CXCL16-Mediated Cell Recruitment to Rheumatoid Arthritis Synovial Tissue and Murine Lymph Nodes Is Dependent Upon the MAPK Pathway

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Objective. Rheumatoid arthritis (RA) is characterized by profound mononuclear cell (MNC) recruitment into synovial tissue (ST), thought to be due in part to tumor necrosis factor α (TNFα), a therapeutic target for RA. Although chemokines may also be involved, the mechanisms remain unclear. We undertook this study to examine the participation of CXCL16, a novel chemokine, in recruitment of MNCs to RA ST in vivo and to determine the signal transduction pathways mediating this process.

Methods. Using a human RA ST–SCID mouse chimera, immunohistochemistry, enzyme-linked immunosorbent assay, real-time reverse transcription–polymerase chain reaction, flow cytometry, and in vitro chemotaxis assays, we defined the expression and func-

tion of CXCL16 and its receptor, CXCR6, as well as the signal transduction pathways utilized by them for MNC homing in vitro and in vivo.

Results. CXCL16 was markedly elevated in RA synovial fluid (SF) samples, being as high as 145 ng/ml. Intense macrophage and lining cell staining for CXCL16 in RA ST correlated with increased CXCL16 messenger RNA levels in RA ST compared with those in osteoarthritis and normal ST. By fluorescence-activated cell sorting analysis, one-half of RA SF monocytes and one-third of memory lymphocytes expressed CXCR6. In vivo recruitment of human MNCs to RA ST implanted in SCID mice occurred in response to intragraft injection of human CXCL16, a response similar to that induced by TNFα. Lipofection of MNCs with antisense oligodeoxynucleotides for ERK-1/2 resulted in a 50% decline in recruitment to engrafted RA ST and a 5-fold decline in recruitment to regional lymph nodes. Interestingly, RA ST fibroblasts did not produce CXCL16 in response to TNFα in vitro, suggesting that CXCL16 protein may function in large part independently of TNFα.

Conclusion. Taken together, these results point to a unique role for CXCL16 as a premier MNC recruiter in RA and suggest additional therapeutic possibilities, targeting CXCL16, its receptor, or its signaling pathways.

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One of the earliest events in rheumatoid arthritis (RA) is the ingress of leukocytes into inflamed synovial tissue (ST). A number of cell-derived factors facilitate this process, including macrophage inflammatory protein 3α (MIP-3α), granulocyte–macrophage colony-stimulating factor, monocyte chemoattractant protein 1 (MCP-1), MIP-1α, epithelial neutrophil–activating peptide 78 (ENA-78), fractalkine, and others (1–6). Thera-

pies designed to block the activity or inhibit the production of these mediators and their corresponding receptors are currently being developed. Some chemokines function in a variety of ways, including initiating angiogenesis (6–8), binding of human immunodeficiency virus (HIV) surface proteins (9), and directly regulating immune responses to antigen (10). However, one of the primary functions of chemokines is their contribution to leukocyte homing (11).

Chemokines are redundant by nature, but they can be subdivided into inducible chemokines produced in response to inflammation and noninducible chemokines. The first group defines chemokines that recruit leukocytes, dendritic cells, and activated T cells to sites of inflammation. The second group defines the noninflammatory, constitutive chemokines expressed in bone marrow, thymus, and secondary lymphoid organs. Chemokines in the latter group are produced for normal physiologic leukocyte trafficking (12). However, regulation of leukocyte recruitment is complex and involves both secretion and cell surface presentation of chemokines, as well as their receptors, during leukocyte differentiation and activation (13). Thus, chemokines are produced in response to a variety of stimuli. For instance, in RA ST fibroblasts, interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα) are well-known stimuli (14–18).

Chemokines are further subdivided into so-called "CXC" (α) or "CC" (β) chemokines. These designations are derived from the location of 2 adjacent amino-terminus cysteine residues. We and others have shown that many CC chemokines and their receptors, such as CCR5, a receptor for the CC chemokines MIP-1α and RANTES, are up-regulated in the RA joint (19–21). The CXC chemokines are also active mediators of inflammation in the RA joint. Examples of this class of chemokines that are important in RA include IL-8 and ENA-78 (6,14,22,23).

Much like fractalkine, CXCL16 has a chemokine domain without the proangiogenic "ELR" (glutamate-leucine-arginine) motif (4,24), and it is flanked by a typical mucin structure that is rich in serine, threonine, and proline. Both fractalkine and CXCL16 contain a hydrophobic transmembrane domain and a short cytoplasmic tail (24). Other similarities exist between CXCL16 and fractalkine, including the expression of a transmembrane domain suspended by a heavily glycosylated mucin stalk and the fact that both proteins exist as either membrane-associated or secreted forms. These chemokines contain a small cytoplasmic domain with a "YXPV" motif that is a potential tyrosine phosphoryla-

tion and SH2-binding site, and is preferentially expressed by type 1 lymphocytes. Finally, CXCL16 has a unique receptor–ligand interaction, much like fractalkine (24–26). These characteristics are important, considering the proinflammatory functions of fractalkine in RA and in rat adjuvant-induced arthritis (AIA) (3,4). Thus, the similar physical properties of fractalkine and CXCL16 are likely to result in similar proinflammatory activity.

CXCR6, the only known receptor for CXCL16, is also known as Bonzo, TYMSTR, and STRL33, and is a newly characterized chemokine receptor initially described as an HIV coreceptor expressed by a subset of memory/T effector cells, natural killer cells, and plasma cells (26–30). The present study directly addresses the participation of both CXCR6 and its ligand CXCL16 in leukocyte migration in RA. CXCR6-expressing leukocytes may also use this receptor to migrate into lymphocyte-rich regions such as lymph nodes (LNs). By expressing a receptor known to actively bind HIV, CXCR6+ lymphocytes may aid in the spread of viral infections (24). We show here that CXCL16 contributes to chronic inflammation, since it is highly expressed in RA synovial fluid (SF), is a potent chemoattractant for mononuclear cells (MNCs) in vitro, and is chemotactic for peripheral blood mononuclear cells (PBMCs) to RA ST and LNs in vivo.

PATIENTS AND METHODS

Patient samples. SF samples were obtained during arthrocentesis from patients with RA, osteoarthritis (OA), and other diseases including juvenile rheumatoid arthritis (JRA), psoriatic arthritis (PsA), polyarthritis, spondylarthropathy, inflammatory myopathy, and gout. Peripheral blood (PB) sera were also obtained from patients with arthritides (RA, OA, JRA, PsA, polyarthritis, and gout), and PB samples were obtained from healthy normal volunteers. STs were obtained from RA patients undergoing total joint replacement who met the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria for the classification of RA (31). Normal STs were obtained from fresh autopsies or from amputations. All specimens were obtained with Institutional Review Board approval.

SCID mice. SCID/NCr mice were purchased from the National Cancer Institute (Bethesda, MD). SCID mice were maintained in a pathogen-free animal facility and given food and water ad libitum.

Depletion of rheumatoid factor (RF) from sera and SF. To avoid any possible confounding effects of RF on assays, RF was immunodepleted from sera and SF samples using anti-IgM antibodies coupled to agarose beads (Sigma, St. Louis, MO) as previously described (3). Removal of RF (IgM) was determined by randomly choosing 5 RA SF samples and measuring RF levels before and after immunodepletion using

an RF enzyme-linked immunosorbent assay (ELISA) kit (RDI, Flanders, NJ). Before immunodepletion, RF levels ranged from 5 IU/ml to 300 IU/ml. After immunodepletion, all samples had RF levels below the detection limit of the assay (0.031 IU/ml; data not shown). Samples immunodepleted of RF were used in the ELISA and chemotaxis studies.

