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Differential Roles of CXCL2 and CXCL3 and Their Receptors in Regulating Normal and Asthmatic Airway Smooth Muscle Cell Migration

Laila A. Al-Alwan,*[,1] Ying Chang,*[,1] Andrea Mogas,* Andrew J. Halayko,† Carolyn J. Baglole,* James G. Martin,* Simon Rousseau,* David H. Eidelman,* and Qutayba Hamid*

Structural cell migration plays a central role in the pathophysiology of several diseases, including asthma. Previously, we established that IL-17–induced (CXCL1, CXCL2, and CXCL3) production promoted airway smooth muscle cell (ASMC) migration, and consequently we sought to investigate the molecular mechanism of CXC-induced ASMC migration. Recombinant human CXCL1, CXCL2, and CXCL3 were used to assess migration of human primary ASMCs from normal and asthmatic subjects using a modified Boyden chamber. Neutralizing Abs or small interfering RNA (siRNA) knockdown and pharmacological inhibitors of PI3K, ERK1/2, and p38 MAPK pathways were used to investigate the receptors and the signaling pathways involved in CXC-induced ASMC migration, respectively. We established the ability of CXCL2 and CXCL3, but not CXCL1, to induce ASMC migration at the tested concentrations using normal ASMCs. We found CXCL2-induced ASMC migration to be dependent on p38 MAPK and CXCR2, whereas CXCL3-induced migration was dependent on p38 and ERK1/2 MAPK pathways via CXCR1 and CXCR2. While investigating the effect of CXCL2 and CXCL3 on asthmatic ASMC migration, we found that they induced greater migration of asthmatic ASMCs compared with normal ones. Interestingly, unlike normal ASMCs, CXCL2- and CXCL3-induced asthmatic ASMC migration was mainly mediated by the PI3K pathway through CXCR1. In conclusion, our results establish a new role of CXCR1 in ASMC migration and demonstrate the diverse mechanisms by which CXCL2 and CXCL3 mediate normal and asthmatic ASMC migration, suggesting that they may play a role in the pathogenesis of airway remodeling in asthma. ***The Journal of Immunology*****, 2013, 191: 2731–2741.**

Migration, or directed cell movement, is a fundamental mechanism involved in a range of physiological and pathophysiological conditions. In recent years, migration of airway smooth muscle cells (ASMCs), including the recruitment of cells with the capacity to differentiate into ASM-like phenotypes (1), has been proposed as a potential mechanism to explain the increase in ASM mass in asthma. Since then, several groups have investigated and established the ability of ASMCs to migrate along a concentration gradient of chemokines (2, 3), cytokines (4, 5), and other inflammatory mediators (6). Very recently, we published that IL-17 cytokines regulate ASMC migration in an autocrine fashion through production of the growth-related oncogene (GRO) chemokines (GRO-α/CXCL1, GRO-β/CXCL2, GRO-γ/CXCL3) (7), which hereafter are collectively referred to as GROs. These findings implicated GROs as new mediators of ASMC migration.

Chemokines are small proteins (8–10 kDa) specialized in attracting inflammatory and structural cells to the sites of injury (8). GROs belong to the CXC family of chemokines bearing (ELR+) motif, glutamic acid (E), leucine (L), and arginine (R). In addition to GROs, this family also comprises epithelium-derived neutrophil-activating peptide 78 (CXCL5), granulocyte chemotactic protein 2 (CXCL6), neutrophil-activating peptide 2 (CXCL7), and IL-8 (CXCL8) (8, 9). CXCL1, CXCL2, and CXCL3 are well known as powerful neutrophil chemoattractants (10) and are involved in cancer metastasis (11), angiogenesis, and wound healing (12). Recently, GROs have been postulated to play a role in asthma severity (13, 14) and asthmatic airway remodeling (7, 15).

