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The Chemokines CXCL9, CXCL10, and CXCL11 Differentially Stimulate Goαi-Independent Signaling and Actin Responses in Human Intestinal Myofibroblasts

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Intestinal myofibroblasts have been implicated in the pathogenesis of chronic inflammatory conditions such as Crohn’s disease via interactions with an elaborate network of cytokines, growth factors, and other inflammatory mediators. CXCR3 is a Goα protein-coupled receptor that binds the proinflammatory chemokines CXCL9, CXCL10, and CXCL11, which are released from the intestinal epithelium. The three CXCR3 ligands shared the ability to activate biochemical (e.g., PI3K and MAPK activation) and functional events (actin reorganization) in intestinal myofibroblasts. However, CXCL11 is unique in its ability to elevate intracellular calcium. Surprisingly, although CXCR3 mRNA is detectable in these myofibroblasts, there is no detectable surface expression of CXCR3. Furthermore, the biochemical responses and actin reorganization stimulated by the CXCR3 ligands in intestinal myofibroblasts are insensitive to the Goα inhibitor, pertussis toxin. This suggests either the existence of differential receptor coupling mechanisms in myofibroblasts for CXCR3 that are distinct from those observed in PBLs and/or that these cells express a modified or variant CXCR3 compared with the CXCR3 expressed on PBLs. The Journal of Immunology, 2005, 175: 5403–5411.

The classical CXC chemokine receptor 3 is a pertussis toxin (PTX)-sensitive, seven-transmembrane domain-spanning G protein-coupled receptor. CXCR3 binds the proinflammatory, non-ELR motif CXC chemokines: monokine induced by human IFN-γ (Mig)/CXCL9, IFN-inducible 10-kDa protein (IP-10)/CXCL10, and IFN-inducible T cell a chemoattractant (I-TAC)/CXCL11. CXCR3 expression can be detected on IL-2 or allogeneically activated T lymphocytes in MLR (1, 2) and on thymocytes during lymphopoesis (3). CXCR3 is also expressed on 35–40% of normal blood T cells, and expression can be enhanced on T cells in culture by IL-2 (4). CXCR3 has been detected preferentially on Th1 cell lines and clones in vitro (5, 6) and has been implicated in the pathophysiology of Th1-type diseases, such as autoimmune disorders and viral infections (7–9), even though CXCR3 did not discriminate between Th1- and Th2-dominated responses in vivo (10). Furthermore, CXCR3 has also been detected in NK cells (11), a small subset of normal circulating B lymphocytes, as well as plasma cells (12), eosinophils (13), endothelial cells (14), pericytes (15), mast cells (16), and glial cells (17).

Although they appear to share the same receptor, there is evidence to support the idea that CXCL9, CXCL10, and CXCL11 have distinct and nonredundant biological roles. For example, freshly isolated T cells respond to CXCL11, but are less responsive to CXCL9 or CXCL10 (18). Moreover, recent studies have shown that the CXCR3 ligands exhibit unique temporal and spatial expression patterns (19, 20). Furthermore, a CXCR3-specific mAb has been reported to inhibit CXCL10 binding to CXCR3, without any effect on CXCL9 binding (21). It has also been demonstrated that CXCL9, CXCL10, and CXCL11 are allotypic ligands for CXCR3 (22). It is interesting to note that an alternative, functional, high affinity receptor for CXCL10 (but not CXCL9 or CXCL11) has been reported to be expressed on epithelial and endothelial cells (23), whereas another spliced variant of CXCR3, termed CXCR3-B, found on endothelial cells (24) binds platelet factor 4/CXCL4 in addition to the other CXCR3 ligands. More recently, a novel variant of CXCR3 has been identified, generated by post-transcriptional exon skipping (25). This new receptor (termed CXCR3-alt), is predicted to have a four- or five-transmembrane domain and is unresponsive to CXCL9 and CXCL10, yet appears to retain full activity for CXCL11. Most chemokines, including CXCL9, CXCL10, and CXCL11, are able to elicit increases in intracellular Ca2+ levels and activate PI3K and MAPK (26–28). PI3K, in particular, is believed to be vital in gradient sensing and directional cell movement for Dictyostelium cells and mammalian leukocytes (29, 30), although it may be dispensable for T cell chemotaxis (31). Interestingly, ligation of CXCR3-B by CXCL9, CXCL10, or CXCL11 does not result in elevation of intracellular calcium (24). In addition, the Goα inhibitor, PTX, had no effect on ligand-stimulated proliferation and survival of CXCR3-B transfectants (24), although it did inhibit CXCR3-mediated proliferation (15).

