

Expression and Function of CXCL16 in a Novel Model of Gout

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Objective. To better define the activity of soluble CXCL16 in the recruitment of polymorphonuclear neutrophils (PMNs) *in vivo*, utilizing a novel animal model of gout involving engraftment of SCID mice with normal human synovial tissue (ST) injected intragraft with gouty human synovial fluid (SF).

Methods. For *in vitro* studies, a modified Boyden chemotaxis system was used to identify CXCL16 as an active recruitment factor for PMNs in gouty SF. Migration of PMNs could be reduced by neutralization of CXCL16 activity in gouty SF. For *in vivo* analyses, fluorescent dye-tagged PMNs were injected intravenously into SCID mice while, simultaneously, diluted gouty SF containing CXCL16, or depleted of CXCL16 by antibody blocking, was administered intragraft. In addition, the receptor for CXCL16, CXCR6, was inhibited by incubating PMNs with a neutralizing anti-CXCR6 antibody prior to injection into the mouse chimeras. Recruitment of PMNs to the gouty SF-injected normal human ST was then examined in this SCID mouse chimera system.

Results. CXCL16 concentrations were highly elevated in gouty SF, and PMNs were observed to migrate in response to CXCL16 *in vitro*. Normal human ST-SCID mouse chimeras injected intragraft with gouty SF that had been depleted of CXCL16 during PMN transfer showed a significant reduction of 50% in PMN recruit-

ment to engrafted tissue as compared with that after administration of sham-depleted gouty SF. Similar findings were achieved when PMNs were incubated with a neutralizing anti-CXCR6 antibody before injection into chimeras.

Conclusion. Overall, the results of this study outline the effectiveness of the human-SCID mouse chimera system as a viable animal model of gout, serving to identify the primary function of CXCL16 as a significant mediator of *in vivo* recruitment of PMNs to gouty SF.

Gout develops as a result of synovial tissue (ST) deposition of monosodium urate monohydrate (MSU) crystals from supersaturated extracellular fluid. Urate crystal deposition in the joints causes acute inflammatory responses, which are set off by the negatively charged crystal surfaces that activate complement (e.g., C5a, C3a), leading to the release of proinflammatory cytokines and chemokines. Interleukin-8 (IL-8; CXCL8), for example, is a chemokine and polymorphonuclear neutrophil (PMN) recruitment factor that is associated with gout (1,2). PMNs respond to CXCL8 via its corresponding receptors, CXCR1 and CXCR2 (3). In addition, in an experimental rabbit model of acute gouty arthritis, studies of the knee joints of rabbits deficient in membrane attack complex (MAC) component C6 revealed that MAC activation appears to play a major role in intraarticular CXCL8 generation and neutrophil recruitment (4).

Inhibition of CXCL8 has been shown to be a principal factor of PMN recruitment in animal models of gout. For example, Nishimura et al showed that intraarticular injection of a neutralizing anti-CXCL8 antibody significantly attenuated crystal-induced joint swelling and neutrophil infiltration in the rabbit knee (5). The overall process is thought to involve MSU crystal deposition that induces CXCL8 secretion and subsequent recruitment of PMNs to the inflamed joints. In addition, multiple CXCR2 ligands may exist in gouty synovial

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fluid (SF) that could account for some of the chemotactic activities beyond those of CXCL8; for example, growth-related oncogene α /CXCL1 exhibited robust neutrophil recruitment activity in a murine subcutaneous air pouch model of urate crystal-induced inflammation (3). Thus, chemokines and their cellular receptors, especially CXCR2, have generated great interest as a foundation for intervention strategies to reduce inflammation.

Although therapeutic approaches to alter or reduce the activity of enzymes required for uric acid formation in gout are currently being improved (6–14), alternative strategies that target the chemokines or other growth factors involved in PMN recruitment may also show clinical potential. Because of the association of MSU crystal formation with PMN recruitment and the observed release of chemokines such as CXCL8 in gout, there continues to be enormous interest in therapies that block not only CXCL8 but also other chemokines that may have proinflammatory activity in gout and many other inflammatory disorders (1,2,15–20).

In the present study, we initially focused on exploring new avenues of cytokine expression in gout, with a principal spotlight on the chemokines involved in the recruitment of mononuclear cells and/or PMNs. To address this, we examined the expression of several CC and CXC chemokines in gouty SF from human patients (16,21). We observed that, in addition to CXCL8, the chemokines expressed in gouty SF were interferon- γ -inducible 10-kd protein/CXCL10, monocyte chemoattractant protein 1/CCL2, and CXCL16. Because of its unusually elevated concentrations in gouty SF, CXCL16 was set apart from the other chemokines for further analysis.

