CXCL12 activation of CXCR4 regulates mucosal host defense through stimulation of epithelial cell migration and promotion of intestinal barrier integrity

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Smith, Jennifer M., Priscilla A. Johanesen, Michael K. Wendt, David G. Binion, and Michael B. Dwinell. CXCL12 activation of CXCR4 regulates mucosal host defense through stimulation of epithelial cell migration and to determine its impact on barrier integrity. CXCL12 stimulated the dose-dependent chemotactic migration of human T84 colonic epithelial cells. Epithelial cell migration was inhibited by CXCR4 neutralizing antibody, pertussis toxin, LY-294002, and PD-98059, thereby implicating Gαi, phosphatidylinositol 3-kinase (PI3-kinase), and the ERK1/2 MAP kinase pathways in CXCR4-specific signaling. CXCL12 was also shown to increase barrier integrity, as defined by transepithelial resistance and paracellular flux across differentiating T84 monolayers. To determine whether CXCL12 regulated epithelial restitution, we used the normal nontransformed intestinal epithelial cell-6 (IEC-6) wound healing model. By using RT-PCR, immunoblot analysis, and immunofluorescence microscopy, we first showed expression of both CXCR4 and its cognate seven transmembrane G protein-coupled receptor CXCR4 (2, 53). CXCL12 is involved in the proliferation of hematopoietic stem cells, movement of maturing thymocytes from the thymus, and inhibition of human immunodeficiency virus (HIV)-1 infection through occupancy of its receptor (2, 39, 51). A further role for CXCL12 in the trafficking of metastatic breast, lung, ovarian, and kidney carcinoma cells has also been demonstrated (43, 45, 48, 55, 56). Notably, mice lacking the CXCL12 gene die perinatally of marked defects in gastrointestinal vascularization, hematopo- and myelopoiesis, and incomplete cardiac septum formation (44). Similar phenotypic defects were noted in mice lacking the CXCR4 gene (59), indicating that this ligand-receptor couple comprises a monogamous signaling unit in vivo.

The single layer of epithelial cells lining the gastrointestinal mucosal surface comprises a physical barrier between the external luminal environment and the internal milieu and is a key component of the mucosal innate immune system. Defects in barrier integrity, a principal pathophysiological feature of mucosal inflammatory diseases, result in increased permeability and, in turn, permit the unrestricted entry of potentially harmful lumen contents (27, 49). Mucosal barrier integrity is preserved through precise epithelial cell migration, a process critically involved in intestinal morphogenesis, enterocyte turnover, epithelial ulceration repair, and adenocarcinoma metastasis (11, 30, 31, 41, 60, 61). In addition, the cells of the intestinal epithelium express receptors for chemokines, cytokines, antimicrobial peptides, and growth factors present within the mucosal milieu (9, 20, 21, 27, 35, 37, 50). These receptors convey signals to regulate genes and cellular effectors important in epithelial functions and innate host defense. We and others (9, 20, 36) have demonstrated the expression of several chemokine receptors, notably CXCR4, CCR5, CCR6, and CX2CR1, by the cells of the human intestinal epithelium, suggesting that cells of the mucosal barrier are likely targets for chemokine signals. Previous studies, including our own (9, 20, 22, 36), indicate that CXCR4 localized to the cell surface of cultured human intestinal epithelial cells activate G proteins, modulate electrogenic ion transport, and upregulate expression of neutrophil chemoattractants and intercellular adhesion molecule-1.

Studies in mice (5, 25, 31, 60, 61) indicate that enterocyte maturation is a multifactorial process requiring, at least in part, tight regulation of epithelial cell migration from the proliferative...
tive zone within the intestinal crypt. Furthermore, enterocyte migration is also a critical process in mucosal wound repair and reestablishment of barrier integrity through epithelial restitution, the rapid and directed migration of cells across the denuded mucosal surface (42, 46, 49). A role for chemotactic migration in the process of epithelial cell migration, barrier formation, and restitution has previously been suggested (3). Because the chemokine CXCL12 and its receptor CXCR4 are critical to normal development and differentiation of embryonic gut tissues and are prominent mediators of immune surveillance, we sought to define the role for these molecules in immune regulation of the epithelial mucosal barrier. We demonstrate herein that CXCL12 activation of CXCR4 stimulates migration of intestinal epithelial cells, maturation of barrier integrity, and restitution of wounded epithelium. These data support the hypothesis that activation of epithelial CXCR4 by its ligand plays a role in constitutive and inducible mucosal innate immunity through regulation of epithelial barrier defense.

MATERIALS AND METHODS

Cytokines. Recombinant human CXCL12 was generated by using an approach analogous to that defined previously (47). Nuclear magnetic resonance spectroscopy was used to confirm purity, folding, and integrity of our recombinant protein. Studies indicated our recombinant CXCL12 was comparable to commercially available chemokine obtained from PeproTech (Rocky Hill, NJ) in inhibiting adenyl cyclase-mediated production of cAMP (22). Recombinant human transforming growth factor (TGF)-β1 and EGF were purchased from R&D Systems (Minneapolis, MN) and TNF-α was from PeproTech.