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1 Abbreviations used in this paper: PTX, pertussis toxin; [Ca2+]i, cytosolic free Ca2+ concentration; CD, Crohn’s disease; IP-10, IFN-inducible 10-kDa protein; I-TAC, IFN-inducible T cell a chemoattractant; Mig, monokine induced by human IFN-γ; PKB, protein kinase B; ROCK, Rho-associated coiled-coil-forming protein kinase; TRITC, tetramethylrhodamine B isothiocyanate.
The intestinal epithelium appears to be a major source of CXCL9, CXCL10, and CXCL11 in the gut (32) as well as other inflammatory mediators implicated in the pathogenesis of inflammatory bowel disease. Consistent with the involvement of Th1 cells in the pathogenesis of Crohn’s disease (CD) (33), the expression of CXCR3 has been reported on T cells infiltrating the inflamed gastrointestinal submucosa of patients (34). However, its expression was also largely found on lymphocytes isolated from patients with ulcerative colitis, which appears to be a Th2 disease (35). Levels of CXCR3 ligands and/or receptor expression have also been reported to be up-regulated in several different animal models of inflammatory bowel disease (36, 37). Myofibroblasts have specialized roles in wound healing, and myofibroblasts present in normal intestinal mucosa are thought to regulate various biological processes that occur in the intestine through interactions with other intestinal cells via an elaborate network of cytokines, growth factors, and other inflammatory mediators (38). In chronic inflammatory conditions, including CD, it is recognized that excessive activation and proliferation of myofibroblasts are responsible for excess deposition of matrix proteins such as collagen, which leads to stricturing and loss of organ function via remodeling (39). Interestingly, many of the growth factors that have been proposed to regulate myofibroblast proliferation (e.g., platelet-derived growth factor and TGF-β) induce the expression of chemokines in other systems (40). Elsewhere, pulmonary fibroblasts (expressing several markers characteristic of myofibroblasts) derived from granulomas express another chemokine receptor, CCR2, which binds MCP-1/CCL2 and is also elevated in granulomas. Although CXCL8 is known to be induced in intestinal myofibroblasts by eosinophil-derived major basic protein (41), very little is known regarding the pattern of chemokine and chemokine receptor expression on these cells.

In this study we report that the three CXCR3 ligands share the ability to activate Gr-γ-independent biochemical (e.g., PI3K and MAPK activation) and actin responses in intestinal myofibroblasts despite the lack of detectable CXCR3 expression. Surprisingly, CXCL11 is unique in its ability to induce calcium mobilization. The observation that signaling responses occur in the absence of detectable surface expression of CXCR3 may simply reflect that CXCR3 is expressed, albeit at levels below detectable limits. Alternately, CXCR3 may undergo some post-translational modification in intestinal myofibroblasts that interferes with its recognition by the CXCR3 Ab, and/or a novel receptor may be expressed on these cells.

Materials and Methods

Reagents

Human rCXCL9, rCXCL10, rCXCL11, and FITC-conjugated mouse anti-CXCR3 mAb (clone 49801) were purchased from R&D Systems. Tetra-methylrhodamine B isothiocyanate (TRITC)-conjugated phalloidin was obtained from Sigma-Aldrich. Phospho-specific polyclonal antibodies recognizing protein kinase B (PKB) phosphoserine 473 (catalogue no. 9271) or phosphothreonine 308 (catalogue no. 9275), ERK1/2 (p42/p44) phosphorylated at threonine 202 and tyrosine 204 (catalogue no. 9101), and p90RSK phosphoserine 380 (catalogue no. 9341) as well as rabbit anti-ERK1/2 (catalogue no. 9102) were purchased from Cell Signaling Technologies. Goat anti-PKB (sc-1618) was purchased from Santa Cruz Biotechnology. The ECL system was obtained from Amersham Biosciences. "LY294002, Y-27632, and PTX were acquired from Calbiochem. Secondary Abs for immunoblotting were purchased from DakoCytomation. Fura 2-AM was obtained from Sigma-Aldrich.