To determine whether inhibition of CXCL16 could influence the migration of PMNs, samples of gouty SF incubated with neutralizing anti-CXCL16 antibodies, as well as sham-depleted gouty SF samples as controls, were tested *in vitro* using a Boyden chemotaxis chamber system. We also utilized a novel model of crystal-induced arthritis that facilitated evaluation of the effects of CXCL16 inhibition on migration of human PMNs from the peripheral blood to engrafted human ST. In this model, we injected gouty SF directly into normal human ST that had been engrafted into SCID mice, followed by intravenous (IV) injections of fluorescent dye-tagged PMNs (17). “Humanizing” the mice in this way facilitated our analysis of actual gouty SF and also provided clinical relevance, because it allowed us to use human ST to quantify PMN recruitment in an *in vivo* setting. The extent of PMN migration to engrafted

normal human ST in response to injected gouty SF was evaluated and linked to the CXCL16 concentrations in gouty SF.

MATERIALS AND METHODS

Collection of human SF and ST samples. For the present study, we obtained SF from patients with gout, using samples that are normally discarded from such patients during therapeutic arthrocentesis, a procedure that was unrelated to the current research. Prior to undergoing this surgery, patients were asked whether they were willing to contribute SF to the study. The presence of gout in the SF was confirmed by crystal detection. Specimens of gouty SF were aliquoted and stored at -80°C until used. All specimens were obtained with Institutional Review Board approval.

For samples of human ST, the Cooperative Human Tissue Network and National Disease Research Interchange were utilized to obtain cadaveric normal human ST specimens. Normal human ST was obtained primarily from the knees of donors, and all samples were processed within 24 hours of the donor's death. The ST samples were screened, and the tissue was considered normal if the donors were not previously diagnosed as having a rheumatic disease, such as rheumatoid arthritis (RA) or osteoarthritis. Under sterile conditions, the normal human ST was isolated from surrounding tissue, cut into 0.5-cm^3 segments, and screened for pathogens before being implanted into SCID mice. All tissue samples were stored frozen at -80°C in a freezing medium (containing 80% heat-inactivated fetal bovine serum with 20% [volume/volume] dimethyl sulfoxide). Prior to insertion into the mice, the frozen samples were thawed and washed 3 times with phosphate buffered saline (PBS). The rate of recovery of tissue using this method is 100%. All specimens of human ST were obtained with Institutional Review Board approval.

SCID mice. SCID/NCr mice were purchased from the National Cancer Institute. All mice were maintained in a pathogen-free animal facility and given food and water *ad libitum*.

Enzyme-linked immunosorbent assay (ELISA) technique. ELISAs were performed in a manner as described previously (20) to assess gouty SF for the presence of chemokines not available on the Luminex panels. Briefly, levels of IL-1 β , CXCL8, and CXCL16 were measured by coating 96-well polystyrene plates with anti-human chemokine antibodies (R&D Systems) followed by a blocking step. All samples were added in triplicate, with recombinant human chemokine (R&D Systems) used as standard. Subsequently, biotinylated anti-human antibody and streptavidin-peroxidase were added, and the concentrations in each sample were measured at 450 nm after developing the reaction with tetramethylbenzidine substrate. The correlation coefficient for detection of each chemokine in the ELISAs was ~ 0.99 , with a sensitivity (usually at a concentration of ~ 15 pg/ml) that was well below the average concentrations of IL-1 β , CXCL8, or CXCL16 in gouty SF.

Luminex assay. Luminex is a technique that combines flow cytometry analysis and ELISA. Luminex was performed with kits from BioSource International and used in accordance with the manufacturer's instructions. The accuracy of the

Luminex data has been confirmed. It should be noted that we did not use the plateau part of the concentration curve; rather, we used the data in the middle of the curve, with a linear correlation coefficient of 0.99. Similar results were obtained by ELISA. A panel of cytokines was measured by Luminex assay in the gouty SF, including measurements of fractalkine (CX3CL1), granulocyte colony-stimulating factor (G-CSF), IL-17, CXCL10, CCL2, macrophage inflammatory protein 1 α (MIP-1 α /CCL3), MIP-1 β /CCL4, tumor necrosis factor α (TNF α), and vascular endothelial growth factor (VEGF).

SF neutralization studies. For neutralization of CXCL16, diluted SF (1:300 with PBS) was preincubated with a neutralizing polyclonal goat anti-human CXCL16 antibody (catalog no. AF976; R&D Systems) at a concentration of 135 ng/100 μ l diluted SF (from undiluted gouty SF samples containing ~25 ng/ml CXCL16). Control (sham-depleted) gouty SF samples were incubated in a similar manner but with a corresponding control nonspecific antibody (goat IgG; R&D Systems), as recommended by the vendor and previously described in experiments with RA SF (20). Similarly, for neutralization of human CXCL10, a goat anti-human CXCL10 antibody was used (catalog no. AF-266-NA; R&D Systems), and the control antibody was also goat IgG. Only those SF samples expressing average concentrations of the chemokines (typically, ~25 ng/ml for CXCL16 and ~304 pg/ml for CXCL10) were used. PMNs were then evaluated in chemotaxis assays *in vitro* and in PMN migration studies *in vivo*, as described below.

