Human type II pneumocyte chemotactic responses to CXCR3 activation are mediated by splice variant A

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Ji R, Lee CM, Gonzales LW, Yang Y, Aksoy MO, Wang P, Brailoiu E, Dun N, Hurford MT, Kelsen SG. Human type II pneumocyte chemotactic responses to CXCR3 activation are mediated by splice variant A. Am J Physiol Lung Cell Mol Physiol 294: L1187–L1196, 2008. First published March 28, 2008; doi:10.1152/ajplung.00388.2007.—Chemokine receptors control several fundamental cellular processes in both hematopoietic and structural cells, including directed cell movement, i.e., chemotaxis, cell differentiation, and proliferation. We have previously demonstrated that CXCR3, the chemokine receptor expressed by Th1/Tc1 inflammatory cells present in the lung, is also expressed by human airway epithelial cells. In airway epithelial cells, activation of CXCR3 induces airway epithelial cell movement and proliferation, processes that underlie lung repair. The present study examined the expression and function of CXCR3 in human alveolar type II pneumocytes, whose destruction causes emphysema. CXCR3 was present in human fetal and adult type II pneumocytes as assessed by immunocytochemistry, immunohistochemistry, and Western blotting. CXCR3-A and -B splice variant mRNA was present constitutively in cultured type II cells, but levels of CXCR3-B greatly exceeded CXCR3-A mRNA. In cultured type II cells, I-TAC, IP-10, and Mig induced chemotaxis. Overexpression of CXCR3-A in the A549 pneumocyte cell line produced robust chemotactic responses to I-TAC and IP-10. In contrast, I-TAC did not induce chemotactic responses in CXCR3-B and mock-transfected cells. Finally, I-TAC increased cytosolic Ca2+ and activated the extracellular signal-regulated kinase, p38, and phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase B (Akt) arms of the mitogen-activated protein kinase (MAPK) pathways (42). Of interest, inhibitor studies in airway epithelial cells indicate that CXCR3-activated signaling pathways include the phosphoinositol 3-kinase (PI 3-kinase) and p38 and extracellular signal-regulated kinase (ERK) arms of the mitogen-activated protein kinase (MAPK) pathways (42). Our recent work indicates that, in addition to inflammatory cells, a major lung structural cell, i.e., the airway epithelial cell, also expresses CXCR3 (3, 28, 42). Specifically, human airway epithelial cells constitutively express CXCR3 and its major splice variants CXCR3-A and -B (30). Moreover, activation of CXCR3 by I-TAC, Mig, and IP-10 induces airway epithelial cell chemotaxis and proliferation (3, 28, 42). In fact, the proliferate response of human airway epithelial cells to I-TAC is approximately equal to the response elicited by epidermal growth factor, a robust mitogen believed to be important in tissue repair following injury (3, 29). Our studies in airway epithelial cells indicate that CXCR3-activated signaling pathways include the phosphoinositol 3-kinase (PI 3-kinase) and p38 and extracellular signal-regulated kinase (ERK) arms of the mitogen-activated protein kinase (MAPK) pathways (42). Of interest, inhibitor studies in airway epithelial cells indicate that chemotactic responses to CXCR3 activation are mediated by PI 3-kinase/protein kinase B (Akt) and p38 but not ERK (42).

Because chemotactically directed cell movement and cell proliferation are believed essential to the airway repair process (27, 29), our studies suggest the possibility that CXCR3 may be involved in repairing airway damage induced by inflammatory cells present in COPD. In fact, IP-10 expression is upregulated in COPD in small airway epithelial cells and whole lung homogenates (21, 40).

COPD is a mix of chronic airway inflammation and remodeling, i.e., chronic bronchitis, and destruction of lung parenchyma, i.e., emphysema (4). Damage and/or destruction of alveolar epithelial cells, the greatest mass of cells resident in the lung, is thought to be the proximal cause of emphysema (4, 13). In fact, apoptosis of alveolar pneumocytes is enhanced in

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subjects with COPD (13). Of note, alveolar epithelial cells like airway epithelial cells produce IP-10, Mig, and I-TAC, and Th1/Th1c lymphocytes from subjects with COPD produce greater than normal amounts of IP-10 and Mig (20, 36).

Accordingly, expression of CXCR3 by alveolar pneumocytes would represent a novel autocrine/paracrine mechanism potentially useful in preventing or reversing lung destruction. The present study, therefore, sought to determine if CXCR3 and its splice variants were expressed by human alveolar type II pneumocytes.

