Chemokines and antisera

Natural human CXCL4 was isolated from stimulated platelets as previously described.[1] Recombinant human CXCL4L1 was produced in *E. coli* and purified by a 4-step chromatographical procedure.[2] Human CXCL11 and CXCL12 were chemically synthesized.[3] Monoclonal anti-human CXCR3/CD183 (Clone 1C6) and anti-human CXCR3/CD183 (Clone 49801) and the corresponding isotype controls were purchased from BD Biosciences (San Diego, CA) and R&D Systems (Abingdon, UK), respectively. Recombinant human and mouse chemokines were ordered from PeproTech (Rocky Hill, NY). TAMRA [5(6)-carboxy-tetramethylrhodamine]-labeled CXCL10 was produced by Invitrogen (Eugene, OR). Polyclonal goat anti-murine CXCR3 was produced by the immunization of a goat with a 16-mer peptide (PYDYGENESDFSDSPP) constituting the NH_2-terminus of murine CXCR3 (mCXCR3).[4] The goat was peptide-immunized at multiple intradermal sites in the presence of CFA followed by at least three boosts in IFA. Direct ELISA was used to evaluate antisera titers, and serum was used for Western blot, ELISA, and neutralization assays when titers had reached values greater than 1/1,000,000. The specificity of anti-mCXCR3 Abs was assessed by Western blot analysis against cells expressing mCXCR3 and a panel of other human and murine chemokine receptor-transfected cell lines. Furthermore, murine splenocyte chemotaxis assays were performed to determine the specificity of the Abs. The anti-mCXCR3 Abs demonstrated a specific neutralizing capacity against CXCR3, as demonstrated in chemotaxis assays with IL-2–stimulated splenocytes. Indeed, migration to mCXCL9 and mCXCL10 (50 ng/ml) was inhibited by pre-incubating the cells with anti-mCXCR3 Abs, whereas migration to either mCXCL12 or mCCL5 (50 ng/ml) was not affected (data not shown). In addition, the Abs raised against murine CXCR3, also recognized and neutralized CXCL11 activities in rats.[5] Analogously, polyclonal Abs were raised in rabbits (against human CXCR3A) and were applied in chemotaxis experiments with human immature DC (*vide infra*).

Tumorigenesis models

The human A549 adenocarcinoma and weakly immunogenic murine Lewis lung carcinoma (LLC) cell lines were obtained from ATCC and cultured as described.[6,7] The LLC cells were transfected with GFP in order to detect metastasis. CB-17 SCID mice (6 to 8 weeks old; UCLA Animal Core Facility, Los Angeles, CA) and C57Bl/6 mice (Jackson Labs, Bar Harbor, ME) were injected subcutaneously into the right flank with 10^6 A549 cells or 10^6 LLC cells in 100 µl, respectively. $CXCR3^{-/-}$ animals on a C57Bl/6 background were developed as described.[8] Starting at the time of tumor inoculation and then three times a week, the tumors were treated with intratumoral injections of 20 µl of chemokine solution (or PBS supplemented with 1 mg/ml human serum albumin as vehicle control). 24 h prior to chemokine treatment (100 ng/injection), mice were injected i.p. with 0.5 ml/injection (4 mg immunoglobulins) of goat anti-mCXCR3 or appropriate control Abs (normal goat serum, NGS) for the duration of the experiment. Once palpable tumors were visible, tumors were measured in two dimensions with calipers on a weekly basis.[2] Tumor volume was calculated as previously described.[2] At 3 to 4 (LLC) or 8 (A549) weeks post-tumor cell injection, animals were euthanized by pentobarbital overdose, the tumors were resected, removed of extraneous tissue and measured. A portion of the resected tumors was fixed in 4% paraformaldehyde for histological analysis. Another portion of the tumors was used for flow cytometry.[2] Briefly, two 6 mm tumor punches were minced and digested in 5 ml of 1 mg/ml type IV collagenase (Roche Applied Sciences, Indianapolis, IN) for 45 min at 37°C with constant agitation in a water bath. Single cell suspensions were obtained by