PMN isolation and fluorescent dye incorporation. Human PMNs were isolated from the peripheral blood (~100 ml) of normal healthy adult volunteers, and the cells were applied to Ficoll gradients in a manner as described previously (22). Both the viability and the purity of the cells were routinely >98%. For *in vivo* studies, PMNs were fluorescent dye-tagged with PKH26 fluorescent dye, in accordance with the manufacturer's instructions (Sigma). Successful labeling of PMNs was confirmed by performing cytospin analysis and observing fluorescing PMNs under a microscope equipped with a 550-nm filter.

***In vitro* migration assay.** Chemotaxis assays were performed using a 48-well modified Boyden chamber system, as previously described (20,23). For studies using gouty SF, all samples were centrifuged, diluted, and centrifuged again before use in *in vitro* assays. Sham-depleted control and antibody-blocked gouty SF samples were processed in a similar manner. Briefly, a stimulant (25 μ l) of either CXCL16 or diluted gouty SF (1:300 in PBS) was added to the bottom wells of the chambers, while 40 μ l of PMNs at 1×10^6 cells/ml was placed in the wells at the top of the chambers. Sample groups were assayed in quadruplicate, with the results expressed as the mean \pm SEM number of cells migrated per high-power field (at 400 \times magnification). Hank's balanced saline solution (HBSS) and FMLP (at 10^{-7} M) were used as negative and positive controls, respectively.

Generation of normal human ST-SCID mouse chimeras. Normal human ST-SCID mouse chimeras represent a unique way to study human tissue *in vivo*. We used this model to study whether gouty SF, some of which was depleted of CXCL16, can recruit PMNs *in vivo*. Six-to-eight-week-old immunodeficient mice were anesthetized with isoflurane under a fume hood, and a 1.5-cm incision was made with a sterile scalpel on the midline of the back. Forceps were used to blunt

dissect a path for insertion of the ST graft. Each ST graft was implanted on the graft bed site and sutured using surgical nylon. Grafts were allowed to "take" and the sutures removed after 7–14 days.

Within 4–6 weeks of graft transplantation, unpooled diluted gouty SF (1:300 in PBS) was injected into the graft. For studies using the gouty SF, all samples were centrifuged, diluted, and centrifuged again before administration into chimeras. Sham-depleted and antibody-blocked gouty SF samples were processed in a similar manner. Purified gouty SF, either CXCL16-depleted (with a specific neutralizing anti-human CXCL16 antibody; R&D Systems) or sham-depleted (with a nonspecific IgG; R&D Systems), was injected directly into the normal human ST grafts using a 23-gauge needle. For some studies, PMNs were incubated with monoclonal mouse anti-human CXCR6 receptor antibodies (125 μ g/ml mouse anti-human CXCR6 antibody added to 0.5 ml PMNs at 5×10^6 PMNs/100 μ l) (catalog no. MAB699; R&D Systems). Control PMNs were incubated in a similar manner, except that a nonspecific mouse IgG was used, before injection into chimeras. Mice were killed, and the grafts were harvested 48 hours later. For all *in vivo* studies, PMNs integrated into the implanted ST were examined using a fluorescence microscope on slides containing cryosections of graft tissue, and scores for PMN migration were assigned. All sections were analyzed by evaluators who were blinded to the experimental setup.

Statistical analysis. Statistically significant differences between groups were calculated using Student's *t*-test. *P* values less than 0.05 were considered significant. All statistical data are expressed as the mean \pm SEM.

RESULTS

Measurement of cytokines in gouty SF by Luminex and ELISA. In gouty SF, the concentrations of chemokines CXCL10 and CCL2 and the angiogenic factor VEGF were significantly up-regulated as compared with the other cytokines measured (Figure 1A). The mean \pm SEM concentrations of CXCL16 and CXCL8, as measured by ELISA (Figure 1B), were at least 100-fold higher for CXCL16 and 10-fold higher for CXCL8 in comparison with the other cytokines measured. In addition, neither TNF α nor the lymphokine IL-17 was detected in the gouty SF.

Induction of PMN migration by CXCL16. Normal human peripheral blood PMNs were assayed in a modified Boyden chamber for their chemotactic response to CXCL16, to determine whether CXCL16 has a proinflammatory role in gout; FMLP and HBSS served as positive and negative controls, respectively. CXCL16 induced chemotaxis of human PMNs in a dose-dependent manner, and PMN migration was maximal in response to CXCL16 concentrations between 1.0 nM and 10.0 nM ($n = 6$ gouty SF samples per group) (Figure 2A). To determine the participation of CXCL16 in PMN migration in gout, samples of gouty SF containing average levels of CXCL16 (~25 ng/ml, before dilution of