Cell culture. The human T84 colon carcinoma cell line (14) was cultured in DMEM/Ham’s F-12 medium (1:1) supplemented with 5% (vol/vol) newborn calf serum and 2 mM L-glutamine as described previously (22). The normal rat small intestinal epithelial cell-6 (IEC-6) cell line (CRL-1592) (52) was purchased from the American Type Culture Collection (ATCC; Rockville, MD) and cultured in DMEM (4 g/l glucose) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 1.5 g/l NaHCO3, and 0.1 U/ml bovine insulin (Invitrogen, Carlsbad, CA). U937 myelomonocytic leukemia cells were supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, L-glutamine, 1.5 g/l NaHCO3, and 0.1 U/ml bovine insulin (Invitrogen, Carlsbad, CA). U937 myelomonocytic leukemia cells were cultured which was then shaken for 5 h at 37°C to allow recycling of protein. Following the initial 5-h incubation, cells were harvested by centrifugation and washed once with PBS/BSA at 4°C.

RT-PCR. Total cellular RNA was isolated from T84 and IEC-6s using TRIZol reagent (Invitrogen) and treated with RNAase-free DNase (Ambion, Austin, TX) to digest genomic DNA. Complementary DNA was synthesized from 1 μg of total cellular DNA using 5 μg/ml oligo(dT)12–18 (Amberson Biosciences, Piscataway, NJ) and 2.5 U Superscript-II reverse transcriptase (Invitrogen) in a final volume of 2 ml containing 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 0.5 μg/ml of oligo(dT)12–18, and 2.5 U Superscript-II reverse transcriptase (Invitrogen).

Immunoblot analysis. To define CXCR4 protein expression and cellular localization, IEC-6 and T84 cells were solubilized in hypotonic lysis buffer [50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, and Protease Inhibitor Cocktail Set III (EMD CalBiochem, San Diego, CA)]. Lysates were passed through a 25-gauge needle and centrifuged at 550 rpm for 5 min at 4°C to pellet nuclei. An aliquot of the postnuclear supernatants was reserved as the total cellular protein, and the remaining supernatants were ultracentrifugated at 40,000 rpm for 45 min at 4°C (rotor model TLA100.3; Beckman). Supernatants containing the cytosolic protein fraction were transferred to prechilled tubes, and the pellets, containing the membrane protein fraction, were resuspended in PBS supplemented with protease inhibitors. Protein concentrations were determined by using the BCA protein assay kit (Pierce Biotechnology, Rockford IL) and 10 μg of protein were size separated by using reducing SDS-PAGE. Proteins were electrophoretically transferred to PVDF (Immobilon-P, Millipore, Bedford, MA) for immunoblot analysis as detailed previously (22, 29). Equal protein loading was confirmed by Coomassie blue staining.

For detection of ERK1/2, confluent T84 monolayers were serum-starved overnight in DMEM/F-12 medium and stimulated with CXCL12 as indicated. Thirty-minute pretreatment with 2 μM PD-98059, a specific inhibitor of the MEK kinase, or vehicle (DMSO), was followed by incubation with 10 ng/ml CXCL12. Cells were lysed in ice-cold modified RIPA buffer [50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.25% (wt/vol) sodium deoxycholate, 1% (vol/vol) Igepal CA-630, 0.1% (wt/vol) sodium dodecylsulfate containing Protease Inhibitor Cocktail III and sodium orthovanadate (10 mM), sodium fluoride (20 mM), and β-glycerophosphate (40 mM)]. Equal protein levels of each sample were analyzed by using reducing SDS-PAGE and transferred to PVDF membrane for immunoblot analysis (29). Blots were incubated with rabbit anti-human phospho-ERK1/2 antibody (Cell Signaling Technology, Beverly, MA) followed by horseradish peroxidase-conjugated donkey anti-rabbit Ig (Amersham Biosciences). After being detected, blots were stripped and reprobed with a rabbit anti-human phospho-ERK1/2 antibody (Cell Signaling) to establish levels of ERK1/2 protein.

Immunofluorescence microscopy. IEC-6 cells plated to glass chamber slides (Labtek; Nalge Nunc, Naperville, IL), were grown to ~70% confluence, and fixed 20 min with ice-cold 4% paraformaldehyde. After two rinses in PBS, fixed cells were blocked overnight in PBS containing 0.1% (wt/vol) saponin (Sigma), 1% (wt/vol) BSA, and 1% (vol/vol) donkey serum at 4°C. On removal of the block solution, cells were incubated with 5 μg/ml of either protein A-purified rabbit anti-mouse CXCL12 (Torrey Pines Bioloabs) or rabbit IgG control antibody. After three washes with PBS containing 0.1% saponin, all cells were incubated with biotin-labeled donkey anti-rabbit antibody (Amersham Biosciences) followed by Alexa Fluor 594-streptavidin conjugate, and were then rinsed and stained with the nuclear dye DAPI. The cells were washed, cover-slipped, and viewed in a LeicaWild microscope (Wetzlar, Germany) with a 40× objective (Vector Laboratories). CXCL12 and IgG images were captured at 500-ms exposure and overlaid with 50-ms exposed DAPI images.

