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CXCR7 Protein Is Not Expressed on Human or Mouse Leukocytes

Robert D. Berahovich,* Brian A. Zabel,* Mark E. T. Penfold,* Susanna Lewén,* Yu Wang,* Zhenhua Miao,* Lin Gan,† Jaime Pereda,‡ Jessica Dias,§ Igor I. Slukvin,§ Kathleen E. McGrath,§ Juan C. Jaen,* and Thomas J. Schall*

Since the discovery that CXCR7 binds to CXCL12/SDF-1α, the role of CXCR7 in CXCL12-mediated biological processes has been under intensive scrutiny. However, there is no consensus in the literature on the expression of CXCR7 protein by peripheral blood cells. In this study we analyzed human and mouse leukocytes and erythrocytes for CXCR7 protein expression, using a competitive CXCL12 binding assay as well as by flow cytometry and immunohistochemistry using multiple CXCR7 Abs. CXCR7−/− mice were used as negative controls. Together, these methods indicate that CXCR7 protein is not expressed by human peripheral B cells, T cells, NK cells, or monocytes, or by mouse peripheral blood leukocytes. CXCR7 protein is, however, expressed on mouse primitive erythroid cells, which supply oxygen to the embryo during early stages of development. These studies therefore suggest that, whereas CXCR7 protein is expressed by primitive RBCs during murine embryonic development, in adult mammals CXCR7 protein is not expressed by normal peripheral blood cells. The Journal of Immunology, 2010, 185: 5130–5139.

The interaction between the chemokine CXCL12/SDF-1 and its receptor CXCR4 has been the subject of experimental investigation for almost 15 years. This receptor–ligand pair has been implicated in a wide range of biological processes, including development, stem cell biology, angiogenesis, leukocyte trafficking, cancer, neurodegenerative disorders, inflammatory disorders, atherosclerosis, and HIV pathology (1–9). Unlike CXCR4, which triggers calcium mobilization and chemotaxis in response to CXCL12, CXCR7 does not effectively initiate these processes. In fact, the CXCR7-mediated cellular responses to CXCL12 and CXCL11 are largely unknown, although ligand binding does trigger β-arrestin2 association with CXCR7 and ligand/receptor internalization in vitro (12–14). CXCR7 regulates CXCR4/CXCL12-mediated processes, such as transendothelial migration of tumor cells and human renal progenitors (14, 15) and primordial germ cell migration during zebrafish development (16, 17), underlining a complex interplay among CXCL12, CXCR4, and CXCR7.

Most leukocyte subsets express CXCR4 and respond functionally to CXCL12 in vitro and in vivo. To determine whether these leukocytes also express CXCR7, we and others have analyzed the cells by flow cytometry or immunocytochemistry with CXCR7-specific mAbs. Four CXCR7 mouse mAbs have been described: 11G8, generated by DNA immunization (10, 18); 9C4, generated by N-terminal peptide immunization (11, 19); and 8F11 and 358426, generated by immunization with CXCR7 cell transfectants (14, 20). Analysis of CXCR7 expression on leukocyte subsets with these Abs has yielded inconsistent results (11, 19–21). Balabanian et al. (11) reported that mAb 9C4 stained a majority of cultured human T cells by immunocytochemistry. Infantino et al. (19) reported that 9C4 stained all human peripheral B cells and monocytes, but only a small fraction of peripheral T cells, by flow cytometry. Hartmann et al. (20) reported that 11G8, 9C4, and 358426 did not stain purified human T cells by flow cytometry. Instead, that group found that the mAbs stained the cells after permeabilization, indicating that the purified T cells expressed CXCR7 only intracellularly (20).

Because CXCL12 is reported to bind only to CXCR4 and CXCR7 with high affinity, CXCR7 protein can be detected using a competitive [125I]–CXCL12 binding assay. In this assay, CXCR7 is distinguished from CXCR4 by the competition of CXCL12, CXCL11, or CXCR7-specific compounds (such as CCX733 and CCX771) for the same binding site, and the lack of competition by the CXCR4 antagonist AMD3100 (10, 18). Indeed, the original hypothesis that there existed a second receptor for CXCL12 emerged from observations at ChemoCentryx that [125I]–CXCL12 could bind to mouse embryonic day (E)13 fetal liver cells from CXCR4−/− mice (10). Although the CXCL12 binding assay has been used to identify CXCR7 protein expression by these fetal liver cells, renal cell progenitors, vascular smooth muscle cells, and certain tumor lines (10, 13, 15), it has not yet been applied to leukocytes.

In this study, we used the competitive [125I]–CXCL12 binding assay and flow cytometry with all four CXCR7 mAbs to evaluate CXCR7 protein expression by normal human and mouse leukocytes. T cells from CXCR7−/− mice were used to assess the specificity of the flow cytometric method employed by other investigators to detect CXCR7 protein after prolonged incubation (20). We also used the competitive CXCL12 binding assay, flow
cytometry, and immunohistochemistry (IHC) to determine which cells in mouse fetal liver express CXCR7.

