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Authors

Phillips, R J
Burdick, M D
Lutz, M
et al.

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The Stromal Derived Factor–1/CXCL12–CXC Chemokine Receptor 4 Biological Axis in Non–Small Cell Lung Cancer Metastases

Roderick J. Phillips, Marie D. Burdick, Marin Lutz, John A. Belperio, Michael P. Keane, and Robert M. Strieter

Division of Pulmonary and Critical Care Medicine, Department of Medicine, and Department of Pathology, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, California

Non–small cell lung cancer is characterized by a specific metastatic pattern. The mechanism for organ-specific metastasis is poorly understood, although evidence has suggested that the chemokine stromal derived factor-1 (CXCL12) and its cognate receptor CXCR4 may regulate breast cancer metastasis. We hypothesized that the CXCL12–CXCR4 biological axis is important in mediating non–small cell lung cancer metastases. Our results indicate that both non–small cell lung cancer tumor specimens resected from patients and non–small cell lung cancer cell lines express CXCR4, but not CXCL12. Non–small cell lung cancer cell lines undergo chemotaxis in response to CXCL12. CXCL12–CXCR4 activation of non–small cell lung cancer cell lines showed intracellular calcium mobilization and mitogen-activated protein kinase activation with enhanced extracellular signal-related kinase-1/2 phosphorylation without change in either proliferation or apoptosis. Target organs in a murine model that are the preferred destination of human non–small cell lung cancer metastases elaborate higher levels of CXCL12 than does the primary tumor, and suggest the generation of chemotactic gradients. The administration of specific neutralizing anti-CXCL12 antibodies to severe combined immunodeficient mice expressing human non–small cell lung cancer abrogated organ metastases, without affecting primary tumor-derived angiogenesis. These data suggest that the CXCL12–CXCR4 biological axis is involved in regulating the metastasis of non–small cell lung cancer.

Keywords: chemokines; chemotaxis; lung cancer metastases

Although lung cancer, and particularly primary non–small cell lung cancer (NSCLC), is the leading cause of malignancy-related mortality in the United States (1, 2), the biology of this devastating disease is complex and poorly understood. NSCLC metastases to regional lymph nodes, liver, adrenal glands, contralateral lung, brain, and bone marrow are a key factor in the virulence of this cancer (3, 4). Experimental data have demonstrated that sites of metastasis are determined not only by the characteristics of neoplastic cells but also by the microenvironment of the specific organ (5). The organ provides growth conditions that are optimized for specific cancers. Moreover, the ability of cancer cells to traffic to these organs depends on the organ's special abilities to attract through chemotactic factors specific types of cancer cells (6). In this context, it appears that NSCLC cells can metastasize to specific organs depending on the ability of the organ to express factor(s) that mediate tumor cell extravasation and recruitment from the vascular to the extravascular tissue compartment. This pattern of tumor cell metastases may be analogous to leukocyte trafficking and tissue extravasation. However, the specific mechanisms that actually promote organ metastases have not been fully elucidated.

Chemokines are a superfamily of small (8–10 kD) proteins, which play a pivotal role in the regulation of leukocyte trafficking and extravasation through the lumenal surface of endothelial cells into sites of tissue inflammation (7–9). The chemokine superfamily includes at least 20 receptors and as many as 40 ligands (7–9). The chemokine ligands can be separated into four categories depending on whether they express a C, CC, CXC, or CX_3C amino acid motif in their N termini. The CXC chemokines bind to a family of G protein-coupled Serpentine (seven transmembrane-spanning) receptors, which are termed CXC chemokine receptors (CXCRs) (8, 10). Currently six of these receptors have been identified (7, 8, 11–13). CXCL12 (stromal cell-derived factor-1, SDF-1) is a member of the CXC chemokine family, and has been found to recruit $CD34^+$ hematopoietic progenitor cells, megakaryocytes, B cells, and T cells (14). CXCL12 binds to CXC chemokine receptor 4 (CXCR4) (14). CXCR4 was originally discovered as the putative coreceptor for lymphotropic strains of human immunodeficiency virus (HIV) (14), and CXCL12 is its lone CXC chemokine ligand. Both $CXCL12^{-/-}$ and $CXCR4^{-/-}$ mice die *in utero*, and both exhibit similar defects in cardiogenesis, vascular development, hematopoiesis, and neuronal development (14–18).

Breast cancer metastases, similar in character to NSCLC metastases, was originally characterized by Paget (19), who demonstrated that this cancer has a distinct metastatic pattern preferentially involving the regional lymph nodes, bone marrow, lung, and liver. Müller and colleagues (20) have provided new insights into potential mechanisms related to organ-specific metastases of breast cancer cells directly related to a CXC chemokine. They found that CXCR4 was the most highly expressed chemokine receptor in human breast cancer. The ligand for CXCR4, CXCL12 mRNA, exhibited peak levels of expression in organs that are preferential destinations of breast cancer metastasis. Moreover, *in vivo* neutralization of CXCR4 resulted in significant inhibition of metastases of breast cancer in an organ-specific manner.

Although the above-cited study has provided evidence that the predominant function of CXCL12–CXCR4 in tumorigenesis is one of inducing metastases, studies have also indicated that this chemokine ligand–receptor pair is important in promoting angiogenesis (21, 22). Evidence suggests that CXCL12α may be involved in upregulating levels of vascular endothelial growth factor and basic fibroblast growth factor, and that subcutaneous injection of CXCL12α into mice induces formation of local small blood vessels (21, 22). However, it has yet to be demonstrated in an *in vivo* tumor model system that endogenous CXCL12 binding to CXCR4

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Correspondence and requests for reprints should be addressed to Robert M. Strieter, M.D., Division of Pulmonary and Critical Care Medicine, David Geffen School of Medicine at UCLA, 900 Veteran Ave., 14-154 Warren Hall, Los Angeles, CA 90024. E-mail: rstrieter@mednet.ucla.edu

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mediates a significant portion of primary tumor angiogenesis and angiogenesis-dependent tumor growth.

In an effort to address the role of the CXCL12–CXCR4 biological axis in NSCLC, we have developed both a tissue culture-based model system and an *in vivo* mouse paradigm to characterize the activity of this ligand–receptor pair. Our results indicate that CXCR4 is significantly expressed on NSCLC cells in freshly isolated tumor specimens from patients, and that CXCL12 is not markedly elevated in primary human NSCLC, as compared with normal adjacent lung tissue. However, *in vitro*, CXCL12 stimulation of CXCR4 on NSCLC cells leads to chemotaxis, calcium mobilization, and activation of mitogen-activated protein kinase p42/44 (extracellular signal-related kinase, ERK-1/2). Using a severe combined immunodeficiency (SCID) mouse system of heterotopic or orthotopic xenoengraftment of human NSCLC cells, we find that CXCL12 protein levels are significantly higher in organs that are known to be highly susceptible to human NSCLC metastases, as compared with either the primary tumor or plasma levels, suggesting that a chemotactic gradient could be established between the site of the primary tumor and those organs that develop NSCLC tumor metastases. Moreover, in this model system, it appears that NSCLCs expressing CXCR4 have a selective advantage for metastasizing to these organs. Furthermore, *in vivo* neutralization of CXCL12 in our heterotopic and orthotopic murine model systems of spontaneous metastasis of human NSCLC results in marked attenuation of NSCLC metastases to several organs including the adrenal glands, liver, lung, brain, and bone marrow. The finding of this study provides evidence to support that the CXCL12–CXCR4 biological axis may be important in orchestrating the metastases of NSCLC.

METHODS

Reagents

Polyclonal goat anti-CXCL12 antibodies were produced by immunization with recombinant CXCL12 (PeproTech, Rocky Hill, NJ) as previously described (23, 24). Five hundred microliters of anti-CXCL12 was sufficient to specifically neutralize 1 μg of either human or murine CXCL12 in leukocyte chemotaxis assays. Anti-CXCL12 inhibited CXCL12-mediated endothelial cell and NSCLC cell chemotaxis. Anti-Factor VIII-related antigen, control antibodies, and proliferating cell nuclear antigen (PCNA) antibodies were purchased from Dako (Carpinteria, CA). The apoptosis detection assay was purchased from Roche Molecular Biochemicals (Indianapolis, IN). C3a levels were measured by the Complement C3a des Arg Correlate-EIA (Assay Designs, Ann Arbor, MI).

Human NSCLC Cell Lines

The Calu-1 and A549 NSCLC cell lines were cultured as previously described (24–27).

Normal Human Lung and NSCLC Tumor Tissue

Tissue specimens were obtained in accordance with University of California Los Angeles (UCLA) internal review board approval and processed as previously described (24, 27). Determination of NSCLC cell type was provided by UCLA pathologists.

Heterotopic and Orthotopic Human NSCLC–SCID Mouse Chimeras

Six- to 8-week-old female CB17-SCID beige mice (UCLA Core Facility) were injected either subcutaneously or transthoracically (orthotopic; 10^4 cells/25 μl) via the left lung with A549 cells, using a modification as previously described (24–27). Tumor-bearing mice were intraperitoneally injected with neutralizing anti-CXCL12 or preimmune serum, or received no treatment, as previously described (25–27). Tissue and blood samples were processed as previously described (24–27).