Sandwich ELISA. CXCL16 levels were measured by coating 96-well polystyrene plates with rabbit anti-human CXCL16 (PeproTech, Rocky Hill, NJ), followed by a blocking step as described previously (3). All samples were added in triplicate. Biotinylated rabbit anti-human antibody (Pepro-Tech) was used to detect CXCL16 using a streptavidin–peroxidase method (PharMingen, San Diego, CA), with a tetramethylbenzidine substrate (Sigma). Assay sensitivity was routinely 125 pg/ml, and rabbit anti-human CXCL16 antibody demonstrated <5% cross-reactivity with other recombinant human chemokines (data not shown).

Fluorescence-activated cell sorting (FACS) analysis. PB samples were obtained from normal volunteers and RA patients. SF cells were obtained from the joints of RA patients undergoing arthrocentesis. CXCR6 expression on CD14+ monocytes was evaluated by FACS analysis as previously described (11). Briefly, cells were incubated with fluorescein isothiocyanate (FITC)–labeled mouse anti-human CD14 (PharMingen) and mouse anti-human CXCR6 antibodies (CXCR6 IgG; kindly provided by Millennium Pharmaceuticals, Cambridge, MA). CXCR6 receptor expression was detected with R-phycoerythrin (R-PE)–labeled goat anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA). For some studies, CXCR6 receptor expression on lymphocyte subsets was evaluated by incubating cells with anti-CXCR6 antibodies or with energy-coupled dye–labeled anti-CD4 or anti-CD8 antibodies (Beckman Coulter, Miami, FL) with the addition of FITC-labeled (anti-CD45RA or anti-CD45RO) and Cy-Chrome–labeled (anti-CD3) antibodies (PharMingen) as previously described (5,32).

Immunohistochemistry. We performed immunohistologic staining on cryosections from RA, OA, and normal STs using immunoperoxidase and avidin–biotin technique as described previously (3). Antibodies, including control antibodies, were used at 10 μg/ml. Polyclonal rabbit anti-human CXCL16 antibodies (PeproTech) or monoclonal mouse anti-CXCR6 antibodies were used as specific antibodies, and tissues were counterstained with Harris' hematoxylin. STs were also stained with the appropriate IgG control antibodies for comparison (CXCL16 control antibody was rabbit IgG and was obtained from R&D Systems [Minneapolis, MN]; CXCR6 control antibody was mouse IgG2α and was obtained from Beckman Coulter). For some immunohistologic experiments, STs were stained with mouse anti-human CXCR6 from a different vendor to check for staining consistency (mouse anti-human CXCR6 was obtained from R&D Systems; control mouse IgG2$\beta\kappa$ antibody was obtained from Beckman Coulter). Various ST cell types were evaluated for staining, including macrophages, lymphocytes, fibroblasts, and endothelial cells. Immunostaining was evaluated in a blinded manner and graded by a pathologist as described previously (33).

For 2-color immunofluorescence histology, serial ST sections (8 μm) were cut, allowed to dry for 2 hours at room temperature, and subsequently fixed in acetone for 10 minutes at 4°C. Following a wash with phosphate buffered saline (PBS),

sections were blocked with 3% fetal bovine serum (FBS)/PBS for 30 minutes at 37°C. Either mouse anti-human CXCR6 antibodies or goat anti-human CXCL16 antibodies (final concentration 10 μg/ml; both from R&D Systems) were added as primary antibodies and incubated for 2 hours at room temperature. Mouse or goat IgG served as controls. After washing twice with PBS, Alexa 488–labeled donkey anti-mouse or anti-goat antibody was added and incubated at room temperature for 1 hour. PE-tagged rat anti-human CD14 antibody (diluted 1:200 in PBS; Ancell, Bayport, MN) was used to detect CD14+ macrophages. After washing with PBS, nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Molecular Probes, Eugene, OR) and coverslipped. Serial sections were examined with a BX51 Fluorescence Microscope System using DP Manager imaging software (Olympus America, Melville, NY). Photographs were merged, and CXCL16 (red)– and/or CXCR6 (red)–expressing CD14+ macrophages (green) were identified by yellow fluorescence microscopy.

Real-time reverse transcription–polymerase chain reaction (RT-PCR) (TaqMan). Total RNA was extracted from joints by the procedure of Chomczynski and Sacchi (34). The final RNA was digested with DNase and with an RNase inhibitor added. Reverse transcription was performed simultaneously using Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) followed by the PCR reactions and preparation of serial dilutions of the purified PCR products (35). PCR primers and TaqMan fluorogenic probes were designed using the Primer Express program, version 1.01 (Applied Biosystems, Foster City, CA). For human CXCL16, the following primers and probe sequences were designed and used: 5′ primer, AAGCCATTGAGACACCAGCTG; 3′ primer, AC-CTCGCTCTGACTCCCAGA; and the probe, ACGT-CACGCGCCGGAGCA. TaqMan probes carry a 5′-FAM reporter dye and a 3′-TAMRA quencher dye (Mega Bases, Evanston, IL). The quantity of complementary DNA (cDNA) of the gene of interest is directly related to the fluorescence detection of FAM. The amount of cDNA was calculated using a comparative cycle threshold method and the standard curve method according to Perkin Elmer ABI Prism 7700 User Bulletin no. 2, 1997 (36). The estimated amount of the gene of interest was normalized to the amount of GAPDH.

Isolation of human MNCs and chemotaxis. PB (~100 ml) was collected from normal healthy adult volunteers to obtain MNCs, and chemotaxis was performed as previously outlined (3) using 48-well chemotaxis chambers (Neuroprobe, Cabin John, MD) with a 5-mm polyvinylpyrrolidone-free polycarbonate filter (Poretics, Livermore, CA). Results were expressed as the number of MNCs migrating per high-power field (hpf). For CXCL16 neutralization studies, SF samples were preincubated either with goat anti-human CXCL16 IgG antibody or with an equivalent amount of a corresponding control antibody (nonspecific goat IgG; Beckman Coulter) for 1 hour at 37°C. Neutralized SF samples were assayed for chemotactic activity for normal PBMCs. All chemotaxis assays included Hanks' balanced salt solution (HBSS) as the negative control and fMLP as the positive control.

RA ST fibroblast isolation and culture. ST fibroblasts isolated from patients with RA were cultured in 24-well plates (Becton Dickinson, Franklin Lakes, NJ) at 100,000/ml for 24 or 48 hours at 37°C in a 5% CO$_2$ atmosphere. RA ST fibroblasts were grown in RPMI medium (Invitrogen, Carlsbad, CA)

supplemented with 10% FBS/1% penicillin–streptomycin. For some cultures, fibroblasts were grown in media containing either TNFα (30 ng/ml; Pfizer, Kalamazoo, MI) or IL-1β (30 ng/ml; Pfizer). Supernatants were assayed for CXCL16 and control chemokines.

Cell culture, cell lysis, immunoblotting, and immuno-precipitation. Cell lysis and immunoblotting were performed as described previously (37,38). Briefly, freshly isolated human MNCs were incubated in 6-well plates for at least 12 hours (2×10^6/well) in serum-free RPMI 1640 prior to stimulation with 10 n*M* CXCL16 for various time periods. Immunoprecipitation was performed according to the protocol of Roche (Basel, Switzerland). Protein A–agarose or protein G–agarose (Roche) was washed twice with 1 ml of lysis buffer. MNC lysate (400 μg) was added to the washed protein A–agarose or protein G–agarose for 1 hour at 4°C. After centrifugation, supernatant was incubated with 7 μl of antiphosphotyrosine antibody (Cell Signaling Technology, Beverly, MA) and 50 μl of protein A–agarose or protein G–agarose overnight at 4°C. Suspensions were washed twice with lysis buffer. Thereafter, pellets were resuspended in 2× Laemmli sample buffer and boiled for 3 minutes. Supernatants were used in Western blot analysis.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Protein lysate (15–20 μg) was run on SDS-PAGE gels and transblotted to nitrocellulose membranes using a semidry transblotting apparatus (Bio-Rad, Hercules, CA). Nitrocellulose membranes were blocked with 5% nonfat milk in Tris buffered saline–Tween 20 (TBST) for 60 minutes at room temperature. Blots were incubated overnight at 4°C with optimally diluted specific primary antibody in TBST containing 5% nonfat milk. Phosphorylation state–specific antibodies to ERK-1/2 (Cell Signaling Technology), Src (BioSource International, Camarillo, CA), and Raf-1 (BioSource International) were used as primary antibodies. Blots were washed 3 times and incubated in horseradish peroxidase–conjugated antibody (1:10,000 dilutions) for 1 hour at room temperature. Protein bands were detected using enhanced chemiluminescence (GE Healthcare, Piscataway, NJ) in accordance with the manufacturer's instructions. Blots were scanned and analyzed for band intensities using UN-SCAN-ITJ version 5.1 software (Silk Scientific, Orem, UT).