repeatedly aspirating the cell suspensions through the bore of a 18 cc syringe. Red blood cell lysis was performed for 3 min at room temperature in lysis buffer (0.15 M NH_4Cl, 0.1 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2). Intermediate wash steps were performed using FACS buffer (1% FCS and 0.1% sodium azide in PBS). Finally, cell suspensions were passed over a 70 µm nylon mesh, the total cell count was determined and cells were resuspended in FACS buffer to 1×10^7 cells/ml. Samples (100 µl) were stained at 4°C for the presence of endothelial cell markers: the panendothelial cell marker MECA32 (BD Biosciences) or Factor VIII-related antigen (Sigma), and then analyzed by FACS. The results from the first LLC experiment (10 mice/group) were confirmed in a second experiment (15 mice/group).

Matrigel assay

Human dermal microvascular endothelial cells (HMVEC-d) were obtained from Clonetics (Walkersville, MD), and were cultured following the manufacturer's instructions in endothelial cell growth medium, i.e. EBM-2 medium supplemented with the EGM-2-MV "bulletkit" containing FCS, ascorbic acid, hydrocortisone, fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), insulin-like growth factor I (IGF-I) and epidermal growth factor (EGF). Plates (48 wells) were coated with 200 µl per well of Matrigel matrix with high growth factor content (BD Biosciences; ½ diluted in endothelial cell growth medium to a final concentration of 10 mg/ml) and placed at 37°C to solidify. Subsequently, 50,000 HMVEC-d were plated on the Matrigel-coated plates in 400 µl of endothelial cell growth medium supplemented with angiostatic CXCL4 or CXCL4L1. In each experiment at least 3 control wells were treated with medium alone to determine the maximal tube length. The angiostatic activity of CXCL4L1 was neutralized by 2.5 µg/ml monoclonal anti-human CXCR3 Abs (Clone 49801 R&D Systems). Reorganization of the endothelial cells into tubular structures was followed using an Axiovert 200 M inverted microscope (Zeiss, Göttingen, Germany) equipped with an XL-3 incubation chamber. Using the motorized table and the 10×/0.3 objective (EC Plan-Neofluar, Zeiss), we constructed for analysis a mosaic compilation (25 microscopic fields) covering nearly the whole well. Photographs were taken every hour. These overviews were analyzed by measuring the total length of tubes present in each well. Results were expressed as percentage inhibition compared to the control cultures containing growth medium alone. Experiments were evaluated 12 h after the initial seeding of the cells when the endothelial networks were developed maximally.

Rat cornea micropocket assay for angiogenesis

In vivo angiogenesis was assessed using the rat cornea micropocket assay as previously described.[1] Chemokines were diluted in PBS plus 0.25% serum albumin to their final concentration with and without anti-murine CXCR3 or control antibody per pellet of Hydron casting solution (Hydro Med Sciences, New Brunswick NJ). 5 µl aliquots were pipetted onto the flat surface of a sterile polypropylene specimen container and were allowed to polymerize overnight under UV light in a laminar flow hood. Prior to implantation, the pellets were rehydrated with normal saline. Animals were given 150 mg/kg ketamine and 250 µg/kg atropine, and the corneas were anesthetized with 0.5% proparacaine hydrochloride ophthalmic solution followed by implantation of the Hydron pellet into an intracorneal pocket (1–2 mm from the limbus). Six days after implantation, animals received heparin (1000 U) and ketamine (150 mg/kg) i.p. 30 min prior to sacrifice, followed by perfusion with 10 ml of colloidal carbon via the left ventricle. Corneas were then harvested and photographed. No inflammatory response was

observed in any of the corneas treated with the cytokines tested. Sustained directional ingrowth of capillary sprouts and hairpin loops toward the implant were considered positive neovascularization responses.