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated with TRIzol (Life Technologies, Rockville, MD) as previously described (23). Reverse transcription-polymerase chain reaction (RT-PCR) was performed (23) with the following primer sequences:

GAPDH sense: 5′-TCCATGACAACTTTGGTATCG
GAPDH antisense: 5′-GTCGCTGTTGAAGTCAGAGGA
CXCR4 sense: 5′-AGTATATACACTTCAGATAAC
CXCR4 antisense: 5′-CCACCTTTTCAGCCAACAG

Antibody Staining and Fluorescence-Activated Cell Sorting Analysis

Fluorescence-activated cell sorting (FACS) analysis was performed as previously described (24, 25) with primary antibodies anti-CXCR4 (R&D Systems, Minneapolis, MN) and anti-human CD49b (BD Biosciences Pharmingen, San Diego, CA); and with secondary antibody Alexa 488 (Molecular Probes, Eugene, OR). For each FACS analysis, 10,000 events per stained condition were analyzed.

Analysis of Tumor-derived Angiogenesis and Angiogenic Activity

Tumor-derived Factor VIII-related antigen was analyzed by FACS as previously described (27). Angiogenic activity was assayed *in vivo* in the cornea micropocket model and in an endothelial cell chemotaxis assay as previously described (23, 24, 26–28).

Detection of Apoptotic and Proliferation NSCLC Cells

NSCLC cell apoptosis was determined with an *in situ* cell death detection kit (Roche Molecular Biochemicals) according to manufacturer specifications. Proliferation of NSCLC cells was assessed using various concentrations of CXCL12 or immunolocalization of PCNA, as previously described (24, 26, 27).

Western Blotting

Western blot analysis was performed with an anti-phospho-ERK-1/2 antibody (Cell Signaling Technology, Beverly, MA) as previously described (23, 24). Equal loading of each lane was determined with anti-ERK-1/2 antibodies. The detection of CXCL12 was made with polyclonal anti-CXCL12 (R&D Systems).

Immunohistochemistry

Immunohistochemistry of tissue specimens was performed as previously described (23–27).

Tumor Cell Chemotaxis

Chemotaxis of NSCLC cell lines was induced according to methods previously described, with modifications (23–27).

Enzyme-linked Immunosorbent Assay Analysis

The quantity of human or murine CXCL12 present in tissue homogenates was determined by specific enzyme-linked immunosorbent assay (ELISA), using a modification of the double-ligand method as previously described (29).

Statistical Analysis

The animal studies involved 10 SCID mice for each treatment group. Data were analyzed with StatView 5.0 (SAS Institute, Cary, NC). All group comparisons were evaluated by analysis of variance with post hoc analysis (i.e., Bonferroni/Dunn) for statistical significance. Data were considered statistically significant if p values were 0.05 or less.

RESULTS

CXCR4, but Not CXCL12, Is Expressed in Specimens of Human NSCLC Tumors

Several studies have now shown that CXCR4 is abundantly expressed on cells from a number of epithelial cells, metastatic cancers including ovarian, pancreatic, neuroblastoma, non–Hodgkin's lymphoma, and multiple myeloma and although these

tumors arise from different tissues they all share the ability to undergo chemotaxis in the presence of CXCL12 (30, 31). One article has further suggested that CXCL12–CXCR4 biology is important in dictating organ-specific metastases of breast cancer (20). Initially, we wanted to establish whether CXCR4 was expressed on NSCLC cells in tumors resected from patients. To do this, we performed immunohistochemical studies with antibodies specific for CXCR4 on 20 specimens of stage I and II squamous cell carcinoma antigen (SCCA) and 20 samples of Stage I and II adenocarcinoma. Our results indicate that CXCR4 is expressed on a majority (more than 80%) of the tumor cells in either SCCA or adenocarcinoma NSCLC tumor specimens (Figures 1IB and 1ID). All of the SCCA or adenocarcinoma NSCLC tumor specimens examined expressed positive staining for CXCR4. There was no evidence for nonspecific staining with the control antibody (Figures 1IA and 1IC). Furthermore, there was evidence of CXCR4 immunolocalization on host responding cells, such as tumor-associated macrophages, fibroblasts, and endothelial cells (Figures 1IB and 1ID). In contrast, we found no difference in protein levels of CXCL12 in NSCLC tumors (SCCA and adenocarcinoma), as compared with normal lung tissue as determined by ELISA analysis (Figure 1II).

CXCR4 Is Expressed on Human NSCLC Cell Lines

Having established that CXCR4 is present on NSCLC cells in human tumors isolated from patients (Figure 1I), we next determined whether the human NSCLC cell lines A549 (adenocarcinoma) and Calu-1 (squamous cell carcinoma) expressed this chemokine receptor. Thus, mRNA was extracted from these cells, reversed transcribed into cDNA, and analyzed for expression of CXCR4 by PCR. We observed that both cell lines constitutively expressed mRNA for CXCR4, but not for its cognate ligand, CXCL12 (Figure 2, and data not shown). Next, we pre-

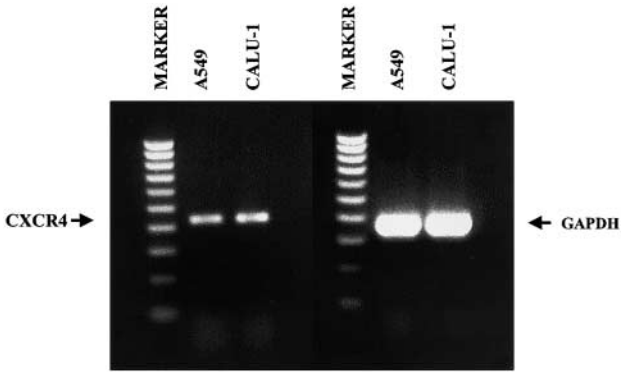

Figure 2. The NSCLC cell lines A549 and Calu-1 express CXCR4 mRNA. RNA was prepared from A549 (lanes 1 and 3) and Calu-1 (lanes 2 and 4) cell lines, reverse transcribed, and then subjected to polymerase chain reaction (PCR) using primer pairs specific for either CXCR4 (lanes 1 and 2) or the housekeeping gene GAPDH (lanes 3 and 4).

