies, SDF-1 antibodies, or AMD3100 significantly decreased it. Pretreatment of primary human AML cells with neutralizing CXCR4 antibodies blocked their homing into the BM and spleen of transplanted NOD/SCID/B2m^{null} mice. Furthermore, weekly administrations of anti-human CXCR4 to mice previously engrafted with primary AML cells led to a dramatic decrease in the levels of human AML cells in the BM, blood, and spleen in a dose- and time-dependent manner. Interestingly, the same treatment did not affect significantly the levels of normal human progenitors engrafted into NOD/SCID mice. Taken together, our findings demonstrated the importance of the SDF-1/CXCR4 axis in the regulation of *in vivo* motility and development of human AML stem cells and identified CXCR4 neutralization as a potential treatment for AML.

INTRODUCTION

The chemokine stromal cell-derived factor-1 (SDF-1, also named CXCL-12) is the only powerful chemoattractant for both human CD34$^+$ progenitors and murine Sca-1$^+$/Thy1^{lo}/Lin$^-$ stem cells (1, 2). In contrast to proinflammatory chemokines, SDF-1 is constitutively produced in many organs including the human bone marrow (BM; Ref. 3), suggesting a major role for SDF-1/CXCR4 interactions in steady-state homeostatic processes such as the control of leukocyte trafficking and retention of undifferentiated and maturing hematopoietic cells within the BM in both normal and pathological conditions. Knockout murine embryos lacking SDF-1 or its receptor CXCR4 similarly show multiple lethal defects, including impaired BM lymphoid and myeloid hematopoiesis (4–6), demonstrating the importance of SDF-1/CXCR4 interactions in definitive fetal liver stem cell seeding of the BM. This defect can be partially corrected by forced expression of SDF-1 in the fetal BM endothelium of SDF-1 knockout mice (7).

We and others have showed that SDF-1/CXCR4 interactions are essential also for adult human stem cell migration *in vivo* (3, 8–10). Homing and subsequent engraftment of human CD34$^+$/CD38$^{-/low}$ SCID-repopulating cells/stem cells in transplanted immune-deficient NOD/SCID mice are critically regulated by cell surface CXCR4 and SDF-1 produced within the murine BM. Interestingly, CXCR4 signaling is also necessary for the repopulation by undifferentiated human CD34$^+$ progenitors directly injected into the murine bone (10). *In vitro* and *in vivo* studies suggest that SDF-1 induces stem cell extravasation to the BM by activation of the adhesion molecules VLA-4, VLA-5, LFA-1, and CD44 (11–14).

In addition, SDF-1/CXCR4 interactions participate in controlling the retention of hematopoietic cells within the BM and their release into the circulation (15, 16). Disruption of SDF-1/CXCR4-mediated cell anchorage results in egress of cells into the circulation (17, 18). BM SDF-1 degradation by proteolytic enzymes causes the release of progenitor and mature cells from the BM into the peripheral blood (PB) during granulocyte colony-stimulating factor-induced mobilization (19, 20). These observations in murine models are of clinical relevance because several studies revealed a correlation between CXCR4 expression and stem cell motility in humans. Higher CXCR4 expression or *in vitro* migratory ability were correlated with faster hematological recovery in patients after allogeneic and autologous CD34$^+$ cell transplantation or in juvenile patients transplanted with cord blood (CB) cells (21–23). Another study reported a correlation between lower levels of SDF-1 and CXCR4 expression in the periphery and higher mobilization rates of immature CD34$^+$ cells in patients treated with Cy and granulocyte colony-stimulating factor (24).

SDF-1 has been found to have numerous biological roles. In addition to controlling cell motility, SDF-1 can regulate cell proliferation, induces cell cycle progression, and acts as a survival factor for both human and murine stem cells (25–28). Finally, SDF-1/CXCR4 interactions can also promote angiogenesis and are regulated by vascular endothelial growth factor (29–31).

Acute myelogenous leukemia (AML) is characterized by uncontrolled proliferation within the BM of myeloid progenitors arrested in their maturation process. It is usually associated with egress of blasts into the circulation and in some AML subtypes dissemination to extramedullar hematopoietic organs such as spleen and liver. Leukemic cells interact with the hematopoietic microenvironment in many ways similar to normal precursors (32). For example, adhesion of AML cells is mediated by the interactions of VLA-4 and VLA-5 with extracellular matrix fibronectin as well as via both VLA-4 and LFA-1 integrin interactions with stromal cells (33, 34). The VLA-4 mediated adhesion of leukemic cells with stromal layers prevents the apoptosis of leukemic cells, attenuating their chemotherapy-induced cell death and may be crucial in BM minimal residual disease and AML prognosis (35, 36). Mohle *et al.* (37, 38) previously showed that some human AML cells express CXCR4 and can also migrate toward a gradient of SDF-1 in a transendothelial migration assay *in vitro*.

In contrast to *in vitro* assays that can only detect progenitors with limited proliferation and replating potential, in the functional preclinical SCID-leukemia model the leukemic stem cells, termed SCID leukemia-initiating cells (SL-ICs), which mostly have a primitive, undifferentiated CD34$^+$/CD38$^-$ expression pattern, can extensively

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repopulate the murine BM. After total body irradiation, intraveinally injected leukemic stem cells reach the BM and proliferate, resulting in a pattern of dissemination and leukemic cell phenotype similar to those seen in the original patients (39–41). In the present study, we explored the importance of SDF-1/CXCR4 interactions for human AML SL-IC/stem cell survival and for *in vivo* homing and repopulation using the NOD/SCID-leukemia model.

MATERIALS AND METHODS

Cell Lines. Human myeloid Meg01, U937, NB4, HL60, ML1, and KG1a cell lines developed from AML patients (kindly provided by Dr. Amnon Peled, Hadassah University Hospital, Jerusalem, Israel) were grown in RPMI with 10% FCS.