Chemotaxis

Human peripheral blood mononuclear cells were purified from buffy coats from healthy volunteers and cultured to generate human activated T lymphocytes and immature DC.[9,10] Chemotaxis with these cells was performed as previously described.[9,10] To identify the G protein type to which CXCR3 couples, DC were incubated with 3 µg/ml pertussis toxin for 1.5 h and subsequently washed before addition to the Boyden microchamber. Desensitization experiments were performed by pretreating cells with the selected chemotactic factor (30 min at 37°C, 5% CO_2), followed by a washing step and application in the chemotaxis assay. Neutralization of CXCR3 was achieved by pre-incubating the cells with anti-human CXCR3 Abs (4°C, 30 min) and subsequent migration in the presence of anti-CXCR3 Abs. The following Abs were used: 10 µg/ml of monoclonal mouse anti-human CXCR3 (Clone 1C6 BD Biosciences), 30 µg/ml of rabbit polyclonal anti-human CXCR3 and 10 µg/ml isotype control monoclonal Ab (see section on chemokines and antisera).

Activated mouse lymphocytes were generated from isolated splenocytes by treatment with IL-2 for at least 10 days. Membranes (5.0-µm polycarbonate filters coated with fibronectin), cell density (2×10^6 cells/ml) and incubation period (3 h) were similar to conditions used for activated human T cells. Expression of CXCR3 was evidenced by FACS analysis.
After isolation of human NK cells from peripheral blood mononuclear cells (EasySep negative selection human NK cell enrichment magnetic beads; StemCell Technologies, Vancouver, Canada) their migration was evaluated using 24-well Costar Transwell chambers (5 µm pore size; Corning, Life Science, Cambridge, MA). Cells were seeded (100 µl; 5×10^6 cells/ml) in the upper chambers, and 600 µl of chemoattractant or control medium (RPMI 1640 medium supplemented with 1% FCS) was added to the lower wells. The chambers were incubated at 37°C for 4 h. The number of migrated cells, recovered from the lower well, was expressed as the percentage of input cells.

Synthesis of fluorescently labeled CXCL4L1 and CXCL10

Synthetic CXCL4L1 was prepared by fluorenylmethoxycarbonyl solid phase peptide synthesis with appropriate side-chain protection groups on a 433A peptide synthesizer (Applied Biosystems) as previously described.[3] Part of the synthetic CXCL4L1 was NH_2-terminally labeled with TAMRA (Novabiochem, Hohenbrunn, Germany) on the peptide synthesizer. Subsequently, unlabeled and TAMRA-labeled CXCL4L1 were deprotected and cleaved from the resin for 2 h at room temperature in 10 ml TFA containing 0.75 g phenol, 0.5 ml thioanisole, 0.25 ml ethanedithiol and 0.5 ml water. Subsequently, the resin was removed by filtration and peptides were precipitated and washed in diethyl ether, dissolved in water and loaded on a 4.6 × 150 mm Source 5RPC column (GE Healthcare). The proteins were eluted from the RP-HPLC column with an acetonitrile gradient in 0.1 % TFA. Part of the column effluent (0.7 v/v %) was split to an ion trap mass spectrometer and averaged profile spectra were deconvoluted to determine the M_r. Proteins with the correct M_r were folded for 2.5 h in 150 mM Tris pH 8.6 containing 1 M guanidinium chloride, 3 mM EDTA, 0.3 mM reduced glutathion and 3 mM oxidized glutathion and repurified by RP-HPLC on a C8 Aquapore RP-300 column

(PerkinElmer). Ion trap mass spectrometry was used to select the fractions that contained the folded peptides. We demonstrated that chemical synthesis and addition of the TAMRA label to the NH_2-terminus of CXCL4L1 did not affect its biological activity. Indeed, TAMRA-CXCL4L1 attracted immature dendritic cells (data not shown).