Generating human ST–SCID mouse chimeras. To generate chimeras, we obtained human ST from RA patients undergoing joint replacement. SCID/NCr mice (6–8 weeks old), having a B lymphocyte and T lymphocyte defect, were anesthetized, and graft beds were prepared subcutaneously in the backs (behind the left ear) of mice. One RA ST specimen ($\sim 1 \times 1 \times 0.05$ cm) was implanted per animal, and the wound was gently sutured. Grafts were allowed to "take" and were used 4–6 weeks after transplantation. Only those animals without gross evidence of inflammation or rejection were used.

After engraftment, freshly isolated human MNCs were dye-tagged with PKH26 fluorescent dye (Sigma, St. Louis, MO). Cytospins were prepared to determine successful labeling of cells using a fluorescence microscope with a 550-nm filter. Labeled cells (5×10^6) were injected intravenously (IV) via the tail vein, and 48 hours later, animals were killed and grafts and LNs were removed and snap-frozen in liquid nitrogen. Cryosections (10-μm thick) were examined in a blinded manner for cell homing using a fluorescence microscope at 550 nm. Migrating fluorescent MNCs were quantified in grafts and LNs by counting the total number of cells per hpf in at least 3 sections per tissue. To examine homing of MNCs in response to chemotactic stimuli, we injected 1 μg recombinant human CXCL16 or 200 ng TNFα directly into implanted grafts prior to IV injection of labeled cells. This is done by gently palpating the mouse around the scruff of the neck for engrafted RA ST and carefully injecting the needle directly into the grafts. The concentrations of intragraft-injected cytokines were those previously shown to be effective in vivo (39). Control chimeras received labeled cells but no stimulus.

Transient transfection of human MNCs. Isolated human PBMCs were plated overnight in 6-well plates with serum-free RPMI 1640 medium and subsequently transfected using Lipofectin reagent (Invitrogen). Oligodeoxynucleotide (ODN) DNA (10 μM) and Lipofectin (5 μl) were incubated separately in 100 μl of serum-free medium for 30 minutes. Solutions were mixed gently, and 880 μl of medium was added. A DNA–Lipofectin mixture was added to the preincubated MNCs with an additional incubation of at least 5 hours before use for the in vitro and in vivo signaling studies. For transient transfection of human MNCs, the following sense and antisense ODNs were used with subsequent CXCL16 stimulation for in vivo migration assays: ERK-1/2 sense, 5′-ATGGCGGCGGCGGCGGC-3′ and ERK-1/2 antisense, 5′-GCCGCCGCCGCCGCCAT-3′. Transfection of ODNs peaked at 5 hours, with an efficiency routinely >80%.

Once transfected, MNCs were labeled with PKH26 dye, washed, and injected IV into RA ST–engrafted SCID mice at a concentration of 5×10^6/100 μl/mouse. We performed viability counts on cells transfected with sense and antisense ODNs for several signaling molecules, including ERK-1/2 (cell viability 82% sense, 92% antisense), Src (cell viability 98% sense, 97% antisense), and phosphatidylinositol 3-kinase (PI 3-kinase) (cell viability 97% sense, 100% antisense). Cell viabilities were evaluated with at least 100 cells counted per group. As described above, some mice received simultaneous intragraft injections of CXCL16 (1 μg/100 μl per mouse) when dye-tagged cells were administered IV.

Statistical analysis. Statistical analysis was done by Student's *t*-test. *P* values less than 0.05 were considered significant.