Human Cells. After informed consent and using protocols approved by the Weizmann ethics committee, PB or BM cells were obtained from 20 newly diagnosed AML patients and 1 resistant AML patient after chemotherapy treatment (Table 1, no. 16). The diagnosis of leukemia was based on routine morphological evaluation, immunophenotyping, and cytochemical smears using the French-American-British classification. The patients were classified as follows: M1: 3; M2: 3; M3: 1 M3v: 1; M4: 9; M4E: 3; and 1 patient was classified as M5 (Table 1).

Human CB cells from full-term deliveries or leftover granulocyte colony-stimulating factor-mobilized PB cells from healthy donors for clinical transplantation were obtained after informed consent. The samples were diluted 1:1 in PBS. Low-density mononuclear cells (MNCs) were collected after standard separation on Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) and washed in PBS. Cells were used freshly or frozen in FCS plus 10% DMSO for storage in liquid nitrogen.

Mice. NOD/LtSz-PrKdc^{scid} (NOD/SCID) mice and β2 microglobulin knockout NOD/LtSz-scid B2m^{null} mice (NOD/SCID/B2m^{null}) were bred and maintained under defined flora conditions in individually ventilated (HEPA-filtered air) sterile microisolator cages (Techniplast, Buguggiate, Italy) at the Weizmann Institute. All of the experiments were approved by the animal care committee of the Weizmann Institute. NOD/SCID and NOD/SCID/B2m^{null} mice 8–12 weeks old were irradiated from a cobalt source with a sublethal dose of 375 or 350 cGy (67 cG/min), respectively, 5–24 h before transplantation. Human AML cells, mobilized PB or CB MNCs at the indicated cell doses, were suspended in 500 μl of RPMI with 10% FCS before injection via the dorsal tail vein. For homing experiments, cells were pretreated with anti-CXCR4 antibodies (Abs) 12G5 (R&D Systems, Minneapolis, MN; 10 μg/ml) for 30 min and i.v. injected without washing into mice. The number of human cells in the BM and spleen was assayed 16 h later as described below. Chimeric mice engrafted with human cells were i.p. injected with anti-CXCR4 Abs 12G5 (10 μg/ml) weekly, starting 2 days or 3 weeks after transplantation. Mice were sacrificed at different time points after transplantation as indicated.

Table 1 *CXCR4 expression on acute myelogenous leukemia primary cells*

Patient no.	French-American-British type	Source	CXCR4$^+$ cells (%)
1	M1	BM[a]	7
2	M1	PB	ND
3	M1	PB	ND
4	M2	BM	16
5	M2	BM	14
6	M2	PB	85
7	M3	PB	0.15
8	M3v	PB	22
9	M4	PB	12
10	M4	PB	32
11	M4	PB	24.4
12	M4	PB	6.3
13	M4	PB	51
14	M4	PB	ND
15	M4	BM	ND
16	M4	PB	ND
17	M4	PB	ND
18	M4E	PB	17.5
19	M4E	PB	74
20	M4E	PB	ND
21	M5	BM	12

[a] BM, bone marrow; PB, peripheral blood; ND, not determined.

Spleen and BM cells, flushed from both femurs and tibias, were resuspended into single-cell suspension. PB from mice asphyxiated with dry ice was collected by cardiac aspiration in heparinized tubes. The percentage of human cells was determined by immunostaining with human-specific Abs.

Homing and Engraftment Analysis. The numbers of human cells in the BM, spleen, and PB from mice assayed for short-term homing (16 h after transplantation) and for engraftment (3–7 weeks after transplantation) were detected with antihuman CD45-FITC (IQ Products, Groningen, the Netherlands) using flow cytometry as described previously (9). Human Fc receptors were blocked with human plasma (1%) and murine Fc receptors by mouse IgG. Isotype control Abs were used to exclude false positive cells (IQ Products). After staining, cells were analyzed on a FACSCalibur (BD) using CellQuest software.

SDF-1 and CXCR4 Immunofluorescence. Expression of external CXCR4 on human AML cells was detected with phycoerythrin-conjugated monoclonal anti-CXCR4 (PharMingen, San Diego, CA). Intracellular staining: After blocking with nonconjugated antihuman CXCR4 mAb (monoclonal antibody) (clone 12G5; 10 μg/ml, 1 h, 4°C), the cells were fixed with 4% paraformaldehyde and then permeabilized with 0.5% Triton X-100 for 10 min. Monoclonal antihuman CXCR4-PE was used to label the cells for flow cytometry. SDF-1 staining: Cells after permeabilization or unmanipulated were incubated for 30 min on ice with monoclonal anti-SDF-1 Abs (R&D Systems), washed, and stained with FITC goat antimouse Abs (Jackson Immunoresearch Laboratories). Because external SDF-1 staining was very low or not detectable, staining of cells after permeabilization reflects mainly intracellular SDF-1 expression.

Survival Assay. Primary AML cells (1×10^6/ml) and AML cell lines ($1–10 \times 10^4$/ml) were grown with or without recombinant human SDF-1 (Peprotech, Rocky Hill, NJ) at indicated concentrations, 12G5 (10 μg/ml) or AMD3100 (10–30 μM; kindly provided by Dr. Nobutaka Fujii, Kyoto University, Kyoto, Japan). Primary AML cells were cultured either in RPMI supplemented with 10% FCS or in serum-free medium supplemented with 2% BSA (Sigma, St. Louis, MO), 10 μg/ml insulin (Biological Industries, Beit Haemek, Israel), 200 μg/ml transferrin (Sigma), 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine (Biological Industries), 100 μg/ml streptomycin (Biological Industries), and 10 mM HEPES (Biological Industries). The number of viable cells was determined using trypan blue exclusion.

Immunohistochemistry. Immunohistochemical staining was performed on chloroma paraffin-embedded sections from AML patients, BM sections from mice engrafted with human AML cells, or CB MNC cells using the avidin-biotin-peroxidase complex method and diaminobenzidine tetrahydrochloride chromogen kit (Dako LSAB2; Dako Corporation, Carpinteria, CA). The sections were stained with the anti-SDF-1 mAb K15C (1:100 dilution; a generous gift from Dr. Fernando Arenzana-Seisdedos, Institute Pasteur, Paris, France) or anti-CXCR4 mAb 12G5 (R&D Systems; 1:100–200 dilution). Slides were counterstained with Mayer's hematoxylin. The specificity of the anti-SDF-1 mAb was confirmed by staining identical tissue sections using an isotype-matched control mAb.