CXCR3 expression was examined at the protein level by immunohistochemistry and Western blotting and at the mRNA level by real-time RT-PCR. CXCR3 function was assessed from the chemotactic response to its ligands. Moreover, the separate functions of the two well-described CXCR3 splice variants, CXCR3-A and -B (30), were assessed in the human pneumocyte cell line A549 by retroviral transfection. Specifically, we assessed the ability of the two isoforms to induce chemotactic responses, to affect cell proliferation, and to scavenge CXCR3 ligands from the extracellular space, i.e., remove exogenous IP-10 from the culture medium. Finally, potential signaling pathways known to be activated by the two isoforms were assessed in CXCR3-A- and -B-transfected cells from changes in cytosolic calcium and phosphorylation of MAPK and PI 3-kinase/Akt kinases (6, 30, 42).

MATERIALS AND METHODS

Several cell culture and explant culture preparations were used.

Type II pneumocyte and lung explant culture. Human type II pneumocytes were obtained from fetal lungs of second-trimester therapeutic abortions (14–23 wk gestational age) under protocols approved by the Committee for Human Research, Children’s Hospital of Philadelphia (19). Pneumocytes were isolated as previously described (19) and cultured in serum-free Waymouth’s medium containing DCF (dexamethasone (10 nM), 8-bromo-cAMP (100 μM) + phosphodiesterase inhibitor, isobutyl methylxanthine (IBMX, 100 μM)) to induce type II cell differentiation. Epithelial cell purity was assessed by cytokeratin immunostaining (19). Dissociated/isolated cells were used to study CXCR3 mRNA and protein expression as well as chemotactic responses to the several CXCR3 ligands (see below). Cells grown in serum-free Waymouth’s medium alone were used to study expression of CXCR3 in undifferentiated pneumocytes.

The human alveolar epithelial cell line, A549 cells [American Type Culture Collection (ATCC), Manassas, VA], was used to selectively overexpress the CXCR3-A and CXCR3-B splice variants. A549 cells were cultured in DMEM with 4.5 g/l glucose (Invitrogen, Carlsbad, CA) plus 4 mM glutamine and 10% FBS. The human alveolar epithelial cell line, A549 cells, were transfected with pCMV-B and pTarget plasmids encoding CXCR3-B (accession no. AF469635) and obtained from Dr. Paola Romagnani (30). Both constructs were sequenced for validation (Fisher Scientific SeqWright, Houston, TX).

Waltham, MA) in 25% sucrose water. Tissues were then frozen over liquid nitrogen fumes for 20–25 min and stored at −80°C (14). Before study, frozen tissues were cut into 1-cm3 blocks, embedded in optimum cutting temperature compound (Tissue Tek, Hatfield, PA), cut into 6-μm-thick sections in a cryostat at −20°C, and stained with hematoxylin and eosin. Normal-appearing tissues were selected for immunohistochemistry.

Immunohistochemistry/fluorescence microscopy. Fetal lung explants, human pulmonary pneumocytes, A549 cells, or frozen sections of adult human lung were fixed with either 100% methanol or 1% paraformaldehyde for 20 min and stained for CXCR3 and surfactant protein (SP)-B as previously described (3, 19, 28).

Sections were first blocked with 3% donkey serum in PBS, then treated with a mouse antihuman CXCR3 antibody that detects both CXCR3-A and -B splice variants (clone 49801.111; 10 μg/ml) (R&D Systems) followed by a Cy2 (1.25 μg/ml)- or Cy3 (0.625 μg/ml)-conjugated donkey, antimouse secondary antibody for 30 min (Jackson ImmunoResearch, West Grove, PA). Type II pneumocytes were identified using a rabbit, anti-bovine SP-B polyclonal antibody (Chemicon, Temecula, CA) (2 μg/ml × 30 min) and a Cy2 (1.25 μg/ml)- or Cy3 (0.625 μg/ml)-conjugated goat antirabbit secondary antibody (Jackson ImmunoResearch).

To control for nonspecific staining, irrelevant isotype antibodies were used. Nuclei were stained with 4’,6’-diamidino-2-phenylindole dilactate (DAPI).

Images were obtained using a fluorescent microscope (Eclipse E800; Nikon) with digital video interface (DEI-750 CE Digital Output) and processed using Adobe Photoshop CS (Adobe Systems).

Western blotting. Cultured cells were washed with cold PBS, scraped, and lysed (1% SDS, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 4 μg/ml pepstatin, 2 mM phenylmethylsulfonyl fluoride, 6 mM NaN3, and 6 mM NaF). Protein concentrations were determined by DC protein assay (Bio-Rad, Hercules, CA). Samples (50–100 μg) were boiled in Tris-SDS buffer (100 mM Tris-HCl, pH 6.8, 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, and 20% glycerol) for 5 min, resolved on 10% SDS-PAGE, and transferred to nitrocellulose membranes in buffer containing 10 mM 3-cyclohexylamino-l-propanosulfonic acid in 15% methanol, pH 10.6. Blots were blocked with 5% nonfat dry milk in 1× TBS buffer with 0.05% Tween 20 for 1 h, incubated with antihuman CXCR3 monoclonal antibody (mAb) (49801.11; R&D Systems) (1–2 μg/ml) overnight at 4°C. Anti-CXCR3 antibody was detected by horseradish peroxidase-conjugated secondary antibody (1:1,000) (Amersham Biosciences), and then exposed using chemiluminescence (Enhanced ECL Detection System; Amersham Biosciences). Equivalence of loading was assessed by stripping and re-probing the blots with anti-human actin mAb (1 μg/ml) (Santa Cruz, Santa Cruz, CA).