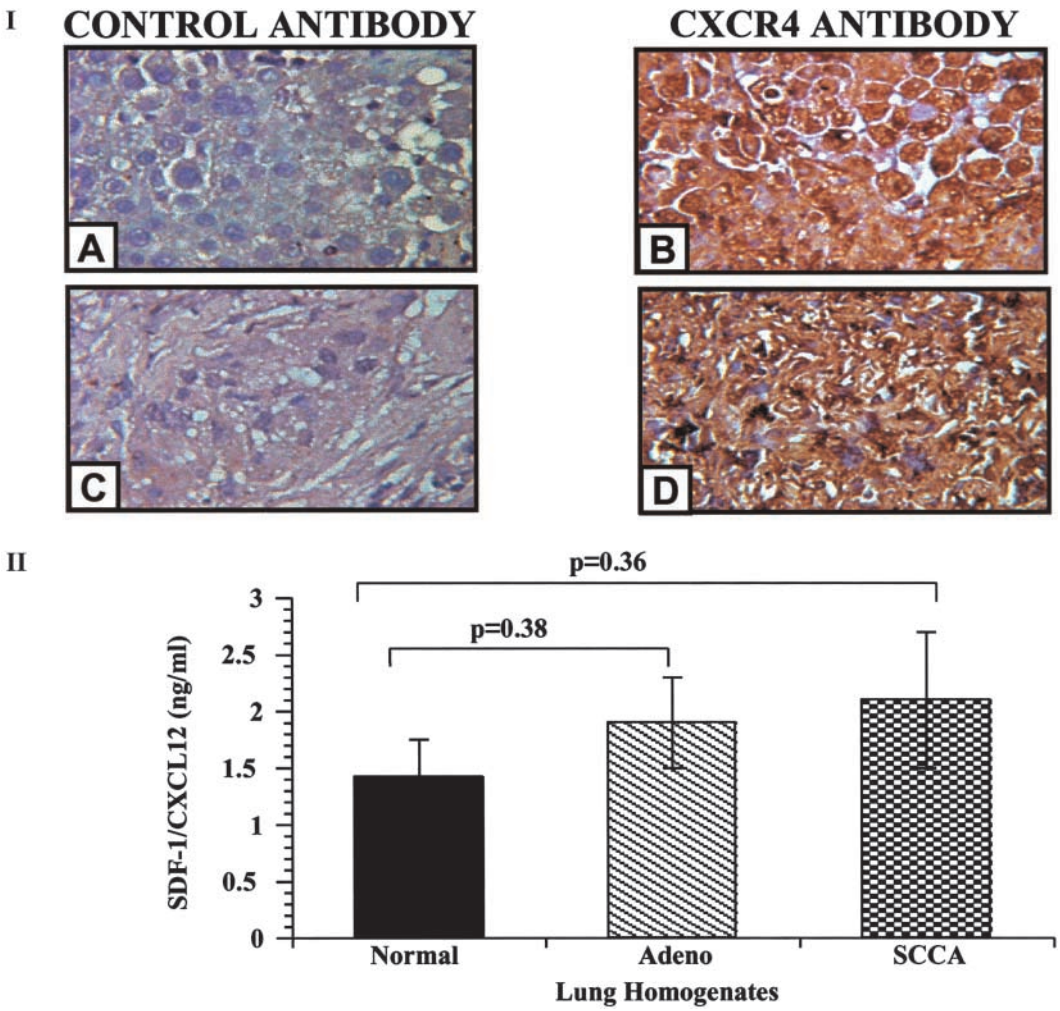

Figure 1. (*Panel I*) Representative photomicrograph (original magnification, ×400) of immunolocalization of CXCR4 in a human non–small cell lung cancer (NSCLC) adenocarcinoma (*A* and *B*) and non–small cell lung cancer (NSCLC) squamous cell carcinoma (*B* and *D*). Tumor biopsies were recovered and stained with either preimmune serum (*A* and *C*) or anti-CXCR4 antibodies (*B* and *D*). (*Panel II*) CXCL12 protein levels measured from 20 specimens of normal lung tissue, adenocarcinoma, and squamous cell carcinoma antigen (SCCA). No significant differences in CXCL12 levels were seen in these specimens ($p > 0.3$).

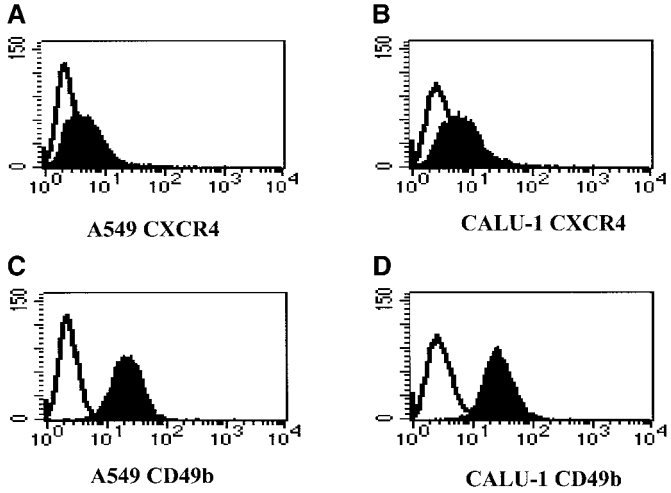

Figure 4. Expression of cell surface CXCR4 protein on A549 and Calu-1 NSCLC cells by fluorescence-activated cell sorting (FACS) analysis. A549 cells (*A* and *C*) or Calu-1 cells (*B* and *D*) stained with CXCR4 (*A* and *B*, *solid histograms*) or CD49b (*C* and *D*, *solid histograms*). Isotype-matched control antibodies were also included and their background staining is represented in each panel by *open histograms*.

pared whole cell extracts of these cell lines and examined them for expression of CXCR4 protein by Western blot analysis, using antibodies specific for CXCR4. As shown in Figure 3A, both A549 and Calu-1 cells constitutively express CXCR4 protein, whereas CXCL12 protein is not observed in these cells (Figure 3B). Finally, A549 and Calu-1 cells were subjected to FACS analysis to look for cell surface expression of CXCR4. Our results show that CXCR4 is observed on a significant number (45 to 50%) of A549 and Calu-1 cells (Figures 4A and 4B). In addition, CD49b, which is a human epithelial cell-specific marker, is strongly expressed on all (more than 99%) of these NSCLC cell lines (Figures 4C and 4D).

CXCR4 Is Functional on Human NSCLC Cell Lines

The above-described results indicated that CXCR4 is expressed on NSCLC cell lines. We next determined whether the receptor was functional. Initially, we loaded A549 and Calu-1 cells with the calcium-sensitive dye Indo-1/AM (2 μM), and then looked for changes in the intracellular calcium concentration in response to either CXCL12α (1 ng/ml) or CXCL12β (1 ng/ml). Transient calcium flux was observed in A549 and Calu-1 cells stimulated with either isoform of CXCL12 (data not shown), thus confirming results observed in other seven-transmembrane receptor systems including human lymphocytes, megakaryoblasts, and epithelial cells (32, 33).

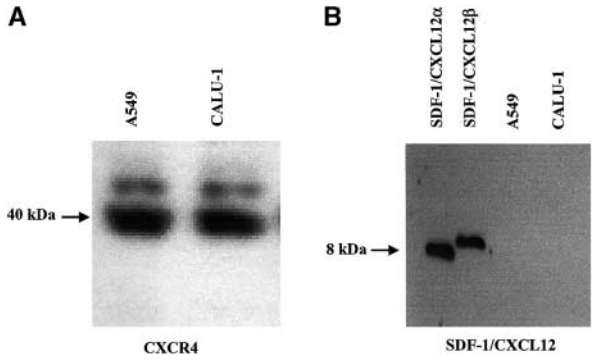

Figure 3. A549 and Calu-1 cells express CXCR4, but not CXCL12, protein: Western blot analysis of CXCR4 (*A*) and CXCL12 (*B*). (*A*) A549 cells (lane 1) and Calu-1 cells (lane 2) stained for anti-CXCR4 antibodies. (*B*) Recombinant CXCL12α (lane 1), CXCL12β (lane 2), A549 cells (lane 3), and Calu-1 cells (lane 4) stained with anti-CXCL12 antibodies. A549 and Calu-1 were negative for CXCL12 expression.

Subsequently, we assessed whether the expression of CXCR4 was functional in promoting NSCLC cell migration. We performed chemotaxis assays with A549 and Calu-1 cells in the presence or absence of CXCL12α (0.001 to 100 ng/ml) and CXCL12β (0.001 to 100 ng/ml) (Figure 5). We observed that both CXCL12α and CXCL12β appeared similarly efficacious in inducing dose-dependent chemotaxis of A549 cells (Figure 5) and Calu-1 cells (data not shown). Finally, to confirm that signal transduction through CXCR4, a G protein-linked receptor, was responsible for the NSCLC cell chemotactic response to CXCL12α and CXCL12β, we tested the ability of pertussis toxin (PTX) to inhibit this specific migration. We observed that chemotaxis of A549 and Calu-1 cells in response to CXCL12α or CXCL12β was attenuated by more than 95% in the presence of PTX. These results suggest that CXCL12 mediates chemotaxis of NSCLC cell lines and that this migration is regulated by a signal transduction pathway involving a PTX-sensitive G protein.

In addition to chemotaxis, we also examined whether CXCL12 altered the proliferative and apoptotic character of A549 and Calu-1 cells. CXCL12 was not found to induce significant proliferation over the background control levels (Table 1). This observation was further confirmed by manually counting cells at the same time point. In other experiments, A549 and Calu-1 cells were cultured in the presence or absence of various concentrations of CXCL12 and examined for evidence of apoptosis (Table 1). Our results indicated that the number of A549 and Calu-1 cells undergoing apoptosis by terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end-labeling assay in the presence of CXCL12 was similar to controls (Table 1). These results suggest that CXCL12 binding to CXCR4 mediates chemotaxis without altering the proliferative or apoptotic indices of the NSCLC tumor cells.

To further delineate the signal transduction events that might be responsible for chemotaxis mediated by the CXCL12–CXCR4 biological axis, we investigated the potential role of the mitogen-activated protein kinase (MAP kinase) pathway. Previous studies have already established that CXCL12 binding to CXCR4 in normal human megakaryocytes induces downstream phosphorylation of mitogen-activated protein kinase p42/44 (MAP kinase ERK-1/2) and the serine/threonine kinase AKT (32). Therefore, A549 cells were exposed to CXCL12β (10 ng/ml) for the times indicated before being solubilized in lysis buffer containing a panel of protease inhibitors. Next, 40 μg of total protein from each sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane. Subsequent Western blot analysis using anti-phospho-p42/44 antibodies (New England BioLabs, Beverly, MA) revealed that whereas A549 cells constitutively expressed the phosphorylated form of ERK-1/2 (p42/44), addition of CXCL12 resulted in further induction in p42/44 MAP kinase phosphorylation that peaked 5 minutes after stimulation (Figure 6A). Reprobing the membrane with anti-p42/44 antibodies (New England BioLabs) revealed that equivalent levels of the ERK-1/2 proteins were present in each lane (Figure 6B). Taken together, these data suggest that ligation of CXCR4 by CXCL12 promotes activation of the MAP kinase cascade, which may in turn regulate CXCR4-mediated NSCLC cell migration via a mechanism that also requires a PTX-sensitive G protein.