RESULTS

Human AML Cells Express Heterogeneous Surface CXCR4 but Store High Levels of Intracellular CXCR4 and SDF-1. Previous studies showed that some AML cells express variable levels of cell surface CXCR4, which correlate with their SDF-1-induced migration (37, 38, 42). We analyzed by fluorescence-activated cell sorting the external and internal expression of CXCR4 and SDF-1 in 6 AML cell lines (Meg01, HL-60, KG1a, ML1, NB4, and U937) and primary AML cells from 14 patients with different FAB subtypes. We observed variable but mostly low levels of external CXCR4 in most of the examined samples (Fig. 1 *A1* and Table 1). However, high expression of internal CXCR4 was found in all cases, including cells that do not express surface CXCR4. Fig. 1 *A2* shows four AML cell lines (ML-1, U937, Kg1a, and NB4) and two primary AML cells (M4E and M3) that expressed high levels of intracellular CXCR4 and heterogeneous surface CXCR4.

SDF-1 is produced and secreted by different hematopoietic cells,

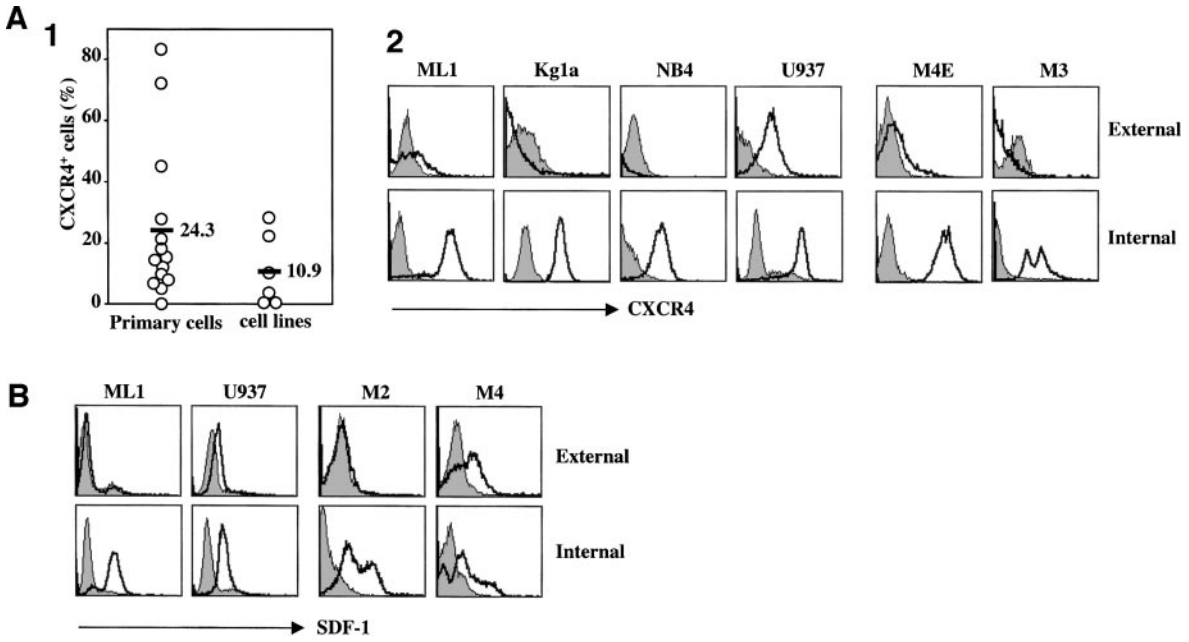

Fig. 1. Human acute myelogenous leukemia (AML) cells express CXCR4 and stromal cell-derived factor-1 (SDF-1). Primary AML cells and cell lines were stained for external and internal CXCR4 and SDF-1. Percentage of external CXCR4 expression on AML primary cells from 14 patients (Table 1) and 6 AML cell lines were measured by fluorescence-activated cell sorting (*A1*). Representative fluorescence-activated cell sorting analyses of AML cell lines (*left panel*) and primary leukemic cells (*right panel*) of external and internal CXCR4 (*A2*) and SDF-1 (*B*). The shaded histograms show staining with isotype-matched control antibodies, and the open histograms show staining with CXCR4 (*A2*) or SDF-1 (*B*) antibodies.

including the more mature $CD34^+/CD38^+$ progenitor cells (43). Secreted SDF-1 can also be cell membrane bound (29). We next analyzed whether human AML cells also express internal and/or external SDF-1. We found that all AML cells tested expressed intracellular SDF-1 (15–95% of cells, average of 53 ± 7.9%). Moreover, in several cases cell surface SDF-1 was also detected in both primary AML cells and cell lines (Fig. 1*B*), suggesting secretion of this chemokine by the malignant cells.

***In Vivo* Staining Revealed Lower CXCR4 Expression on AML Cells as Compared with Normal Progenitor Cells.** Recently, it was shown that normal human BM hematopoietic cells express high level of CXCR4 (44). To study human CXCR4 expression *in vivo*, we compared CXCR4 staining on BM sections of NOD/SCID mice engrafted either with primary AML cells or normal CB MNC. We observed that leukemic cells displayed lower CXCR4 labeling as compared with the normal progenitors (Fig. 2*A–D*). Chloromas are rare infiltration of AML cells into non hematopoietic tissues such as the skin or gum and are found usually often in M4, M5 FAB subtypes. We were interested to examine the SDF-1/CXCR4 expression in AML cells infiltrated to these unusual sites. We therefore stained paraffin-embedded sections of human chloroma for CXCR4 and SDF-1. Fig. 2, *E* and *F*, shows a representative specimen of chloroma from the gum of AML patient with predominantly infiltration of leukemic cells (white arrows) and few normal squamoepithelial cells (black arrows). Immunostaining for human CXCR4 (Fig. 2*E*) and SDF-1 (Fig. 2*F*) revealed a diffuse staining on leukemic cells with lower CXCR4 and higher SDF-1 immunoreactivity than the normal surrounding tissue.