Cell culture

Studies were performed at passages 4–10 on myofibroblasts isolated from normal tissue and from endoscopic biopsies or surgical specimens derived from six patients at Royal United Hospital (Bath, U.K.). Tissues were collected with the approval of local ethical committees. Primary cultures of human intestinal myofibroblasts were prepared according to the method reported by Mahida et al. (42). Briefly, mucosal specimens were treated with 1 mM DTT for 15 min and washed three times in HBSS. They were then incubated three times for 30 min at 37°C in the presence of 1 mM EDTA, pH 8.0. At the end of the EDTA treatment, the mucosal samples were completely denuded of epithelial cells and were subsequently cultured in RPMI 1640 medium supplemented with antibiotics. During culture, numerous cells appeared both in suspension and adhered to the culture flask. The cells in suspension were removed every 24–72 h, and the denuded mucosal tissue was maintained in culture for up to 4 wk until myofibroblasts appeared attached to the bottom of the culture flask. Tissue specimens were then removed, and intestinal myofibroblasts were cultured in DMEM supplemented with 10% (v/v) FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), 0.5 µg/ml fungizone, and 1% (v/v) nonessential amino acids. Cells were passaged when fully confluent in a 1:2 to 1:3 split ratio. Before all experiments, the myofibroblast phenotype of the cells isolated from the biopsies was verified by confirming the expression of smooth muscle actin and vimentin and the lack of expression of desmin by immunofluorescence microscopy and immunoblottting (data not shown).

Isolation and preparation of previously activated human PBL

Heparinized blood samples were separated on a Histopaque (1.077) density gradient, and PBMC (106 cells/µl) removed from the gradient were stimulated with staphylococcal enterotoxin B (1 µg/ml) for 72 h. The cells were washed, and growth was maintained by supplementing cultures every 2 days with 0.1 nM IL-2. Cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. After 10 days, cells were deprived of IL-2 for at least 2 days and allowed to accumulate at the G0/G1 stage as described previously (43).

Measurements of cytosolic free Ca2+ concentration ([Ca2+]i)

Measurements of cytosolic calcium with the acetoxyethyl ester of the Ca2+-sensitive fluorescent probe fura 2-AM were performed in intestinal myofibroblasts on an inverted fluorescence microscope setup. The system allows fluorescence measurements at the single-cell level at three excitation wavelengths. The field of measurement can be set between a diameter of 2 and 300 µm with an adjustable pinhole. A time resolution of up to 200 Hz was achieved by using a high speed filter wheel and a single photon-counting tube (H63460-04; Hamamatsu). The autofluorescence signal of cells that had not been loaded with fura 2 was measured and subtracted from the results obtained in fura-2-loaded cells. This had no effect on the bandwidth of the measurements. A calibration of the fura 2 fluorescence signal was attempted at the end of each experiment using Ca2+ ionophore ionomycin (1 µM) and low and high Ca2+ buffers. [Ca2+]i was calculated from the fluorescence ratio 340/380 nm according to the equation described by Grynkiewicz et al. (44).