RESULTS

Up-regulation of CXCR6 expression in CD14+ monocytes and memory lymphocytes in RA SF. Normal or RA PB monocytes expressed virtually no CXCR6, while 50% of RA SF monocytes expressed this receptor (Figure 1A). This suggests that monocytes in RA SF might be a target for CXCL16. While only a small minority of normal or RA PB CD3+ lymphocytes expressed CXCR6, a mean of 33% of RA SF CD3+ lymphocytes expressed this receptor, as determined by 4-color flow cytometry (Figure 1B). Similar results were obtained for CD3+,CD8+,CD45RO+,CXCR6+ lym-
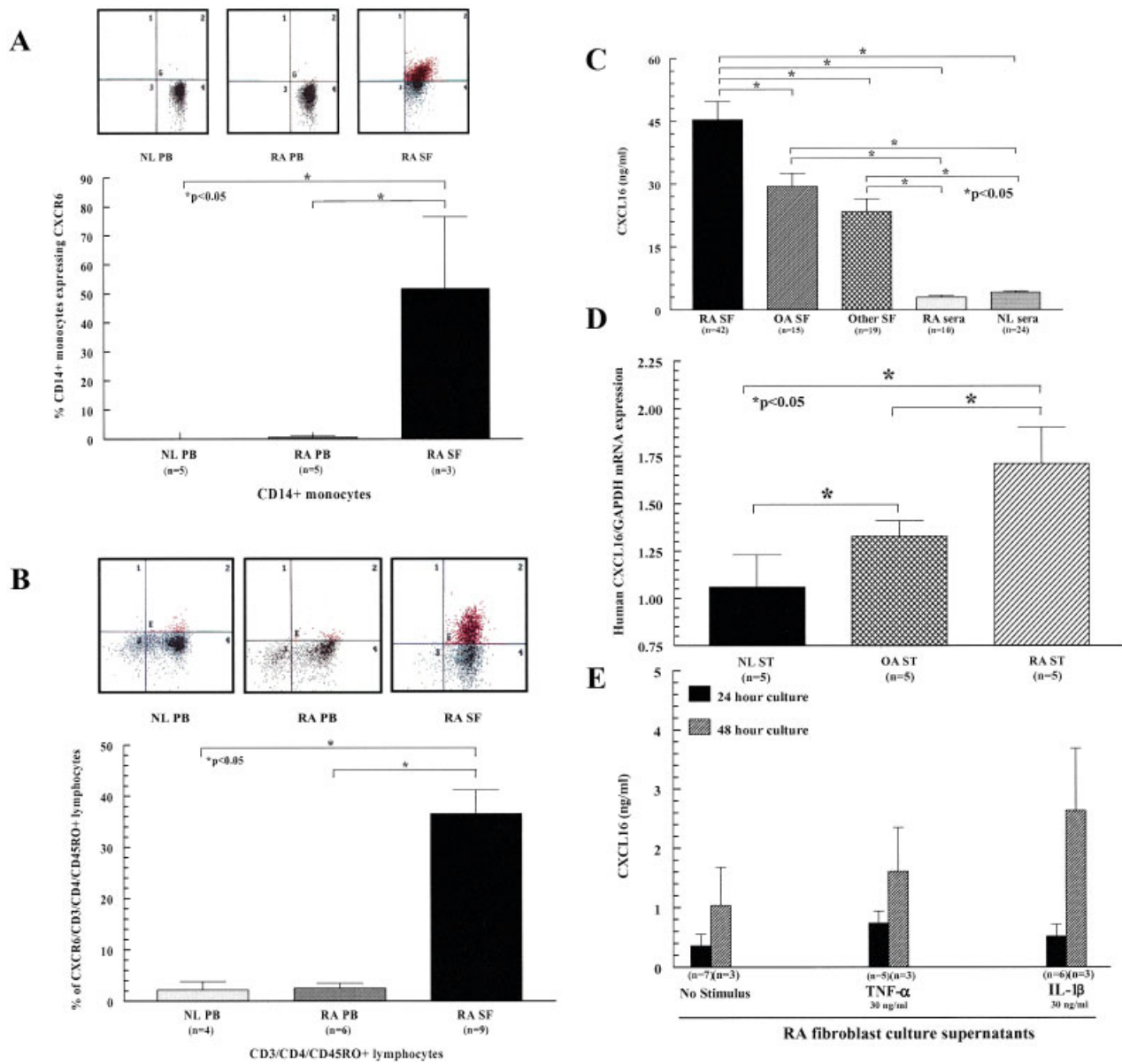
Figure 1. Flow cytometric analysis identifying the percentage of CD14+ monocytes expressing CXCR6 (red cells in dot plots) in normal (NL) peripheral blood (PB), PB from patients with rheumatoid arthritis (RA), and synovial fluid (SF) from RA patients. **A,** Representative dot plot of CD14+ monocyte CXCR6 expression with corresponding graph of results. CD14+ monocytes did not significantly express CXCR6 in normal or RA PB. However, ~50% of CD14+ monocytes from RA SF expressed CXCR6. **B,** Four-color fluorescence-activated cell sorting analysis on memory lymphocytes (with corresponding graph of results) identifying elevated percentages of memory CD3+,CD4+,CD45RO+,CXCR6+ lymphocytes in RA SF compared with normal and RA PB. Similar results were obtained for memory CD3+,CD8+,CD45RO+,CXCR6+ lymphocytes (data not shown). **C,** CXCL16 concentrations in RA SF were significantly elevated compared with those in normal PB, RA PB, SF from patients with osteoarthritis (OA), and other SF samples. **D,** Normal, OA, and RA synovial tissue (ST) CXCL16 mRNA expression was also evaluated by real-time reverse transcription–polymerase chain reaction. RA ST had elevated expression of CXCL16 mRNA, indicating that factors in the RA joint support CXCL16 expression at the level of transcription. **E,** Fibroblasts are one source of CXCL16 in RA. RA fibroblasts produced CXCL16 after culture for 24 or 48 hours and did not respond significantly to exogenous cytokine (tumor necrosis factor α [TNFα] or interleukin-1β [IL-1β]) stimulation at either time point. Values are the mean and SEM (n = number of patient samples). Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.
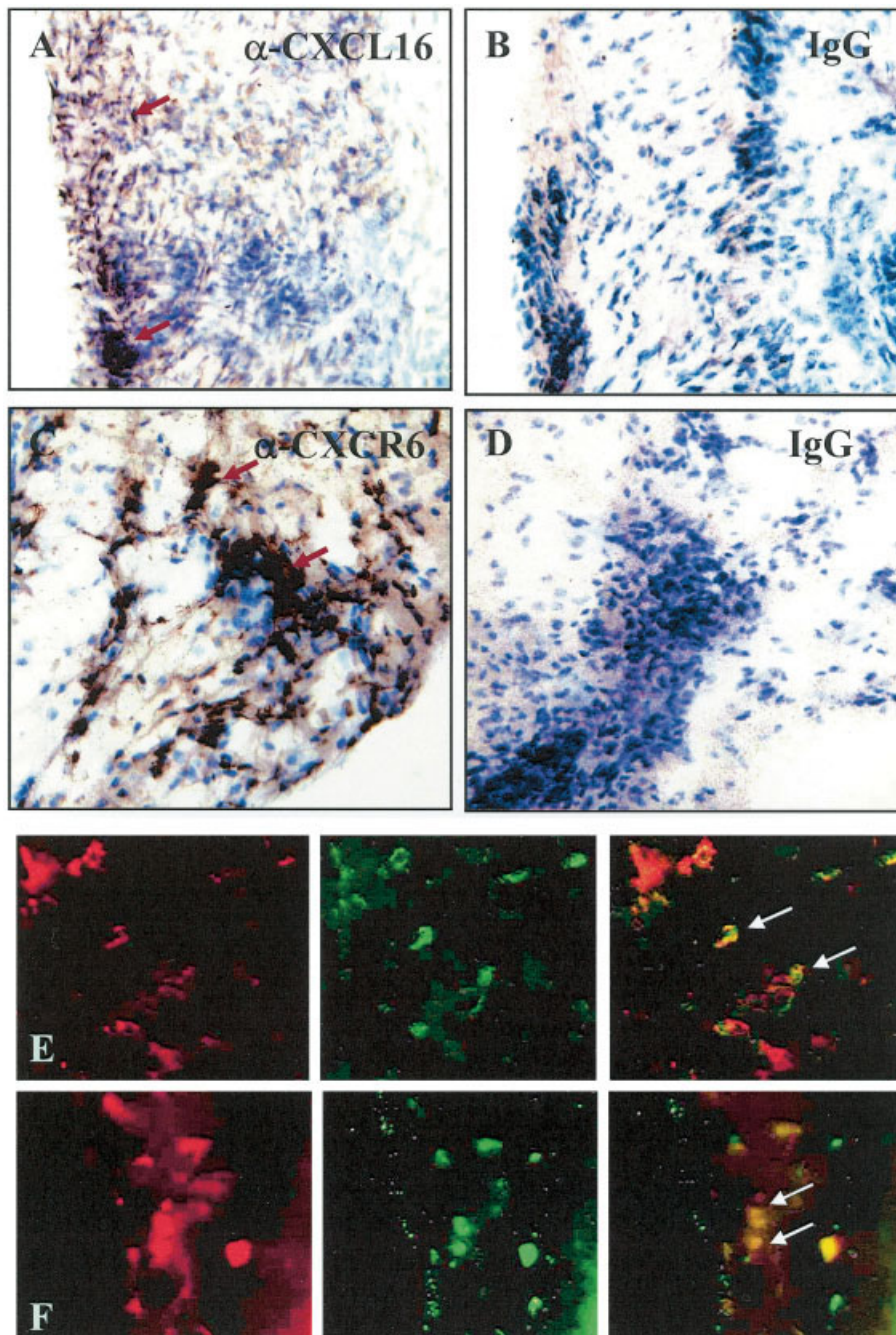
Figure 2. A and **C**, Immunostaining of RA ST for CXCL16 and CXCR6, respectively. **B** and **D,** Staining with the IgG control antibody. Macrophages stained positive for both CXCL16 and CXCR6 in RA ST (**arrows** in **A** and **C**). **E** and **F,** Phycoerythrin-tagged rat anti-human CD14 antibody was added to detect CD14+ macrophages. Photographs were merged, and CXCL16-expressing (red in **E**) and/or CXCR6-expressing (red in **F**) CD14+ macrophages (green) were identified by yellow fluorescence microscopy (**arrows**). RA ST CD14+ macrophages expressed both CXCL16 and CXCR6. Table 1 summarizes the various cell types staining positive for CXCL16 and CXCR6 in RA, OA, and normal ST. α-CXCL16 = antibody to CXCL16; α-CXCR6 = antibody to CXCR6 (see Figure 1 for other definitions). (Original magnification × 200 in **A–D**; × 400 in **E** and **F.**)

phocytes (data not shown). This indicates that CXCR6 receptor expression is on both monocytes and T lymphocytes in the RA joint.

Elevated CXCL16 concentration in RA. We found that the CXCL16 concentration in RA SF was significantly elevated compared with that in OA and

Table 1. Summary of immunostaining shown in Figure 2*

	CXCL16+ cells, %		CXCR6+ cells, %	
	Lining cells	Macrophages	Lining cells	Macrophages
Normal ST†	29.9 ± 8.2	64.3 ± 6.2	9.4 ± 2.7	41.7 ± 4.9
OA ST†	20.6 ± 7.2	41.0 ± 8.6‡	7.2 ± 2.9	49.6 ± 4.5
RA ST†	78.3 ± 6.9§	77.5 ± 3.0	38.1 ± 6.6§	72.6 ± 4.6§

* Values are the mean ± SEM. ST = synovial tissue; OA = osteoarthritis; RA = rheumatoid arthritis.
† CXCL16+ cells were obtained from 4 healthy normal volunteers, 5 OA patients, and 4 RA patients. CXCR6+ cells were obtained from 5 healthy normal volunteers, 5 OA patients, and 7 RA patients.
‡ $P < 0.05$ versus normal and RA ST.
§ $P < 0.05$ versus normal and OA ST.

other SF samples ($P < 0.05$) and 8 times greater than that in sera from normal healthy people (Figure 1C). These results point toward a significant function of CXCL16 in the inflammatory environment of the RA joint.