ELR+ CXC chemokines act through CXCR1 and CXCR2 chemokine receptors (16). Receptor binding studies have demonstrated that only CXCL6 and CXCL8 use both CXCR1 and CXCR2 for their signaling, whereas CXCL1, CXCL2, CXCL3, CXCL5, and CXCL7 act mainly through CXCR2 (17). CXCR1 and CXCR2 belong to the class A of rhodopsin-like, seven-transmembrane G protein–coupled receptors (18) that may form hetero- or homodimers (19–21).

ASMCs express both CXCR1 and CXCR2 (22) and produce GROs, which together may result in the autocrine promotion of ASMC migration through CXCR2 (7). However, little is known about the mechanism by which GROs direct ASMC migration. In

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Abbreviations used in this article: ASMC, airway smooth muscle cell; GRO, growth-related oncogene; PDGF-BB, platelet-derived growth factor subunit B; siRNA, small interfering RNA.

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the present study, we have examined the particular effect of each of CXCL1, CXCL2, and CXCL3 on normal and asthmatic ASMC migration and the cellular pathways mediating this effect. In addition to CXCR2, we also investigated the contribution of CXCR1 receptor in GRO-induced ASMC migration and signaling pathway activation. Our results provide strong evidence that CXCL2 and CXCL3 mediate normal and asthmatic ASMC migration via different mechanisms.

Materials and Methods

Culture of human ASMCs

Human ASMCs were isolated from lung transplant donors and prepared as described previously (22). The protocol was approved by an Institutional Research Ethics Board of the McGill University Health Centre and the Université de Montréal. Informed consent for ASMC harvesting was obtained from all participants. Cultured cells were identified as smooth muscle cells by immunohistochemical staining for smooth muscle–specific α-actin, myosin H chain, and calponin by Western blot and flow cytometry. ASMCs were cultured in DMEM/F12 (1:1; Life Technologies/Invitrogen, Grand Island, NY) supplemented with 10% FBS and 100 U/ml penicillin/streptomycin (Clonetics, Walkersville, MD) at 37°C with 5% CO_2 and were used between passages four and eight. Primary human ASMCs from four asthmatic and four normal subjects used in our protocols were purchased from Lonza (Basel, Switzerland). In each experiment that required using normal primary ASMCs, both cells purchased from Lonza and those isolated in our laboratories were used simultaneously. A description of the medical history of the asthmatic subjects used in this study is provided in Table I.

Migration assay

ASMC migration was assessed as previously described (23) using a 48-well microchemotaxis chamber (Neuro Probe, Cabin John, MD). Briefly, 0.01–100 ng/ml recombinant human CXCL1, CXCL2, or CXCL3 or 0.125–8 ng/ml (0.0313–64 ng/ml where applicable) of CXCL2 or CXCL3 was added to the lower chamber and 1×10^6 cells/ml of serum-starved nonasthmatic AMSCs (or asthmatic ASMCs where applicable) were added to the upper chamber. A 10 μm-pore polycarbonate membrane (Neuro Probe) treated with 0.01% collagen type I (rat tail; Nalgene Culture 3D Matrix; Nalgene, Rochester, NY) was placed between the two chambers. After 4 h, the membrane was removed, the upper face (containing nonmigrated cells) was wiped, and the cells that had migrated to the lower side of the membrane were fixed with 4% paraformaldehyde and stained using a Hema 3 kit (Fisher Scientific, Kalamazoo, MI). The number of migrated cells was counted in five random fields at a magnification of ×20. The molar concentrations of CXCL2 and CXCL3 chemokines at repeatedly used concentrations are 0.0125 ng/ml (=15.6 pM), 4 ng/ml (=0.5 nM), and 8 ng/ml (=1 nM). There was no evidence of cell migration off the filter into the lower chamber. The magnitude of 4 ng/ml CXCL2- and CXCL3-induced migration of normal ASMCs was compared with other factors that are known to induce ASMC migration, namely, platelet-derived growth factor subunit B (PDGF-BB) (10 ng/ml) (24), CXCL8 (100 ng/ml) (22), and CCL11 (100 ng/ml) (25).