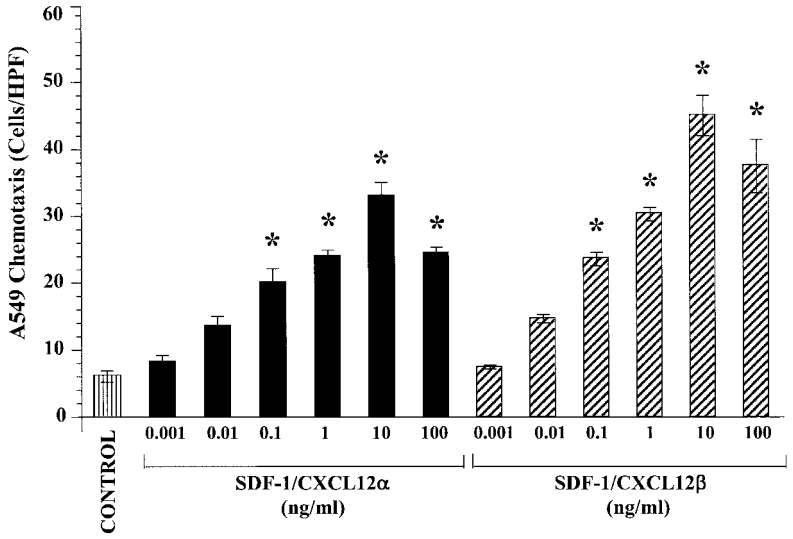

Figure 5. A549 chemotaxis in the presence of CXCL12α (0.001 to 100 ng/ml) and CXCL12β (0.001 to 100 ng/ml). Results are expressed as the number of A549 cells that migrated per high-power field (HPF). *$p < 0.05$.

CXCL12 Protein Levels Are Elevated in Those Organs That Display NSCLC Metastases

Having established *in vitro* that A549 cells undergo directional movement in response to a CXCL12–CXCR4 chemotactic gradient (Figure 5), we next wanted to determine whether a potential CXCL12 chemotactic gradient existed between the primary tumor and the organ sites of metastases *in vivo* in SCID mice. Thus, we analyzed CXCL12 protein in tissue homogenates from the primary tumor and a panel of murine organs by ELISA. Our results indicate that significantly higher levels of CXCL12 protein were observed in the adrenal glands, lung, liver, and bone marrow, which are target organs for NSCLC metastasis, whereas CXCL12 expression is barely detectable in the kidney and heart, which favors the idea that these organs are not necessarily sites for NSCLC tumor metastasis (Figure 7). Furthermore, the levels of CXCL12 protein observed in the adrenal glands, liver, lung, and bone marrow, but not the kidney and heart, were significantly higher than both the primary tumor and plasma concentrations of this chemokine (Figure 7). Therefore, it is possible that specific chemotactic gradients could exist between the primary tumor/plasma and organ sites of metastases.

TABLE 1. LACK OF PROLIFERATION* AND APOPTOSIS† IN A549 CELLS STIMULATED WITH STROMAL DERIVED FACTOR–1/CXCL12α AND STROMAL DERIVED FACTOR 1/CXCL12β

Conditions	Proliferation: [^{3}H]Thymidine Uptake (*cpm*)	Apoptosis: TUNEL$^+$ Cells (%)
Unstimulated control	7,321 ± 23	7 ± 1.0
HGF, 30 ng/ml	9,351 ± 35	10 ± 2.3
SDF-1/CXCL12α		
1 ng/ml	7,059 ± 60	6 ± 1.2
3 ng/ml	7,307 ± 90	5 ± 2.0
10 ng/ml	7,324 ± 25	7 ± 0.9
30 ng/ml	7,686 ± 102	6 ± 2.0
SDF-1/CXCL12β		
1 ng/ml	7,015 ± 38	9 ± 2.5
3 ng/ml	7,692 ± 26	6 ± 1.0
10 ng/ml	7,535 ± 28	7 ± 2.4
30 ng/ml	7,592 ± 54	6 ± 0.8

Definition of abbreviations: cpm = counts per minute; HGF = human growth factor; SDF-1 = stromal cell-derived factor-1; TUNEL = terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick end-labeling.

* [^{3}H]Thymidine uptake.

† TUNEL.

NSCLC Metastases Express Significant Levels of CXCR4

We next wanted to assess whether NSCLC metastases *in vivo* demonstrate a selective advantage for the expression of CXCR4, and are capable of utilizing potential CXCL12 chemokine gradients that exist between the primary tumor and metastases. Indeed, previous studies have already shown that A549 cells injected into the flank of mice do spontaneously metastasize (24, 26–28). Thus, to determine whether NSCLC metastases express CXCR4, we used the heterotopic and orthotopic tumor models of human A549 cells in SCID mice and 8 weeks later killed them to examine their metastatic pattern. Cell suspensions of the primary tumor, adrenal glands, liver, lung, brain, and bone marrow were isolated, stained with antibodies specific for CD49b and CXCR4, and analyzed by FACS (Figure 8; n = 6 animals/6 organs for each type). The strategy implemented here takes advantage of the fact that all A549 cells express human CD49b (Figure 4), thus detection of human CD49b-positive cells in murine organs was used as a tool for the quantitative detection of human NSCLC cells. Our data revealed that only about 65%

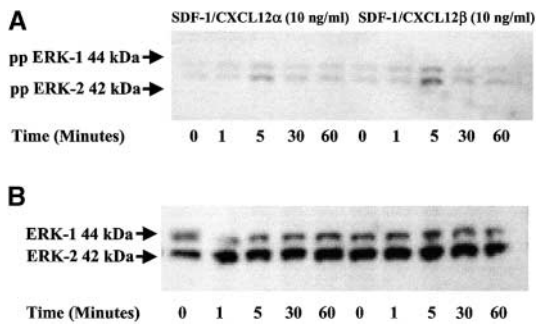

Figure 6. Phosphorylation of ERK-1/2 in A549 cells by CXCL12α and CXCL12β, as shown by Western blot analysis. (*A*) A549 cells stimulated with CXCL12α (10 ng/ml) or CXCL12β (10 ng/ml) for the times indicated, and probed with an anti-phospho-ERK-1/2 antibody. (*B*) To show equal loading, membranes were stripped and reprobed with an anti-ERK-1/2 (p42/p44) antibody.

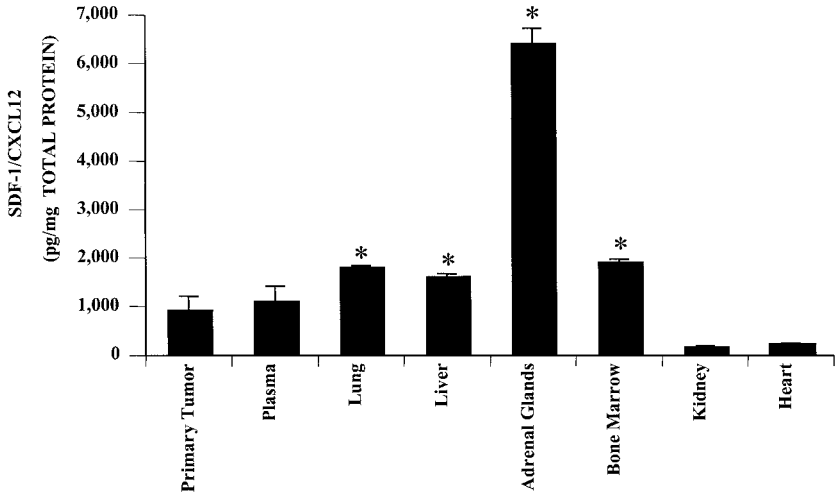

Figure 7. Expression of CXCL12 in tissues that are targets for NSCLC metastasis. Data are presented as picograms of CXCL12 protein per milligram of total protein. *$p < 0.05$.

of the CD49b$^+$ A549 cells in the primary tumor were CXCR4$^+$ (Figure 8I). Thus about 35% of the CD49b$^+$ A549 cells were CXCR4$^-$ (Figure 8I). In addition, when we examined metastases from primary heterotopic and orthotopic tumors isolated from the adrenal glands, liver, brain, bone marrow, and lung (heterotopic only) of A549-xenoengrafted SCID mice, we observed that more than 99% of the NSCLC tumor cells were CD49b$^+$CXCR4$^+$ double positive (Figures 8II–8VI). Moreover, analysis of the heart and kidney revealed few if any metastases. Therefore, it seems likely that, in common with breast cancer cells, CXCR4 plays an important role in NSCLC tumor metastasis.

Depletion of CXCL12 Inhibits NSCLC Metastases

On the basis of the above-described results, we wanted to determine whether the systemic depletion of CXCL12 by specific neutralizing antibodies to CXCL12 in SCID mice bearing A549 heterotopic and orthotopic tumors would attenuate NSCLC tumor metastasis. We used anti-CXCL12 rather than anti-CXCR4 antibodies to eliminate the possibility that anti-CXCR4 antibodies could promote clearance of CXCR4-expressing A549 cells from the circulation by the reticuloendothelial system.

Having established the specificity of the anti-CXCL12 antibody, we next investigated whether the administration of neutralizing anti-CXCL12 antibodies to heterotopic or orthotopic A549 tumor-bearing SCID mice attenuated NSCLC tumor metastasis. We injected A549 cells heterotopically or orthotopically into SCID mice, and then treated them with intraperitoneal injections (500 μl) of either neutralizing goat anti-CXCL12 or preimmune serum, or did not treat them, Monday through Friday for 8 weeks, starting at the time of xenoengraftment.