RNA extraction and RT-PCR

Cellular RNA was extracted from the different cell sources, using RNXol B (AMS Biotechnology), as described by the manufacturer. Polyadenylated RNA was purified using a Quick Prep minipurification kit (Amerham Biosciences). mRNA (2.5 µg) was denatured at 70°C for 10 min in the presence of 5 µM oligo(dT)12-18 primer. It was then reverse transcribed in a 10-µl volume with SuperScript II (Invitrogen Life Technologies), 1 X RT buffer, 5 mM DTT, and 2.5 U/µl RNase (Promega) at 42°C for 60 min. Control reactions were performed identically, except reverse transcriptase was replaced with RNase-free water. This gave 20 µl of cDNA or -RT control reaction. PCR was performed using ABgene ReddyMix PCR tubes (50 µl) using 3 µl of cDNA or -RT control reactions or 0.2 µg of human genomic DNA (from HEK293 cells). One hundred nanograms of each primer was added. PCR was performed using CXCR3 (accession nos. X95876) or CXCR3B (accession no. AF469435) specific primers (CXCR3 3’ primer, TTCATGCTCACCAGCTCTAC; CXCR3 3’ primer, TGGAGGCTTCAGACAGGATAG (which yield products of 622 bp for CXCR3 and 285 bp for CXCR3-alt); CXCR3B 3’ primer, GACGATTTAGGAGGCTCTC; CXCR3B 3’ primer, CAGTGCTCAGCACGACG (which yield a product of 420 bp). Mixing tubes were incubated at 94°C for 1 min, then 35 cycles of 20 s at 94°C, 30 s at 58°C, 1 min at 72°C, with a final incubation of 7 min at 72°C. PCR products were resolved by electrophoresis on 2% agarose gels and viewed by ethidium bromide staining.
Flow cytometry

Cells (1 × 10⁶) were washed twice in PBS and resuspended in 100 μl of FITC-conjugated anti-CXCR3 (5 μg/ml) or the corresponding IgG1 isotype control, diluted in PBS/10% PBS, and incubated for 45 min on ice in the dark. After washing, cells were analyzed by flow cytometry on a FACS Vantage cell sorter (BD Biosciences), using excitation at 488 nm and fluorescence emission at 520 nm.

Cell lysis

Cells (1 × 10⁶/sample) were incubated in 1.5-ml microfuge tubes at 37°C, and after the indicated treatment, reactions were terminated by pelleting the cells in a microfuge for 10 s, aspirating the supernatant, and then resuspending in 50 μl of ice-cold lysis buffer (20 mM Tris (pH 7.4), 137 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 10% glycerol (w/v), 1% Nonidet P-40 (w/v), and protease inhibitors, 1 mM PMSF; 2 μg/ml aprotinin. 2 μg/ml leupeptin, 2 μg/ml pepstatin A, and 1 mM sodium orthovanadate). Lysates were incubated on a rotator for 20 min, and nonsoluble material was removed by centrifugation at 14,000 rpm for 10 min. The protein levels in supernatants were determined by the Bradford protein assay (Bio-Rad) and adjusted to equal protein concentration by dilution with lysis buffer where necessary.

Immunoblotting

Cell lysates were boiled for 5 min in sample buffer, and samples were loaded, at volumes corresponding to equivalent amounts of protein, onto 10% SDS-PAGE gels and transferred by electroblotting onto nitrocellulose membranes. Membranes were incubated for 1 h with 25 ml of blocking solution (1% nonfat milk/0.05% sodium azide in TBS (10 mM Tris (pH 7.5) and 100 mM NaCl)), then incubated overnight with 10 ml of a 1/1000 dilution (in TBS and 0.1% Tween) of the appropriate Ab, as described previously (45). When necessary, membranes were stripped for repробинг by incubation in stripping buffer (62.5 mM Tris (pH 6.8), 2% SDS, and 100 mM 2-ME) at 60°C for 30 min.

Cell staining for immunofluorescence microscopy

Cells were grown on 22-mm glass cover slips and treated for different lengths of time with various combinations of chemokines and/or inhibitors. After treatment, cells were fixed in 4% paraformaldehyde (w/v) and 1% glutaraldehyde (w/v) for 10 min and permeabilized with 0.2% Triton X-100 (w/v) at room temperature for 5 min. After washing three times with PBS, the cells were blocked in 1% BSA in PBS for 30 min at 37°C and then incubated with TRITC-conjugated phalloidin (0.1 μg/ml) for 1 h at room temperature. Fluorescent cells were visualized using an epifluorescence imaging system comprised of an Olympus IX70 inverted epifluorescence microscope (Olympus America) with a TRITC (excitation, 547 nm; emission, 580 nm) filter, an Ultraview camera with Kodak KAF1400 chip (Eastman Kodak; 6.7 μm physical pixels, giving 67 nm/image pixel with a ×100 oil immersion objective), and a personal computer with Fluoview imaging software (Olympus America).