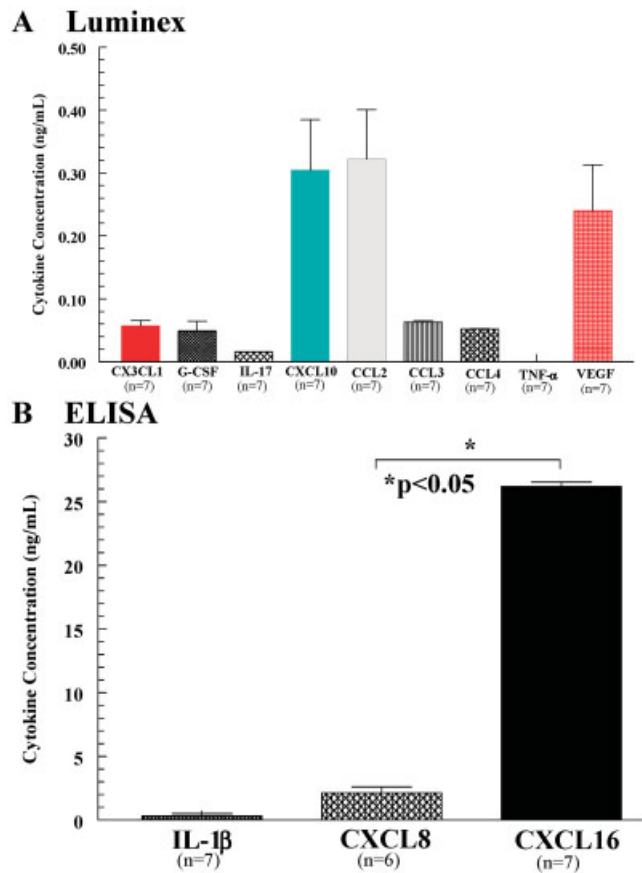


Figure 1. Cytokine measurements in gouty synovial fluid (SF) by Luminex assay and enzyme-linked immunosorbent assay (ELISA). **A**, In the Luminex analyses, chemokines CXCL10 and CCL2 and the angiogenic factor vascular endothelial growth factor (VEGF) were found to be significantly up-regulated as compared with the other cytokines measured. All values shown are the final concentrations observed in undiluted gouty SF. **B**, ELISA measurements indicated that the concentration of CXCL16 was at least 100-fold higher than that of the other cytokines measured in gouty SF. The concentration of CXCL8 was also elevated as compared with many other cytokines, but it was >10-fold less abundant than CXCL16 in gouty SF. Of note, tumor necrosis factor α (TNF α) and interleukin-17 (IL-17) were not expressed in the gouty SF. Bars show the mean and SEM. G-CSF = granulocyte colony-stimulating factor. Color figure can be viewed in the online issue, which is available at <http://www.arthritisrheum.org>.

1:300 in PBS) were incubated with an anti-human CXCL16 antibody. The SF samples were then tested in an in vitro migration assay and compared with sham-depleted controls. Gouty SF showed an ~20% decrease in PMN migratory activity ($P < 0.05$ versus controls) after incubation with the neutralizing CXCL16 antibody (Figure 2B).

We also examined the role of CXCL10 as an alternative CXC chemokine, since, similar to the expression patterns of CXCL8 and CXCL16, it was also