Real-time RT-PCR. Total cellular RNA was extracted using TRI reagent (Sigma-Aldrich), treated with DNase I (Promega, Madison, WI). Reverse transcription was performed at 25°C for 10 min followed by 48°C for 30 min. The following primers and probes were used for real-time PCR: CXCR3-A (accession no. NM_001504.1): forward primer 5′-CCCAAGCGACCAGACACC-3′, reverse primer 5′-ACCAGACGACGAGACACC-3′, forward primer 5′-CTCATAAGAGAGGTGAATCTCCCA-3′, reverse primer 5′-CGCCCTGCGCTCAGGC-3′; CXCR3-B (accession no. AF469635): forward primer 5′-CGGGCTTCATTTACAGGC-3′, reverse primer 5′-TGCCGGCTTTACACAGC-3′; forward primer 5′-GACCCGAGGAGCTGAC-3′, reverse primer 5′-GACCCGAGGAGCTGAC-3′. Real-time TaqMan PCR was performed using Universal PCR Master Mix (Applied Biosystems). Two-step PCR was performed: denaturing at 95°C for 15 s and elongation at 60°C for 60 s; cycle number 40–45.

The two sets of primers displayed similar amplification efficiency and specificity when tested on plasmids encoding CXCR3-A (pCMV) or CXCR3-B (pTarget) cDNA sequences, respectively. CXCR3-A cDNA in pCMV was purchased from ATCC. CXCR3-B cDNA in pTarget was a gift from Dr. Paola Romagnani (30). Both constructs were sequenced for validation (Fisher Scientific SeqWright, Houston, TX).
mRNA levels were quantitated by comparing experimental levels with standard curves generated using dilutions of the plasmids.

**Chemotaxis assay.** Chemotaxis of cultured pneumocytes was assessed using a 96-well system (ChemoTx, Neuro probe, Gaithersburg, MD). Chemotaxis medium (serum-free RPMI 1640/0.1% BSA, 30 μl) containing I-TAC/CXCL11 (1–1,000 ng/ml; R&D Systems), the most potent CXCR3 ligand (23, 30), Mig/CXCL9 (1–1,000 ng/ml; R&D Systems), or IP-10/CXCL10 (1 to 1,000 ng/ml; R&D Systems) was placed in the bottom wells of the chamber. A polycarbonate, fibronectin-coated membrane with 8-μm size pores was placed on top of the lower wells. Fetal pneumocytes or A549 cells (70,000 or 20,000 cells, respectively) and 50-μl chemotaxis medium were seeded on the surface of the membrane. Chambers were incubated at 37°C for 2 h for A549 cells and 18–20 h for human fetal pneumocytes. The membrane was then fixed and stained. Migrated cells attached to the bottom surface of the membrane were counted under a microscope (×40 magnification).

In several experiments, the effect of blocking changes in cytosolic calcium were assessed using a combination of Xestospongin C (5 μM; Calbiochem, San Diego, CA) and ryanodine (10 μM; Calbiochem), which block the inositol trisphosphate (IP3) receptor and ryanodine receptor, respectively (16, 18). Cells were pretreated with the calcium blockers 15 min before addition of I-TAC (10 ng/ml).

**Regulation of CXCR3 expression by dexamethasone or cAMP/IBMX stimulation.** The role of glucocorticoids, intracellular cAMP, or the combination on CXCR3 expression was assessed in cultured fetal pneumocytes. Cells were cultured in serum-free Waymouth’s medium for 2–5 days with one of the following treatments: 1) dexamethasone (10 or 100 nM); 2) 8-bromo-cAMP (100 μM) and IBMX (100 μM); or 3) DCl (dexamethasone (10 nM) plus 8-bromo-cAMP (100 μM) and IBMX (100 μM)). Serum-free Waymouth’s medium served as control.