SDF-1 Enhances *in Vitro* Survival of AML Cells. SDF-1 was recently found to be a survival factor for normal human $CD34^+$ cells and murine stem cells (25, 26, 28). We assessed whether also AML cells respond to exogenous SDF-1 in culture. Primary AML cells (1×10^6) and AML cell lines ($1–10 \times 10^4$) were cultured in serum-free media or RPMI supplemented with 10% FCS, respectively, in the presence of 100 ng/ml SDF-1. When AML cells were cultured with SDF-1 (3 days for primary cells and ML-1 cell line and 7 days for U937 and HL-60), we observed a significant 1.5–2.2-fold increase in the number of viable cells as compared with control cells (Fig. 3*A*). Of note, different growth patterns were observed between primary AML cells and cell lines. The number of primary cells was reduced after 3 days in serum-free conditions; however, when cells were cultured in the presence of SDF-1, a small but significant increase in the numbers of viable cells was observed (Fig. 3*B*). After 5–7 days in RPMI supplemented with 10% FCS, the number of viable U937 cells was increased and cell proliferation was additionally enhanced by 100 ng/ml SDF-1 (Fig. 3*C*). Notably, exogenous SDF-1 had no effect on cell proliferation and survival on culture of AML cells, which did not express cell surface CXCR4 (patient no. 7; data not shown). To determine the effect of various doses of exogenous SDF-1 on AML cell proliferation, ML-1 cells were cultured for 3 days in the absence or presence of increasing concentrations of SDF-1 (1, 100, or 1000 ng/ml). The stimulatory effect of SDF-1 on ML-1 cell proliferation was maximal for 100 ng/ml, whereas addition of 1000 ng/ml SDF-1 had only a minimal effect (Fig. 3*D*). At high concentrations (1–2 μg/ml), SDF-1 provokes internalization and desensitization of CXCR4 in normal hematopoietic progenitor cells (8, 16). Indeed, we observed maximal CXCR4 internalization on ML-1 and U937 AML cell lines after incubation with 1000 ng/ml SDF-1 (data not shown).

Neutralizing Anti-CXCR4, AMD3100, or Anti-SDF-1 Abs Reduce AML Cell Survival. We showed that AML cells express SDF-1 and that they can efficiently respond to exogenous SDF-1. To investigate the eventual role of endogenous SDF-1 on AML cell survival, we incubated AML cells with CXCR4 or SDF-1 blocking agents. Treatment for 3–4 days with the neutralizing anti-CXCR4 Abs 12G5 (10 μg/ml) or AMD3100 (10–30 μM), an antagonist of SDF-1 binding to CXCR4, reduced by 20–58% the proliferation of AML cell lines and the survival of primary AML cells (Fig. 4). We next tested the effect of blocking the endogenous SDF-1 on survival of AML cells by treating AML cells from patients and ML-1 cell line with neutralizing