On sacrifice, cells from the lungs (heterotopic model only), adrenal glands, liver, bone marrow, and brain of A549 heterotopic and orthotopic tumor-bearing mice were isolated and examined for signs of spontaneous metastases by FACS analysis of CD49b. Depletion of CXCL12 in the heterotopic NSCLC tumor model resulted in markedly attenuated metastases to the lung (Figures 9I and 9II). Depletion of CXCL12 in both heterotopic and orthotopic NSCLC tumor models significantly impaired metastases to the adrenal glands, bone marrow, liver, and brain (Figure 10). In control SCID mice that lacked human tumors, CD49b-positive cells were not detected. These data support the notion that CXCR4 is expressed on human NSCLC cells, and

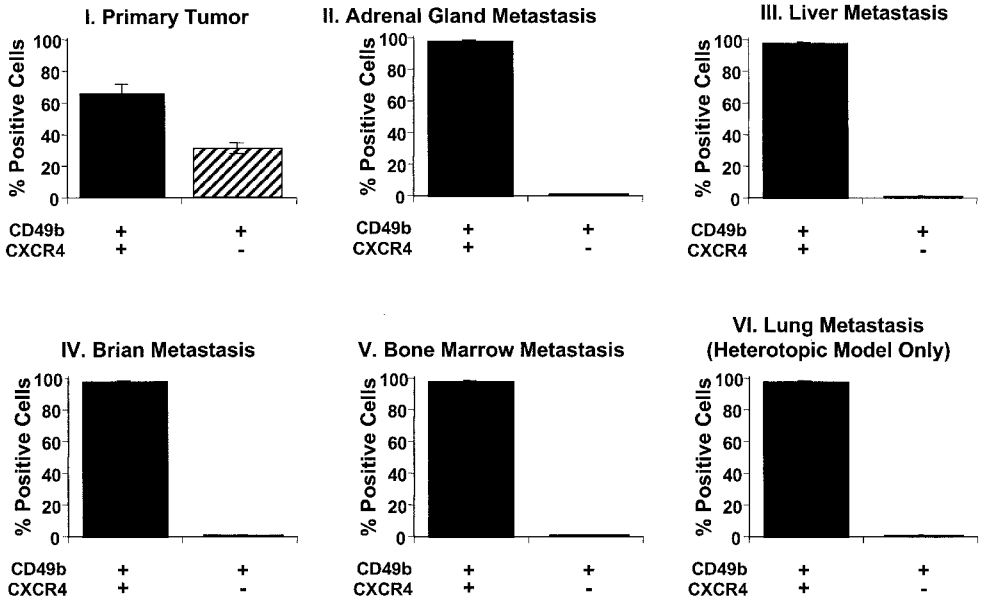

Figure 8. Selective advantage for CD49b$^+$ CXCR4$^+$ double-positive A549 cells in organ-specific metastases. Data are presented as the percentage of CD49b$^+$ CXCR4$^+$ (*solid columns*) and CD49b$^+$ CXCR4$^-$ (*hatched columns*) A549 cells found in each organ examined.

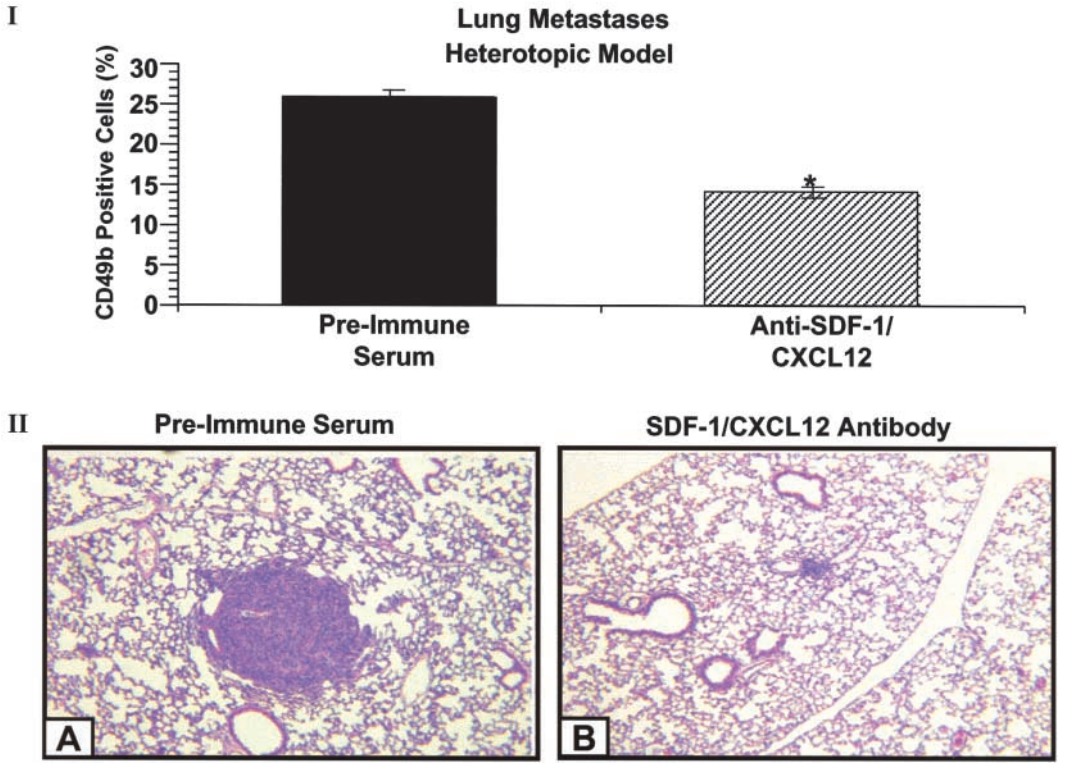

Figure 9. (*I*) Neutralization of CXCL12 in animals bearing heterotopic A549 tumors resulted in attenuated metastases to the lung. *$p < 0.05$. These findings represent a total of six lungs. (*II*) Representative photomicrograph (original magnification, ×20) of reduced tumor metastases to the lungs of heterotopic tumor-bearing animals treated with anti-CXCL12 antibodies (*B*), as compared with preimmune serum (*A*).

that the CXCL12–CXCR4 biological axis is important in mediating metastases in an organ-specific manner.

Depletion of CXCL12 *in Vivo* Does Not Alter Primary Tumor Size or Tumor-derived Angiogenesis

Studies have indicated that the CXCL12–CXCR4 biological axis may be involved in mediating angiogenesis (21, 22). This in turn has led to speculation that the predominant function of this ligand–receptor pair in tumorigenesis results from this perceived angiogenic capability rather than from an ability to mediate metastasis. Thus, we wanted to determine whether there was any evidence of CXCL12–CXCR4-mediated angiogenesis in our *in vivo* model system of heterotopic or orthotopic human NSCLC tumor growth and metastasis. On this basis, we analyzed the microvascular density of primary heterotopic and orthotopic A549 tumors in SCID mice that were treated with specific neutralizing CXCL12 antibodies, as compared with preimmune serum. Factor VIII-related antigen was used as a marker of endothelial cells. We found no difference in the vascular density of primary tumors from mice treated with neutralizing anti-CXCL12, as compared with preimmune serum (Figure 11A).

To further confirm that CXCL12–CXCR4 axis was not mediating tumor-derived angiogenic activity, we assessed A549 tumor homogenates from mice treated with either anti-CXCL12 antibodies or preimmune serum in the cornea micropocket assay of neovascularization and endothelial cell chemotaxis assays. There was no difference in tumor-derived angiogenic activity between the two treatment groups. To confirm that anti-CXCL12 antibod-

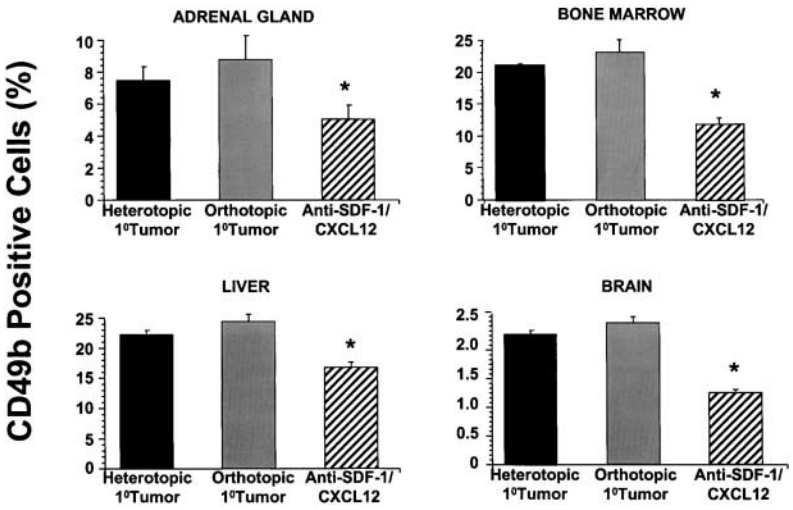

Figure 10. Neutralization of CXCL12 in animals bearing heterotopic and orthotopic A549 tumors resulted in attenuated metastases to specific organs. *$p < 0.05$. These findings represent a total of six organs of each type.