Chemoattractant assay. T84 cells were treated with 0.05% trypsin/0.53 mM Na2EDTA (Invitrogen) to generate a nonadherent single-cell culture, which was then shaken for 5 h at 37°C to allow recycling of CXCR4 to the cell surface (2). T84 cells resuspended to 5 × 106 cells/ml in PBS plus 0.1 mg/ml BSA (PBS/BSA) were incubated with 2 μM calcine AM (Molecular Probes, Eugene, OR) at 37°C for 30 min, during which time the hydrolysis of intracellular calcine AM and or for CXCL12, 35 cycles of 94°C for 30 s, 53°C for 45 s, 72°C for 45 s and yielded PCR products of 197 bp and 125 bp, respectively. RNA isolated from human intestinal microvascular endothelial cells (6) or rat liver was used as positive controls for CXCL12 and CXCR4 mRNA expression. As negative controls, PCR reactions without cDNA (H2O) and reverse transcription reactions without RNA (no RNA) were performed. Aliquots of each PCR reaction were analyzed by gel electrophoresis.

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forms a fluorescent product. After washes in PBS/BSA, calcein AM-loaded cells were resuspended in serum-free, phenol red-free DMEM/F-12 medium supplemented with 0.1 ng/ml BSA. A chromo-
tactic gradient was created by adding titrated doses of CXCL12 diluted in phenol red-free DMEM/F-12 medium plus 0.1 ng/ml BSA at 29 µl/well to a disposable ChemToX instrument (8-µm pore size, 3.2-mm diameter; Neuro Probe, Gaithersburg, MD). After the framed filter on the ChemToX plate was secured, 25 µl of calcein AM-loaded cells were placed on the filter over each well and incubated for 2 h at 37°C. The top surface of the filter was gently washed with PBS to remove nonmigrated cells and then centrifuged at 1,000 rpm for 2 min to dislodge migrated cells adherent to the underside of the filter. After the filter was removed, fluorescence was measured in a plate reader using 485-nm excitation and 535-nm emission wavelengths. Each condition was assessed in triplicate chemotaxis plate wells. The number of migrated cells was determined from a standard curve of calcein AM-labeled cells.

To define receptor specificity in epithelial cell chemotaxis, T84 cells were pretreated for 30 min with 5 µg/ml murine monoclonal neutralizing antibody to CXCR4 (clone 12G5, BD Biosciences PharMingen, San Diego, CA) or, as a control, 5 µg/ml murine monoclonal IgG3, (BD Biosciences PharMingen). To define the role of Gαi-proteins coupled to the receptor, T84 cells were incubated with 100 ng/ml pertussis toxin (CalBiochem) for 12 h before and during the surface CXCR4 recycling, cell labeling, and assay periods. In separate studies, the participation of the phosphatidylinositol 3-kinase (PI3- kinase) and ERK1/2 signaling pathways in CXCL12-stimulated epi-
thelial cell migration was assessed. For those experiments, calcein AM-labeled T84 cells were treated 30 min at 37°C with either the PI3-kinase-specific inhibitor LY-294002 (IC50 = 1.4 µM) or the specific MEK inhibitor PD-98059 (IC50 = 2 µM; CalBiochem) before placement on the filter.

Transepithelial resistance and paracellular permeability. To assess the role for CXCL12-stimulated migration in regulating barrier integrity, T84 cells were seeded to 12-mm mixed cellulose ester filter inserts (Millicell-HA; Millipore) and stimulated with 10 ng/ml CXCL12, or, as a control, 5 ng/ml TGF-β1, in both the apical and basolateral insert chambers in triplicate wells. Unstimulated cells were used as a negative control. A hand-held Millicell-ERS volt ohmmeter (Millipore) was used to measure resistance at three reproducible locations on each insert. Basal resistance of the filter and medium alone were subtracted from experimental values, and transepithelial resistance (TER) was calculated and presented as Ohms by centimeter squared (Ω·cm²).

Paracellular permeability of 4 kDa FITC-dextran was measured in T84 cells grown on filter inserts. TER was measured before the addition of 4 kDa FITC-dextran at a final concentration of 0.2 mM (10) to the apical insert compartment. After 4 h of incubation, aliquots of conditioned medium were removed from both chambers and transferred to a 96-well microtiter plate. Fluorescence was measured in a plate reader using 485-nm excitation and 535-nm-emission wave-

lengths and compared with a standard curve of known concentrations of 4 kDa FITC-dextran. Paracellular permeability was calculated as picomoles per hour by square centimeters surface area, normalized to unstimulated controls and expressed as percent change from control.

Proliferation assays. Two complementary approaches were used to evaluate epithelial cell proliferation. Epithelial cell viability was measured by using the colorimetric MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazo-
mol salt] assay (Promega, Madison, WI), whereas DNA synthesis was measured by using [3H]thymidine incorporation. For the MTS assay, 5 × 10⁴ T84 cells were plated in serum-containing growth medium to a 96-well tissue culture plate and allowed to adhere for 24 h before addition of titrated doses of CXCL12. Cells remained untreated or were treated for 5 days with 10 ng/ml EGF as negative and positive controls, respectively. Growth medium was refreshed with serum-free medium containing the appropriate stimulus after an initial 2-day incubation. After a final 3-day incubation, 20 µl CellTiter Aqueous One Solution (Promega) was added to each well, the plates were incubated for 4 h at 37°C in 5% CO₂, and absorbance at 490 nm was assessed.