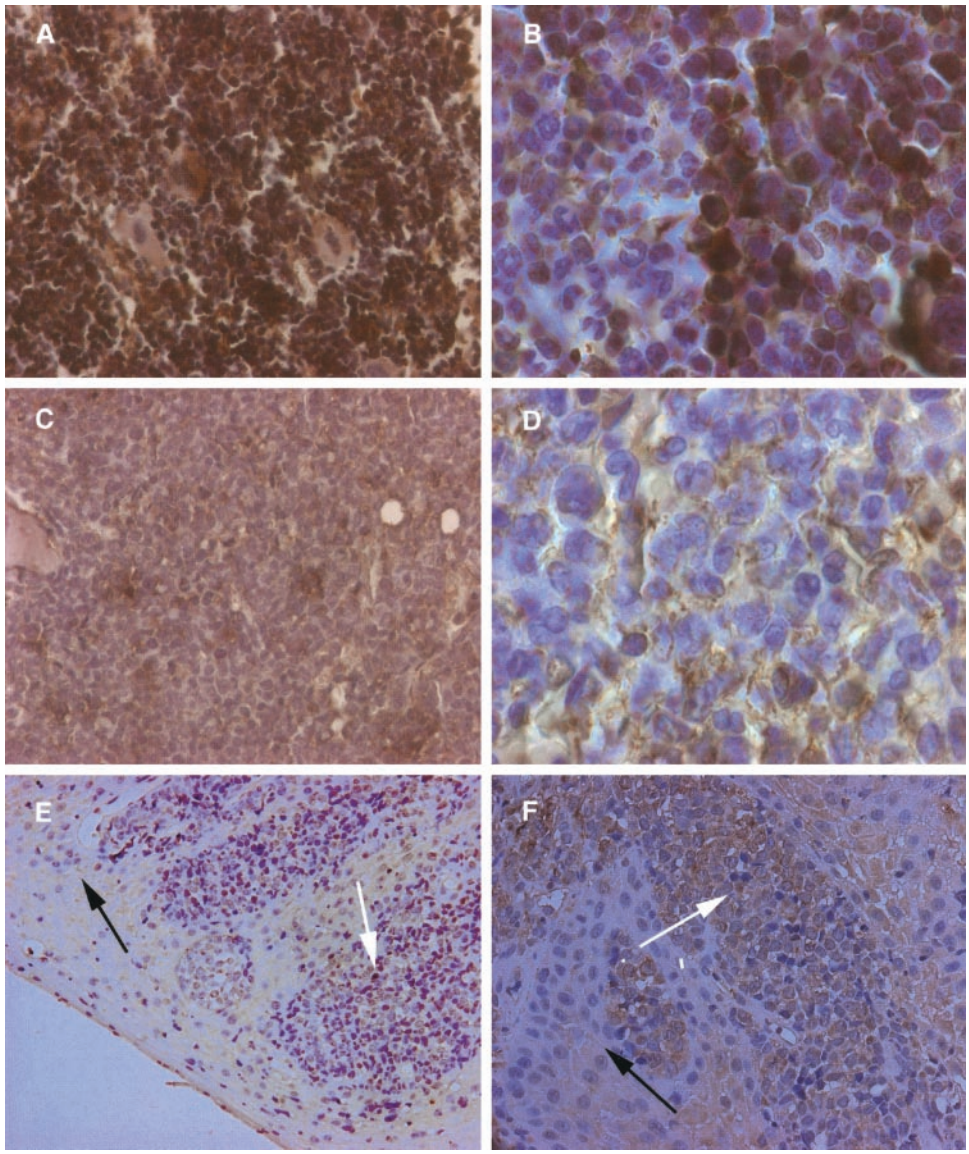

Fig. 2. *In vivo* acute myelogenous leukemia (AML) cells express lower CXCR4 levels than normal $CD34^+$ cells. Bone marrow sections from NOD/SCID mice engrafted with human cord blood $CD34^+$ cells (*A* and *B*) or primary AML cells (*C* and *D*) were immunohistochemically stained for human CXCR4. Human chloroma sections from the gum were stained for CXCR4 (*E*) and stromal cell-derived factor-1 (*F*). Rabbit antimouse IgG conjugated with peroxidase was used as secondary antibody, and color reaction was developed with 3,3′-diaminobenzidine as substrate. *Black arrows* represent leukemic cells, whereas *white arrows* show normal surrounding tissues. Original magnification, ×160 (*A*, *C*, *E*, and F); ×400 (*B* and *D*).

anti-SDF-1 Abs (1 μg/ml); the treated cells showed a decrease of 25–70% in the number of viable cells as compared with untreated cells (Fig. 4). These data suggest that survival and proliferation of AML cells are mediated by SDF-1/CXCR4 interactions and autocrine secretion of SDF-1.

Homing of AML Cells into NOD/SCIDB2m^{null} Mice Is CXCR4 Dependent. SDF-1/CXCR4 interactions play major roles in the homing and repopulation of human SCID-repopulating cell/stem cells transplanted into immune-deficient NOD/SCID mice (8, 9). Previous studies showed that transplantation of primary AML SL-IC/stem cells into SCID and NOD/SCID mice resulted in their engraftment and proliferation in the murine hematopoietic tissues (39–41). To study the regulation of AML stem cell trafficking and homing by SDF-1/CXCR4 interactions, primary human AML cells (5×10^6) from 6 patients were injected into sublethally irradiated NOD/SCIDB2m^{null} mice, either untreated or after 30 min incubation with neutralizing antihuman CXCR4 12G5. Sixteen h after transplantation, human AML cells could efficiently home to the BM and spleen of the recipients (Fig. 5). For all samples examined, anti-CXCR4 pretreatment inhibited the homing of human leukemic cells into the murine BM and spleen (Fig. 5). These results demonstrate that, similarly to normal stem and progenitor cells, human AML cells also home to the murine BM and spleen in an SDF-1/CXCR4-dependent manner.

Neutralizing Anti-CXCR4 Abs Preferentially Decreases the Number of AML Cells in the BM of Engrafted NOD/SCID Mice. AML is characterized by a deregulated and increased cell proliferation of leukemic blasts. Our findings demonstrated that neutralizing the CXCR4 receptor could partially decrease *in vitro* AML cell proliferation. We hypothesized that anti-CXCR4 treatment may also interfere *in vivo* with the repopulation of AML SL-IC/stem cells transplanted into NOD/SCID mice, a process that requires active cell proliferation. Primary AML cells ($14–30 \times 10^6$) were injected into sublethally irradiated NOD/SCID mice, and anti-CXCR4 12G5 was administrated 2 days later (allowing the cells to home into the BM). Weekly treatment with 10 μg of anti-CXCR4 for 3 or 7 weeks significantly decreased the engraftment levels of AML cells as compared with untreated mice (Fig. 6*A*). Notably, in the mouse that was treated for 7 weeks, human AML cells were almost undetectable. In addition to this inhibitory effect on AML engraftment in the BM, treatment with anti-CXCR4 Abs also resulted in severe reduction in the number of AML cells disseminated in the PB, as well as in the spleen (Fig. 6*B*). These results demonstrate the importance of SDF-1/CXCR4 interac-