Binding and signaling assays

CHO-K1 cells were transfected with CXCR3A, CXCR3B or CXCR7 as described previously.[11,12] These transfected cell lines were used in calcium[11] and ERK signaling[11] assays to identify the human CXCL4L1 receptor.

For endothelial cell binding tests with TAMRA-CXCL10 and TAMRA-CXCL4L1, HMVEC-L (human microsvascular endothelial cells isolated from lung tissue) were plated into 96-well black tissue culture plates (3000 cells/well) and allowed to adhere overnight. The following day the cultures were washed with HBSS and then treated with 2% BSA in HBSS (1 h at 37°C). Subsequently, the labeled and unlabeled chemokines were added and the cells were incubated (1 h at 37°C), washed and fixed with 2% paraformaldehyde. Fluorescence (excitation 546 and emission 574) was quantified using a fluorescent plate reader (DYNEX Technologies, Chantilly, VA).

Binding of TAMRA-CXCL4L1 to receptor-transfected cells was performed using a different protocol. Confluent monolayers of CXCR3-transfected CHO cells (6-well plates) were pre-chilled on ice for 30 min. Subsequently, monolayers were washed twice with binding buffer [PBS containing 0.5% (w/v) BSA] and incubated on ice for 2 h with 300 ng/ml TAMRA-CXCL4L1 in the absence or presence of competing unlabeled chemokines (750 µl chemokine solution/well). After four washes with binding buffer, monolayers were treated with 150 µl/well of lysis buffer [1mM EDTA, 0.5% (v/v) Triton X-100, 5 mM NaF, 6 M urea, protease inhibitor cocktail for mammalian tissues and phosphatase inhibitor cocktails 1 and 2 from Sigma, pH 7.4]. Cell lysates were cleared by centrifugation and supernatants were stored at −20°C. To visualize fluorescent chemokine in the lysates, 20 µl samples were subjected to SDS-PAGE under reducing conditions and the gels were afterwards scanned using the Ettan DIGE scanner (GE Healtcare, Uppsala, Sweden).

To determine the binding affinity of CXCL4 and CXCL4L1 to glycosaminoglycans (GAG) low molecular weight heparin (Sigma-Aldrich) or chondroitin sulphate-E (US Biologicals, Swampscott, MA) was immobilized (25 µg/ml solutions in PBS, incubated overnight at 4°C) on a GAG binding 96-well plate (BD Biosciences). Next, the plates were washed three times [wash buffer: 100 mM NaCl, 50 mM NaAc, 0.2% (v/v) Tween 20, pH 7.2] and remaining binding sites were blocked at 37°C [blocking buffer: wash buffer supplemented with 0.2% (w/v) gelatin or 1% (w/v) BSA for heparin or chondroitin sulphate-E, respectively]. Chemokines were diluted in blocking buffer, added to the wells and incubated for 2 h at 37°C. Subsequently, biotinylated polyclonal rabbit anti-human CXCL4[13] or anti-human CXCL4L1 Ab[13] was added and plates were incubated for 1 h at 37°C. After removal of excess Ab with wash buffer, immune complexes were detected by streptavidin-coupled peroxidase.

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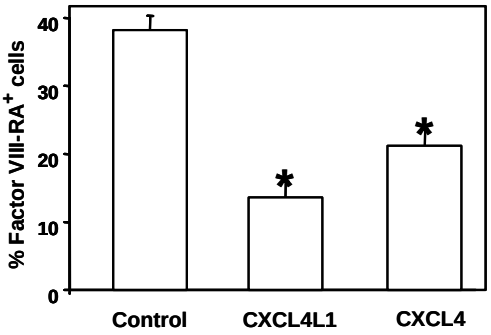