Materials and Methods

**Primary Abs and reagents**

CXCR7 mAb 11G8 was generated at ChemoCentryx (Mountain View, CA) and has been described previously (10, 18). 293-mCXCR7 cells were generated by electroporation of HEK293 cells with the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) expressing murine CXCR7 and cultured in DMEM containing 10% FBS (Mediatech, Washington, DC). B lymphoblast Raji and prostate cancer PC3 cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI 1640 medium (Mediatech) containing 10% FBS. The osteosarcoma K7M2 cell line was obtained from the American Type Culture Collection and cultured in DMEM containing 10% FBS. The glioma U343 cell line was donated by Christine Hsieh (University of California, San Francisco, CA) and cultured in DMEM containing 10% FBS. Human normal bone marrow cells were purchased from AllCells (Emeryville, CA). Human thymocytes were obtained by mechanical disruption of fetal thymus tissue purchased from Advanced Bioscience Resources (Alameda, CA). Human PBMCs were collected from buffy coats (Stanford Blood Center, Stanford, CA) by centrifugation on Ficoll-Paque (GE Healthcare, Freiburg, Germany) and rinsed with PBS. T cells, B cells, and monocytes were isolated from the PBMCs with CD3, CD19, and CD14 (respectively) magnetic microbeads according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). PBLs were isolated from the PBMCs by depletion with CD14 microbeads. Mouse peripheral blood leukocytes were prepared from freshly drawn blood of BALB/c mice by erythrocyte lysis with Pharmlyse (BD Biosciences). Spleenocytes were prepared from mouse E13 embryos by mechanical disaggregation of splenic tissue and washing with 0.05% trypsin + 0.5 EDTA (Invitrogen) for 20 min at 37˚C. Cells were then transferred to PBS containing 12.5 µg/ml heparin, and the tweezers were removed, allowing the embryo blood to flow out of the umbilical vessels. Afterward, the blood cells were collected by centrifugation and resuspended in PBS. Freshly collected c57BL/6 mouse and human blood were mixed with Pharmlyse (BD Biosciences) to lyse erythrocytes, after which the leukocytes were collected by centrifugation and resuspended in PBS. Collection of cells from the umbilical vessels was performed in accordance with the ChemoCentryx Institutional Animal Care and Use Committee. An intact human embryo with normal appearance was obtained from an emergency salpingectomy performed for tubal ectopic pregnancy at the Department of Obstetrics and Gynecology, Barros-Luco Hospital, Santiago, Chile. The age of the embryo was determined to be 32 d postconception by extrapolation from the patient’s last menstrual period, and then, with use of a stereomicroscope, confirmed by the anatomical characteristics of the specimen. The study was approved by the Ethics Committee of the Faculty of Medical Sciences, Santiago de Chile University. Human prenatal erythrocyte cells were generated in culture as follows. The human embryonic stem cell (ESC) line H1 (passage 31–47; WiCell Research Institute, Madison, WI) was maintained in an undifferentiated state by coculture with human embryonic fibroblasts (WiCell Research Institute). To induce hematopoietic differentiation, H1 cells were cocultured with OP9 cells (provided by Toru Nakano at Osaka University, Osaka, Japan), as previously described (22). After 8 d of coculture, cells were harvested by treatment with 1 mg/ml collagenase (Invitrogen) for 25 min, followed by 0.05% EDTA (Invitrogen) for 20 min at 37˚C. Cells were washed with a-modified Eagle medium (Invitrogen) containing 10% FBS, filtered through a 70-µm cell strainer and cultured in a poly 2-hydroxyethyl methacrylate (Sigma-Aldrich)-coated flask with serum-free medium (Stem Cell Technologies, Vancouver, BC, Canada) containing 0.3% Ex-Cyte (Millipore, Bedford, MA), 1 mg/ml iron-saturated transferrin (Sigma-Aldrich), 10⁻⁶ M dexamethasone (Sigma-Aldrich), 20 ng/ml insulin (Sigma-Aldrich), 50 ng/ml stem cell factor, 2 U/ml erythropoietin, 50 ng/ml thombopepoitin, 5 ng/ml IL-3, 10 ng/ml IL-6, and 200 ng/ml Flt3L (all from PeproTech, Rocky Hill, NJ). After 2 d, the culture was harvested and mechanically disaggregated. Subsequently, the cells were spun over

FIGURE 1. CXCR7 mAb 11G8 stains mouse primitive erythroid cells by IHC. Whole-mount sections of E13 mouse embryos were analyzed by IHC using mAb 11G8. Staining in liver sinususes was detected by fluorescence IHC, using the Alexa Fluor 488 fluorochrome (green, top). Staining in heart lumen was detected by enzymatic IHC, using the fuchsin stain (red, bottom). Mouse IgG1 was used as an isotype control Ab. Original magnification ×400.

FIGURE 2. Mouse primitive erythroid cells express CXCR7. A, Freshly isolated, nonpermeabilized blood cells from mouse E13 embryos were analyzed by flow cytometry. The cells were stained by mAb 11G8 (purple histogram), but not by the mouse IgG1 isotype control Ab (green overlay). B, Freshly isolated blood cells from mouse E13 embryos were analyzed by the competitive [125I]–CXCL12 binding assay. [125I]–CXCL12 binding was inhibited by CXCL12, CXCL11, and the CXCR7 antagonist CCX733, but not by the CXCR4 antagonist AMD3100. The means ± SE of four wells for each concentration of inhibitor are shown.
20% Percoll (Sigma-Aldrich) to remove dead cells and cell aggregates, as described previously (23). The viable cells were then cultured on a layer of irradiated MS-5 stromal cells (DSMZ, Braunschweig, Germany) using the medium described above but lacking thrombopoietin, IL-3, and IL-6. Half-medium changes were performed every 2 d thereafter, maintaining the cell density near 1–2 × 10⁶ cells/ml. Cells were collected after 15 d of expansion for analysis.