To estimate the role of CXCR1 and CXCR2 receptors on GRO-induced ASMC migration, ASMCs were treated for 1 h at 37°C with 10 μg/ml anti-CXCR1 or anti-CXCR2 neutralizing Abs (R&D Systems, Minneapolis, MN) or small interfering RNA (siRNA; Santa Cruz Biotechnology, Santa Cruz, CA) prior to addition of GROs to the microchemotaxis chamber. Species-specific isotypes or scrambled siRNAs were used as controls for the proper experiments.

To investigate the role of signaling pathways in GRO-induced asthmatic and nonasthmatic ASMC migration, ASMCs were treated for 1 h at 37°C with the p38 MAPK inhibitor BIRB0796 (0.1 μM) (26, 27) (Axon Medchem, Groningen, The Netherlands), the ERK 1/2 MAPK inhibitor PD184352 (2 μM) (28, 29) (United States Biological, Swampscott, MA), and the PI3K inhibitor PI103 (5 μM) (30, 31) (Cayman Chemical, Ann Arbor, MI) prior to addition of GROs to the microchemotaxis chamber. All inhibitors were dissolved in DMSO. Controls were exposed to the same concentration of DMSO (1:1000).

CXCR1 and CXCR2 knockdown by siRNA transfection

ASMCs were plated in 6-well plates and grown to 60–80% confluence in medium containing 10% FBS. The cells were then transfected with siRNA targeting CXCR1 or CXCR2 (Santa Cruz Biotechnology) using the siRNA