**Generation of CXCR3-A and CXCR3-B transfectants.** Overexpression of CXCR3-A or -B was performed in A549 cells by retroviral infection. The full-length cDNAs of CXCR3-A and CXCR3-B (pCMV-CXCR3-A and pTarget-CXCR3-B, respectively) were subcloned into an RNA retrovirus vector, pFBneo (Stratagene, La Jolla, CA). Briefly, CXCR3-A cDNA was inserted between the Sal I and Not I restriction sites in the multicloning site of pFBneo (i.e., pFBneo-CXCR3-A). CXCR3-B cDNA was inserted between EcoRI and Not I restriction sites (i.e., pFBneo-CXCR3-B). The cloned constructs were tested for proper sequence and orientation by restriction digests and sequencing. To produce encapsulated, competent viral particles, 10 μg pFBneo-CXCR3-A or pFBneo-CXCR3-B were transfected in virus-packaging cells (Bing cells) using calcium phosphate precipitation. Bing cells were cultured in DMEM with 4.5 g/l glucose plus 4 mM glutamine and 10% FBS. Retrovirus-containing medium from Bing cell cultures was harvested after 24 h, and centrifuged before infecting A549 cells in the presence of 8 μg/ml hexadimethrine bromide (Sigma-Aldrich). Infection of A549 cells was performed in this manner for three consecutive days. Infection efficiency, assessed using pFBneo constructs expressing green fluorescent protein or luciferase, was >95%.

Expression of CXCR3-A and CXCR3-B protein was assessed by flow cytometry and immunocytochemistry using the 49801.11 anti-human CXCR3 mAb as previously described (3). Transfected cells were then grown in DMEM with 0.5 mg/ml G418 (Sigma-Aldrich) for selection of stably transfected cells.

**Cytosolic calcium.** Cytosolic Ca²⁺ concentrations ([Ca²⁺]) were measured by the microfluorimetric technique in cultured A549 cells grown to 50–60% confluence, as previously described (8). Cells were loaded with fura 2-AM (3 μM) in Hanks’ balanced salt solution (HBSS) for 45 min, and HBSS alone for an additional 15–60 min to allow dye deesterification. Cover slips were mounted in a 500-μl bath on the stage of a TE2000U Eclipse Nikon inverted microscope equipped with a Photometrics CoolSnap HQ charge-coupled device camera and perfused with HBSS at 2.5 ml/min.

Fura 2 fluorescence (emission = 520 nm) following alternate excitation at 340 and 380 nm was acquired at a frequency of 0.2 Hz using Metafluor software.

The effect of a combination of calcium blockers that affect calcium release from the endoplasmic reticulum, i.e., Xestospongin C (5 μM) and ryanodine (10 μM), on I-TAC-induced [Ca²⁺], was assessed. Cells were pretreated with the two drugs simultaneously 15 min before addition of I-TAC (10 nM).

**MAPK and PI 3-kinase/Akt stimulation.** Cells were grown in full medium until subconfluent and then changed to serum-free DMEM for 24 h. Cells were stimulated with I-TAC (100 ng/ml) or PF4 (100 ng/ml) for 1–120 min, washed in ice-cold PBS, and then harvested in lysis buffer. Western blotting was performed as described above. Blots were incubated with rabbit antihuman polyclonal antibodies against either phospho- or total ERK, p38, or Akt at 1:1,000 dilution (Cell Signaling, Beverly, MA).

**Scavenging analysis.** Several chemokine receptors, including CXCR3-B, are capable of removing their ligands from the extracellular compartment, thereby diminishing the intensity of the chemotactic stimulus, a process termed scavenging (12, 17, 41). Accordingly, we examined the rate of removal of exogenous IP-10 from the culture medium of CXCR3-A- and -B-transfected A549 cells. Cells at confluence were serum starved for 24 h after which fresh medium containing IP-10 (200 ng/ml) was added. IP-10 concentration in the culture medium was assessed over a 30-min period by enzyme-linked immunosorbent assay (R&D System).

**Cell proliferation assay.** To examine the effect on cell proliferation of CXCR3-A, CXCR3-B, and mock vector control transfected A549 cells, DNA-related fluorescence was measured over time (CyQUANT Cell Proliferation Assay; Invitrogen) using a fluorimeter (Victor Multilabel Counter, Shelton, CT). The following two types of experiments were performed: 1) cells were grown over a 6-day period in the absence of ligand stimulation; and 2) cells were treated with I-TAC (0, 10, or 100 ng/ml) over a 3-day period. Cells (2,000) were plated on 96 well. Triplicate wells were used for each time point.

**Statistical analysis.** Results are presented as group means ± SE. Statistical significance of differences in group mean values was assessed using one-way or two-way ANOVA. Post hoc analysis was performed using the Student’s t-test. The level of statistical significance was set at P < 0.05. Curve fitting for ligand-induced chemotactic responses was performed by least mean squares regression using a third-order polynomial equation.

**RESULTS**

**CXCR3 protein and mRNA expression in fetal and adult human pneumocytes.** Fetal human lung explants strongly expressed CXCR3 and SP-B immunoreactivity. Of interest, CXCR3 and SP-B immunoreactivity colocalized, indicating that CXCR3 is expressed by type II pneumocytes (Fig. 1A; 1 experiment representative of 3). CXCR3 immunoreactivity was also present in the normal adult lung and colocalized with SP-B (Fig. 1B; 1 subject representative of 4).