**Construction and analysis of CXCR7 knockout mice**

To generate a CXCR7lacZ targeting construct, the 2.9-kb 5′ flanking sequence immediately upstream of the CXCR7 translation initiation codon and the 4.3-kb 3′ flanking sequence starting 1.1 kb downstream of the CXCR7 translation initiation codon were inserted at the NheI site and the SalI–KpnI sites of the pKII-lacZ vector containing lacZ (L. Gan, unpublished observations), respectively. The targeting construct thus contains the lacZ reporter gene under the control of 5′ and 3′ regulatory sequences of CXCR7. To generate CXCR7lacZ knockin mice, the targeting construct was linearized at the KpnI site and inserted into W4 ESCs (Taconic Farms, Germantown, NY) by electroporation. Five targeted mouse ESC clones were obtained from a total of 192 G418-resistant ESC clones. The targeted clones were confirmed by Southern blotting and PCR genotyping, and injected into C57BL/6J blastocysts to generate mouse chimeras. CMV-Cre mice (The Jackson Laboratory, Bar Harbor, ME) were used to remove the neomycin resistance gene to generate the CXCR7lacZ allele. CXCR7lacZ heterozygous and homozygous mice were generated and maintained in a mixed 129S6 and C57BL/6J background.

**RT-PCR**

Total RNA was isolated from human PBLs, purified human leukocyte subsets, mouse peripheral blood leukocytes, mouse splenocytes, human ESC-derived erythroid cells, and endogenous CXCR7-expressing human and mouse cell lines, using the RNAeasy Kit (Qiagen, Valencia, CA). First-strand cDNA was generated using the GeneAmp RNA PCR Core Kit with poly dTTT primers (Applied Biosystems, Foster City, CA). PCR of cDNA samples was performed using Taq PCR Master Mix Kit (Qiagen). Primer sets were as follows: human CXCR7, 5′-AGCACAGCCAGGAAGCGAGGCGAG-3′ and 5′-TCATAGCCTGTGGTCTTGGC-3′; mouse CXCR7, 5′-GGAGCCTGCAGCGCTCACCG-3′ and 5′-CTTAGCCTGGATATTCCACCG-3′; human ε-globin, 5′-GCCTGTGGAGCAAGATGAAT-3′ and 5′-GCGGGCTTGAGGTTGT-3′; universal GAPDH, 5′-CATTGACCTCAA-CTACATGG-3′ and 5′-GGGCCATCCACAGTCTTCTG-3′.

**QuantiGene analysis**

435-CXCR7 cells, untransfected 435 cells (20,000 cells per reaction), and human PBLs (150,000 cells per reaction) were analyzed for CXCR7.
CXCR4, and β-actin mRNA with the branched DNA-based Quantigene Assay according to the manufacturer’s instructions (Genospectra, Fremont, CA). Briefly, cell lysates were hybridized with specific probe sets overnight at 53°C, after which amplifier reagents and labeled probe sets were added and incubated for 1 h at 53°C after washing. Substrate reagents were added and incubated for 30 min, after which the plates were analyzed for chemiluminescence.

**Flow cytometry**

Mouse E13 blood cells, human thymocytes, and cells derived from human ESCs were stained with 11G8 or mouse IgG1 isotype control mAbs at 10 μg/ml on ice for 20 min and then rinsed with cold buffer (PBS containing 2% FBS). Cells were then incubated on ice for 20 min with PE-conjugated F(ab’)_2 fragments of donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and rinsed with cold buffer. Human peripheral blood leukocytes were stained with CXCR7 mAbs, isotype control mAbs, or Bcl-2 mAbs, as described above, then incubated on ice for 10 min with mouse serum (final concentration 5%) (Jackson ImmunoResearch Laboratories) and mixed with fluorochrome-conjugated Abs (FITC anti-human CD3, PE-Cy5 anti-human CD19, Alexa Fluor 647 anti-human CD16, and allophycocyanin-Cy7 anti-human CD14, all from BD Biosciences) on ice for 30 min. Human bone marrow leukocytes were stained with 11G8 in a similar fashion, except the lineage Abs were FITC anti-human CD3, allophycocyanin anti-human CD19, allophycocyanin-Cy7 anti-human CD14, and PE-Cy5 anti-human CD34 (all from BD Biosciences). Mouse leukocytes were stained with CXCR7 mAbs or isotype control mAbs at 10 μg/ml on ice for 20 min and then rinsed with cold buffer. Cells were then incubated on ice for 30 min with FITC-conjugated anti-mouse Ly6G, PE-conjugated anti-mouse CD3, PE-Cy5-conjugated anti-mouse B220 (all from BD Biosciences), and allophycocyanin-conjugated F(ab’)_2 fragments of donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories). Cells were rinsed with cold buffer and analyzed with a FACScan (BD Biosciences). For permeabilization experiments, human leukocytes were first incubated in PBS containing 3% paraformaldehyde (Sigma-Aldrich) for 5 min, collected by centrifugation, and then incubated in PBS containing 0.2% saponin (Sigma-Aldrich) for 10 min. Cells were then rinsed with buffer and stained with Abs, as indicated above. For prolonged incubation experiments, allophycocyanin-conjugated 11G8 (R&D Systems) was added to total leukocytes from CXCR7+/+ and CXCR7−− mixed background mice (see above) at 10 μg/ml in the absence or presence of CXCR7-specific compounds at 2.5 μM, and the cells were incubated at 37°C for 2 h. Cells were collected by centrifugation, suspended in cold buffer, and incubated on ice for 30 min with FITC-conjugated anti-mouse CD3 (BD Biosciences). Cells were rinsed cold with buffer and analyzed with the FACScan.