For [3H]thymidine incorporation, 1 × 10⁵ T84 cells or separately, 1 × 10⁶ IEC-6 cells were seeded to 12-well plates and cultured overnight at 37°C and 5% CO₂. The cells were then serum-starved for 16 h before addition of titrated doses of CXCL12 or, as a control, 10 ng/ml EGF to quadruplicate wells. After 12 h of stimulation at 37°C, 1 µCi [3H]thymidine/well (Amersham Biosciences) was added and the cells were incubated for an additional 6 h. Subsequently, cells were washed with ice-cold PBS, incubated in 5% (wt/vol) TCA for 10 min on ice, and solubilized in 1.0 ml 0.5 N NaOH. An 800-µl aliquot of each sample was added to scintillation fluid, and radioactivity was counted in a Beckman LS-6000TA liquid scintillation counter.

Wounding assay. Confluent IEC-6 cell monolayers grown in 60-mm dishes were incubated overnight in serum-free medium and subsequently wounded with a sterile razor blade as described previ-
ously (16, 33, 42). Wounded monolayers were washed twice with PBS before incubation with serum-free medium alone, 10 ng/ml CXCL12, or, as a control, 5 ng/ml TGF-β1 for 24 h at 37°C in 5% CO₂. To assess the CXCL12 signaling mechanisms, monolayers were wounded and unstimulated controls or 10 ng/ml CXCL12-treated cells were incubated with pertussis toxin (100 ng/ml), LY-294002 (20 µM), or PD-98059 (2 µM), specific inhibitors of Gαi, PI3-kinase, and ERK1/2, respectively. Photomicrographs at ×100 magnification were taken at 4–5 locations per wound at 0 and 24 h. The number of migrated cells was determined by counting cells that crossed the wound edge.

Statistical analysis. Differences between unstimulated control and experimental samples were analyzed by unpaired Student’s t-test using SigmaStat (Jandel Scientific Software, San Rafael, CA). Statistical significance was defined as P < 0.05.

RESULTS

CXCL12 stimulates chemotaxis of T84 intestinal epithelial cells. Human intestinal epithelial cells in vitro and in vivo express the chemokine receptor CXCR4 (20, 36). CXCL12 has been reported to stimulate migration of CXCR4-expressing hematopoietic stem cells and mature leukocytes, as well as carcinoma cells (7, 43, 48, 51, 55, 56). Embryonic lethality in mice genetically deficient in CXCL12 and CXCR4 prevents assessment of the signaling and function of this ligand-receptor pair in the intestinal mucosa in vivo. Therefore, we used a culture model of the intestinal epithelium to test the hypothesis that CXCL12 regulates intracellular signals to modulate intes-
tinal epithelial cell migration and barrier maturation. To define the ability of CXCL12 to stimulate chemotactic migration in intestinal epithelial cells, we used the T84 human colonic epithelial cell line that we (22) previously demonstrated to signal through CXCR4 and regulate epithelial cell functions.

To determine whether the CXCR4 ligand could stimulate chemotaxis, T84 epithelial cells were incubated with titrated doses of CXCL12 in modified Boyden chambers. As shown in Fig. 1A, the 1, 10, and 100 ng/ml doses of CXCL12 stimulated T84 cell migration with a maximal chemotactic response observed at the 10 ng/ml chemokine concentration. Migration was not restricted to T84 epithelial cells, because a similar CXCL12 dose response was observed in leukemic U937 cells used as a positive control, with a comparable maximal re-
sponse at 10 ng/ml (Fig. 1B). In addition, the structurally related chemokine CXCL8/IL-8, whose receptor is not ex-
pressed by T84 cells (20), failed to stimulate T84 chemotaxis.


Together these data indicate T84 colonic epithelial cells migrate in response to the homeostatic chemokine CXCL12. CXCL12-induced chemotactic migration occurs via the G_{i} coupled receptor CXCR4. CXCL12 engagement of CXCR4 expressed by human T84 epithelial cells and leukocytes activates the G_{i} subunit of the heterotrimeric G protein complex (22, 53). To determine the receptor specificity and involvement of G proteins in the CXCL12-stimulated chemotactic response of T84 cells, we analyzed migration in the presence of monoclonal CXCR4-neutralizing antibody, clone 12G5. Cells were incubated with 10 ng/ml of CXCL12, the chemokine concentration shown to be optimal for inducing migration. As shown in Fig. 2A, CXCR4 neutralization with 5 μg/ml 12G5 completely reversed CXCL12-induced chemotaxis. Migration of cells treated with CXCL12 and incubated with an isotype-matched control, or, separately, and irrelevant monoclonal antibody were not similarly inhibited (Fig. 2A), suggesting that T84 migration required engagement of its sole cognate receptor CXCR4. We then investigated the requirement of G_{i} activation in intracellular signaling during CXCL12-stimulated T84 cell chemotaxis. As shown in Fig. 2B, CXCL12-stimulated epithelial cell migration was abolished in cells pretreated with 100 ng/ml pertussis toxin, a specific inhibitor of G_{i} proteins. Epithelial cell chemotaxis in chemokine and pertussis toxin-treated cells was not significantly different from cell movement observed in those cells treated with pertussis toxin alone, or untreated control cells (Fig. 2B). These data implicate G_{i} protein activation in signal transduction from CXCR4 resulting in intestinal epithelial cell migration and agree with our prior report of CXCL12 signaling in T84 cells (22).