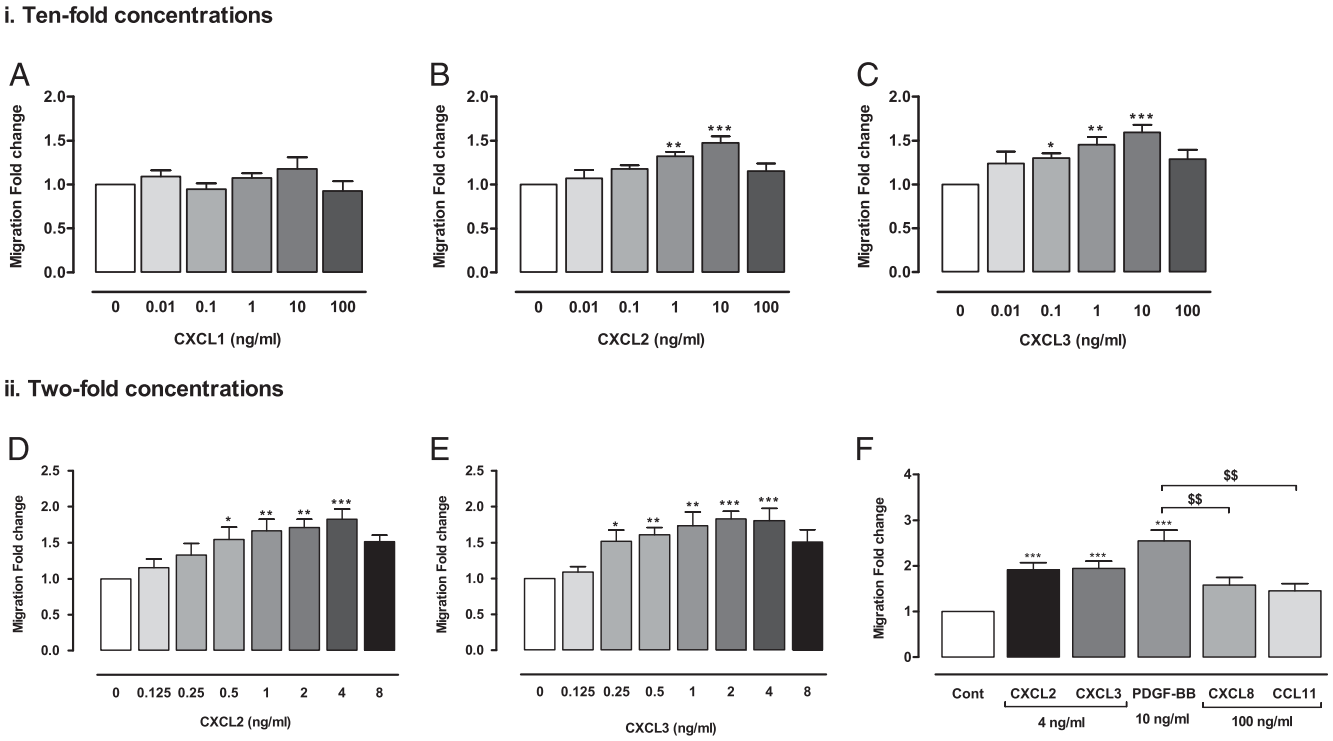

FIGURE 1. CXCL2 and CXCL3, but not CXCL1, induce ASMC migration. Migration of normal ASMCs was investigated by Boyden chamber with either 1) 10-fold doses (0.01–100 ng/ml) of recombinant human (**A**) CXCL1, (**B**) CXCL2, or (**C**) CXCL3, or 2) 2-fold doses (0.125–8 ng/ml) of (**D**) CXCL2 and (**E**) CXCL3. CXCL2 and CXCL3, but not CXCL1, were able to induce significant migration of ASMCs. (**F**) The magnitude of ASMC migration induced by CXCL2 and CXCL3 is comparable to that induced by PDGF-BB, CXCL8, and CCL11. Studies included four to six independent experiments using four to six subjects. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with nonstimulated control; $^{\$\$}p < 0.01$ compared with PDGF-BB. Results are expressed as means ± SEM.

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reagent system (Santa Cruz Biotechnology) according to the manufacturer's protocol. Scrambled siRNAs (Santa Cruz Biotechnology) were used as a negative control. After 24 h starvation in 0.1% FBS, ASMCs were either used directly for migration experiments or stimulated with CXCL2 or CXCL3 for 0, 5, 15, 30, 45, and 60 min and prepared for Western blot assay.

An aliquot of the protein lysates from transfected, nonstimulated ASMCs was retained to evaluate the efficiency of knockdown by Western blotting using rabbit polyclonal anti-CXCR2 (Abcam, Cambridge, MA) or mouse monoclonal anti-CXCR1 (R&D Systems) Abs. GAPDH (Millipore, Billerica, MA) was used as a loading control.

Western blot for signaling pathway analysis

Prior to experimentation, normal ASMCs (or transfected ASMCs where applicable) were cultured in reduced-serum DMEM/F12 (0.1% FBS) for 24 h and then treated with 4 ng/ml recombinant human CXCL2 or CXCL3 for 0, 5, 15, 30, 45, or 60 min. Cells were then washed twice with cold 1× PBS and lysed. Whole-cell lysate (10–15 μg) was loaded on 10% acrylamide SDS-PAGE Next Gel (Amresco, Solon, OH), followed by transfer to nitrocellulose membranes (Bio-Rad, Hercules, CA). The membranes were then blocked for 1 h at room temperature and then incubated for 24 h at 4°C with Abs specific to phosphorylated p38 MAPK (38 kDa) (Millipore, Temecula, CA), GAPDH (38 kDa) (Millipore), total ERK1/2 MAPK (42 and 44 kDa) (Cell Signaling Technology, Danvers, MA), phosphorylated ERK1/2 MAPK (42 kDa and 44 kDa) (Cell Signaling Technology), and phospho-Ser[473] Akt Ab (~60 kDa) (GenScript, Piscataway, NJ) for PI3K pathway activation. After washing (0.1% Tween 20/PBS), the membranes were incubated with a 1:15,000 dilution of IRDye 680 goat anti-mouse IgG or IRDye 680 goat anti-rabbit IgG (Rockland Immunochemicals, Philadelphia, PA) in blocking buffer and analyzed with an Odyssey IR scanner using Odyssey imaging software 3.0 (LI-COR Biosciences).