Results

Expression of CXCR3 on human intestinal myofibroblasts

First, we examined the expression of mRNA for the known CXCR3 variants in human intestinal myofibroblast. Primers were designed that specifically amplify CXCR3A or CXCR3B, as shown by PCR using genomic DNA as a template (Fig. 1A). The CXCR3A primers should also amplify CXCR3alt, generating a smaller product (285 bp rather than 622 bp). RT-PCR from myofibroblast mRNA was performed using the specific mouse FITC-conjugated anti-CXCR3 mAb (1 μg/ml; gray line) or with FITC-conjugated mouse IgG at the same concentration (black), as an isotype-matched control, and examined by flow cytometry as described in Materials and Methods. Data are from individual experiments representative of three others, and myofibroblast data are representative of at least three different donors.

Effect of CXCR3 ligands on changes in [Ca²⁺]i in human intestinal myofibroblasts

Increased intracellular calcium is one of the best-characterized and most ubiquitous biochemical response to chemokine stimulation (46). Consequently, we used this biochemical readout to further investigate the disparity between the presence of CXCR3 mRNA and the lack of detectable surface expression in our system. Administration of 10 nM CXCL9 and CXCL10 to myofibroblasts loaded with fura 2-AM had no effect on [Ca²⁺]i (Fig. 2, A and B, respectively). Concentrations up to 100 nM were examined and were found to be ineffective at eliciting a rise in [Ca²⁺]i (data not shown). CXCL11 resulted in a transient increase in [Ca²⁺]i (Fig. 2C). Some random increases in [Ca²⁺]i were observed in some cells independent of application of the chemokines, which were due to calcium oscillations that occur naturally in primary intestinal myofibroblasts. Addition of endothelin-1 verified that myofibroblasts were responsive to calcium-mobilizing stimuli in settings where CXCL9 and CXCL10 were without effect (data not shown).

CXCL9, CXCL10, and CXCL11 stimulate PI3K-dependent phosphorylation of PKB in human intestinal myofibroblasts

In addition to elevation of intracellular Ca²⁺, another well-characterized biochemical signal attributed to most chemokine receptors, including CXCR3, is activation of the PI3K-dependent signaling cascade (15, 27). The major downstream effector of PI3K activity is PKB, a serine/threonine kinase, whose recruitment, phosphorylation, and subsequent activation are entirely dependent upon the lipid products of PI3K (47). Monitoring PKB phosphorylation at Thr³⁰⁸ and Ser³⁸⁵ is therefore an indirect readout of PI3K activation. CXCL9, CXCL10, and CXCL11 all stimulated phosphorylation of PKB at both Ser³⁸⁵ and Thr³⁰⁸ in intestinal myofibroblasts (Fig. 3, left panels) and previously activated PBL (Fig. 3, right panels). Phosphorylation of PKB in intestinal myofibroblasts was detected 1 min after the addition of the chemokines and was sustained for 10–20 min, whereas phosphorylation in PBL was much more transient, and was not detectable after 5 min...
Phosphorylation of PKB at Ser473 in response to CXCL9, CXCL10, and CXCL11 was completely abrogated by the PI3K inhibitor LY294002 in both intestinal myofibroblasts (Fig. 4A) and activated PBL (Fig. 4B).

CXCR3 ligands activate the MAPK pathway in human intestinal myofibroblasts

Another well-characterized intracellular signaling target of the CXCR3 ligands is the MAPKs p42/44 (15, 26). Indeed, CXCL9, CXCL10, and CXCL11 all result in phosphorylation of ERK1/2 in intestinal myofibroblasts and PBL (Fig. 5A, left and right panels, respectively). ERK1/2 phosphorylation in intestinal myofibroblasts is generally detectable at 2 min after stimulation and is maintained up to 10 min, declining toward the basal level thereafter. Phosphorylation levels in PBL are generally detected within 1 min, but are much more transient, having reached maximum levels at 2 min and declined back to basal levels within 5–10 min. That the phosphorylation of ERK1/2 compares with its activation in both cell types is confirmed by phosphorylation of its downstream target p90RSK (Fig. 5B), the kinetics of which are slightly delayed compared with those reported for ERK1/2.