**Immunohistochemistry**

Mouse and human embryos were fixed in 10% neutral-buffered formalin (Sigma-Aldrich), dehydrated, and embedded in paraffin, and then 5-μm sections were cut and transferred to glass slides. Sections were deparaffinized with xylenes (Sigma-Aldrich) and hydrated through graded alcohols (Sigma-Aldrich) by conventional methods. For enzymatic IHC, sections were rinsed in water and immersed in TBS containing 1% BSA (Sigma-Aldrich) for 10 min. CXCR7 mAbs or isotype control Abs were added to the sections at 10 μg/ml and incubated at room temperature for 1 h. Sections were rinsed thoroughly in TBST and incubated with biotinylated anti-mouse IgG (Jackson ImmunoResearch Laboratories) for 30 min. Sections were rinsed thoroughly in TBST and incubated with streptavidin-conjugated alkaline phosphatase (Dako, Glostrup, Denmark) for 20 min. Sections were rinsed thoroughly in TBST and incubated with Fuchsīn reagent (Dako) for 5–10 min. Sections were rinsed with water and counterstained with Mayer’s hematoxylin (Sigma-Aldrich) for 3 min. Sections were rinsed with water and coverslipped with Faramount (Dako). For fluorescence IHC, sections were rinsed in water and immersed in TBS containing 1% BSA, 0.1% Triton X-100, and 0.05% Tween-20 (all from Sigma-Aldrich) for 30 min. CXCR7 mAbs or isotype control Abs were added to the sections at 10 μg/ml and incubated at room temperature for 1 h. Sections were rinsed thoroughly in TBST and incubated with Alexa Fluor 488-conjugated anti-mouse IgG (Invitrogen) for 30 min. Sections were rinsed thoroughly in PBS and incubated with DAPI (Invitrogen) for 15 min. Sections were rinsed thoroughly in PBS, transferred to water, and coverslipped with GelMount (WVR, West Chester, PA). All images were acquired on a Nikon Eclipse E800 microscope and captured with a SPOT RT-3 camera and imaging software (Diagnostic Instruments, Twinsburg, OH).

**FIGURE 5.** Human 5-wk embryo primitive erythroid cells do not express CXCR7. A, Sections of caudal pole tissue from a 5-wk postconception human embryo were analyzed by IHC. Staining was detected by enzymatic IHC, using the fuchsin stain (red). Primitive erythroid cells stained with a mAb specific for the erythroid marker CD235a, but not with mAb 11G8. Original magnification ×400. B, Cells generated in culture from human ESCs were analyzed by flow cytometry. The cells were stained by the erythroid marker CD235a (purple histogram), but not by 11G8 or the isotype control Abs (green overlays). Blood cells from mouse E13 embryos were included as a positive control. C. Cells generated in culture from human ESCs were analyzed in the competitive [125I]–CXCL12 binding assay. [125I]–CXCL12 binding was not inhibited by CXCL12, CXCL11 (100 nM each), or the CXCR7 antagonist CCX771 (10 μM). Blood cells from mouse E13 embryos were included as a positive control. The means ± SE of four wells for each inhibitor are shown. D, Cells generated in culture from human ESCs and CXCR7+/− PC3 prostate cancer cells were analyzed by RT-PCR for the presence of CXCR7, ε-globin, and GAPDH mRNAs.
CXCL12 binding assay

Cells were suspended at 1–5 million/ml in HBSS containing 0.1% BSA and transferred to 96-well plates (100 µl/well) containing 5 µl/well recombinant CXCL11 or CXCL12 (final concentration 100 nM in single-dose assays) or compounds (final concentration 10 µM in single-dose assays). Then 100 µl/well cold buffer containing 0.025 µCi (~1 nM) [125I]–CXCL12 was added, and the plates were shaken at 4°C for 3 h. Cells were transferred onto polyethyleneimine-treated GF/B glass fiber filters (PerkinElmer) with a cell harvester (Tomtec, Hamden, CT) and washed twice with washing buffer (25 mM HEPES, 500 mM NaCl, 1 mM CaCl₂, 5 mM MgCl₂, pH 7.1). Next 50 µl/well MicroScint-20 (PerkinElmer) was added to the filters, and counts per minute were measured on a Packard TopCount Scintillation Counter (PerkinElmer).

Results

CXCR7 protein is expressed on mouse primitive erythroid cells

We previously reported that total liver cells from mouse E13 embryos express CXCR7 protein, as determined by a competitive [125I]–CXCL12 binding assay and by flow cytometry with mAb 11G8 (10). We next sought to identify the cell type in the fetal liver that expresses CXCR7 protein. By IHC on E13 mouse embryonic liver sections (not shown), CXCR7 was expressed in the primitive erythroid cells. As a positive control, a competitive [125I]–CXCL12 binding assay was performed on mouse hepatic cells isolated from E13 embryonic liver sections (not shown).