PI3-kinase signaling is required for CXCL12-directed T84 cell migration. After engagement of ligand to receptor, G_{i}, as well as its concordant G_{βγ}-subunits, is known to directly activate the heterodimeric p85/p100 α-PI3-kinase and p100/p110 γ-PI3-kinase signaling elements (26, 40). Moreover, mice genetically deficient in the p110 subunit are characterized by a pronounced decrease in leukocyte migration, suggesting a critical role for PI3-kinase in that process (34). Those in vivo conclusions are supported by several in vitro studies, including our prior analyses of human intestinal microvascular endothelial cell migration and endothelial tube formation (28, 29). Therefore, we hypothesized that PI3-kinase signaling would play a key role in CXCL12-induced T84 cell migration. As shown in Fig. 2C, T84 cells treated with 20 μM LY-294002, a PI3-kinase inhibitor, significantly diminished CXCL12-stimulated chemotaxis.

Fig. 1. CXCL12 stimulates chemotaxis of T84 intestinal epithelial cells. A nonadherent, single-cell suspension of calcein AM-labeled T84 cells (A) or, as a control, U937 cells (B) was added to a chemotaxis plate containing the indicated doses of CXCL12 in the bottom wells. Migration through the filter was determined after 2-h incubation. Each of the 1, 10, and 100 ng/ml doses of CXCL12 stimulated cell migration (A; gray bars), with the 10 ng/ml dose significantly increasing chemotaxis. This chemokine dose was similarly maximal for U937 migration (A; black bar). Values are means ± SE of 3–5 independent experiments. *Significant difference from unstimulated control (P < 0.05).

Fig. 2. CXCR4 linked to G_{i} and phosphoinositide 3-kinase (PI3-kinase) signals CXCL12-induced chemotactic migration. Calcein AM-labeled T84 cells were incubated with 5 μg/ml CXCR4 neutralizing antibody (clone 12G5), or isotype-matched (IgG2a), or, separately, and irrelevant monoclonal control antibody (A), 100 ng/ml of the specific G_{i} inhibitor pertussis toxin (PTx) (B), or 20 μM of the PI3-kinase inhibitor LY-294002 (C) before addition to a chemotaxis plate containing medium alone (Ctrl) or 10 ng/ml CXCL12. CXCR4 neutralization, G_{i}, and PI3-kinase inhibition significantly diminished CXCL12-stimulated chemotaxis. The number of migrating T84 cells was calculated, and the fold change from unstimulated control migration was determined. Values are means ± SE of 3–5 independent experiments. *Significant difference from unstimulated control; **significant difference from CXCL12-stimulated cells (P < 0.05).
specific inhibitor of both α- and γ-PI3-kinase isoforms (26), significantly inhibited T84 cell migration evoked by CXCL12. PI3-kinase inhibitor or irrelevant controls had no impact on epithelial cell chemotaxis, collectively indicating a key role for PI3-kinase signaling in CXCL12-directed epithelial cell migration.

**CXCL12-stimulated ERK1/2 phosphorylation is required for T84 cell chemotaxis.** In addition to activation of Gαi proteins, CXCL12 binding to CXCR4 has been shown to stimulate other signal transduction cascades, including the ERK1/2 pathway, a signaling intermediate linked to the regulation of leukocyte, endothelial cell, and epithelial cell locomotion (15, 28, 29, 38, 54, 58). To determine whether CXCL12 was able to stimulate ERK1/2 phosphorylation in human intestinal epithelial cells, immunoblot analysis was performed on whole cell lysates from chemokine-stimulated T84 cells. Increased phosphorylation of ERK1/2 proteins was evident 1 min after stimulation with 10 ng/ml CXCL12, with maximal phosphorylation achieved and subsequently sustained after 5-min stimulation (Fig. 3A). In contrast, CXCL12 did not stimulate phosphorylation of p38 MAPK or JNK/SAPK in human T84 colonic epithelial cells (not shown). Pretreatment of T84 cells with 2 μM PD-98059, a MEK-specific inhibitor, abolished ERK1/2 phosphorylation in response to either CXCL12 or TNF-α used as a control (Fig. 3B). Immunoblot analysis with antibodies to total ERK1/2 proteins verified equal protein loading (Fig 3, A and B).

Having demonstrated CXCL12-stimulated phosphorylation of ERK1/2 in T84 cells, we then evaluated the role for that signaling intermediate in intestinal epithelial cell chemotaxis. T84 cells were incubated with 2 μM PD-98059 before measurement of migration. As shown in Fig. 3C, the inhibitor of ERK1/2 phosphorylation markedly decreased CXCL12-induced migration compared with T84 cells stimulated with chemokine alone. Although this dose of PD-98059 was sufficient to prevent ERK1/2 phosphorylation (Fig. 3B), it did not completely abolish the migratory response (Fig. 3C). Nonetheless, these data suggest that the ERK1/2 signaling pathway is one of the key participants in intestinal epithelial cell migration in response to CXCL12. In agreement with our phosphospecific immunoblot analysis, we found that the specific p38 MAPK inhibitor SB-203580 had no effect on T84 chemotaxis induced by CXCL12 (not shown).