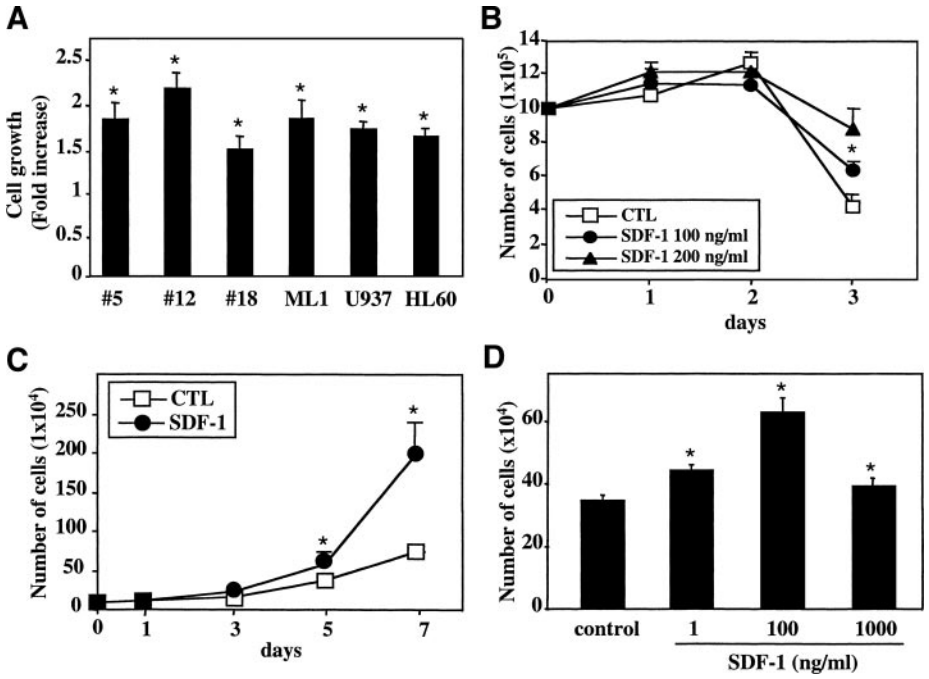

Fig. 3. Stromal cell-derived factor-1 (SDF-1) enhances the survival of acute myelogenous leukemia (AML) cells. *A*, primary AML cells and AML cell lines were grown in serum-free conditions or RPMI with 10% FCS, respectively, in presence or not of recombinant SDF-1 (100 ng/ml). The number of viable cells was determined after 3 (primary AML cells and ML-1) or 7 days (U937 and HL-60). Results are shown as fold increase in the number of cells as compared with control. *, $P < 0.05$; **, $P < 0.01$. *B*, representative figure of survival of primary mononuclear cells (1×10^6) from AML patient (M2, no. 5) grown in serum-free medium for 3 days with or without SDF-1 (100 or 200 ng/ml). Cultures were done in triplicate. *C*, U937 cells (1×10^4/ml) were cultured up to 7 days with or without SDF-1 (100 ng/ml), and viable cells were counted at days 0, 1, 3, 5, and 7. Three independent experiments were performed in triplicate, and the mean values of one representative experiment with SDs are shown. *D*, the ML-1 cell line (1×10^5) was grown in RPMI/10% FCS in the presence of various concentrations of SDF-1 (1, 100, or 1000 ng/ml) and counted by trypan blue exclusion on day 3. Results indicate mean (±SD) of two independent experiments done in triplicate.

tions for the retention and repopulation of the murine BM by the malignant AML clone. However, similar weekly treatment with neutralizing anti-CXCR4 Abs for 4 weeks also significantly reduced the engraftment of normal human CB MNC (Fig. 6*A*) and mobilized PB MNC (data not shown).

We next examined the therapeutic potential of CXCR4 neutralization on NOD/SCID mice already engrafted with AML cells from 3 patients. Three weeks after transplantation with AML cells, engrafted NOD/SCID mice were treated with anti-CXCR4 once a week for a period of 3–4 additional weeks. This anti-CXCR4 treatment resulted in significant reduction in the levels of AML cells present within the BM as compared with untreated chimeric mice (Fig. 6*C*).

Surprisingly, when this treatment was given to NOD/SCID mice engrafted with CB MNC starting 3 weeks after transplantation, no significant effect on the levels of engraftment was noticed in the murine BM (Fig. 6C, $P = 0.35$) and spleen (data not shown) in contrary to the effect observed with neutralizing CXCR4 treatment starting after 2 days. These results suggest that the initial stage of the engraftment process by normal progenitors is more dependent on CXCR4 than their long-term repopulation ability and that AML SL-IC/stem cells are more sensitive to CXCR4 neutralization than their normal counterparts.

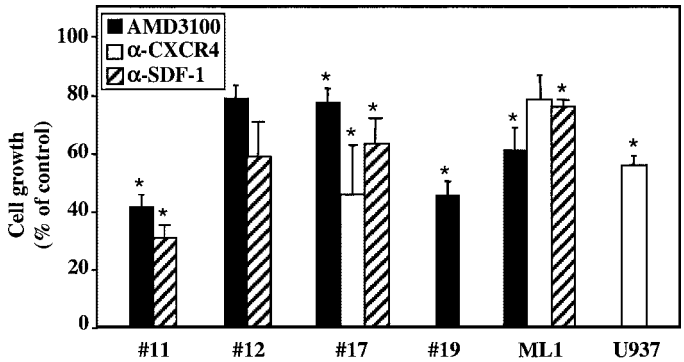

Fig. 4. Effect of neutralizing CXCR4 and stromal cell-derived factor-1 (SDF-1) on acute myelogenous leukemia (AML) cell survival *in vitro*. Primary AML cells and AML cell lines were cultured for 3–6 days with or without AMD3100 (10–30 μM), neutralizing antibody to CXCR4 (12G5; 10 μg/ml), or anti-SDF-1 antibodies (1 μg/ml). The total number of viable cells was counted by trypan blue exclusion. The results show the percentage of treated cells as compared with control (*, $P < 0.05$). The results are average ± SD of two independent experiments (cell lines) or single experiments (primary AML cells) done in triplicate.

DISCUSSION

In recent years, a number of studies emphasized the idea that tumor cell migration and organ-specific metastasis are critically regulated by chemokines and their receptors. Abnormal expression of CXCR4 or SDF-1 has been observed in solid tumors such as prostate cancer (45), kidney cancer (46), neuroblastoma (47), gilomas (48), pancreatic cancer (49), colon (50), and breast cancer. Muller *et al.* (51) showed that CXCR4 was highly expressed in some breast malignant cells but not in normal mammary tissue and that SDF-1 was expressed in organs where breast cancer metastases are frequently found (BM, lymph nodes, lung, and liver). Most importantly, anti-CXCR4 treatment could efficiently reduce the tumor load of a human breast cancer cell line in the lungs and lymph nodes of SCID mice (51).