Figure S1. Inhibition of intratumoral angiogenesis by CXCL4L1
CB-17 SCID mice were injected subcutaneously with A549 adenocarcinoma cells and divided into three groups (n=6 per group), which received vehicle control, 0.1 µg CXCL4L1 or 0.1 µg CXCL4 per injection, respectively. Treatment started at the time of tumor inoculation and chemokines were injected intratumorally 3 times a week. After 8 weeks, mice were sacrificed and tumors were minced into single cell suspensions for flow cytometric analysis. In these cell suspensions the % of Factor VIII-related antigen (Factor VIII-RA) positive cells was determined to monitor angiogenesis in the tumors. P values (* $p < 0.05$) indicate a statistically significant difference (t-test) between the control group and the mice treated with CXCL4L1 or CXCL4. The angiostatic activity of CXCL4L1 in the A549 model was confirmed in a second experiment (not shown).

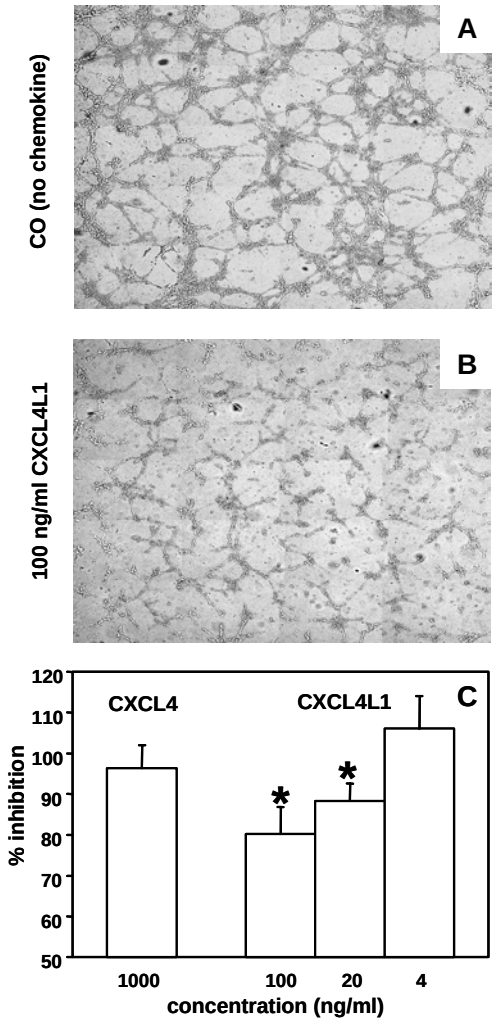

Figure S2. Inhibition of in vitro angiogenesis by CXCL4L1

HMVEC-d were seeded in 48-well plates on matrigel in the presence of CXCL4 (1000 ng/ml) or CXCL4L1 (100, 20 and 4 ng/ml). After 12 h, rearrangement of the endothelial cells into tubular structures was evaluated by microscopy. **(A) and (B) Representative photographs of inhibition of tube formation by 100 ng/ml CXCL4L1 when compared to a control culture.** (C) For statistical analysis the total length of tubes in each well was determined and results are expressed as the percentage inhibition of tube formation compared to control cultures stimulated with growth medium alone. Results (mean ± SEM of 5 independent experiments performed in duplicate or triplicate) were analyzed by the Mann Whitney test to detect differences between control and CXCL4L1-treated cultures (* $p<0.05$).