To estimate the capacity of CXCL2 and CXCL3 to activate the PI3K pathway in asthmatic ASMCs, cells were treated with CXCL2 (4 and 8 ng/ml) or CXCL3 (0.125 and 4 ng/ml) for 0, 5, 15, 30, 45, and 60 min prior to collecting cell lysates and running Western blots as described above. To investigate the role of CXCR1 and CXCR2 in mediating PI3K pathway activation in asthmatic ASMCs, cells were treated for 1 h at 37°C with 10 μg/ml anti-CXCR1 or anti-CXCR2 neutralizing Abs (R&D Systems) prior to stimulation with CXCL2 (4 and 8 ng/ml) or CXCL3 (0.125 and 4 ng/ml) for 5 min. Appropriate isotypes were used as controls.

Flow cytometric assay (FACS)

Serum-starved human ASMCs from healthy control and asthmatic subjects were trypsinized, washed, and then permeabilized using BD Cytofix/Cytoperm solution (BD Biosciences Pharmingen, San Diego, CA). Cells then were incubated with 1;100 of mouse allophycocyanin-conjugated anti-CXCR1 (R&D Systems), mouse monoclonal anti-CXCR2 (Invitrogen), or nonimmune isotype control (IgG) for 1 h at 4°C. After staining, cells were washed and, where applicable, incubated with 1:200 Alexa 647–conjugated goat anti-mouse IgG (H+L) (Invitrogen) for 30 min at 4°C. Finally, cells were fixed in 1% paraformaldehyde and analyzed by flow cytometry (BD FACSCalibur; BD Biosciences, Franklin Lakes, NJ). The results are expressed as a percentage of positive cells expressing the receptor obtained using CellQuest Pro software (BD Biosciences). Data analysis was performed by gating on live cells based on forward versus side scatter profiles.

Apoptosis assays

Flow cytometry (BD FACSCalibur; BD Biosciences) for annexin V and propidium iodide staining was used to examine the ASMCs for possible toxicity of the pharmacological inhibitors or siRNA used. Serum-starved human ASMCs from healthy control and asthmatic subjects were treated for 1 h at 37°C with medium alone, DMSO solvent control (1:1000), or with the p38 MAPK inhibitor BIRB0796 (0.1 μM), the ERK1/2 MAPK inhibitor PD184352 (2 μM), and the PI3K inhibitor PI103 (5 μM). Where applicable, ASMCs from healthy controls were subjected to scrambled (CXCR1 or CXCR2) siRNA transfection according to the aforementioned protocol. Subsequently, cells were trypsinized, washed, and then stained using annexin V and a propidium iodide staining kit (BD Annexin V FITC apoptosis detection kit; BD Biosciences) to quantify the percentage of cells undergoing apoptosis following the suggested protocol by the manufacturer. In the concentrations of inhibitors or siRNA used there was no evidence of cytotoxicity (data not shown).

Statistical analysis

All data are presented as means ± 1 SEM. Statistical analysis was performed by one-way ANOVA followed by the post hoc Dunnett test. For statistical analysis within and between stimulated conditions, a Bonferroni multiple comparison post hoc test was performed. A p value of <0.05 was regarded as statistically significant. Statistical analysis was performed using the Graphpad Prism 5 software.