In contrary to solid tumors that invade into the BM usually in the late stages of the disease, AML originates in the BM. SDF-1 is constitutively produced in the BM by immature osteoblasts lining the endosteum region, stromal and endothelial cells (3, 52). It was found in different studies to enhance the survival of human $CD34^+$ cells and murine stem cells (25, 26, 28). We hypothesized that the SDF-1/CXCR4 axis may also play essential roles in the migration, development, abnormal proliferation, and anchorage of human AML stem cells in the BM. In the present study, we reveal the importance of SDF-1 in the regulation of AML stem cells trafficking and survival. We show that 100 ng/ml exogenous SDF-1 moderately enhances *in vitro* survival of AML cells that express CXCR4 on their surface. This effect was observed even at a low concentration of 1 ng/ml. Interestingly, the increased survival was mainly observed after deprivation conditions of cultured AML cell lines for at least 5–7 days or primary AML cells cultured in serum-free medium. These results are in accordance with the previous studies demonstrating that SDF-1 enhanced *in vitro* survival of normal human $CD34^+$ cells and murine stem cells in the absence of growth factors (25, 27, 28). This effect of

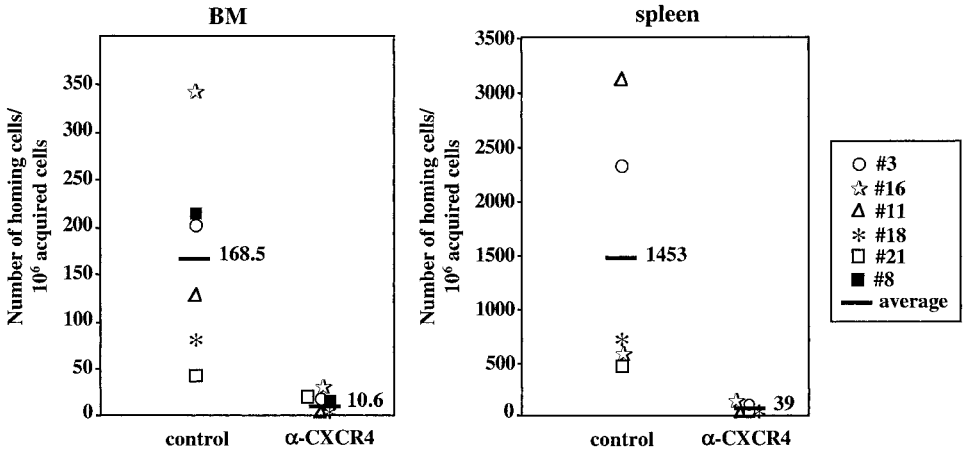

Fig. 5. Anti-CXCR4 prevents the homing of acute myelogenous leukemia (AML) cells into NOD/SCIDB2m^{null} mice. Primary AML cells from 6 different patients, either untreated or after 30 min incubation with anti-CXCR4 antibodies (12G5; 10 μg/ml), were injected into sublethally irradiated NOD/SCIDB2m^{null} mice. Data shown represent the number of human CD45$^+$ cells/10^6 total acquired cells from bone marrow (BM; *A*) and spleen (*B*) 16 h after transplantation.

SDF-1 in stress deprivation conditions may give an advantage in the growth and dissemination of myeloid blast cells, especially in view of the fact that BM SDF-1 levels increase after irradiation and chemotherapy (3). Because VLA-4 activation was shown to be involved in the pathogenesis of AML minimal residual disease (36) and SDF-1 can activate the major integrins VLA-4 and VLA-5 (12), therefore SDF-1 may be important for the development of BM minimal residual disease that causes AML relapse after chemotherapy. Furthermore, we found that AML cells express SDF-1 and that *in vitro* treatment of AML cells with neutralizing anti-CXCR4 Abs, anti-SDF-1 Abs, or AMD3100 decrease cell survival, implying autocrine regulation of AML cell survival by endogenous SDF-1. However, the fact that blocking CXCR4 or SDF-1 reduced but did not completely inhibit the proliferation and survival of AML cells suggest that other factors and pathways than CXCR4/SDF-1 interactions are also involved in the regulation of these processes.

Our results show that the expression of cell surface CXCR4 on AML cells is variable and in most cases lower than the level of CXCR4 on normal CD34$^+$ progenitor cells, 40% of which express external CXCR4 (43). SDF-1/CXCR4 interactions are crucial for retention of hematopoietic progenitors within the BM. Therefore, low CXCR4 expression on AML cells together with secretion of the ligand and proteolytic enzymes may impair their anchorage and facilitate their egress into the circulation and their dissemination. In some AML cells from different FAB subtypes, the receptor is not expressed on the surface; however, all of the leukemic cells that we analyzed express internal CXCR4 receptor. The reasons for the low or absent levels of cell surface CXCR4 expression despite the presence of internal CXCR4 are not clear but could result from cleavage by matrix metalloproteinases or other proteolytic enzymes, which are abnormally secreted by AML cells (53). The levels of CXCR4 expression usually correlate with cell motility toward SDF-1. In childhood acute lymphoblastic leukemia, patients with high expression of CXCR4 develop a more aggressive phenotype of the disease with extramedullar organ infiltration (54). Moreover, Mohle *et al.* (37) found that higher expression of CXCR4 on AML M4 and M5 FAB subtypes is associated with increased SDF-1-induced transendothelial migration *in vitro*. Because human chloromas are usually seen in M4 and M5