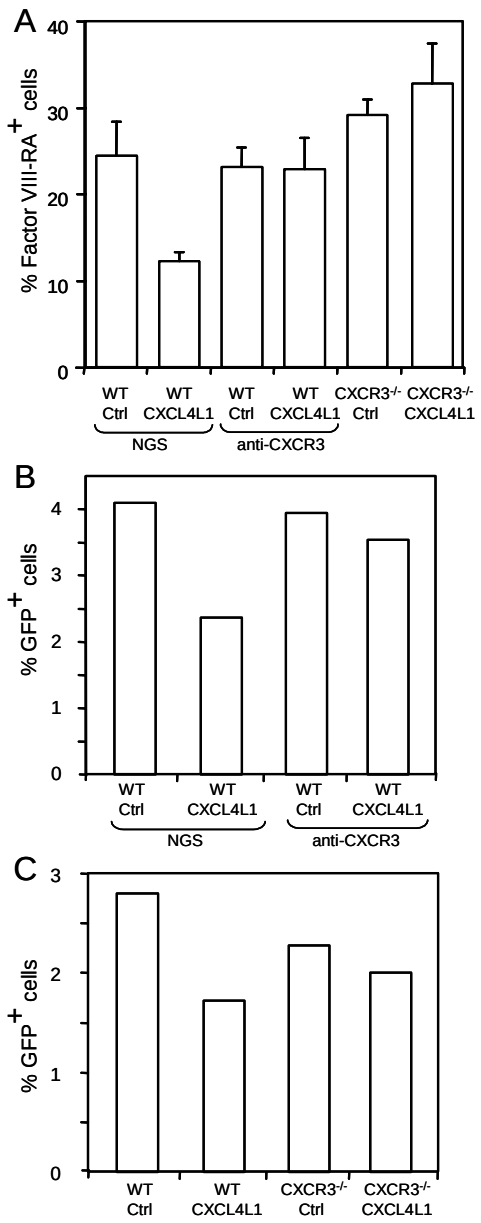

Figure S3. Treatment with anti-CXCR3 reduces the inhibitory capacity of CXCL4L1 on intratumoral neovascularization and tumor metastasis

GFP-LLC cells were subcutaneously implanted in C57Bl/6 mice in two separate experiments. In the first experiment 6 treatment groups were included [Ctrl versus CXCL4L1 NGS (normal goat serum)-treated; Ctrl versus CXCL4L1 anti-CXCR3-treated and Ctrl versus CXCL4L1 in CXCR3$^{-/-}$ mice]. In the second experiment two additional control groups of wt mice (WT Ctrl and WT CXCL4L1) were included that received no antibody treatment. Intratumoral chemokine treatment (vehicle control or 0.1 µg of human CXCL4L1 per injection) started at the time of tumor inoculation and was repeated 3 times a week. Neutralization of CXCR3 was obtained by intraperitoneal injection of 0.5 ml of polyclonal antiserum (4 mg Ig), 3 times a week, starting at the time of tumor inoculation. Panel A represents FACS analysis for the endothelial cell marker Factor VIII-related antigen (Factor VIII-RA), which was performed to asses the vascularization in the tumors from the first experiment (n=5/group). Panel B and C show the percentage of GFP-positive cells detected in the lungs of mice from the second experiment (n=5/group).

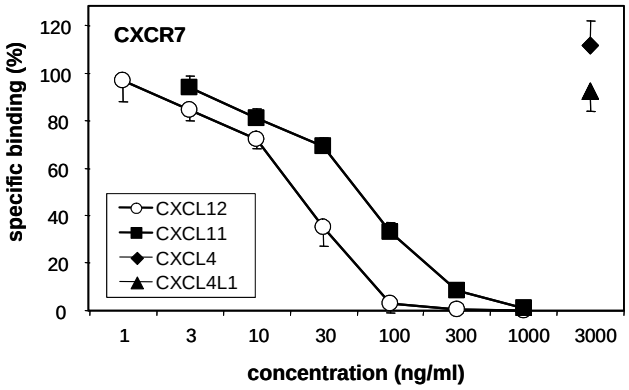

Figure S4. Receptor binding properties of CXCL4 and CXCL4L1

Competition for binding of ^{125}I-CXCL11 (PerkinElmer, Waltham, MA) to CXCR7-transfected CHO cells was evaluated in the presence of CXCL4, CXCL4L1, CXCL11 or CXCL12. Results (from 3 independent experiments) are expressed as the % of remaining specific binding of labeled chemokine. Experiments were performed according to the protocol described by Proost *et al.* (Blood, 2007).