Comparison of the signaling potency of CXCR3 ligands in human intestinal myofibroblasts

Even though CXCL11 appears to be more potent and efficacious than either CXCL9 or CXCL10 as a chemotactic factor in activated T cells (48), studies comparing the abilities of these chemokines to initiate intracellular signals are lacking. Cells were stimulated with CXCL9, CXCL10, or CXCL11 over a time course of up to 10 min, a period within which maximal phosphorylation of PKB and ERK1/2 had previously been demonstrated for both cell types. In intestinal myofibroblasts, no differences could be detected in the magnitude of the signals elicited by those three ligands, suggesting that their efficacies for the signaling events examined are similar in these cells (Fig. 6A). In marked contrast, stimulation of PBL revealed that CXCL11 appeared to be the most potent of the three CXCR3 ligands (Fig. 6B), confirming previous studies.
Effect of PTX on CXCR3-ligand mediated signaling events in human intestinal myofibroblasts

Most chemokine receptors, including CXCR3 (26), are coupled to the heterotrimeric protein subunit G<sub>q</sub> and are inhibited by PTX. Because we could detect no discernable differences in potency among CXCL9, CXCL10, and CXCL11 in the biochemical responses of myofibroblasts, we focused on the use of CXCL10 to examine the PTX sensitivity of signaling responses and actin responses in intestinal myofibroblasts (Fig. 7, left panels). Surprisingly, CXCL10-stimulated phosphorylation of PKB, ERK1/2, and p90RSK in intestinal myofibroblasts was found to be insensitive to pretreatment with PTX. In contrast, we observed complete inhibition of CXCL10-stimulated phosphorylation of ERK1/2, PKB, and p90RSK in activated PBL (Fig. 7, right panels).

CXCR3 ligands stimulate Rho-associated coiled coil-forming protein kinase (ROCK)-dependent, but PI3K- and G<sub>q</sub>-independent, actin reorganization in human intestinal myofibroblasts

Myofibroblasts possess specialized contractile features that are essential for connective tissue remodeling during normal and pathological wound healing. Reorganization of the actin cytoskeleton and formation of axial bundles of F-actin and associated proteins is a key component of the contractile apparatus (49). To examine the ability of the CXCR3 ligands to induce actin reorganization, intestinal myofibroblasts were stimulated with CXCL9, CXCL10, or CXCL11 for various times, and cells were stained with TRITC-conjugated phalloidin, which binds specifically to polymerized actin. The chemokines induced a transient increase in actin polymerization in the form of stress fiber formation, which peaked 10 min after addition of the chemokines (Fig. 8A). In many cells, the small GTPases, Rho, Rac, and Cdc42, have key roles in regulating the morphology of migrating cells through effects on the actin cytoskeleton. There is an overlap in cellular functions regulated by Rac and class I PI3K, and PI3K can activate Rac indirectly by phosphatidylinositol 3,4,5-triphosphate-dependent Rac-guanosine nucleotide exchange factors (27). However, pretreatment of the cells with the PI3K inhibitor LY294002 had little or no effect on chemokine-stimulated responses (Fig. 8B). Similarly, the G<sub>q</sub> inhibitor PTX had little or no effect on CXCL9-, CXCL10-, or CXCL11-stimulated actin reorganization (Fig. 8B). RhoA-induced stress fiber formation in fibroblasts is associated with contractility (49, 50). RhoA activation induces changes in the actin cytoskeleton through a large number of downstream targets, of which the best characterized are the ROCKs. Pretreatment of intestinal myofibroblast cells with the ROCK inhibitor Y27632 markedly reduced chemokine-induced actin reorganization (Fig. 8B).