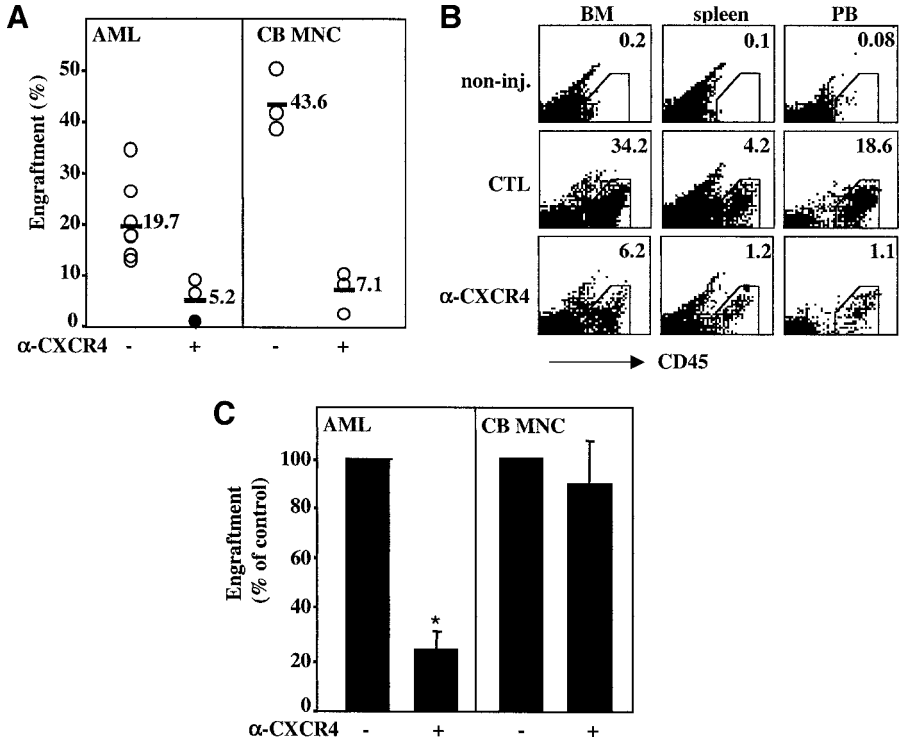

Fig. 6. Anti-CXCR4 significantly inhibits human acute myelogenous leukemia (AML) cell engraftment. NOD/SCID mice were treated with neutralizing antihuman CXCR4 (12G5; 10 μg/week) starting 2 days (*A* and *B*) or 3 weeks (*C*) after transplantation of human primary AML or cord blood mononuclear cell (CB MNC) cells. Percentage of human CD45$^+$ cells engrafted in the murine bone marrow (BM) was determined by fluorescence-activated cell sorting immunostaining. *Left panel* shows the percentage of AML cell engraftment in 7 control mice and 3 anti-CXCR4-treated mice from two independent experiments. In the treated mice, ○ represents mice treated with 3 weekly injections, whereas ● represents 1 mouse treated with 7 weekly injections ($P = 0.002$). *Right panel* shows the percentage of CB MNC cell engraftment in control mice and CXCR4-treated mice for 4 weeks. *B* shows a representative fluorescence-activated cell sorting analysis of AML cell engraftment in BM, spleen, and peripheral blood (PB) of control untreated mouse and mouse treated with anti-CXCR4 for duration of 4 weeks starting 2 days after cell injection. Analysis of a noninjected mouse is also shown. Numbers represent percentage of human cell engraftment. *C* represents average ± SD from 4 (AML) and 4 (CB) independent experiments (*, $P = < 0.0001$).

subtypes, we hypothesized that infiltration of the leukemia cells to nonhematopoietic sites could be associated with increased motility due to overexpression of CXCR4. Surprisingly, we found low levels of CXCR4 in human chloromas but higher SDF-1 expression as compared with the surrounding normal tissues, suggesting that secretion of SDF-1 by AML stem cells may facilitate, in an autocrine manner, their infiltration and tumor growth in nonhematopoietic tissues such as gum and skin. In support of this hypothesis, Sun *et al.* (55) recently showed that aggressive phenotype and metastasis of human prostate carcinoma into the patient bone is also associated with higher SDF-1 expression by the cancer cells and not only with the level of CXCR4.

Many observations suggest that cancer stem cells are regulated by genes and pathways similar to the normal stem cells (56). SDF-1/CXCR4 interactions are crucial for homing and repopulation of normal human $CD34^+/CD38^{-/low}$ SCID-repopulating cells into the BM of NOD/SCID mice (8, 57). Previous studies showed that also the more primitive AML $CD34^+/CD38^-$ cells with the SL-IC phenotype express CXCR4 receptor (37, 42). In the present study, we demonstrate that homing of primary human AML cells into NOD/SCID/$B2m^{null}$ mice is CXCR4 dependent, similarly to normal human stem cells.

In addition, we found that CXCR4 neutralization had a remarkable effect on engraftment of human AML cells. Chimeric mice treated with anti-CXCR4 starting 2 days or even 3 weeks after transplantation show a dramatic reduction of AML cell engraftment. As little as 10 μg of anti-CXCR4 weekly injected was sufficient to reduce the number of AML cells engrafted in the BM, spleen, and PB of NOD/SCID mice. Recently, Bertolini *et al.* (58) showed decrease in human non-Hodgkin's lymphoma growth *in vivo* and prolonged survival of transplanted NOD/SCID mice after 3 weekly treatment of mice with 100 μg of antihuman CXCR4 Abs. The different doses needed to abrogate the growth of AML and non-Hodgkin's lymphoma cells in NOD/SCID mice might be due to the diverse level of surface CXCR4 expression that is high in non-Hodgkin's lymphoma and lower in human AML cells. Interestingly, we previously showed that a single injection of anti-CXCR4 Abs within the first 24 h after transplantation of normal human $CD34^+$ cells into NOD/SCID mice severely reduced their BM engraftment. On the other hand, when the single injection of anti-CXCR4 was given 4 days after transplantation, no effect was observed, suggesting that neutralizing CXCR4 interferes only with the initial step of homing of normal human stem cells (8). In the present study, we further addressed this issue and showed that prolonged, repeated neutralizing CXCR4 treatment starting 2 days after engraftment dramatically reduces the repopulation and survival of normal progenitors *in vivo*. However, once the cells were engrafted, no significant impairment of normal hematopoiesis by CXCR4 inhibition was observed, even after repetitive weekly injections. These results suggest that AML stem cells are more sensitive to anti-CXCR4 treatment than normal human stem cells and are of clinical relevance when considering CXCR4 neutralization for treatment of AML as well as for various tumor types as suggested previously (52, 58, 59). Especially, this treatment could be relevant in combination with chemotherapy/radiotherapy that increase BM SDF-1, which itself may promote growth and angiogenesis of residual malignant cells (3).

Taken together, our data indicate that SDF-1/CXCR4 interactions participate in the migration, repopulation, and development of AML SL-IC/stem cells in the BM by regulating their anchorage to the stromal microenvironment and cell survival. We additionally propose CXCR4 neutralization as a potential therapeutic approach, which may inhibit AML stem cell proliferation and migration while only minimally affecting normal stem cells.

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