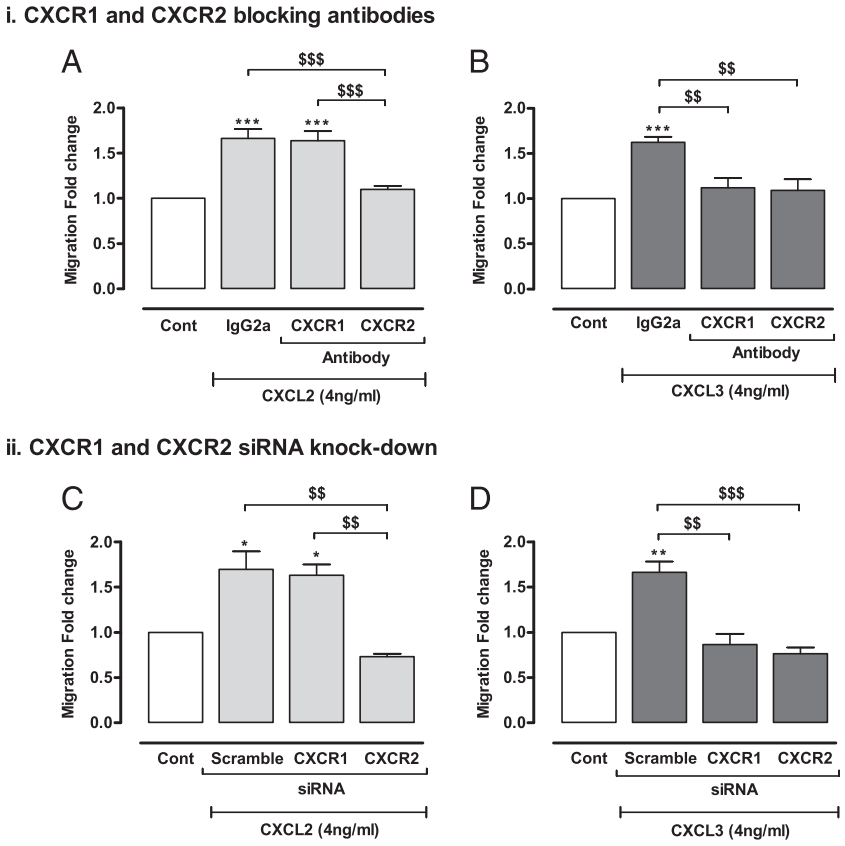

FIGURE 2. CXCR1 and CXCR2 differentially regulate CXCL2- and CXCL3-induced ASMC migration. To inhibit the functionality of CXCR1 and CXCR2, serum-starved ASMCs were either 1) incubated with Abs against CXCR1 or CXCR2 for 1 h prior to migration experiment, or 2) transfected with siRNA to knock down CXCR1 or CXCR2 expression. ASMC migration toward (**A**) CXCL2 was completely abolished following treatment with CXCR2, but not CXCR1, neutralizing Ab. Similar results were shown after using (**C**) CXCR2, but not CXCR1, siRNA knockdown. In contrast, (**B**) CXCL3-induced ASMC migration was inhibited after neutralization of either CXCR1 or CXCR2. The results were similar with (**D**) CXCR1 and CXCR2 siRNA knockdown. Studies included three to four independent experiments using three to four subjects. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with nonstimulated controls; $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ compared with isotype or scrambled siRNA controls. Results are expressed as means ± SEM.