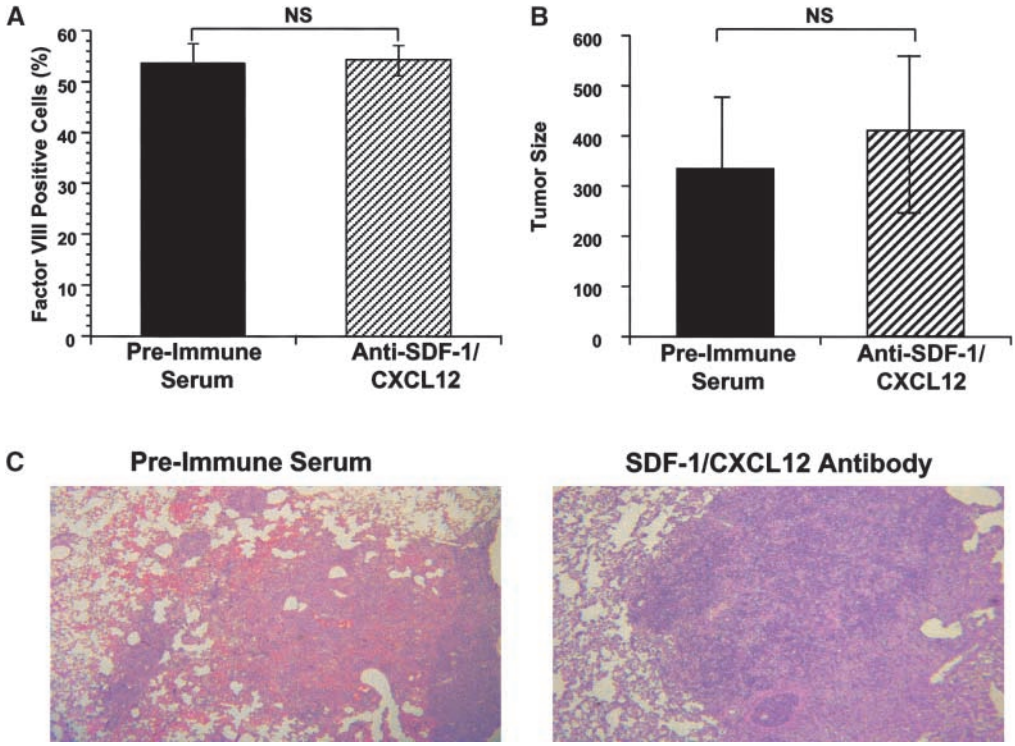

Figure 11. (*A*) Depletion of CXCL12 does not alter vascular density in primary NSCLC tumors. Data are presented as the percentage of Factor VIII-related antigen-positive cells observed in each sample. Six tumors for each treatment were assessed. (*B*) Depletion of CXCL12 does not alter tumor size in heterotopic primary NSCLC tumors. Ten tumors for each treatment were assessed. NS = No significant difference. (*C*) Representative photomicrograph (original magnification, ×20) of orthotopic A549 tumors in animals treated with anti-CXCL12 antibodies or preimmune serum.

ies were found within the tumors, we performed immunohistochemical localization of goat IgG. Goat IgG was found to be immunolocalized within the tumors.

Furthermore, there was no significant difference in size of the heterotopic A549 tumors in mice treated with neutralizing anti-CXCL12 antibodies, as compared with preimmune serum (Figure 11B; $p = 0.64$). There was no significant difference in the number of proliferating cells, as determined by immunolocalization of PCNA in tumors from mice treated with neutralizing anti-CXCL12 antibodies, as compared with preimmune serum. The number of PCNA-positive cells per field (five fields per tumor from six tumors per group) at ×400 was 206 ± 17 and 210 ± 7 for the tumors from mice treated with neutralizing anti-CXCL12 antibodies, as compared with preimmune serum, respectively ($p = 0.76$). In addition, there was no significant difference in the levels of CXCL12 protein expressed by the primary tumor versus the adjacent normal skin. In a similar manner, we found in the orthotopic tumor model no significant difference in the magnitude of tumor burden in the lungs of animals treated with neutralizing anti-CXCL12 antibodies or preimmune serum (Figure 11C).

DISCUSSION

In this article we examined the interrelationship between primary NSCLC tumor growth and metastases and the chemokine ligand–receptor pair, CXCL12–CXCR4, in human NSCLC tumor samples, human NSCLC cell lines, and human NSCLC tumors growing in SCID mice. In keeping with the results of another study that demonstrated CXCR4 expression on a panel of NSCLC cell lines (34), we confirmed that A549 cells and Calu-1 cells express both mRNA and surface levels of this chemokine receptor. This is also consistent with the findings of Murdoch and associates for the expression of CXCR4 on human epithelial cells (30). Furthermore, immunohistochemical staining showed that Stage I and II NSCLC tumor specimens resected from patients strongly expressed CXCR4. Additional studies revealed that although the NSCLC primary tumor and plasma have measurable levels of CXCL12, higher levels of CXCL12 were observed in organs that are known to be the preferred sites of NSCLC tumor metastases (3, 4). Moreover, several studies have now shown that CXCR4 is abundantly expressed on cells from a number of metastatic cancers including ovarian cancer, pancreatic cancer, neuroblastoma, non-Hodgkin's lymphoma, and multiple myeloma, and although these tumors arise from different tissues they all share the ability to migrate in response to CXCL12 (31, 35–38). Some of these tumors have also been shown to upregulate adhesion molecules, notably members of the integrin family (37, 38), which may have an important role to play in the process of metastasis.

Although a great deal of work has contributed to our understanding of the basic pathophysiology of CXCL12–CXCR4, the signaling properties of CXCR4 and CXCL12 in the proposed regulation of organ-specific metastasis have not been well characterized. Neither is it clear whether the pathways activated by CXCL12–CXCR4 in these metastatic cancers will be similar to those activated in other noncancerous CXCL12–CXCR4 systems, notably T cells, neuronal cells, and hematopoietic cells (32, 39–41). Therefore, we began our study by examining the signal transduction mechanisms that may regulate the migratory process of NSCLC metastasis. Our data indicate that CXCL12 activation of CXCR4 on NSCLC cell lines results in the following: (*1*) mobilization of calcium from intracellular stores, (*2*) chemotaxis that is mediated by a PTX-sensitive G protein, and (*3*) enhanced phosphorylation of the downstream signaling molecules ERK-1/2.

These initial results show some differences from the other noncancerous systems. For example, ERK-1/2 in the NSCLC cell line A549 and in primary astrocyte cultures, but not in T cells, are constitutively phosphorylated (41, 42). Furthermore, the kinetics of phosphorylation differ, because in both T cells and astrocytes phosphorylated forms of ERK-1/2 appear within a few minutes of stimulation with CXCL12 and remain elevated

for more than 20 minutes (41, 42), whereas in NSCLC cells CXCL12-mediated phosphorylation of ERK-1/2 peaks abruptly at 5 minutes and then quickly returns to basal levels. Reports in T cells and astrocytes have shown that ligation of CXCR4 by CXCL12 activates a multitude of signaling pathways including Janus kinase-signal transducer and activator of transcription (JAK-STAT), NF-κB, protein kinase C, phosphoinositide 3-kinase, and protein kinase B (32, 33, 41–44), and detailed study of these pathways in NSCLC versus noncancerous systems may reveal important future clues to the mechanisms underlying CXCL12–CXCR4-mediated NSCLC tumor metastasis.

The expression of CXCR4 on NSCLC tumor cells *in vitro* and *in vivo* in primary tumors is heterogeneous. It is possible, therefore, that the expression of this receptor is sensitive to the local milieu in which the tumor cell resides. In the confined environment of a tumor the concentration of appropriate chemokines, cytokines, and other potential factors are likely to be elevated, and thus CXCR4 expression can be maintained at high levels. Thus, it will be important to characterize which factors found within the tumor microenvironment have the ability to modulate CXCR4 expression. Once we have a greater appreciation of the mechanisms that govern the expression of CXCR4 on NSCLC tumor cells we will improve our understanding of how CXCR4, itself, regulates NSCLC tumor metastasis.

Müller and colleagues (20) have provided direct evidence to support the notion that the CXCL12–CXCR4 biological axis can actually mediate human carcinoma metastasis *in vivo*. Their article provided evidence that the CXCL12–CXCR4 biological axis is involved in the metastatic propensity of breast cancer to specific organs (20). This group has demonstrated that CXCR4 is highly expressed on human breast cancer cells, malignant breast tumors, and metastases. Furthermore, CXCL12 mRNA exhibited peak levels of expression in organs that are the preferred sites of breast cancer metastasis. Signaling through CXCR4 in breast cancer cells mediated actin polymerization and pseudopodia formation and, subsequently, induced chemotactic and invasive responses at the local level. Finally, *in vivo* neutralization of CXCR4 with specific anti-CXCR4 antibodies resulted in significant inhibition of metastasis of breast cancer cells to regional lymph nodes and the lung (20).