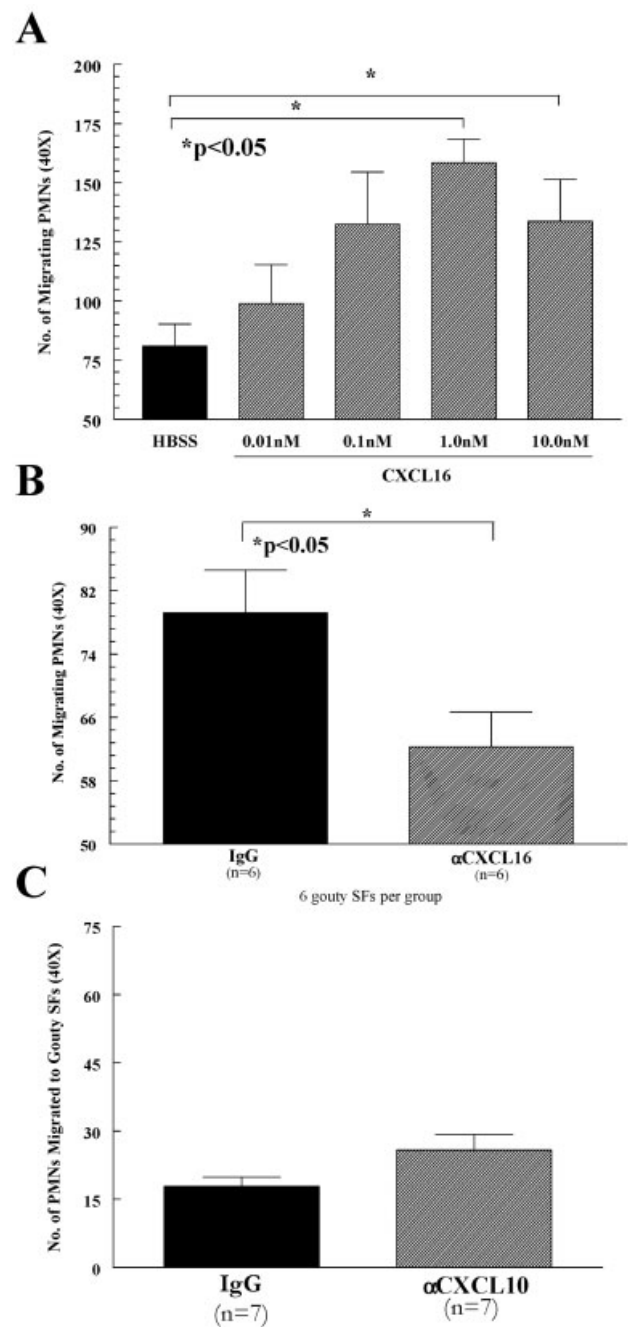


Figure 2. Migration of polymorphonuclear neutrophils (PMNs) toward CXCL16 in vitro. **A**, CXCL16 induced chemotaxis of human PMNs in gouty synovial fluid (SF) in a dose-dependent manner and was maximal at CXCL16 concentrations between 1.0 nM and 10.0 nM ($n = 6$ samples per group). Hank's balanced saline solution (HBSS) served as a negative control. **B**, Blocking of CXCL16 with a neutralizing anti-CXCL16 antibody in gouty SF reduced the in vitro PMN chemotactic activity of the gouty SF. **C**, Antibody blocking of CXCL10 in gouty SF showed that PMNs failed to migrate toward CXCL10 in vitro, and thus CXCL10 did not induce chemotaxis of human PMNs at concentrations similar to those observed in gouty SF. Goat IgG served as a control nonspecific antibody. Bars show the mean and SEM.

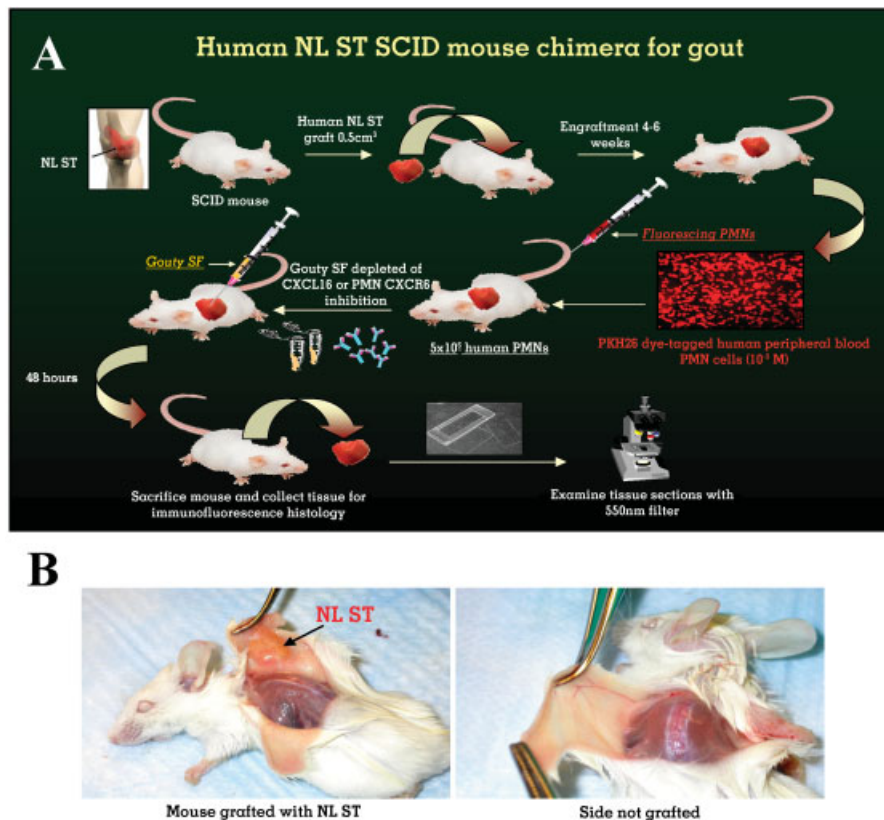


Figure 3. A, Procedure used to produce an animal model of gout involving SCID mouse chimeras. Normal (NL) human synovial tissue (ST) was engrafted into SCID mice, followed by intra-graft administration of diluted gouty human synovial fluid (SF) (diluted 1:300 with phosphate buffered saline; 100 μ l/graft) and intravenous injection of PKH26 red fluorescent dye-tagged human polymorphonuclear neutrophils (PMNs) (5×10^6). Simultaneous inhibition experiments involved antibody blocking of CXCL16 in the gouty SF or depletion of CXCR6 from the PMNs. B, A representative normal human ST-SCID mouse chimera at 4–6 weeks after engraftment is shown.

observed to be highly up-regulated relative to the other cytokines analyzed in gouty SF (Figure 1A). Because the role of the CXCL8–CXCR2 ligand–receptor pair in PMN chemotaxis is already well established (3), we compared CXCL16-mediated chemotaxis with that of CXCL10 in gouty SF, with the use of antibody-blocking studies. Comparison of CXCL16-mediated PMN chemotaxis with the activity of CXCL10, the latter of which is not known to recruit PMNs but, rather, is known to recruit monocytes, T cells, natural killer cells, and dendritic cells, serves as a natural negative control for CXCL16-mediated PMN chemotaxis. Accordingly, unlike the findings with regard to CXCL16, we observed no effect on PMN chemotaxis *in vitro* upon blocking of CXCL10 (Figure 2C), confirming that not all CXCL chemokines are effective PMN recruitment factors in gout.