Western blots of cultured fetal lung epithelial cells also demonstrated the characteristic 35- to 40-kDa bands of CXCR3. CXCR3 expression was similar in fetal pneumocytes and human airway epithelial cells but was less than that of human Th1 lymphocytes (Fig. 2A; 1 experiment representative of 3). CXCR3 mRNA expression was also present in the normal adult lung and not detected in fetal lung explants (Fig. 2B, n = 3 experiments). However, in both fetal pneumocytes and airway epithelial cells, expression of CXCR3-B exceeded expression of CXCR3-A (P < 0.05 for both types of cells by ANOVA). In contrast, in Th1 cells, CXCR3-A exceeded
CXCR3-B transcripts \[965 \pm 162 \text{ and } 107 \pm 8 \text{ (SE) pg/100 ng RNA for CXCR3-A and -B, respectively, } P < 0.05\].

CXCR3 mediates chemotaxis in fetal human pneumocytes. The CXCR3 ligands I-TAC/CXCL11 IP-10/CXCL10, and Mig/CXCL9 induced concentration-dependent chemotactic responses in human fetal pneumocytes (Fig. 3). For I-TAC, the response was maximal at 10 ng/ml and equaled 250 \pm 48\% \text{(SE) of the medium control values} (P < 0.05 \text{ by ANOVA, } n = 6 \text{ experiments}). \text{ The absolute number of migrated cells in control medium was } 271 \pm 48 \text{ (SE); range } 175–388 \text{ cells. For Mig and IP-10, the response was maximum at } 100 \text{ ng/ml and equaled } 240 \text{ and } 250\% \text{ of medium control, respectively (} n = 2 \text{ experiments for each ligand).} \text{ Glucocorticoids upregulate CXCR3 expression in fetal human pneumocytes. CXCR3 expression, like that of SP-B, was reduced in cultured lung explants in which DCI was removed from the medium (Fig. 1). Dexamethasone, a component of the DCI mix, dose dependently (10 and 100 nM) increased CXCR3 protein expression as assessed by Western blot (Fig. 4A; 1 experiment representative of 3). In contrast, 8-bromo-cAMP/IBMX did not alter CXCR3 protein levels (Fig. 4A). Of interest, removal of DCI did not affect CXCR3-A and -B mRNA expression (Fig. 4B, } n = 6 \text{ experiments, } P = 0.883 \text{ by ANOVA).} \text{ Functions of CXCR3-A and -B in human pneumocytes. CXCR3-A or -B transfection markedly increased CXCR3-A or -B expression in A549 cells when assessed by flow cytometry and immunocytochemistry (Fig. 5; 1 representative of 3). CXCR3-A-transfected A549 cells demonstrated strong chemotactic responses to I-TAC (Fig. 6A; } P < 0.01 \text{ by ANOVA;}

Fig. 1. A: cultured human fetal lung explants labeled for CXCR3 (green, Cy2) and surfactant protein (SP)-B (red, Cy3). Nuclei stained with 4',6'-diamidino-2-phenylindole dilactate (DAPI) (blue). Top: differentiated pneumocytes cultured in the presence of DCI [i.e., dexamethasone (10 nM), 8-bromo-cAMP (100 \text{ \mu M}), and isobutyl methylxanthine (IBMX, 100 \text{ \mu M})] stain positively for both CXCR3 and SP-B. Middle: isotype control for both CXCR3 and SP-B shows nuclear staining only. Bottom: explants cultured in the absence of DCI have lower expression of both CXCR3 and SP-B. One experiment representative of 3. B: immunofluorescent labeling of CXCR3 and SP-B in serial, frozen sections from normal adult human lung. Left: double labeling for CXCR3 (Cy3, red) and SP-B (Cy2, green). Nuclei are stained with DAPI (blue). White arrows show cells in which CXCR3 and SP-B colocalize. Of note, both type II and type I pneumocytes are positive for CXCR3. Right: isotype control for both CXCR3 and SP-B showing nuclear staining only. Original magnification \times 400.

Fig. 2. A: Western blot for CXCR3. Lanes from left to right show lysates from phytohemagglutinin/interleukin (IL)-2-treated T lymphocytes (lane 1), human airway epithelial cells (16-HBE cells, lane 2), and human fetal pneumocytes (lane 3). One experiment representative of 3. B: comparison of CXCR3-A and CXCR3-B mRNA expression in human fetal pneumocytes and 16-HBE cells. CXCR3 mRNA levels were assessed by real-time RT-PCR and calculated from standard curves generated using CXCR3-A and -B plasmids. Note that CXCR3-B mRNA expression is greater than CXCR3-A in both types of lung epithelial cells (} P < 0.05 \text{ for comparison of CXCR3-B vs. -A in both cell types by ANOVA). Mean \pm \text{ SE of 3 experiments.} \text{ AJP-Lung Cell Mol Physiol • VOL 294 • JUNE 2008 • www.ajplung.org}
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ATP (10 μM) increased $[Ca^{2+}]_{i}$, dramatically and induced $[Ca^{2+}]_{i}$ oscillations in both CXCR3-A- and -B-transfected cells (Fig. 7).