Our results complement this work, and address two potential criticisms of Müller's original findings. First, by using antibodies targeted to CXCR4, it could be argued that breast cancer metastasis was reduced because the cells were being eliminated by the reticuloendothelial system, rather than because their migratory ability had been abrogated though loss of CXCR4 function. Our study obviates that criticism, because the antibodies we used are directed toward CXCL12 rather than to CXCR4-bearing NSCLC cells. Second, studies have suggested a possible role of the CXCL12–CXCR4 biological axis in mediating tumorigenesis via the promotion of angiogenesis, rather than the regulation of metastasis (21, 22). However, our studies revealed that tumor-derived angiogenic activity remained unchanged in primary tumors of animals depleted of CXCL12, indicating that the function of this chemokine may not be as important in promoting angiogenesis in the local tumor microenvironment. One possible explanation for this is that there are many more CXCR4-bearing tumor cells than $CXCR4^+$ endothelial cells in the local microenvironment of the tumor, and the tumor cells are able to outcompete the endothelial cells for the available CXCL12. In contrast, known angiogenic factors (i.e., epithelial neutrophil activating peptide-78, interleukin-8, and vascular endothelial growth factor) are found to be elevated in human NSCLC, as compared with normal lung tissue (26, 28, 45, 46). Furthermore, the addition of neutralizing antibodies to these angiogenic factors resulted in a net reduction of angiogenesis, and a consequent reduction in tumor size and metastatic propensity (26, 28, 45, 46). Our data therefore suggest a dichotomy in the function for CXCL12 versus the other angiogenic factors, such that epithelial neutrophil activating peptide-78, interleukin-8, and vascular endothelial growth factor promote metastasis through their stimulatory effects on angiogenesis, whereas CXCL12 mediates metastasis through direct effects on tumor cell migration.

In agreement with the work of Müller and coworkers (20), our study shows that the CXCL12–CXCR4 biological axis can regulate the overall metastatic propensity of tumors without changing their proliferative capacity, and also provides the first *in vivo* evidence to suggest that this chemokine ligand–receptor pair can mediate the metastasis of NSCLC tumor cells. In our murine paradigms of heterotopic and orthotopic tumor growth, A549 tumor cells found at the sites of organ metastases were almost exclusively $CD49b^+CXCR4^+$ double positive. By contrast, in the primary tumor itself, only 65% of A549 cells were found to be $CD49b^+CXCR4^+$ double positive, leaving 35% as $CD49b^+CXCR4^-$. These results suggest that in spite of the phenotypic heterogeneity of the A549 cells within the primary tumor, only those A549 cells that are $CD49b^+CXCR4^+$ double positive were destined to metastasize to distant organs.

Data from SCID mice heterotopically and orthotopically xenoengrafted with A549 cells revealed that NSCLC tumor metastases to specific organs were significantly reduced, although not completely abrogated, in the presence of neutralizing anti-CXCL12 antibodies. There are at least two possibilities to account for the continued expression of NSCLC metastases in mice treated with neutralizing anti-CXCL12 antibodies. First, the anti-CXCL12 antibody administration was incomplete in blocking all CXCL12 to its cognate CXCR4 receptor, and second, it is possible that other mechanisms, in addition to the CXCL12–CXCR4 biological axis, could be involved in NSCLC tumor metastasis.

CXCR4-mediated NSCLC metastasis to distant organs requires a CXCL12 chemotactic gradient. On measuring levels of CXCL12 protein in a variety of organs, we found that the majority of CXCL12 protein was elaborated in organs correlated with organ-specific metastases of NSCLC. On the other hand, little or no CXCL12 protein was found in the heart or kidneys, which contained few, if any, metastases. Furthermore, when we compared CXCL12 levels from these organs to the primary tumor and plasma levels, we observed that CXCL12 expression in adrenal glands, lung, liver, and bone marrow, but not in kidneys and heart, was significantly higher than in either primary tumor or plasma. Under these conditions, once the tumor cell has escaped the primary tumor, it would be possible for a CXCL12 chemotactic gradient to develop that would facilitate metastasis of A549 $CD49b^+CXCR4^+$ double-positive cells from the primary tumor to specific organs. In addition, it should be borne in mind that the levels of CXCL12 observed in these organs represent an average concentration, and it is entirely possible that within the organ microenvironment the local concentration of CXCL12 could be substantially higher, creating an even greater chemotactic gradient to further attract metastatic A549 $CD49b^+CXCR4^+$ double-positive cells.

Our work, together with that of others, correlates well with an extensive body of research showing that chemokines play a pivotal role in leukocyte trafficking and homeostasis (8, 12, 47–51). The nature of this regulation has shown that chemokines not only mediate tissue-specific homing of leukocytes, they also dictate microenvironmental separation of lymphocyte subsets within primary and secondary lymphoid organs (48, 52). In this regard CXCL12 is known to be involved in directing progenitor cells into the appropriate maturation sites in the bone marrow (53). In addition, CXCL12 may support the colonization of the

bone marrow by hematopoietic precursors during embryogenesis (54). Clearly the bone marrow is a sensitive site for CXCL12 activity, and this may partly explain why the introduction of anti-CXCL12 antibodies to our *in vivo* model significantly abrogates NSCLC metastasis to this organ. Moreover, it seems likely that metastasis is not a passive and random process, but rather is a result of an active migratory process by a tumor cell that has co-opted a system already in place for leukocyte trafficking.

Metastatic cancer is not the only process that has utilized the CXCL12–CXCR4 biological axis against its host. The HIV-1 virus has also successfully utilized the CXCR4 receptor to mediate infection of host immune cells (54–56). From that perspective, CXCR4 has become an important target for therapeutic intervention to prevent the deleterious effects of HIV infection and some drugs already in development are aimed at disrupting the HIV–CXCR4 interaction. Drugs of this nature may also have a role to play in ameliorating or even defeating some metastatic cancers such as NSCLC. One such drug is a bicyclam derivative called AMD3100 (AnorMED, Langley, BC, Canada), which has been shown to be a small molecule inhibitor of HIV-1 entry via the CXCR4 coreceptor (57). Thus, in future *in vivo* studies, we will determine the efficacy of this compound for inhibition of NSCLC metastases.