PMN recruitment to engrafted human ST in a SCID mouse chimera. To test PMN migration *in vivo*, we used engraftment of a SCID mouse chimera with normal human ST (diagram of the procedure is shown in Figure 3A). After 4–6 weeks, the mice engrafted with normal human ST and showing no signs of graft rejection were used (Figure 3B). To determine homing of normal human PMNs, freshly isolated cells were fluorescent dye-tagged with PKH26, and 5×10^6 cells/100 μ l/mouse were injected IV by tail vein 48 hours before the mice were killed. Cryosections (10 μ m) of the normal human ST grafts were then examined using a fluorescence microscope. SCID mice engrafted with normal human ST that was injected intra-graft with diluted (1:300 in PBS) gouty SF showed robust recruitment of IV-administered, fluorescent dye-tagged PMNs.

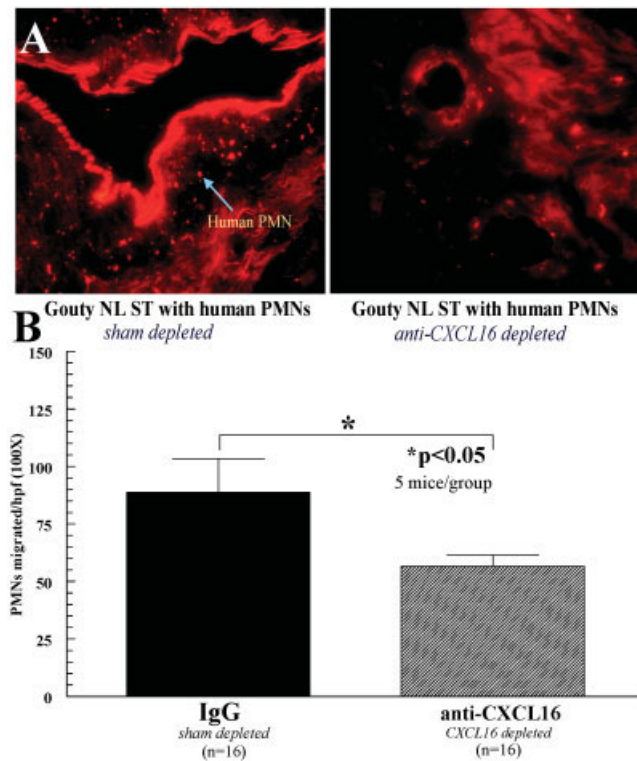


Figure 4. Reduction of polymorphonuclear neutrophil (PMN) recruitment in vivo by inhibition of CXCL16 with a neutralizing anti-CXCL16 antibody in gouty synovial fluid (SF). **A**, PKH26 red fluorescent dye-tagged human PMNs were injected intravenously into SCID mice engrafted for 4–6 weeks with normal (NL) human synovial tissue (ST). Before administration of the cells, ST grafts were injected with gouty SF that was either blocked with neutralizing anti-CXCL16 antibodies or sham-depleted with nonspecific IgG. At 48 hours, grafts were harvested and tissue sections were examined using immunofluorescence microscopy at an optical density of 550 nm (original magnification $\times 400$). **B**, Migration of the PMNs was quantified by dividing the number of cells per high-power field (hpf) (counted at $100\times$ magnification) in each ST tissue section. Bars show the mean and SEM number of ST sections counted (from a total of 5 mice per group). Color figure can be viewed in the online issue, which is available at <http://www.arthritisrheum.org>.

These results indicate that exogenously administered gouty SF containing CXCL16, as well as many other PMN chemotactic factors, will induce recruitment of human PMNs in the SCID mouse chimera system.

Reduction in PMN recruitment in vivo by antibody blocking of CXCL16 in gouty SF. Dye-tagged PMNs were allowed to migrate for 48 hours to human ST grafts injected with gouty SF (diluted 1:300 in PBS). The tissue grafts were then harvested, snap-frozen, and cryosectioned. Dye-tagged migrated cells were observed, using a fluorescence microscope, in the normal human ST grafts (Figure 4A). Normal human ST–SCID mouse

chimeras injected intragraft with gouty SF that had been preincubated with blocking antibodies to CXCL16 at the time of PMN transfer showed a 50% reduction in PMN recruitment to the grafts as compared with controls injected with sham-depleted gouty SF (Figure 4B). This finding is consistent with the results from our in vitro studies and further demonstrates that the extent of CXCL16 expression is correlated with PMN recruitment to gouty SF.