We also measured CXCL16 messenger RNA (mRNA) expression in RA ST by real-time RT-PCR. Normal ST as well as ST obtained from patients with OA and RA was snap-frozen and prepared for mRNA analysis. Samples were reverse-transcribed into cDNA and examined for CXCL16 mRNA by real-time RT-PCR. RA ST contained elevated levels of CXCL16 mRNA compared with normal and OA ST, confirming that CXCL16 was up-regulated in RA joint inflammation (Figure 1D). We identified one source of CXCL16 expression by culturing RA fibroblasts for 48 hours with and without cytokine stimulation. Figure 1E shows that unstimulated RA fibroblasts produced significant amounts of CXCL16 (up to 0.5 ng/ml at 24 hours and 1 ng/ml at 48 hours), which were not significantly up-regulated with the addition of TNFα or IL-1β. This is contrary to our previous findings with fractalkine and MIP-3α, since we showed that RA ST fibroblasts could be stimulated to produce significantly elevated levels of these chemokines when stimulated with TNFα (fractalkine and MIP-3α) or IL-1β (MIP-3α) (3,5).

Expression of CXCL16 and CXCR6 in RA ST by immunohistology. As shown in Figure 2, CXCL16 and CXCR6 were immunolocalized in ST obtained from patients with active RA. Figure 2A shows representative immunostaining for CXCL16 in macrophages, while this was not observed in RA ST stained with the IgG control antibody (Figure 2B). Figure 2C shows RA ST stained with antibody to CXCR6. CXCL16 and CXCR6 were also immunolocalized primarily on macrophages in RA ST, while RA ST stained with control IgG antibody did not show significant reactivity (Figure 2D).

For 2-color immunofluorescence histology, serial ST sections were cut and stained either with mouse anti-human CXCR6 antibody or with goat anti-human CXCL16. Alexa 488–labeled donkey anti-mouse or donkey anti-goat antibody was also added as described in Patients and Methods. PE-tagged rat anti-human CD14 antibody was added to detect CD14+ macrophages. Photographs were merged, and CXCL16 (red)– and/or CXCR6 (red)–expressing CD14+ macrophages (green) were identified by yellow fluorescence microscopy (Figures 2E and F, respectively). As shown, RA ST CD14+ macrophages expressed both CXCL16 and CXCR6.

Table 1 summarizes the immunostaining shown in Figure 2. Although CXCL16 and CXCR6 were expressed in normal, OA, and RA ST, we observed significantly elevated macrophage staining for CXCL16
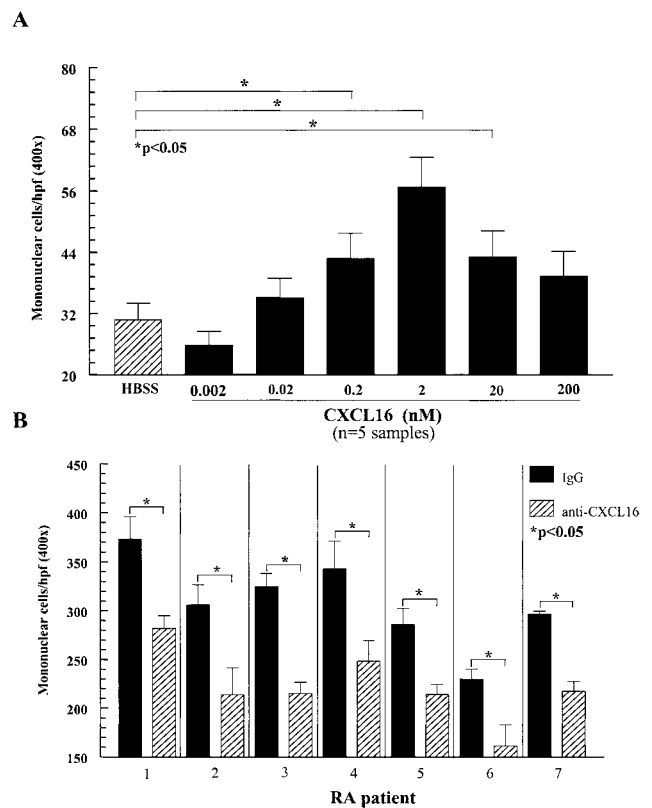
Figure 3. A, CXCL16 induces chemotaxis of human mononuclear cells in a dose-dependent manner and is maximal between concentrations of 2 nM and 20 nM. **B,** Mononuclear cell migration was also measured in vitro in a chemotaxis assay using RA SF as a chemotactic agent after incubation with a neutralizing anti-CXCL16 antibody. Goat IgG served as a negative control (sham depletion). CXCL16 depletion resulted in an ~30% reduction in RA SF–induced mononuclear cell migration. Note the similar reductions in mononuclear cell chemotaxis in every sample tested. Values are the mean and SEM (n = number of cell isolations from different blood donors). hpf = high-power field; HBSS = Hanks' balanced salt solution (see Figure 1 for other definitions).
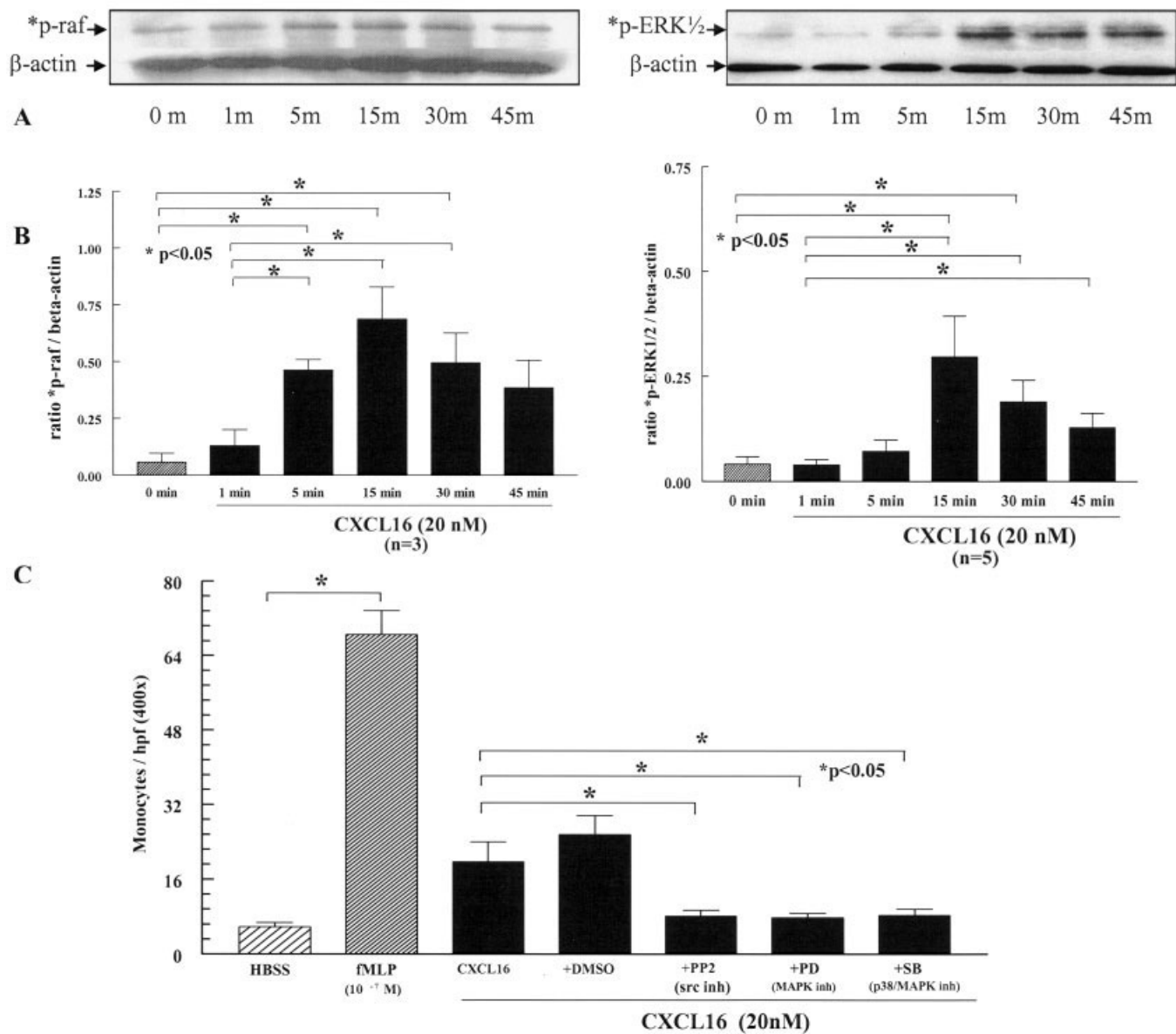
Figure 4. CXCL16 activates Raf and ERK-1/2 in a time-dependent manner. Monocytes (2.5×10^6) were stimulated with 20 nM recombinant human CXCL16. Cell lysates were probed for p-Raf and p–ERK-1/2 by Western blotting, showing a marked increase in phosphorylation after 5 and 15 minutes, respectively. **A,** Representative blots for both p-Raf and p–ERK-1/2. **B,** Graphs corresponding to blots in **A**. Values are the mean and SEM (n = number of blood donors). **C,** Chemotaxis for human monocytes was also measured in the presence of signaling pathway inhibitors (inh). Values are the mean and SEM (n = 4–7 donors per group). hpf = high-power field; HBSS = Hanks' balanced salt solution; PP2 = a Src inhibitor; PD = PD98059 (a MAPK inhibitor); SB = SB203508 (a p38 MAPK inhibitor).