**FIGURE 6.** Adult human and mouse peripheral blood cells do not bind to CXCR7-specific mAbs. A, Freshly isolated mouse blood cells were analyzed by flow cytometry using CXCR7 mAbs 11G8, 9C4, and 8F11. Abs specific for T cells (CD3) and neutrophils (Ly6G) were included in the stains. Open histograms indicate CXCR7 mAb staining; color-matched filled histograms indicate isotype control Ab staining. HEK293 cells transfected with mouse CXCR7 (293-mCXCR7) were included as a positive control. CXCR7 mAb 358426 did not stain 293-mCXCR7 cells, indicating that this mAb does not recognize mouse CXCR7. B and C, Freshly isolated human blood was depleted of erythrocytes and analyzed by flow cytometry using CXCR7 mAbs 11G8, 9C4, 8F11, and 358426 as well as a mAb specific for the intracellular protein Bcl-2. Abs specific for T cells (CD3), B cells (CD19), monocytes (CD14), and NK cells (CD16) were included in the stains. Neutrophils were identified based on light scatter properties and CD16 expression. Open histograms indicate CXCR7 mAb staining; color-matched filled histograms indicate isotype control Ab staining. MDA MB 435 cells transfected with human CXCR7 (435-CXCR7) were included as a positive control. Surface staining is shown in B, and staining following permeabilization is shown in C. D, Freshly isolated blood from CXCR7−/− mice or CXCR7+/+ littermates was depleted of erythrocytes, incubated with CXCR7 mAb 11G8 or isotype control mAb at 37°C for 2 h in the presence or absence of CXCR7-specific compounds CCXT33 or CCXT71, and analyzed by flow cytometry for mAb binding to T cells. The means ± SE of four wells for each inhibitor are shown.
embryos, 11G8 did not stain fetal hepatocytes, but rather stained nearly all of the primitive erythroid cells present in the large sinuses of the liver (Fig. 1). 11G8 also stained primitive erythroid cells elsewhere in the embryo, both inside (Fig. 1) and outside the vasculature (data not shown). 11G8 stained primitive RBCs by flow cytometry as well (Fig. 2A). [125I]–CXCL12 binding to primitive erythroid cells was inhibited by CXCL12 (IC50 value 0.2 nM), CXCL11 (IC50 value 0.4 nM), and the CXCR7-selective compound CCX733 (IC50 value 2.4 nM) but not by the CXCR4 antagonist AMD3100, thus confirming CXCR7 protein expression (Fig. 2B). In contrast, definitive (fetal) erythrocytes did not stain with 11G8 by IHC, either during development in erythroblast islands in the fetal liver or after release into the vasculature (data not shown).

To determine if CXCR7 is expressed early in the mouse primitive erythroid cell lifespan, we analyzed embryos from E9 to E12 by IHC. 11G8 did not stain primitive erythroblasts in mouse E9 embryos, and only weakly stained these cells in E10 embryos (Fig. 3). The intensity of staining increased thereafter, with strong staining in primitive erythroblasts in E12 embryos (Fig. 3). These data indicate that CXCR7 is not expressed by immature primitive erythroblasts, but is instead induced as these cells mature over time during embryonic development.

To investigate the role of CXCR7 on primitive erythroid cells, we generated CXCR7-deficient mice. Primitive erythroblasts, defined as nucleus-containing TER-119+ cells, were present in both CXCR7−/− and CXCR7+/− E13 embryos (Fig. 4). 11G8 did not stain primitive erythroid cells of E13 CXCR7−/− embryos by either IHC or flow cytometry (Fig. 4). E13 CXCR7−/− embryos did not exhibit any gross histological or morphological changes compared with CXCR7+/− or CXCR7+/+ embryos, and contained normal numbers of primitive erythroid cells with unaltered morphology (data not shown). These results confirm that CXCR7 protein is expressed by mouse primitive erythrocytes, and indicate that CXCR7 expression is not required for the development or survival of these cells.

To determine whether CXCR7 is expressed on human primitive erythroid cells, we used two approaches. First, we performed IHC on sections of the caudal pole from a human embryo 5 wk postconception, an age at which all blood cells are primitive erythroblasts. Human primitive erythroblasts were identified by staining with CD235a, a surface sialoglycoprotein expressed during all stages of erythropoiesis, and by the presence of a nucleus. 11G8 did not stain human primitive erythroblasts present in this tissue (Fig. 5A). Next, we analyzed erythroid cells generated in vitro from human ESCs. CD235a+ cells generated from the H1 ESC line did not stain with 11G8 (Fig. 5B), did not exhibit a CXCR7 profile in the competitive CXCL12 binding assay (Fig. 5C), and did not contain detectable CXCR7 mRNA (Fig. 5D). These cells were confirmed to be primitive erythrocytes by the presence of hemoglobin ε mRNA (Fig. 5D).