**CXCL12 modulates intestinal epithelial barrier maturation.** ERK1/2 signaling, in addition to its role in cell migration, has a documented role in tightening the epithelial barrier in the T84 cell model (37). Furthermore, studies in transgenic mice examining intestinal crypt-villus development demonstrate that intestinal barrier maturation is tightly linked with cellular cell migration (5, 25, 30, 31, 60, 61). Therefore, we then examined intestinal epithelial differentiation in response to CXCL12 stimulation by measuring development of epithelial barrier function using T84 cells. Those cells spontaneously differentiate into a polarized monolayer when cultured on semipermeable filter inserts (14). Two complementary measures of barrier function, TER and paracellular permeability of an inert marker, 4 kDa FITC-dextran, were assessed. As shown in Fig. 4A, TER was significantly increased with a concomitant decrease in paracellular permeability after 3, 5, and 7 days of CXCL12 addition. CXCL12-treated cell monolayers also more rapidly reached the hallmark electrical resistance of 1,000 Ω·cm², an indicator of a fully developed T84 epithelial cell barrier, and maintained an elevated TER compared with untreated control monolayers over time (Fig. 4B).

We then determined whether the observed barrier tightening reflected CXCL12 engagement to its sole cognate receptor CXCR4. Consistent with our studies of T84 chemotaxis, we found that 5 μg/ml of the neutralizing CXCR4 antibody clone 12G5 prevented CXCL12-induced changes in paracellular flux (Table 1). Isotype control or irrelevant monoclonal antibodies were significantly less effective in blunting CXCL12’s ability to decrease apical-to-basolateral transit of 4-kDa FITC-dextran. Similarly, the TER of those maturing T84 monolayers was modulated in a reciprocal fashion (not shown), together suggesting a role for CXCL12 binding to CXCR4 in magnifying T84 barrier integrity.

Those data suggested a role for CXCL12-stimulated migration in strengthening of the barrier in differentiating epithelial monolayers. As additional support, we then asked whether TGF-β1, a cytokine with a well-documented role in stimulating epithelial cell migration in vitro and barrier maturation in vivo (5, 16, 17), could modulate barrier establishment in T84 cells as we had shown for CXCL12. Consistent with that hypothesis,
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Table 1. CXCL12 enhances barrier formation by binding to its cognate receptor CXCR4

<table>
<thead>
<tr>
<th>Antibody</th>
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<tr>
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</tr>
<tr>
<td>Anti-CXCR4</td>
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</tr>
<tr>
<td>Isotype control</td>
<td>2,646 ± 1080</td>
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<tr>
<td>10 ng/ml CXCL12</td>
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<tr>
<td>1 ng/ml CXCL12</td>
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Values are means ± SD of 4 kDa FITC dextran in picomoles per hour by centimeter surface area of replicate monolayers from a representative of 3 separate experiments. Unstimulated or CXCL12 (10 ng/ml)-stimulated T84 cells remained untreated as a control (none) or were incubated with either 5 μg/ml neutralizing anti-CXCR4 antibody (clone 12G5) or 5 μg/ml of the irrelevant monoclonal isotype control antibody (IgG2a). Paracellular permeability was assessed on day 5 after plating to cellulosic ester inserts. *Significant differences between unstimulated, no antibody control cells, and T84 cells incubated with CXCL12 alone or with anti-CXCR4 neutralizing antibody (P < 0.05). ND, not determined.

Table 2. Results from T84 cell proliferation assays following CXCL12 stimulation

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>MTS, A490nm</th>
<th>[3H]thymidine, cpm</th>
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<tbody>
<tr>
<td>None</td>
<td>0.71 ± 0.05</td>
<td>2,297.3 ± 239</td>
</tr>
<tr>
<td>CXCL12 (1 ng/ml)</td>
<td>0.76 ± 0.04</td>
<td>2,600.6 ± 347</td>
</tr>
<tr>
<td>CXCL12 (10 ng/ml)</td>
<td>0.71 ± 0.04</td>
<td>2,122.3 ± 289</td>
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<tr>
<td>CXCL12 (100 ng/ml)</td>
<td>0.72 ± 0.07</td>
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<tr>
<td>EGF (10 ng/ml)</td>
<td>0.83 ± 0.03*</td>
<td>7,971.3 ± 865*</td>
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</table>

Values are means ± SD of 3 wells from representative experiments. MTS assay, n = 3; [3H]thymidine incorporation, n = 3. *Significant difference between EGF-treated cells or control unstimulated or CXCL12-stimulated cells (P < 0.05). MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt.

as shown in Fig. 4A, 5 ng/ml TGF-β1 was an equally potent stimulator of intestinal epithelial cell barrier maturation. Thus treatment of T84 cells with TGF-β1 induced an increase in TER and a decrease in paracellular permeability comparable to cells stimulated with CXCL12. Taken together, these data suggest that epithelial cell migration induced by CXCL12 or TGF-β1 plays a role in strengthening barrier development.