in RA ST compared with OA ST, and we observed elevated macrophage staining for CXCR6 in RA ST compared with both normal and OA ST. RA ST lining cells, containing macrophages and fibroblasts, expressed significantly more CXCL16 and CXCR6 than did normal or OA ST.

Induction of MNC migration by CXCL16. Normal human PBMCs were assayed for their chemotactic response to CXCL16 in a modified Boyden chamber to determine a proinflammatory role for CXCL16. HBSS and fMLP served as negative and positive controls, respectively. We found a dose-dependent migration of

human MNCs to CXCL16, with a peak between 2 nM (20 ng/ml) and 20 nM (200 ng/ml) (Figure 3A). These levels were consistent with those found in RA SF (mean $\pm$ SEM 44.4 $\pm$ 4.4 ng/ml). To determine the participation of CXCL16 in MNC migration in RA, RA SF samples containing average levels of CXCL16 (45 ng/ml) were incubated with anti-human CXCL16 antibody. SF samples were then tested in an in vitro migration assay and compared with sham-depleted controls. RA SF samples showed a 28% decrease ($P < 0.05$) in MNC migratory activity after incubation with neutralizing antibody to CXCL16 (Figure 3B).

Activation of Raf and ERK-1/2 in monocytes by CXCL16. To determine the signaling pathways mediating CXCL16 function, isolated normal human monocytes were cultured overnight and stimulated with CXCL16 for various time periods. Cell lysates were prepared and probed with anti–phospho–ERK-1/2 and anti–phospho-Raf by Western blotting. The blots were stripped and reprobed with β-actin to confirm equal loading of the samples. Monocyte stimulation with CXCL16 induced a marked increase in an early phosphorylation of Raf at 5 minutes and of ERK-1/2 at 15 minutes (Figure 4A). The graphs of 3 experiments with Raf and 5 experiments with ERK-1/2 are also shown (Figure 4B). These results were consistent with MAPK signaling, since Raf is upstream of ERK-1/2, and they indicated that CXCL16 may induce some of its proinflammatory properties through this pathway.

To further analyze the relevance of the MAPK pathway for monocyte recruitment, migration assays were performed with 20 nM CXCL16 in the presence of various signaling pathway inhibitors at a final concentration of 10 μM (40). As shown in Figure 4C, monocyte migration was significantly blocked by PP2 (an inhibitor of Src), by PD98059 (a MAPK inhibitor), and by SB203508 (a p38 MAPK inhibitor), suggesting participation of Src and MAPK pathways for monocyte chemotaxis toward CXCL16 in vitro.

Recruitment by CXCL16 of MNCs in vivo to engrafted human ST in a SCID mouse chimera. To test MNC migration in vivo, we used a human ST–SCID mouse chimera. After 4–6 weeks, animals engrafted with human RA ST were killed. Well-engrafted tissues with no signs of rejection were observed and were used at this time point (Figure 5A). To determine homing of normal human MNCs, freshly isolated cells were dye-tagged with PKH26, and 5 $\times$ 10^6 cells/100 μl/mouse were injected IV via the tail vein 48 hours before mice were killed. Cryosections (10 μm) of the RA ST grafts were examined using a fluorescence microscope with a
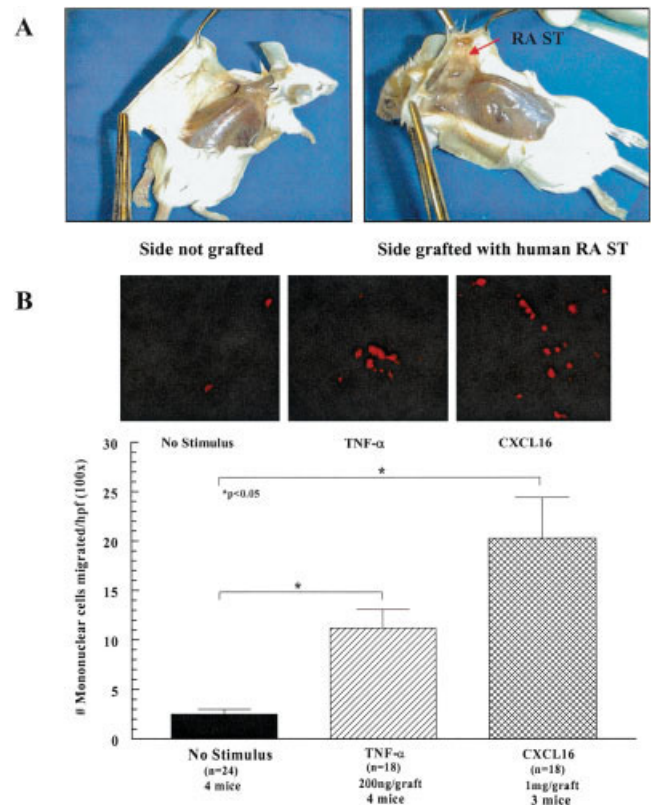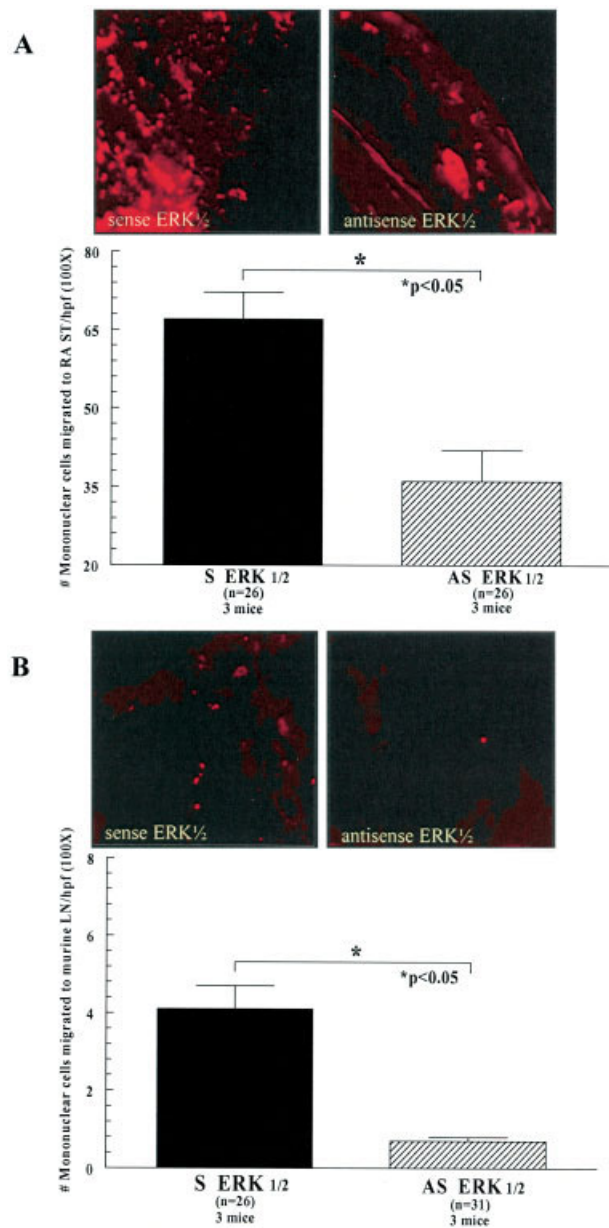