CXCR7 protein is not expressed on or in primary leukocytes

We next evaluated CXCR7 protein expression in definitive (adult) mouse erythrocytes and in mouse and human leukocyte subsets by flow cytometry, using all available CXCR7 mAbs. First, we determined the specificity of the mAbs, using CXCR7− cells transfected with CXCR7 or empty expression vectors. For mouse CXCR7, mAbs 11G8, 9C4, and 8F11 stained 293-mCXCR7 cells (Fig. 6A), but not 293-empty vector cells (data not shown). mAb 358426 did not stain 293-mCXCR7 cells (Fig. 6A), indicating that it does not bind to mouse CXCR7. For human CXCR7, mAbs 11G8, 9C4, and 8F11, and 358426 stained 435-CXCR7 cells (Fig. 6B), but not 435-empty vector cells (data not shown). On primary cells, mAbs 11G8, 9C4, and 8F11 did not stain mouse peripheral blood erythrocytes, T cells, or neutrophils (Fig. 6A). Similarly, mAbs 11G8, 9C4, and 8F11 did not stain T cells, B cells, NK cells, monocytes, or neutrophils from freshly drawn human blood (Fig. 6B). mAb 358426 stained human monocytes but did not stain the other leukocyte subsets (Fig. 6B). In addition, all four CXCR7 mAbs failed to stain each human leukocyte subset after per-
meabilization; effective permeabilization was confirmed by positive staining for the intracellular protein Bcl-2 (Fig. 6C). mAb 358426 failed to stain 435-CXCR7 cells and monocytes after permeabilization, likely indicating that the target epitope is altered by fixation (Fig. 6C).

Other investigators have reported that, although 11G8 failed to stain intact purified human T cells under normal conditions, the Ab did stain the cells if they were incubated together for 2 h at 37°C (20). We attempted to reproduce this finding but also employed leukocytes from both wild-type and CXCR7−/− mice as a specificity control. After 2 h at 37°C, 11G8 stained wild-type mouse T cells to a small degree (Fig. 6D). However, not only was staining not inhibited by CXCR7-specific compounds, but staining also occurred on T cells from CXCR7−/− mice (Fig. 6D). These data suggest that the 11G8 staining seen after prolonged incubation at 37°C is artifactual or nonspecific.

In addition, we evaluated plasma membrane expression of CXCR7 on purified human T cells, B cells, and monocytes, as well as total mouse peripheral blood leukocytes, with the competitive [125I]–CXCL12 binding assay. Although CXCL12, CXCL11, and the CXCR7-specific small molecule CCX733 blocked [125I]–CXCL12 binding to 435-CXCR7 cells, none of the primary cell types displayed a CXCR7 binding profile, confirming that these cells do not express cell-surface CXCR7 protein (Fig. 7).

We also analyzed leukocytes in human bone marrow and thymus for CXCR7 protein expression (Fig. 8). By flow cytometry, CXCR7 mAbs 11G8, 9C4, and 8F11 did not stain any cell subset either (not shown). 435-CXCR7 cells (C) were used as a positive control. CXCR7 mAbs 9C4 and 8F11 did not stain any cell subset either (not shown). 435-CXCR7 cells (C) were used as a positive control.

![FIGURE 8.](http://example.com/figure8.png) Human bone marrow leukocytes and thymocytes do not express CXCR7 protein. Freshly isolated human bone marrow cells (A) and freshly isolated human fetal thymocytes (B) were analyzed in the competitive [125I]–CXCL12 binding assay (left) and by flow cytometry using CXCR7 mAb 11G8 (right). [125I]–CXCL12 binding was not inhibited by CXCL12, CXCL11 (100 nM each), or the CXCR7 antagonists CCX754 or CCX771 (10 μM) in either sample. 435-CXCR7 cells (C) were used as a positive control. The means ± SE of four wells for each inhibitor are shown. 11G8 (purple histograms) did not stain bone marrow hematopoietic progenitor cells (CD34+), T cells (CD3+), B cells (CD19+), monocytes (CD14+), or granulocytes (high side scatter), nor did 11G8 stain CD4+CD8+ immature thymocytes or CD4+CD8− or CD4−CD8+ mature thymocytes. Green overlays indicate isotype control Ab staining. CXCR7 mAbs 9C4 and 8F11 did not stain any cell subset either (not shown). 435-CXCR7 cells (C) were used as a positive control.

![FIGURE 9.](http://example.com/figure9.png) CXCR7+/+ and CXCR7−/− mouse leukocytes respond equally to CXCL12. Freshly isolated mouse bone marrow leukocytes from CXCR7+/+ and CXCR7−/− mice were tested for chemotaxis to varying concentrations of CXCL12 in vitro. Equal numbers of CXCR7+/+ and CXCR7−/− cells migrated to CXCL12, and the potency of CXCL12 was equal for CXCR7+/+ and CXCR7−/− cells.
Our initial observation that cells in mouse E13 embryo liver express CXCR7 protein (10) led us to explore the expression of CXCR7 protein in human and mouse leukocytes. We found that human and mouse leukocytes express virtually no CXCR7 mRNA, which suggests that CXCR7 protein expression might be limited to specific subpopulations or stages of hematopoiesis.

### Discussion

In these studies, we used a competitive $[^{125}\text{I}]$-CXCL12 binding assay, flow cytometry, and IHC to assess human and mouse normal blood cells for expression of CXCR7 protein. We first followed up on our initial observation that cells in mouse E13 embryo liver express CXCR7 protein (10). In this paper, we show that these cells are primitive erythroid cells. Primitive erythroid cells collected from wild-type, but not CXCR7$^{-/-}$, mouse E13 embryos exhibited the signature CXCR7 binding profile in the $[^{125}\text{I}]$-CXCL12 binding assay: $[^{125}\text{I}]$-CXCL12 binding was inhibited by CXCL11- and CXCR7-specific compounds, but not by the CXCR4 antagonist AMD3100. In addition, CXCR7 mAb 11G8 stained wild-type, but not CXCR7$^{-/-}$, primitive erythroid cells by IHC and flow cytometry.