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Results

CXCL2 and CXCL3, but not CXCL1, induce ASMC migration

Depending on health and/or disease status, the physiological levels of chemokines may range between ≈100 and 10,000 pg/ml (32–38). Therefore, we started first by testing the effect of CXCL1, CXCL2, and CXCL3 on ASMC migration using a broad range of concentrations (0.01, 0.1, 1, 10, and 100 ng/ml). We found that CXCL1 (Fig. 1A) failed to induce ASMC migration at any of the concentrations tested. However, CXCL2 (Fig. 1B) and CXCL3 (Fig. 1C) induced significant migration of ASMCs, predominantly at 1 and 10 ng/ml for CXCL2 and at 0.1, 1, and 10 ng/ml for CXCL3. We then tested CXCL2 and CXCL3 effects on ASMC migration over a narrower range (2-fold) of concentrations between 0.1 and 10 ng/ml. We found that ASMC migration was induced significantly by CXCL2 (0.5–4 ng/ml) and CXCL3 (0.25–4 ng/ml) with a peak response at 4 ng/ml (CXCL2, 1.83 ± 0.14-fold increase, $p < 0.001$, Fig. 1D; CXCL3, 1.81 ± 0.17-fold increase, $p < 0.001$, Fig. 1E). Similar significant results were obtained when we analyzed the absolute numbers of migrated cells (Supplemental Fig. 1). The effect of CXCL2 and CXCL3 on ASMC migration was not significantly different from that of PDGF-BB (10 ng/ml), CXCL8 (100 ng/ml), or CCL11 (100 ng/ml) (Fig. 1F). These results establish that CXCL2 and CXCL3 are potent mediators of ASMC migration, even when present only at relatively low concentrations.

CXCR1 and CXCR2 are both required for CXCL3, but not CXCL2, induced ASMC migration

ASMCs express both CXCR1 and CXCR2 receptors (22). CXCR2 is the putative receptor that is known to mediate CXCL2 and CXCL3 function. However, CXCL6 and CXCL8, from the same family as CXCL2 and CXCL3, could bind to both CXCR1 and CXCR2 (17). Hence, we sought to investigate the contribution of both receptors in CXCL2- and CXCL3-induced ASMC migration. Blockade of CXCR2, but not CXCR1, significantly inhibited CXCL2-mediated ASMC migration (IgG2a, 1.67 ± 0.11; anti-CXCR2, 1.10 ± 0.04; $p < 0.001$, Fig. 2A). In contrast, neutralizing either one of these receptors significantly reduced CXCL3-induced ASMC migration (IgG2a, 1.62 ± 0.06; anti-CXCR1, 1.12 ± 0.11, $p < 0.01$ and anti-CXCR2, 1.09 ± 0.12, $p < 0.01$) (Fig. 2B). Similar results were obtained when we used siRNAs to knockdown CXCR1 or CXCR2 expression (Fig. 2C, 2D). We validated the siRNA knockdown of receptors by Western blots and found significant decrease in the expression of CXCR1 (scrambled siRNA, 1.13 ± 0.09; CXCR1 siRNA, 0.66 ± 0.1; $p < 0.01$, Supplemental Fig. 2A) and CXCR2 (scrambled siRNA, 1.10 ± 0.06; CXCR2 siRNA, 0.67 ± 0.04; $p < 0.001$, Supplemental Fig. 2B). Taken together, these results suggest that CXCR1 and CXCR2 play different roles in CXCL2- and CXCL3-induced ASMC migration.

Differential regulation of CXCL2- and CXCL3-induced ASMC migration by p38 and ERK1/2 MAPK pathways

PI3K (39), p38 MAPK (4, 40) and ERK1/2 MAPK (41) pathways are involved in the migration of smooth muscle cells of different origins. To investigate which of these signaling pathways governs the effect of CXCL2 and CXCL3 on ASMC migration, we compared the responses of ASMCs treated with or without inhibitors targeting p38 MAPK (BIRB0796), ERK1/2 MAPK (PD184352), and PI3K (PI103). We found significant inhibition of CXCL2-induced ASMC migration after treating ASMCs with the p38 MAPK pathway inhibitor BIRB0796 (DMSO, 1.44 ± 0.08; BIRB0796, 0.98 ± 0.05, $p < 0.01$, Fig. 3A). However, CXCL3-induced ASMC migration was significantly inhibited after treating ASMCs with either BIRB0796 or the ERK1/2 MAPK inhibitor PD184352 (DMSO, 1.64 ± 0.05; BIRB0796, 0.92 ± 0.07, $p < 0.001$; and PD184352, 1.2 ± 0.16, $p < 0.01$) (Fig. 3B). These results indicate that CXCL2 and CXCL3 act through different signaling pathways to induce ASMC migration.