CXCR3-A mediates mitogenic/extracellular signal-regulated kinase/MAPK and PI 3-kinase/Akt phosphorylation. I-TAC (100 ng/ml) increased phospho-ERK, phospho-p38, and phospho-Akt in CXCR3-A-, CXCR3-B-transfected, and mock vector-transfected A549 cells (Fig. 8, n = 3 experiments). However, increases in phospho-ERK, phospho-p38, and phospho-Akt were greater and more sustained in CXCR3-A-transfected than in CXCR3-B-transfected or mock vector-transfected cells. In contrast, PF4 induced little or no increase in phospho-ERK, -p38, or -Akt (data not shown).

Effect of inhibition of $[Ca^{2+}]_{i}$ on chemotactic and MAPK responses to I-TAC. In CXCR3-A-transfected cells (n = 43 cells in 1 experiment), the combination of Xestospongin C (5 μM) and ryanodine (10 μM) completely blocked I-TAC-induced increases in $[Ca^{2+}]_{i}$, (Fig. 9, inset) and partially blocked I-TAC-induced chemotaxis (n = 3 experiments; Fig. 9). Of interest, Xestospongin C and ryanodine did not appear to affect the magnitude or time course of phospho-ERK, -p38, or -Akt (data not shown).

DISCUSSION

The present study demonstrates that the chemokine receptor CXCR3 is expressed in human pneumocytes in vitro and

\[ n = 4 \text{ experiments} \] and IP-10, but not to the putative CXCR3-B selective ligand PF4 (Fig. 6B; $P = 0.957$ by ANOVA; n = 4 experiments). Cell migration in response to I-TAC was maximum at 10 ng/ml and equaled 643 ± 201% (SE) of the medium control (Fig. 6, A and B; $P < 0.01$ by ANOVA; n = 4 experiments). Cell migration in response to IP-10 was maximum at 100 ng/ml and equaled 229 ± 30% (SE) of the medium control ($P < 0.05$ by ANOVA; n = 3 experiments). In contrast, CXCR3-B-transfected and mock vector-transfected cells did not demonstrate a chemotactic response to either I-TAC or PF4 (Fig. 6, A and B; $P = 0.355$ by ANOVA; n = 4 experiments).

In contrast to the effects of CXCR3 transfection on cell chemotactic responses, cell proliferation was similar in CXCR3-A-, CXCR3-B-, and mock vector-transfected A549 cells in the presence and absence of I-TAC ($P = 0.74$, data not shown).

Moreover, the concentration of IP-10 in culture medium assessed over time was similar in CXCR3-A-, CXCR3-B-, and mock vector-transfected A549 cells (n = 3 experiments; data not shown). These data suggest that neither CXCR3-A nor CXCR3-B scavenged IP-10.

Activation of CXCR3-A alters cytosolic calcium. Mean basal $[Ca^{2+}]_{i}$, was 47 ± 2.6 nM. Administration of I-TAC (1, 10, and 100 nM) increased CXCR3-A-transfected cells induced a concentration-dependent increase in $[Ca^{2+}]_{i}$, (Fig. 7, top and bottom). The response was fast and transitory and did not generate subsequent oscillations. In contrast, administration of I-TAC to CXCR3-B-transfected cells did not alter $[Ca^{2+}]_{i}$, (Fig. 7).

Administration of PF4 had no effect on $[Ca^{2+}]_{i}$, in CXCR3-A-, CXCR3-B-, or mock-transfected cells.

Fig. 4. A: Western blot showing effects of dexamethasone or cAMP/IBMX on CXCR3 protein expression in cultured human fetal pneumocytes. Blot stripped and reprobed for β-actin is shown on bottom. Dexamethasone (10 and 100 nM) increased CXCR3 expression in a concentration-dependent fashion. 8-Bromo-cAMP/IBMX did not alter CXCR3 expression. One experiment representative of 3. B: effects of DCI on CXCR3-A and -B mRNA in cultured human fetal pneumocytes assessed by real-time RT-PCR. DCI did not affect CXCR3-A and -B mRNA expression (mean ± SE of 6 experiments; $P = 0.883$ by ANOVA).
CXCR3 expression and function were assessed in four distinct human preparations: fetal lung explants, cultured type II pneumocytes, adult lung, and the pneumocyte cell line A549 cells. CXCR3 expression was demonstrated at the protein level by immunohistochemistry and Western blotting and at the message level by real-time RT-PCR. CXCR3 expression in pneumocytes was comparable to that of airway epithelial cells and less than that observed in Th1 lymphocytes.