T84 cells do not proliferate in response to CXCL12. Because CXCL12 has been shown to increase proliferation of hematopoietic progenitor (39) and endothelial cells (29), we then sought to determine whether the CXCL12-stimulated increase in barrier integrity reflected enhanced cell locomotion or proliferation. To distinguish this possibility, we used the MTS assay of cell viability and [3H]thymidine incorporation to determine cell proliferation in T84 cells treated with CXCL12. As shown in Table 2, CXCL12 stimulation did not modulate T84 cell growth, whereas EGF, used as a positive control, significantly increased cell proliferation. These data indicate that the tightening of the T84 epithelial barrier, as assessed by TER and paracellular permeability, is not a function of cell proliferation. Taken together, these data suggest that the strengthened barrier formation noted in T84 cells resulted from enhanced cell migration induced by CXCL12.

Normal rat intestinal epithelial IEC-6 cells express CXCR4 and CXCL12. Mucosal wound repair parallels homeostatic barrier maturation and requires the sequential processes of migration/restitution, proliferation, and maturation of epithelial cells at the site of injury. Thus we then sought to test the hypothesis that CXCL12 stimulates epithelial restitution. Furthermore, we sought to determine whether the CXCL12 migration response was a function of the transformed nature of the T84 colon carcinoma cell line. For these studies, we focused on the normal nontransformed rat IEC-6 epithelial cells, because they are a well-established model epithelium used extensively to define mechanisms regulating intestinal epithelial cell migration and restitution (15–17, 33, 42). Before testing our hypothesis, we first used RT-PCR analysis to assess chemokine receptor and ligand expression by IEC-6 cells. As shown in Fig. 5A, IEC-6 epithelial cells constitutively expressed CXCR4 and CXCL12 mRNA. Consistent with our
Fig. 5. Normal rat intestinal epithelial cells-6 (IEC-6) express CXCR4 and CXCL12. A: CXCR4 and CXCL12 mRNA expression. cDNAs reverse transcribed from IEC-6 and T84 intestinal epithelial cells were amplified in PCR reactions using sequence-specific primers for CXCR4, CXCL12, and β-actin. PCR reactions containing no cDNA (H2O), no RNA, or rat liver or human intestinal microvascular endothelial cell cDNA were used as negative and positive controls (Ctrl), respectively. CXCR4 and CXCL12 mRNA expression was observed in normal nontransformed rat IEC-6 cells. Lysates from IEC-6 cells were separated into membrane (M), cytosolic (C), and total (T) protein fractions by ultracentrifugation. Immunoblot analysis demonstrated both membrane and cytosolic expression of CXCR4 protein in IEC-6 cells. Lysates from T84 epithelial cells were used as positive controls. Data shown are representative of lysates from 3 separate experiments. B: CXCR4 protein expression. 

CXCL12 stimulates epithelial migration and wound repair in IEC-6 cell monolayers. To determine whether IEC-6 cells migrate in response to CXCL12 stimulation, we used a well-characterized in vitro cell wounding model (17, 42). Confluent IEC-6 cell monolayers were wounded and subsequently incubated with CXCL12 (Fig. 5B), indicating that those cells may be functional targets for CXCL12. CXCL12 mRNA expression in IEC-6 cells was paralleled with intracellular protein localization, as demonstrated by using immunofluorescence microscopy. CXCL12 protein was localized to intracellular organelles in permeabilized IEC-6 cells (Fig. 5C). Lack of immunofluorescence was noted in matching cultures immunostained with the isotype control monoclonal antibody (Fig. 5D). Taken together, these data indicate that normal nontransformed epithelial cells parallel the in vivo human colonic epithelium in CXCR4 and CXCL12 mRNA and protein expression (1).

DISCUSSION

CXCR4 and its sole cognate ligand CXCL12 have a documented role in vascularization of the developing gastrointestinal tract and in myelopoiesis and hematopoiesis as well as embryonic development and are the only chemokine receptor or ligand shown to be critical for life (44, 62). The CXCL12-CXCR4 signaling axis is thought to be an essential regulator of immune surveillance via the directed trafficking of leukocytes throughout the body (53). We show herein that CXCL12,
through engagement of cell surface-localized CXCR4, stimulates intestinal epithelial cell migration and enhances the integrity of the mucosal barrier. These findings are commensurate with a key role for the CXCL12-CXCR4 signaling axis in regulating the fundamental innate barrier protection function of the gastrointestinal mucosal epithelium.

Our investigation using the T84 colonic epithelial cell line demonstrates that CXCL12 stimulated chemotactic migration in a dose-dependent manner with a maximal motogenic response observed at 10 ng/ml of chemokine. These data agree closely with our prior report (22) indicating that a comparable CXCL12 dose effectively activates Goi proteins and modulates electrogenic ion transport in T84 cells. Furthermore, the optimal concentration for T84 chemotaxis parallels our previous findings (29) defining angiogenesis regulation by CXCL12 in the human intestinal microvascular endothelium. The optimal chemotactic dose we observed is within the characterized range of CXCL12 serum concentrations reported for normal healthy humans (13). The dose-response curve observed for epithelial cell migration agrees with data shown by others for CXCL12-induced chemotaxis, with a similar optimal concentration for migration of varying CXCR4-expressing leukocyte populations (43, 48, 51). Differences between epithelial cell and leukocyte migration are consistent with the size of the chemotaxis chamber pores and likely reflect physiological differences in motogenic potential of those cell types.