The role of CXCR7 on murine primitive erythroid cells is uncertain. CXCR7$^{-/-}$ embryos were unaltered in the numbers of primitive erythroid cells, and no gross changes in morphology were observed. Indeed, most of our CXCR7$^{-/-}$ mice did not die until soon after birth, similarly to other CXCR7$^{-/-}$ mice (25, 26). Hence CXCR7 is not required for the development, proliferation, or survival of primitive erythroid cells. CXCR7 is also not likely necessary for primitive erythrocyte function, as mutations that disrupt primitive erythrocyte function cause mouse embryo lethality by E10.5 (27). Interestingly, CXCR7 protein was not expressed by wild-type mouse primitive erythroid cells as they develop in the yolk sac and enter circulation (which occurs at E9). Instead, low levels of CXCR7 protein appeared on the cell surface at E10 (Fig. 9).

To determine whether human and mouse leukocytes lack CXCR7 protein because they lack CXCR7 mRNA, we analyzed these cells for CXCR7 mRNA expression (Fig. 10). In total human PBLs, CXCR7 mRNA was undetectable by RT-PCR and QuantiGene analysis. In addition, little to no CXCR7 mRNA was detected in purified T cells, B cells, and monocytes by RT-PCR. CXCR7 mRNA was either not detected or detected at low levels in each sample. Positive controls included CXCR7$^+$ Raji cells and U343 cells. GAPDH control reactions demonstrated that the substrate RNAs were intact. Negative control reactions were analyzed by RT-PCR and agarose gel electrophoresis. Two donors are shown for each cell population. CXCR7 mRNA was either not detected or detected at low levels in each sample. Positive controls included CXCR7$^+$ Raji cells and U343 cells. GAPDH control reactions demonstrated that the substrate RNAs were intact.

### FIGURE 10.

Human and mouse leukocytes express virtually no CXCR7 mRNA. Human and mouse leukocyte subsets were assessed for CXCR7 mRNA expression by RT-PCR and QuantiGene analysis. A, RNA preparations from human PBLs (left gel) and purified T cells, B cells, and monocytes (right gel) were analyzed by RT-PCR and agarose gel electrophoresis. Two donors are shown for each cell population. CXCR7 mRNA was either not detected or detected at low levels in each sample. Positive controls included CXCR7$^+$ Raji cells and U343 cells. GAPDH control reactions demonstrated that the substrate RNAs were intact. B, RNA preparations from mouse peripheral blood leukocytes and splenocytes were analyzed by RT-PCR and agarose gel electrophoresis. Mouse CXCR7 mRNA was either not detected or detected at low levels in each sample. The positive control was K7M2 cells. GAPDH control reactions demonstrated that the substrate RNAs were intact. C, QuantiGene analysis was performed on human PBLs, with 435-CXCR7 cells and untransfected 435 cells serving as positive and negative control cells, respectively. CXCR7 mRNA was not detected in any PBL sample. CXCR4 and β-actin control reactions demonstrated that the substrate RNAs were intact. The means ± SE of three wells for each reaction are shown.

(T cells, B cells, monocytes, granulocytes, and CD34$^+$ progenitor cells) in bone marrow. In addition, 11G8, 9C4, and 8F11 did not stain immature CD4$^+CD8^+$, mature CD4$^+CD8^+$, or mature CD4$^-CD8^+$ thymocytes. Bone marrow leukocytes and thymocytes did not display the CXCR7 profile in the competitive $[^{125}\text{I}]$-CXCL12 binding assay, indicating that leukocytes in these two organs do not express cell-surface CXCR7 protein.

Because CXCR7 has been shown to affect CXCR4-mediated responses to CXCL12 (14, 15, 20, 24), we reasoned that if mouse leukocytes do not express CXCR7, then genetic ablation of CXCR7 should not affect leukocyte responses to CXCL12. Indeed, bone marrow leukocytes and thymocytes did not display the CXCR7 profile in the competitive $[^{125}\text{I}]$-CXCL12 binding assay, indicating that leukocytes in these two organs do not express cell-surface CXCR7 protein.

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primary erythrocyte lifespan, although it is not known whether primary erythrocytes are actively removed from the embryo or are simply swamped out by the definitive erythrocytes (28). In contrast to mouse primitive erythroid cells, human primitive erythroid cells do not appear to express CXCR7 at the same stage of development. Primitive erythroblast present in a 5-week-old human fetus (Theiler stage 15, which corresponds to mouse E12) did not stain with CXCR7 mAb 11G8. In addition, primitive erythroblasts generated in culture from human ESCs did not stain with 11G8 or exhibit the CXCR7 profile in the competitive CXCL12 binding assay. However, it is possible that CXCR7 protein is induced on human primitive erythroid cells at earlier or later stages of erythropoiesis than in the mouse. We were unable to procure human embryos containing primary erythroid cells at these other stages.