Figure 5. A, A SCID mouse grafted with RA ST. Left, An area where the RA ST graft was not inserted. Right, Side of the mouse where the RA ST graft was inserted, clearly showing a viable, growing RA ST graft (**arrow**). The photograph was taken 5 weeks after engraftment. **B,** PKH26 red fluorescent dye–tagged human PB mononuclear cells (5 $\times$ 10^6) were injected intravenously into SCID mice engrafted for 6–8 weeks with human RA ST. Before administering cells, ST grafts were injected with TNFα (200 ng/graft) or CXCL16 (1,000 ng/graft) or were sham injected (no stimulus). After 48 hours, grafts were harvested and tissue sections were examined by immunofluorescence microscopy at 550 nm. Top, PKH26 dye–tagged mononuclear cells (original magnification $\times$ 100). Bottom, Numbers of dye-tagged cells migrating to engrafted RA ST in response to the various stimuli. Cell migration was quantified by dividing the number of cells per high-power field (hpf). Values are the mean and SEM (n = number of tissue sections counted). See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.

550-nm filter. Injection of CXCL16 or TNFα (positive control) into the engrafted RA ST prior to adoptive cell transfer resulted in a significant increase of MNC migration (39) (Figure 5B). To ensure that cell recruitment was induced by CXCL16 and not by contaminants, only additive-free and sterile-filtered CXCL16 was used. In addition, the soluble CXCL16 used in all experiments contained <0.1 ng/μg (1 endotoxin unit/μg) endotoxin. These results indicate that exogenously administered CXCL16 is chemotactic for human MNCs in vivo.

Figure 6. Intravenous administration of fluorescent dye–tagged human mononuclear cells to SCID mice engrafted with human RA ST. **A,** Fluorescent human mononuclear cells lipofected with sense (S) oligodeoxynucleotides (ODNs) to ERK-1/2 migrated readily to engrafted human RA ST in response to an intragraft injection of human CXCL16, whereas those cells lipofected with antisense (AS) ODNs to ERK-1/2 showed reduced migratory activity. **B,** Fluorescent human mononuclear cells lipofected with sense ODNs to ERK-1/2 migrated readily to murine lymph nodes (LNs) in response to an intragraft injection of human CXCL16, whereas those cells lipofected with antisense ODNs to ERK-1/2 showed reduced migratory activity. Values in **A** and **B** are the mean and SEM (n = number of tissue sections counted from 3 mice per group). **C,** Mononuclear cells transfected with antisense ODNs to ERK-1/2, but not nontransfected (NT) or sense-transfected mononuclear cells, showed significant inhibition of p–ERK-1/2 expression. **D,** A significant percentage of mononuclear cell inhibition of ERK-1/2 expression using antisense ODNs to ERK-1/2 is shown compared with sense-transfected mononuclear cells. Values are the mean and SEM. The experiment was repeated 3 times. β-actin served as the loading control. hpf = high-power field (see Figure 1 for other definitions). (Original magnification $\times$ 100.) Color figure can be viewed in the online issue, which is available at http://www.arthritisrheum.org.

MNC chemotaxis to exogenously administered intragraft CXCL16 is reduced by 50% to RA ST, and 5-fold to murine LNs, in cells transfected with antisense ODNs for ERK-1/2. We further examined the participation of CXCL16 in the recruitment of MNCs in vivo by inhibiting the signaling pathways it utilizes. To do this, we transfected MNCs with sense or antisense ODNs for ERK-1/2, and then we labeled the transfected cells with PKH26 fluorescent dye. Before dye-tagging the cells, we performed viability counts in order to make certain that
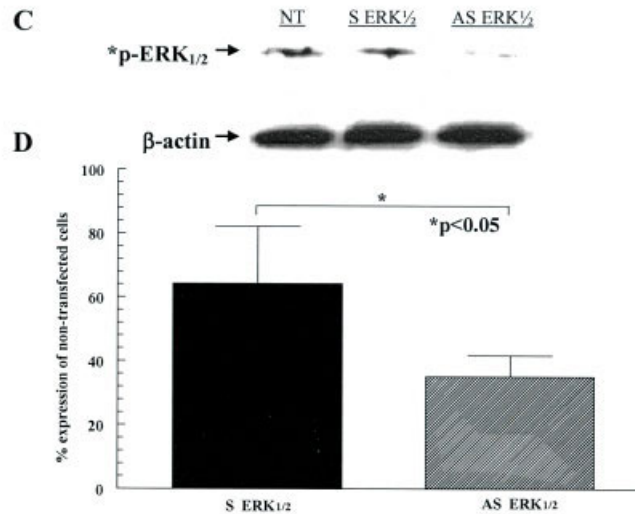
cells did not experience an increased rate of cell death. Transfected dye-tagged cells were injected into tail veins of SCID mice bearing RA ST receiving simultaneous intragraft injections of CXCL16. Engrafted RA ST and murine inguinal LNs were removed 48 hours later. Transfection with antisense ODNs resulted in significant reductions of MNC migration compared with sense-treated controls (Figures 6A and B). The attenuated migration to LNs was especially impressive considering the elevated mean $\pm$ SEM concentrations of soluble

human CXCL16 in the serum of mice receiving intra-graft injections of CXCL16 (CXCL16-injected mice 1.91 ± 0.23 ng/ml, n = 7 mice; noninjected mice 0.84 ± 0.21 ng/ml, n = 6 mice) ($P < 0.05$) (data not shown).

To ensure that ERK-1/2 expression was inhibited by antisense ODNs, we performed Western blot analysis for ERK-1/2 expression in MNCs as described in Patients and Methods. As shown in Figure 6C, MNCs transfected with antisense ODNs to ERK-1/2, but not nontransfected or sense-transfected MNCs, showed significant inhibition of ERK-1/2 expression. These data, graphed in Figure 6D, show a significant percentage of MNC inhibition of ERK-1/2 expression using antisense ODNs to ERK-1/2 compared with sense-transfected MNCs. These results show that targeting CXCL16-mediated signaling pathways influences MNC migration to RA ST and regional LNs in vivo.