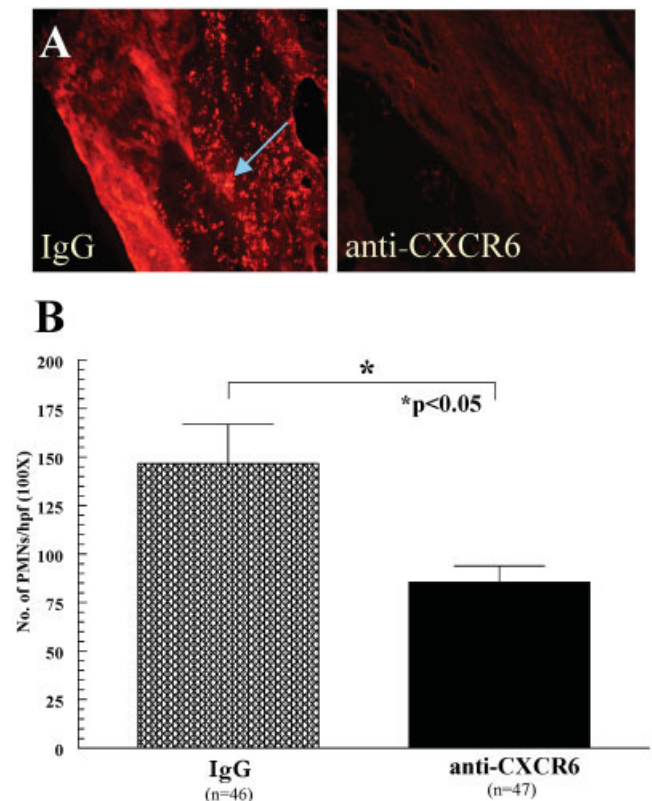


Figure 5. Reduction of polymorphonuclear neutrophil (PMN) recruitment to normal human synovial tissue by inhibition of CXCR6 in PMNs in the gouty SCID mouse chimera model. **A**, The number of PMNs (arrow) per high-power field (hpf), counted at $100\times$ magnification on fluorescence microscopy, was dependent on the size and composition of the graft and corresponding section. The entire area of each tissue section was counted. Approximately 60–90 hpf were counted per implant, from at least 5 independently grafted mice in each group (IgG controls versus anti-CXCR6–treated mice). The total number of sections from each mouse constituted the number of samples (e.g., ~ 15 sections/graft from 3 grafts is 45 sections). Grafts were surgically removed and set in histology blocks containing OCT. Grafts composed of significant amounts of murine tissue were excluded from the study. **B**, Migration of the PMNs was quantified by dividing the number of cells per high-power field. Bars show the mean and SEM number of synovial tissue sections counted. Color figure can be viewed in the online issue, which is available at <http://www.arthritisrheum.org>.

Reduction in PMN recruitment to normal human ST injected with gouty SF by antibody blocking of CXCR6. PMNs were incubated with a neutralizing anti-CXCR6 antibody, and the cells were then fluorescent dye-tagged and injected IV into SCID mouse chimeras that had received intragraft injections (100 μ l/graft) of diluted gouty SF (1:300 in PBS). After 48 hours, the mice were killed, and the grafts were removed for cryosectioning. Control mice received the same intragraft injection of gouty SF but were administered fluorescent dye-tagged PMNs preincubated with nonspecific IgG (Figure 5A). As shown in Figure 5B, mice administered PMNs preincubated with anti-CXCR6 showed a 42% reduction in PMN migration, which was a significantly greater reduction as compared with that in control mice.

DISCUSSION

Cytokine networks in gout have been insufficiently characterized, although some attempts have been made in animal models (6,24–27). To identify significant cytokine and chemokine systems, we examined several cytokines by performing Luminex assays on gouty SF, which allowed us to profile the cytokine expression patterns in human patients. These analyses included measurement of chemokines, a subset of cytokines that work to induce cell migration to sites of inflammation (16,19). The CC chemokines measured were CCL2, CCL3, and CCL4. The CXC chemokines included CXCL10, CXCL8, and CXCL16; the latter 2 were measured by ELISA. We also examined CX3CL1, a chemokine that is associated with mononuclear cell recruitment and angiogenic activity in RA (20,28)

We showed that the concentrations of CXCL10, CCL2, CXCL16, and the proangiogenic factor VEGF were all up-regulated relative to a panel of other cytokines measured. The levels of CCL2 measured in the gouty SF samples were comparable with those described in a previous study (29), and the extent of CCL2 expression likely accounts for many of the recruited monocytes found in gouty SF. We also observed that expression of the PMN growth factor G-CSF was up-regulated in gouty SF.

Several of these cytokines are proinflammatory mediators that are associated with cell recruitment to joint tissue (16,19). VEGF and CXCL16 are associated with angiogenesis, or new blood vessel growth. Moreover, VEGF has been implicated in wound repair (30), and CXCL16 has been shown to be a very active mononuclear cell recruitment factor in RA (17) and has been shown to have angiogenic activity (31). According

to our preliminary profile, it is likely that, in addition to CXCL8, the mediators measured in the present study may all be important for cell infiltration to the joints of patients with gout (3).

We also noted that IL-17 was not significantly expressed in the samples of gouty SF, in accordance with the paucity of lymphocytes observed in the gouty SF (results not shown). Interestingly, TNF α was also not expressed in the gouty SF (n = 7 different specimens examined), suggesting that the cytokines up-regulated in gout possibly function in a manner independent of TNF α . Expression of IL-1 β was also relatively low in the gouty SF, compared with the concentrations of CXCL8 or CXCL16, but has been shown to play a more central role than TNF α in experimental urate crystal-induced inflammation (32,33). Indeed, favorable results with IL-1 inhibitors to suppress pain and inflammation have been observed in small, open-label studies in patients with chronic, treatment-refractory gouty inflammation (32–34).