Discussion

In this study the expression of CXCR3 in intestinal myofibroblasts and various biochemical responses to the CXCR3 ligands were investigated. We were unable to detect any surface expression of CXCR3 in intestinal myofibroblasts, even though they express mRNA for this receptor. There was no detectable expression of mRNA for the variant CXCR3-B or CXCR3-alt. Nevertheless, these cells responded both biochemically and functionally to the CXCR3 ligands, as demonstrated by activation of the PI3K and ERK pathways and chemokine-stimulated actin reorganization. Additionally, CXCL11 was the only ligand capable of inducing calcium mobilization in intestinal myofibroblasts. There are several explanations for the apparent disparity between the presence of CXCR3 mRNA and the lack of detectable protein expression in intestinal myofibroblasts. Firstly, the receptor may be expressed on the cell surface, but below the sensitivity limits of the Ab used in

FIGURE 4. Chemokine-induced PKB phosphorylation in human intestinal myofibroblasts is PI3K dependent. Human intestinal myofibroblasts (A: 1 × 10<sup>6</sup>) or activated PBL (B: 1 × 10<sup>6</sup>) were left untreated or were treated with 10 μM LY294002 (30-min pretreatment). Cells were then stimulated with CXCL9 (upper panels), CXCL10 (middle panels), or CXCL11 (lower panels), all at 10 nM at 37°C for the times indicated. Cells were lysed by the addition of lysis buffer as described in Materials and Methods. Cell lysates (20 μg of protein) were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and immunoblotted with an antiphospho-PKB<sup>473</sup> Ab as described in Materials and Methods. The blots were stripped and reprobed with anti-PKB Ab to verify equal loading and efficiency of protein transfer. Data are representative of three separate experiments.
this study. Second, the receptor may be post-translationally modified in these cells, which might impede its recognition by the CXCR3 Ab. Indeed, some human chemokine receptors are reported to be sulfated and/or glycosylated at their N-terminal extracellular domains (51, 52). Third, CXCR3 could be expressed at the mRNA level, but the protein may either not be translated at all or be degraded post-translationally. In this instance, a novel receptor expressed on intestinal myofibroblasts may be responsible for the biochemical and functional responses observed. Finally, a combination of the above possibilities may explain the results described in this study.

The observation that only CXCL11 (not CXCL9 or CXCL10) stimulated a rapid and transient elevation in \([\text{Ca}^{2+}]_{i}\) was another surprising finding, especially because all three CXCR3 ligands have been reported to induce mobilization of intracellular \([\text{Ca}^{2+}]_{i}\) in activated T cells (53), glial cells (54), and primary podocytes (55). However, it is interesting to note that ligation of CXCR3-B does not result in elevation of intracellular calcium and also appears to be resistant to the \(G_{\alpha/\text{i}}\) inhibitor PTX (24), whereas CXCR3-mediated signaling has been found to be PTX sensitive (26). In intestinal myofibroblasts, PTX appeared to have no effect on CXCL10-mediated signaling, suggesting coupling of the receptor to G proteins other than \(G_{\alpha/\text{i}}\). PTX-insensitive heterotrimeric G proteins include members of the \(G_{12}\) and \(G_{q}\) families and the \(G_{i}\) family member \(G_{\alpha_{i}}\), although the latter is predominantly expressed in neurons and platelets (56).

The activation of PI3K/PKB, ERK, and p90RSK, which have been implicated in multiple biological functions and transcriptional events, fits well with the role of CXCR3 in regulating migration, proliferation, and angiostatic events in other settings. Interestingly, PKB, ERK, and p90RSK phosphorylation in intestinal myofibroblasts was more sustained compared with phosphorylation events observed in activated PBL. This may reflect differences in the expression of regulatory components such as phosphatases between the two cell types. The robustness and duration of the activation of a given signaling pathway have far-reaching biological consequences. For example, in T cells, persistent activation of PKB by CXCL12 through CXCR4 is thought to occur because CXCL12 and CXCR4 are involved in homeostasis rather than inflammation; sustained activation could protect CXCR4 cells from undergoing apoptosis (57). In contrast, CXCR3 ligands are up-regulated in the intestine under inflammatory conditions (32). Sustained PKB activation in intestinal myofibroblasts by these chemokines could, therefore, not just regulate and guide the migration of CXCR3-bearing myofibroblasts, but also have a protective role in apoptosis in the case of high local expression of CXCR3 ligands.