In this study, we also determined that CXCR7 protein is not expressed on adult human or mouse leukocytes. T cells, B cells, NK cells, monocytes, and neutrophils in freshly isolated human blood and bone marrow each failed to stain with CXCR7 mAbs 11G8, 9C4, and 8F11 by flow cytometry. These three mAbs also failed to stain human thymocytes and mouse peripheral blood erythrocytes, T cells, and neutrophils. CXCR7 mAb 358A26, sold by R&D Systems, stains human monocytes but also binds to cells lacking cell-surface CXCR7 and fails to bind to tumor cell lines endogenously expressing CXCR7 (21). CXCR7 polyclonal Abs ab12870 and ab38039, sold by Abcam (Cambridge, MA), stains tissues from CXCR7+/+ and CXCR7–/– mice by IHC (21), indicating that not all CXCR7 Abs are reactive or specific for CXCR7. Moreover, by IHC, none of the CXCR7 mAbs (including 9C4) stained leukocytes in human tissue sections (R.D. Berahovich, unpublished observations), in contrast to a previous report wherein 9C4 was observed to stain follicular B cells in human tonsil (19). Finally, purified human T cells, B cells, and monocytes, as well as total leukocytes in human bone marrow, thymus, and mouse peripheral blood, each did not display the CXCR7 profile in the competitive [125I]–CXCL12 binding assay. In fact, most human and mouse leukocyte samples did not contain detectable CXCR7 mRNA either, as assessed by RT-PCR and QuantiGene analysis. CXCR7 mRNA expression levels in human leukocytes have previously been reported, but with inconsistent results (19, 29–31). These results differ from results in several published reports (11, 19, 20), in which CXCR7 is expressed on the surface of most T cells (where it was reported to mediate chemotaxis) (11); on all B cells and monocytes, but only a small subset of T cells (19); and inside (but not on the surface of) all T cells (20). In the last of these reports, the authors performed flow cytometry on T cells several hours after blood draw, after multiple steps of enrichment (20). Because we did not observe CXCR7 mAb staining inside T cells in freshly drawn blood, it is possible that the staining seen by these investigators (20) was due to extensive manipulation of the cells. The authors also showed that 11G8 could stain their purified T cells without permeabilization if incubated for 2 h at 37°C. We showed that under these unusual conditions, 11G8 equally stains T cells from wild-type and CXCR7–/– mice.

Finally, we used a functional assay to explore whether mouse CXCR7 protein is expressed by normal mouse or mouse peripheral blood leukocytes. In contrast to the human studies, most leukocytes in human bone marrow, thymus, and mouse peripheral blood express CXCR7 mRNA either, as assessed by RT-PCR and QuantiGene analysis. CXCR7 mRNA expression levels in human leukocytes have previously been reported, but with inconsistent results (19, 29–31). These results differ from results in several published reports (11, 19, 20), in which CXCR7 is expressed on the surface of most T cells (where it was reported to mediate chemotaxis) (11); on all B cells and monocytes, but only a small subset of T cells (19); and inside (but not on the surface of) all T cells (20). In the last of these reports, the authors performed flow cytometry on T cells several hours after blood draw, after multiple steps of enrichment (20). Because we did not observe CXCR7 mAb staining inside T cells in freshly drawn blood, it is possible that the staining seen by these investigators (20) was due to extensive manipulation of the cells. The authors also showed that 11G8 could stain their purified T cells without permeabilization if incubated for 2 h at 37°C. We showed that under these unusual conditions, 11G8 equally stains T cells from wild-type and CXCR7–/– mice. In contrast to the human studies, most leukocytes in human bone marrow, thymus, and mouse peripheral blood express CXCR7 mRNA either, as assessed by RT-PCR and QuantiGene analysis. CXCR7 mRNA expression levels in human leukocytes have previously been reported, but with inconsistent results (19, 29–31). These results differ from results in several published reports (11, 19, 20), in which CXCR7 is expressed on the surface of most T cells (where it was reported to mediate chemotaxis) (11); on all B cells and monocytes, but only a small subset of T cells (19); and inside (but not on the surface of) all T cells (20). In the last of these reports, the authors performed flow cytometry on T cells several hours after blood draw, after multiple steps of enrichment (20). Because we did not observe CXCR7 mAb staining inside T cells in freshly drawn blood, it is possible that the staining seen by these investigators (20) was due to extensive manipulation of the cells. The authors also showed that 11G8 could stain their purified T cells without permeabilization if incubated for 2 h at 37°C. We showed that under these unusual conditions, 11G8 equally stains T cells from wild-type and CXCR7–/– mice. In contrast to the human studies, most leukocytes in human bone marrow, thymus, and mouse peripheral blood express CXCR7 mRNA either, as assessed by RT-PCR and QuantiGene analysis. CXCR7 mRNA expression levels in human leukocytes have previously been reported, but with inconsistent results (19, 29–31). These results differ from results in several published reports (11, 19, 20), in which CXCR7 is expressed on the surface of most T cells (where it was reported to mediate chemotaxis) (11); on all B cells and monocytes, but only a small subset of T cells (19); and inside (but not on the surface of) all T cells (20). In the last of these reports, the authors performed flow cytometry on T cells several hours after blood draw, after multiple steps of enrichment (20). Because we did not observe CXCR7 mAb staining inside T cells in freshly drawn blood, it is possible that the staining seen by these investigators (20) was due to extensive manipulation of the cells. The authors also showed that 11G8 could stain their purified T cells without permeabilization if incubated for 2 h at 37°C. We showed that under these unusual conditions, 11G8 equally stains T cells from wild-type and CXCR7–/– mice.

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Disclosures
The authors have no financial conflicts of interest.

References