CXCL16 was implicated in the early phases of this study as a possible mediator of angiogenesis and cell recruitment in gout. Our findings confirmed the presence of highly elevated levels of CXCL16 in gouty SF, with measurements that were ~100-fold greater than those of many of the other cytokines present. CXCL16 also showed chemotactic properties with regard to PMNs. Indeed, our *in vitro* chemotaxis findings showed that CXCL16 appeared to be a very active PMN recruitment factor in the 1.0 nM (10 ng/ml) to 10.0 nM (100 ng/ml) concentration range, consistent with the concentrations of CXCL16 observed in the gouty SF (Figure 2A).

For the *in vivo* phase of this study, we depleted the cytokines that were observed to be up-regulated in gouty SF and tested their PMN recruitment ability in the SCID mouse chimera system. We observed that depleting either CXCL16 or membrane-bound CXCR6 in gouty SF significantly inhibited the recruitment of PMNs *in vivo*. Of note was the failure to observe complete inhibition of PMN recruitment in the SCID mouse chimera system. A plausible explanation for this may be that there is incomplete up-regulation of CXCR6 in PMNs, such that only basal levels of CXCR6 in PMNs account for just a fraction of the total cellular CXCR6 (35). The findings from our experiments using unstimulated PMNs, before incubation with anti-CXCR6, support this notion. Other factors contributing to incomplete *in vivo* suppression of PMN migration may be the expression of complement byproducts or numerous uninhibited chemokines, such as CXCL8, that are also prominent in gouty SF.

Detection of VEGF in gouty SF was somewhat unexpected. Gout is not thought of as a disease that is dependent on a high degree of vasculature. However, the cellular infiltrate observed in the gouty SF seemed to indicate that the cells were migrating freely from the peripheral bloodstream into the joint tissue, presumably aided by additional vasculature mediated by VEGF expression. Furthermore, monocytes respond, produce, and migrate toward VEGF, providing further evidence that recruited monocytes may amplify the angiogenic process in gout.

It is tempting to speculate that the effects of VEGF on vascular growth may exacerbate gout pathology. A review of the current literature indicates only a paucity of information linking gout and angiogenesis. However, in a small study by Lapkina et al, possible laboratory markers of vascular endothelium activation were identified as being of clinical significance in gout (36). Although those authors did not examine circulating VEGF levels, they did, interestingly, show increased serum concentrations of soluble vascular cell adhesion molecule 1 and von Willebrand factor in patients with gout. This reflected not only the activation of vascular endothelium but also the development of atherosclerotic processes in these patients (36). In accordance with this, Schumacher demonstrated the expected robust neutrophil infiltrates in a gouty synovial biopsy specimen, and also observed high vascularization of gouty human ST (37). We propose that recruitment of PMNs and monocytes to gouty tissue may rely heavily on neoangiogenic responses induced by VEGF in gouty synovium, and also possibly those induced by CXCL16 in gouty SF. Since CXCL16 is an angiogenic mediator (31), monocyte recruitment factor (17), memory T cell recruitment factor (38), and PMN recruitment factor (35), CXCL16 may be a premiere proinflammatory mediator in gout, and this role of CXCL16 would support our premise of significant CXCL16 involvement in gout pathology.

With these experiments, we determined the expression and function of secreted chemotactic factors and their impact on local PMN recruitment in gouty SF. We have presented evidence to suggest that several chemokines and chemokine receptors, apart from CXCL8, may be at work in gout pathology. We observed that CXCL16 concentrations are elevated in gouty SF and that soluble CXCL16 is expressed at concentrations much higher than those of CXCL8. This suggests that soluble mediators other than CXCL8 may play a significant role in PMN recruitment caused by MSU crystal-induced inflammatory responses. For this study, we tested our initial in vitro findings with a novel in vivo model of crystal-induced arthritis and related these

findings to the extent of CXCL16 expression. Although other investigators have characterized some rudimentary cytokine networks in gout (24), this study was novel in that we examined the nature of PMN recruitment and homing in a normal human ST-SCID mouse chimera. These in vivo experiments built on our initial findings by examining the activity of CXCL16 and its receptor, CXCR6, in an in vivo setting. This allowed for the evaluation of the architectural changes in engrafted normal human ST due to PMN migration in response to injected gouty fluid. Thus, we were able to mimic gout pathology with an in vivo model that reproduces this disease with accuracy, which will further the development of therapeutic strategies designed to inhibit proinflammatory factors, such as CXCL16, that enhance PMN recruitment to gouty tissue.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Ruth had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Ruth, Koch.

Acquisition of data. Ruth, Arendt, Amin, Ahmed, Marotte, Rabquer, Lesch, Lee.

Analysis and interpretation of data. Ruth, Arendt, Amin.

ROLE OF THE STUDY SPONSOR

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