Both CXCR3-A and CXCR3-B mRNA were demonstrated in fetal pneumocytes with CXCR3-B mRNA 75-fold more abundant than CXCR3-A mRNA. The excess of CXCR3-B mRNA relative to CXCR3-A mRNA seen in human pneumocytes resembles results obtained previously in airway epithelial cells (3), salivary duct epithelial cells (41), and endometrial epithelial cells (24). In contrast, our results, like those of others (30), indicate that CXCR3-A mRNA predominates in human Th1 lymphocytes.

The CXCR3 expressed by human pneumocytes is functional and causes directed cell movement when activated by the potent IFN-γ-inducible CXCR3 ligands I-TAC/CXCL11, IP-10/CXCL10, and Mig/CXCL9.

Overexpression experiments performed with retroviral-transfected A549 demonstrated that CXCR3-A mediates the chemotactic responses to I-TAC. In contrast, overexpression of CXCR3-B did not elicit chemotactic responses to either I-TAC or the putative CXCR3-B selective ligand PF4, indicating that CXCR3-B does not affect cell movement in human pneumocytes.

Fig. 5. Overexpression of CXCR3-A or -B by pFBneo retrovirus transfection in A549 cells. A: CXCR3-A or -B protein expression (CXCR3-FITC) assessed by flow cytometry. CXCR3 protein was increased in both pFBneo-CXCR3-A- and pFBneo-CXCR3-B-transfected cells but not in mock vector-transfected cells (pFBneo). B: CXCR3-A and -B expression (Cy3, red) assessed by immunofluorescence microscopy. Nuclei stained with DAPI (blue). CXCR3 immunoreactivity was increased in pFBneo-CXCR3-A- and pFBneo-CXCR3-B-transfected cells compared with mock vector (pFBneo) (fields 1–3). Isotype controls are shown in fields 4–6. One experiment representative of 3 for each vector.

![Graph A](http://ajplung.physiology.org/)  ![Graph B](http://ajplung.physiology.org/)

**Fig. 6.** A: chemotactic responses to I-TAC in CXCR3-A- and -B-transfected A549 cells. Cell migration was expressed as a percentage of control values with medium alone. pFBneo-CXCR3-A-transfected cells (○) demonstrate strong responses to I-TAC (P < 0.005 by ANOVA). In contrast, neither pFBneo-CXCR3-B (●) nor mock vector-transfected cells (♦) respond to I-TAC. Mean ± SE of 4 experiments. B: chemotactic responses to platelet factor 4 (PF4). PF4 elicited no chemotactic responses in any of the three transfection conditions. Mean ± SE of 4 experiments (P = 0.355 by ANOVA).
Given the inhibitory effect of CXCR3-B on human endothelial cell movement (30), this finding is of considerable interest. However, it has been observed that the functional response to CXCR3-B activation may be cell type dependent (30).

CXCR3-A activation increases T cell proliferation while CXCR3-B inhibits endothelial cell proliferation (30, 48). Accordingly, we examined the role of CXCR3-A and CXCR3-B on cell proliferation in transfected A549 cells. However, cell growth curves of CXCR3-A- and CXCR3-B-transfected cells were similar to those of mock transfectant cells both in the presence and absence of I-TAC, supporting the idea that neither CXCR3-A nor CXCR3-B regulates growth in this cell type.

It has been reported recently that CXCR3-B, which is selectively expressed in cultured human salivary gland epithelial cells, removes IP-10 from the extracellular space into the cytoplasm (41) as is the case with the chemokine receptors D6 and CCR11/CCX-CKR (12, 17). Ligand “scavenging” is believed to decrease chemokine bioavailability and, hence, the magnitude of the chemotactic stimulus (12, 17). Our experiments failed to demonstrate a scavenging role for either CXCR3-B or CXCR3-A in A549 cells.

Previous studies in other cell types indicate that CXCR3 signals through changes in cytosolic calcium and activation of ERK, p38, or PI 3-kinase/Akt kinases (3, 6, 42, 43). In fact, increases in [Ca\textsuperscript{2+}]i, are believed to play an essential role in affecting cell movement and may activate downstream signaling pathways such as MAPK and possibly PI 3-kinase (1, 5, 22, 26, 43, 46, 47, 49). Our data indicate that, in human pneumocytes, the CXCR3-A splice variant but not the CXCR3-B splice variant increases [Ca\textsuperscript{2+}]i. Of interest, the monotonic shape of the [Ca\textsuperscript{2+}]i response induced by I-TAC differed from the oscillatory response induced by the P2Y agonist ATP. These results are not surprising, however, since CXCR3 couples to a G protein, G\textsubscript{i0}, different from that of the P2Y receptor, which is thought to couple to G\textsubscript{i0} (35, 45).