Experiments with neutralizing antibody demonstrated the specificity of signaling through CXCR4, because 12G5 abrogated CXCL12-induced epithelial cell migration and barrier maturity. In agreement with our prior findings in T84 cells (30) and intestinal microvessels (29), we have shown that epithelial cell migration, through sensitivity to the specific inhibitor pertussis toxin, requires signaling via Goi proteins. Similarly, we found that PI3-kinase, a key downstream target of Goi and Gβγ signaling (26, 40), played a significant role in T84 chemotaxis and IEC-6 restitution. Of note, our data support previous observations, suggesting a role for Goi and PI3-kinase activation in endothelial and epithelial barrier formation and epithelial migration (12, 24). CXCL12-mediated chemotaxis was also decreased, although not abolished, by PD-98059, a specific inhibitor of ERK1/2 activation. Therefore, our data suggest a mechanism whereby CXCL12 signaling via CXCR4 induces intestinal epithelial cell chemotaxis through the activation of a receptor-coupled Goi protein, PI3-kinase, and the ERK1/2 MAP kinase pathway. We also determined that those signaling pathways regulating epithelial cell chemotaxis were similarly needed for CXCL12-induced IEC-6 wound restitution. Furthermore, the ERK1/2 signaling cascade has also been shown to modulate tight junction proteins, thereby strengthening the mucosal barrier, as well as participating in epithelial restitution (15, 37). Additional mechanisms for CXCL12 stimulation of intestinal epithelial cell chemotaxis and migration may include pathways known to be activated by CXCR4 and linked to leukocyte locomotion (29, 58). Our data herein suggest that chemokine-evoked epithelial cell migration uses comparable signaling pathways to those observed for leukocyte trafficking, endothelial angiogenesis, and epithelial restitution.

Within the intestinal epithelium, the processes of cellular migration and maturation are closely linked, as evidenced by the increasing state of differentiation achieved by cells as they migrate from the crypt base to the villus tip (8, 30, 31, 60). Studies utilizing transgenic mice with altered expression of E-, N-, or β-catenin, or adenomatous polyposis coli proteins exhibit modulated migratory phenotypes, which may lead to defects in barrier maturation or adenoma formation (31). Those data provide important mechanistic insights into the essential relationship between directed cell migration and enterocyte differentiation (4, 60, 61). Based on those findings in murine models, we examined CXCL12 regulation of barrier formation as a measure of cellular differentiation. Consistent with this, Fedyk et al. (23) suggested that CXCL12 and its receptor

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**Fig. 6.** CXCL12 stimulates IEC-6 cell restitution. Serum-starved confluent monolayers of IEC-6 cells were wounded and then incubated 24 h in serum-free medium alone (A and D), with 10 ng/ml CXCL12 (B and E), or, as a control, 5 ng/ml TGF-β1 (C and F). Photomicrographs at ×100 magnification were taken at 0 (A–C) and 24 h (D–F) after wounding, and the number of cells migrated across the wound margin were counted. Photomicrographs shown are representative of 3 independent wounding experiments. G: quantitation of IEC-6 cell migration across the wound demonstrated CXCL12 significantly stimulated wound repair. CXCL12-induced restitution was not significantly different from that induced by the TGF-β1-positive control. Values are means ± SE of 3 independent experiments. H: CXCL12 does not enhance IEC-6-cell proliferation as measured by [H]thymidine incorporation. Values are means ± SD from a representative of 6 independent experiments. *Significant difference from unstimulated controls (P < 0.05).
CXCR4 function as physiological regulators of dermal architecture. In T84 cells cultured on permeable cellulose ester filter inserts, CXCL12, via binding to its cognate receptor CXCR4, significantly increased the electrical resistance across the monolayer and, in turn, decreased paracellular permeability. This enhanced barrier morphogenesis was not due to regulation of T84 proliferation, because CXCL12 did not alter cell survival or [3H]thymidine incorporation in those cells. Lack of T84 proliferation, because CXCL12 did not alter cell surface density. In the absence of CXCL12, T84 cell migration was comparably to TGF-β/H9252, IL-1, IL-2, IL-8, IFN-γ, EGF, hepatocyte growth factor, polyamines, lysophosphatidic acid, short-chain fatty acids, and trefoil factors known to be active in mucosal immune responses (reviewed in Ref. 49). However, none of those mediators has been shown to be essential for regulation of epithelial restitution, implying a degree of necessary redundancy in that process. Our findings demonstrate that CXCL12 increased IEC-6 cell locomotion comparably to TGF-β1, a potent stimulator of intestinal epithelial cell restitution in vitro and barrier maintenance in vivo (5, 17, 25). Thus our data support a role for the CXCL12-CXCR4 axis in intestinal epithelial wound repair by enhancing cellular migratory responses.

Together, these data suggest that the epithelial cells lining the gastrointestinal mucosa express CXCR4 and are functional immunoregulatory targets of CXCL12. Through stimulation of intestinal epithelial cell migration, barrier development, and epithelial wound repair, our data implicate the CXCL12-CXCR4 chemokine-receptor pair in the regulation of innate mucosal barrier defense. The significance of these findings is underscored by the ongoing development of specific CXCR4 receptor antagonists (18, 19), which may provide novel targets for future combinatorial therapies to aid in mucosal healing as well as preventing metastasis in colorectal adenocarcinoma.

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