Regardless of the identity of the receptor(s) that binds to CXCL9, CXCL10, and CXCL11, it appears to be functional, because all three CXCR3 ligands were able to promote actin reorganization and stress fiber formation in intestinal myofibroblasts. Our findings indicate that the three CXCR3 ligands could profoundly influence the function of intestinal myofibroblasts in providing a contractile force within granulation tissue of healing wounds. Actin cytoskeleton reorganization is also important for cellular motility. This is best demonstrated in leukocytes, where binding of the chemokine to the receptor results in cytoskeleton rearrangement, integrin-mediated focal adhesion formation, and cell detachment from the substrate in a coordinated manner, with extension and retraction of pseudopods to execute the directional migration (46). However, myofibroblasts are not well characterized in terms of migratory responses. In fact, there is only one report about intestinal myofibroblast migration, which demonstrated that platelet-derived growth factor, insulin-like growth factor, epidermal growth factor,
and TGF-β1 stimulate the modest migration of these cells (58). The identification of additional migration-inducing factors is required to elucidate the network of interactions and complex mechanisms involved in intestinal wound healing. CXCR3 ligand-induced actin polymerization in intestinal myofibroblasts is strongly indicative of a role for these chemokines in this process.

It is now well established that the dynamics of actin cytoskeleton are closely regulated by the activation of members of the Rho GTPase family, including RhoA and Rac1, and their activities control cell migration and adhesion. Rho, in particular, has been associated with stress fiber formation and cell contractility (50). Several proteins have been isolated as putative Rho effectors on the basis of their selective interaction with GTP-bound Rho. Among them, the ROCK family of kinases has been shown to be involved in Rho-induced formation of actin stress fibers and focal adhesions (59, 60). In agreement with this, pretreatment of intestinal myofibroblasts with Y27632 abolishes chemokine-induced actin polymerization and stress fiber formation. Regulation of actin polymerization, downstream from G protein-coupled receptors, is thought to depend on PI3K activation and involves the activation of PKB and the GTPases Cdc42 and Rac2 (61). Recently an alternative, PI3K-independent mechanism for actin polymerization...
Results are from single experiments representative of three replicate experiments.

In human neutrophils has been proposed (62). The PI3K-independent pathway in neutrophils is dependent on Src tyrosine kinases, NADPH oxidase and protein kinase A, as well as RhoA and ROCK. Our findings tend to support the existence of a PI3K-independent pathway, because LY294002 did not appear to have any effect on CXCL9-, CXCL10-, or CXCL11-induced actin polymerization. Actin polymerization is also PTX insensitive in intestinal myofibroblasts. Most of the G protein-coupled receptors agonists shown to activate Rho are thought to be coupled to G12-mediated signaling pathways (63). However, there is evidence to suggest that G proteins of the PTX-insensitive G12/13 family are also able to control Rho-dependent stress fiber formation (64), in agreement with our data indicating PTX-insensitive signaling pathways.

The data presented in this study suggest that although intestinal myofibroblasts do not express detectable surface expression of CXCR3, they may express either a modified or variant CXCR3 receptor with different signaling properties and pharmacological profiles compared with the CXCR3 expressed on T cells. The existence of multiple ligands and CXCR3 receptor forms is exciting because it allows for heterogeneity in signaling and function, although this makes a one-fits-all design of a CXCR3 antagonist rather more difficult. In contrast, given the role of myofibroblasts in fibrosis and stricture formation during chronic inflammatory diseases, the potential for tailored receptor responses to CXCL9, CXCL10, and CXCL11 via one or more receptors may provide exciting alternative therapeutic opportunities for future drug intervention in intestinal inflammatory diseases such as CD.

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Disclosures

The authors have no financial conflict of interest.

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