Previous studies, including our own in airway epithelial cells, indicate that the p38 and PI 3-kinase/Akt signaling pathways mediate the chemotactic response to CXCR3 ligands while ERK1/2 plays no role (42). Data from this study, therefore, suggest that activation of both MAPK and PI 3-kinase/Akt likely contribute to the chemotactic response of human pneumocytes to CXCR3 ligands.

In CXCR3-A-transfected cells, Xestospongin C and ryanodine completely blocked I-TAC-induced increases in [Ca\textsuperscript{2+}]i, suggesting that calcium release in response to CXCR3 activation is regulated by IP3 and/or ryanodine receptors in the endoplasmic reticulum. [We did not examine the separate effects of the two inhibitors and, hence, do not know the relative importance of these two classes of receptors.] Of interest, complete blockade of changes in cytosolic calcium inhibited chemotactic responses to I-TAC by ~50% but did not eliminate them. Moreover, Xestospongin C and ryanodine treatment had no effect on I-TAC-induced ERK, p38, or Akt phosphorylation. These results suggest that both calcium-dependent and calcium-independent signaling pathways drive CXCR3-A-mediated cell migration. In addition, our data support the possibility that ERK, p38, or Akt may mediate the calcium-independent pathways.

In contrast to results obtained with CXCR3-A, our data indicate that CXCR3-B, which does not induce chemotaxis,
also does not affect [Ca\(^{2+}\)], or the p38 and PI 3-kinase/Akt signaling pathways.

It has been reported that I-TAC is a high-affinity ligand for the newly described chemokine receptor CXCR7 in some cells, including A549 cells (9). CXCR7 has been reported to promote cell growth and survival but, unlike other conventional chemokine receptors, does not affect cell movement or cytosolic calcium (9). This raises the possibility that I-TAC-induced responses were mediated by CXCR7 rather than CXCR3. However, we believe that our results are not mediated by CXCR7 for several reasons. First, our experiments in transfected A549 cells indicate that chemotaxis, changes in [Ca\(^{2+}\)]; and greater more sustained phosphorylation of ERK, p38, and Akt occur only in CXCR3-A transfectants and not in CXCR3-B or mock transfectants. The fact that I-TAC-mediated effects occur only in CXCR3-A-transfected cells tends to rule out an effect of CXCR7, since CXCR7 expression should be the same in all three transfectants. Second, IP-10, which does not activate CXCR7, produced robust chemotactic responses in CXCR3-A-transfected cells. Third, CXCR7 activation by I-TAC does not appear to increase phosphorylation of ERK or Akt (37).

The biological function of CXCR3 expressed by structural cells in the human lung is unclear. However, its ligands, IP-10/CXCL10, Mig/CXCL9, and I-TAC/CXCL11, are produced by airway and alveolar epithelial cells and are believed to play important roles in lung inflammation by selectively recruiting Th1/Tc1 lymphocytes, which highly express CXCR3 (4, 7, 36, 40). In fact, an inflammatory infiltrate comprised primarily of CXCR3-expressing Th1/Tc1 lymphocytes is present in several lung diseases in which CXCR3 ligands are overexpressed (e.g., COPD, sarcoidosis) (2, 35, 38). Of particular interest, in subjects with COPD, IP-10 expression is constitutively upregulated in whole lung homogenates and in small airway epithelial cells (21, 38). Moreover, CXCR3 knockout mice demonstrate greater bleomycin-induced pulmonary fibrosis than wild-type animals, suggesting that CXCR3 inhibits pulmonary fibrosis and remodeling, perhaps by affecting the behavior of lung structural cells (25). In addition, viral infection and the proinflammatory cytokines tumor necrosis factor-\(\alpha\), IL-1\(\beta\), and IFN-\(\gamma\) increase production of the CXCR3 ligands (40, 44). Therefore, expression of CXCR3 by lung structural cells raises the possibility of autocrine or paracrine responses to CXCR3 ligands.

We have shown that CXCR3 induces directed cell movement in human pneumocytes. In airway epithelial cells, CXCR3 not only mediates chemotaxis but also induces DNA synthesis and cell proliferation (3). Because cell chemotaxis and proliferation are essential mechanisms in tissue repair (27, 29), the CXCR3 receptor expressed by human pneumocytes is likely to play a role in lung homeostasis and responses to injury.

In summary, the present study demonstrates that human alveolar type II pneumocytes constitutively express a functional CXCR3 receptor. In human pneumocytes, the CXCR3-A splice variant mediates chemotactic responses, increases cytosolic calcium, and activates ERK, p38, and PI 3-kinase/Akt
kinases. The function of the CXCR3-B splice variant, whose mRNA is present in greater abundance, is unknown.

GRANTS

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