References

1. American Cancer Society. Selected cancers. In: Cancer facts & figures 2000. Atlanta, GA: American Cancer Society; 2000.
2. Devesa SS, Blot WJ, Stone BJ, Miller BA, Tarone RE, Fraumeni JF Jr. Recent cancer trends in the United States [see comments]. *J Natl Cancer Inst* 1995;87:175–182.
3. Fidler IJ. Critical factors in the biology of human cancer metastasis: twenty-eighth G. H. A. Clowes Memorial Award Lecture. *Cancer Res* 1990;50:6130–6138.
4. Fidler IJ. Critical determinants of cancer metastasis: rationale for therapy. *Cancer Chemother Pharmacol* 1999;43:S3–S10.
5. Hart IR, Fidler IJ. Role of organ selectivity in the determination of metastatic patterns of B16 melanoma. *Cancer Res* 1980;40:2281–2287.
6. Stetler-Stevenson WG, Kleiner DEJ. Molecular biology of cancer: invasion and metastases. In: DeVita VTJ, Hellman S, and Rosenberg SA, editors. Cancer: principles and practice of oncology, 6th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2001. p. 123–136.
7. Rossi D, Zlotnik A. The biology of chemokines and their receptors. *Annu Rev Immunol* 2000;18:217–242.
8. Premack BA, Schall TJ. Chemokine receptors: gateways to inflammation and infection. *Nat Med* 1996;2:1174–1178.
9. Baggiolini M. Chemokines and leukocyte traffic. *Nature* 1998;392:565–568.
10. Power CA, Wells TN. Cloning and characterization of human chemokine receptors. *Trends Pharmacol Sci* 1996;17:209–213.
11. Murphy PM. The molecular biology of leukocyte chemoattractant receptors. *Annu Rev Immunol* 1994;12:593–633.
12. Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, Power CA. International Union of Pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev* 2000;52:145–176.
13. Murphy PM. International Union of Pharmacology. XXX. Update on chemokine receptor nomenclature. *Pharmacol Rev* 2002;54:227–229.
14. Luster AD. Chemokines: chemotactic cytokines that mediate inflammation. *N Engl J Med* 1998;338:436–445.
15. Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* 1998;393:595–599.
16. Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushima K, Yoshida N, Nishikawa S, *et al.* The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature* 1998;393:591–594.
17. Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, Kitamura Y, Yoshida N, Kikutani H, Kishimoto T. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* 1996;382:635–638.
18. Ma Q, Jones D, Borghesani PR, Segal RA, Nagasawa T, Kishimoto T, Bronson RT, Springer TA. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci USA* 1998;95:9448–9453.
19. Paget SL. The distribution of secondary growths in cancer of the breast. *Lancet* 1889;1:571.
20. Müller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, *et al.* Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001;410:50–56.
21. Kijowski J, Baj-Krzyworzeka M, Majka M, Reca R, Marquez LA, Christofidou-Solomidou M, Janowska-Wieczorek A, Ratajczak MZ. The SDF-1–CXCR4 axis stimulates VEGF secretion and activates integrins but does not affect proliferation and survival in lymphohematopoietic cells. *Stem Cells* 2001;19:453–466.
22. Salcedo R, Wasserman K, Young HA, Grimm MC, Howard OM, Anver MR, Kleinman HK, Murphy WJ, Oppenheim JJ. Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells: in vivo neovascularization induced by stromal-derived factor-1α. *Am J Pathol* 1999;154:1125–1135.
23. Addison CL, Daniel TO, Burdick MD, Liu H, Ehlert JE, Xue YY, Buechi L, Walz A, Richmond A, Strieter RM. The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR$^+$ CXC chemokine-induced angiogenic activity. *J Immunol* 2000;165:5269–5277.
24. Addison CL, Arenberg DA, Morris SB, Xue YY, Burdick MD, Mulligan MS, Iannettoni MD, Strieter RM. The CXC chemokine, monokine induced by interferon-γ, inhibits non–small cell lung carcinoma tumor growth and metastasis. *Hum Gene Ther* 2000;11:247–261.
25. Arenberg DA, Kunkel SL, Burdick MD, Polverini PJ, Strieter RM. Treatment with anti-IL-8 inhibits non–small cell lung cancer tumor growth [meeting abstract]. *J Invest Med* 1995;43(Suppl 3):479A.
26. Arenberg DA, Kunkel SL, Polverini PJ, Morris SB, Burdick MD, Glass MC, Taub DT, Iannettoni MD, Whyte RI, Strieter RM. Interferon-γ-inducible protein 10 (IP-10) is an angiostatic factor that inhibits human non–small cell lung cancer (NSCLC) tumorigenesis and spontaneous metastases. *J Exp Med* 1996;184:981–992.
27. Arenberg DA, Keane MP, DiGiovine B, Kunkel SL, Morris SB, Xue YY, Burdick MD, Glass MC, Iannettoni MD, Strieter RM. Epithelial-neutrophil activating peptide (ENA-78) is an important angiogenic factor in non–small cell lung cancer. *J Clin Invest* 1998;102:465–472.
28. Arenberg DA, Kunkel SL, Polverini PJ, Glass M, Burdick MD, Strieter RM. Inhibition of interleukin-8 reduces tumorigenesis of human non–small cell lung cancer in SCID mice. *J Clin Invest* 1996;97:2792–2802.
29. Rot A. Endothelial cell binding of NAP-1/IL-8: role in neutrophil emigration. *Immunol Today* 1992;13:291–294.
30. Murdoch C, Monk PN, Finn A. CXC chemokine receptor expression on human endothelial cells. *Cytokine* 1999;11:704–712.
31. Bertolini F, Dell'Agnola C, Mancuso P, Rabascio C, Burlini A, Monestiroli S, Gobbi A, Pruneri G, Martinelli G. CXCR4 neutralization, a novel therapeutic approach for non-Hodgkin's lymphoma. *Cancer Res* 2002;62:3106–3112.
32. Majka M, Ratajczak J, Kowalska MA, Ratajczak MZ. Binding of stromal derived factor-1α (SDF-1α) to CXCR4 chemokine receptor in normal human megakaryoblasts but not in platelets induces phosphorylation of mitogen-activated protein kinase p42/44 (MAPK), ELK-1 transcription factor and serine/threonine kinase AKT. *Eur J Haematol* 2000;64:164–172.
33. Vicente-Manzanares M, Rey M, Jones DR, Sancho D, Mellado M, Rodriguez-Frade JM, del Pozo MA, Yanez-Mo M, de Ana AM, Martinez AC, *et al.* Involvement of phosphatidylinositol 3-kinase in stromal cell-derived factor-1α-induced lymphocyte polarization and chemotaxis. *J Immunol* 1999;163:4001–4012.
34. Soejima K, Rollins BJ. A functional IFN-γ-inducible protein-10/CXCL 10-specific receptor expressed by epithelial and endothelial cells that is neither CXCR3 nor glycosaminoglycan. *J Immunol* 2001;167:6576–6582.
35. Geminder H, Sagi-Assif O, Goldberg L, Meshel T, Rechavi G, Witz IP, Ben-Baruch A. A possible role for CXCR4 and its ligand, the CXC chemokine stromal cell-derived factor-1, in the development of bone marrow metastases in neuroblastoma. *J Immunol* 2001;167:4747–4757.
36. Koshiba T, Hosotani R, Miyamoto Y, Ida J, Tsuji S, Nakajima S, Kawaguchi M, Kobayashi H, Doi R, Hori T, *et al.* Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: a possible role for tumor progression. *Clin Cancer Res* 2000;6:3530–3535.
37. Scotton CJ, Wilson JL, Milliken D, Stamp G, Balkwill FR. Epithelial cancer cell migration: a role for chemokine receptors? *Cancer Res* 2001;61:4961–4965.
38. Sanz-Rodriguez F, Hidalgo A, Teixido J. Chemokine stromal cell-derived

factor-1α modulates VLA-4 integrin-mediated multiple myeloma cell adhesion to CS-1/fibronectin and VCAM-1. *Blood* 2001;97:346–351.
39. Bajetto A, Barbero S, Bonavia R, Piccioli P, Pirani P, Florio T, Schettini G. Stromal cell-derived factor-1α induces astrocyte proliferation through the activation of extracellular signal-regulated kinases 1/2 pathway. *J Neurochem* 2001;77:1226–1236.
40. Rincon M. MAP-kinase signaling pathways in T cells. *Curr Opin Immunol* 2001;13:339–345.
41. Tilton B, Ho L, Oberlin E, Loetscher P, Baleux F, Clark-Lewis I, Thelen M. Signal transduction by CXC chemokine receptor 4: stromal cell-derived factor 1 stimulates prolonged protein kinase B and extracellular signal-regulated kinase 2 activation in T lymphocytes. *J Exp Med* 2000;192:313–324.
42. Sotsios Y, Whittaker GC, Westwick J, Ward SG. The CXC chemokine stromal cell-derived factor activates a G_i-coupled phosphoinositide 3-kinase in T lymphocytes. *J Immunol* 1999;163:5954–5963.
43. Vila-Coro AJ, Rodriguez-Frade JM, Martin De Ana A, Moreno-Ortiz MC, Martinez AC, Mellado M. The chemokine SDF-1α triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. *FASEB J* 1999;13:1699–1710.
44. Han Y, He T, Huang DR, Pardo CA, Ransohoff RM. TNF-α mediates SDF-1α-induced NF-κB activation and cytotoxic effects in primary astrocytes. *J Clin Invest* 2001;108:425–435.
45. Smith DR, Polverini PJ, Kunkel SL, Orringer MB, Whyte RI, Burdick MD, Wilke CA, Strieter RM. Inhibition of interleukin 8 attenuates angiogenesis in bronchogenic carcinoma. *J Exp Med* 1994;179:1409–1415.
46. Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, Strieter RM. CXC chemokines in angiogenesis. *J Leukoc Biol* 2000;68:1–8.
47. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, Lipp M. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 1999;99:23–33.
48. Campbell JJ, Butcher EC. Chemokines in tissue-specific and microenvironment-specific lymphocyte homing. *Curr Opin Immunol* 2000; 12:336–341.
49. Butcher EC, Williams M, Youngman K, Rott L, Briskin M. Lymphocyte trafficking and regional immunity. *Adv Immunol* 1999;72:209–253.
50. Peled A, Petit I, Kollet O, Magid M, Ponomaryov T, Byk T, Nagler A, Ben-Hur H, Many A, Shultz L, *et al.* Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science* 1999;283:845–848.
51. Strieter RM. Chemokines: not just leukocyte chemoattractants in the promotion of cancer. *Nat Immunol* 2001;2:285–286.
52. Reif K, Ekland EH, Ohl L, Nakano H, Lipp M, Forster R, Cyster JG. Balanced responsiveness to chemoattractants from adjacent zones determines B-cell position. *Nature* 2002;416:94–99.
53. D'Apuzzo M, Rolink A, Loetscher M, Hoxie JA, Clark-Lewis I, Melchers F, Baggiolini M, Moser B. The chemokine SDF-1, stromal cell-derived factor 1, attracts early stage B cell precursors via the chemokine receptor CXCR4. *Eur J Immunol* 1997;27:1788–1793.
54. Bleul CC, Fuhlbrigge RC, Casasnovas JM, Aiuti A, Springer TA. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor (SDF-1). *J Exp Med* 1996;184:1101–1109.
55. Bleul CC, Farzan M, Choe H, Parolin C, Clark-Lewis I, Sodroski J, Springer TA. The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* 1996;382:829–833.
56. Feng Y, Broder CC, Kennedy PE, Berger EA. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 1996;272:872–877.
57. Donzella GA, Schols D, Lin SW, Este JA, Nagashima KA, Maddon PJ, Allaway GP, Sakmar TP, Henson G, De Clercq E, *et al.* AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. *Nat Med* 1998;4:72–77.