DISCUSSION

Substantial progress in characterizing chemokines and chemokine receptor function in vitro has led to better understanding of cell migration (3,5,11,32), but the limitations of the in vitro environment make it difficult to interpret how chemokines and corresponding receptors function in vivo. Limitations of in vitro migration assays include evaluating the role of a particular chemokine as it relates to other chemokines and chemokine receptors, and ranking the importance of chemokines and chemokine receptors in a particular disease. Indeed, many investigators have used a similar SCID mouse chimera to study the effects of gene therapy and RA ST fibroblast infiltration in vivo (41–43). Therefore, to evaluate the proinflammatory function of chemokines in RA, we developed an in vivo cell migration assay to determine the relative importance of chemokines and their respective cellular receptors in a human RA ST–SCID mouse chimera.

Manipulation of chemokines has shown promise for RA therapy. We previously showed that ENA-78, a CXC chemokine, is up-regulated in RA SF as well as in a rodent model of arthritis (22,44). Immunodepletion of human ENA-78 resulted in decreased ability to chemoattract polymorphonuclear cells in vitro (22). Furthermore, anti–ENA-78 given systemically ameliorated signs and symptoms of rat AIA (44). Inhibition of CC chemokines also reduced joint inflammation. For example, in vivo inhibition of the CC chemokine MIP-1α by IL-10 delayed the onset and reduced the severity of collagen-induced arthritis (CIA) in mice (45). A neutralizing antibody to MCP-1, another CC chemokine, sig-

nificantly reduced ankle swelling in a rat model of RA (46). Chemokine antagonists have similarly shown promise. For instance, a 67–amino acid sequence of MCP-1 that specifically antagonizes MCP-1 abrogated arthritis in MRL-*lpr* autoimmune mice, and an antagonist of RANTES ameliorated CIA in mice (47–49). IL-1 receptor antagonist protein, a naturally produced specific antagonist of IL-1β, has consistently shown potent antiinflammatory properties in animal models of inflammation (50–52) and is used in the treatment of RA (53–58). CXCL16 is a novel chemokine that has not been fully characterized in chronic disease, and it is especially of interest because its only known receptor to date has been shown to be highly expressed in chronic Th1 diseases such as RA (28).

Initial reports on CXCL16 in mice and humans showed predominant gene expression in spleen, LNs, and Peyer's patches, with little or no expression in other organs (24). Immunohistologic analysis revealed that macrophages in RA ST stained intensely for both CXCL16 and CXCR6. This staining is consistent with a recent report that CXCL16 is produced by macrophages (59). This was also confirmed by real-time RT-PCR analysis showing elevated CXCL16 mRNA in RA ST compared with OA and normal ST. CXCL16 was also strongly expressed on a subset of PB leukocytes, including CD14+ monocytes and macrophages, from which functional CXCL16 has been shown to be shed (25). These findings are completely consistent with FACS results indicating that ~40% of CD14+ monocytes and 33% of memory CD4+ and CD8+ lymphocytes express CXCR6 in RA SF. The FACS results suggest that both monocytes and lymphocytes may use the CXCR6 receptor to migrate from the normal PB to the joints in response to CXCL16 expression.

In combination with expression of its receptor Bonzo/CXCR6 on T cell subsets, these results suggest a unique role for CXCL16 in chronic inflammation. Recent studies showing expression of both CXCL16 and its receptor on T lymphocytes, akin to IL-2 and its receptor, point to a possible mechanism of autocrine stimulation (60). Our LN data support this role for CXCL16 as a possible T lymphocyte regulatory factor. We observed elevated serum levels of CXCL16 when CXCL16 was injected intragraft into SCID mice (data not shown), explaining in part the impressive recruitment of MNCs to murine LNs.

RA patient data show that CXCL16 is highly expressed in RA SF, is a potent chemoattractant for MNCs in vitro, and is chemotactic for PBMCs to RA ST in vivo. These findings suggest that inhibition of CXCR6

expression or CXCL16 signaling pathways may substantially halt migration of inflammatory cells to RA ST. Our hypothesis was formulated on the basis of strong preliminary data suggesting that CXCL16 is abundant in chronic inflammation and may be associated with type 1 inflammatory responses (28). We have reason to believe that although CXCL16 may play a dominant role in RA, it may very well be important in other chronic diseases such as OA, since we observed elevated mRNA expression of CXCL16 in OA compared with normal ST, and elevated soluble CXCL16 in OA SF compared with normal serum. Chandrasekar et al (61) demonstrated that CXCL16 signals via Gi, PI 3-kinase, Akt, IκB kinase, and NF-κB in aortic smooth muscle cells, pointing to specific signaling pathways after CXCL16–CXCR6 binding. We found that stimulation of monocytes with CXCL16 induced dose-dependent increases in phosphorylation of the signaling molecules Raf and ERK-1/2. This correlates with in vitro MNC migration assays. For example, in the presence of ERK-1/2 signaling pathway inhibitors, CXCL16-induced MNC migration was significantly blocked. Interestingly, FACS analysis showed that normal PB CD14+ monocytes had little or no expression of CXCR6.

These findings may indicate that other yet-unknown receptors may also bind and respond to CXCL16. Indeed, our observation of robust in vitro recruitment of PB monocytes toward CXCL16 may be the result of monocytes using an alternative receptor for CXCL16. Also, it is possible that in the inflammatory milieu of the RA SF, SF monocytes predominantly use the CXCR6 receptor while inactivated PB monocytes use another receptor. Our data cannot completely rule out this possibility. We recently observed a similar phenomenon with MIP-3α, finding that PB monocytes do not express the only known receptor for MIP-3α, namely, CCR6, but that they readily migrate dose dependently toward MIP-3α in vitro (5).

We tested the capacity of CXCL16 to recruit MNCs in vivo using the human RA ST–SCID mouse chimera. This model has been used successfully by other groups to show that engrafted RA ST not only keeps many of the morphologic and pathologic characteristics of RA ST (62), but also can be used as an appropriate model of RA (63). We found that tail vein–injected cells migrate to engrafted RA ST and murine LNs in response to intragraft injections of human CXCL16. We examined possible signaling mechanisms underlying this response. We found that preincubation of MNCs with antisense ODNs against ERK-1/2 resulted in a pronounced decline in cell recruitment to engrafted RA ST

and inguinal LNs in CXCL16-injected SCID mice. Although other signaling pathways may certainly be involved, we report here that inhibition of a chemokine-induced signaling cascade can temper the inflammatory infiltrate in vivo.

These findings have twofold importance. First, we observed an increase in MNC migration in response to exogenously added chemokine. Second, the primary function of this chemokine could be reversed in vivo by inhibiting its signaling cascade. CXCL16 may have chemotactic potency apart from other chemokines shown to be expressed in RA (1–3,5,6,14,16,21–23,44,64). This is supported by the finding that CXCL16 may function independently of TNFα and IL-1β, at least in vitro. This was initially unexpected, considering the potent proinflammatory activity of these cytokines in RA (8). We found that cultured RA ST fibroblasts constitutively produce large amounts of CXCL16 (~1 ng/ml), explaining in part the highly elevated levels of CXCL16 in RA SF. Previous reports show that TNFα further stimulates RA synovial fibroblasts to release a variety of chemokines, suggesting that cell recruitment is largely due to TNFα, and implicating TNFα as an ultimate downstream eliciting agent (3,5).

Interestingly, we found that incubation of RA ST fibroblasts with either TNFα or IL-1β failed to further increase CXCL16 expression, contrary to previous findings with fractalkine and MIP-3α (3,5). This is especially important when considering the amounts of CXCL16 found in patient samples, reaching levels as high as 145 ng/ml in some cases. It suggests that large amounts of CXCL16 may be produced in RA independently of established proinflammatory cytokines. This finding is consistent with results of a recent study showing that RA fibroblasts express CXCL16 directly when stimulated by way of the Toll-like receptor 2 ligand peptidoglycan (65).

In conclusion, we evaluated the expression and function of CXCL16 and its receptor CXCR6 in RA. We found both the ligand and receptor to be very highly expressed in RA, and we observed that CXCL16 functions to aggressively recruit MNCs in vivo via the Raf and ERK-1/2 kinase pathways. These results point to CXCL16 as a novel, potent MNC recruiter in RA inflammation.

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