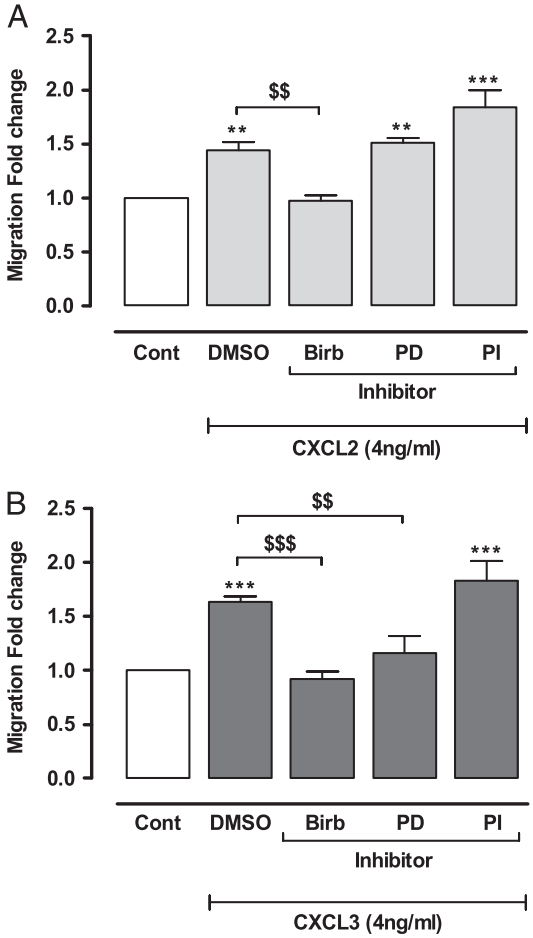

FIGURE 3. Differential regulation of CXCL2- and CXCL3-induced ASMC migration by p38 and ERK1/2 MAPK pathways. Serum-starved ASMCs were incubated with the following pharmacological inhibitors; BIRB0796 (BIRB, for p38 MAPK), PD184352 (PD, for ERK1/2), or PI103 (PI, for PI3K) for 1 h prior to migration experiment with 4 ng/ml CXCL2 or CXCL3. Both (**A**) CXCL2- and (**B**) CXCL3-induced ASMC migration were significantly inhibited after inhibition of p38 MAPK pathway using BIRB0796 (BIRB). However, only (B) CXCL3-induced ASMC migration was significantly reduced after inhibition of ERK1/2 MAPK pathway using PD184352 (PD). Studies included independent experiments using five to seven subjects. $^{**}p < 0.01$, $^{***}p < 0.001$ compared with nonstimulated control; $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ compared with vehicle control (DMSO). Results are expressed as means ± SEM.

CXCL2 and CXCL3 treatment to ASMCs activate p38 and ERK1/2 MAPK pathways

The above results suggest that, in contrast to CXCL2, CXCL3 may be able to activate multiple signaling pathways. Hence, we examined the activation of p38 MAPK, ERK1/2 MAPK, and PI3K in response to CXCL2 and CXCL3 stimulation. We found that both CXCL2 and CXCL3 induced significant and rapid activation of p38 (CXCL2, 2.43 ± 0.6 and CXCL3, 2.2 ± 0.4, $p < 0.001$, Fig. 4A) and ERK1/2 (CXCL2, 3.99 ± 0.85, $p < 0.05$ and CXCL3, 4.66 ± 1.07, $p < 0.01$, Fig. 4B) MAPK pathways at 5 min after exposure to agonist. However, activation of MAPKs by CXCL3 lasted longer (up to 15 min with p38 and up to 30 min with ERK1/2). In contrast, the PI3K pathway was not significantly activated by

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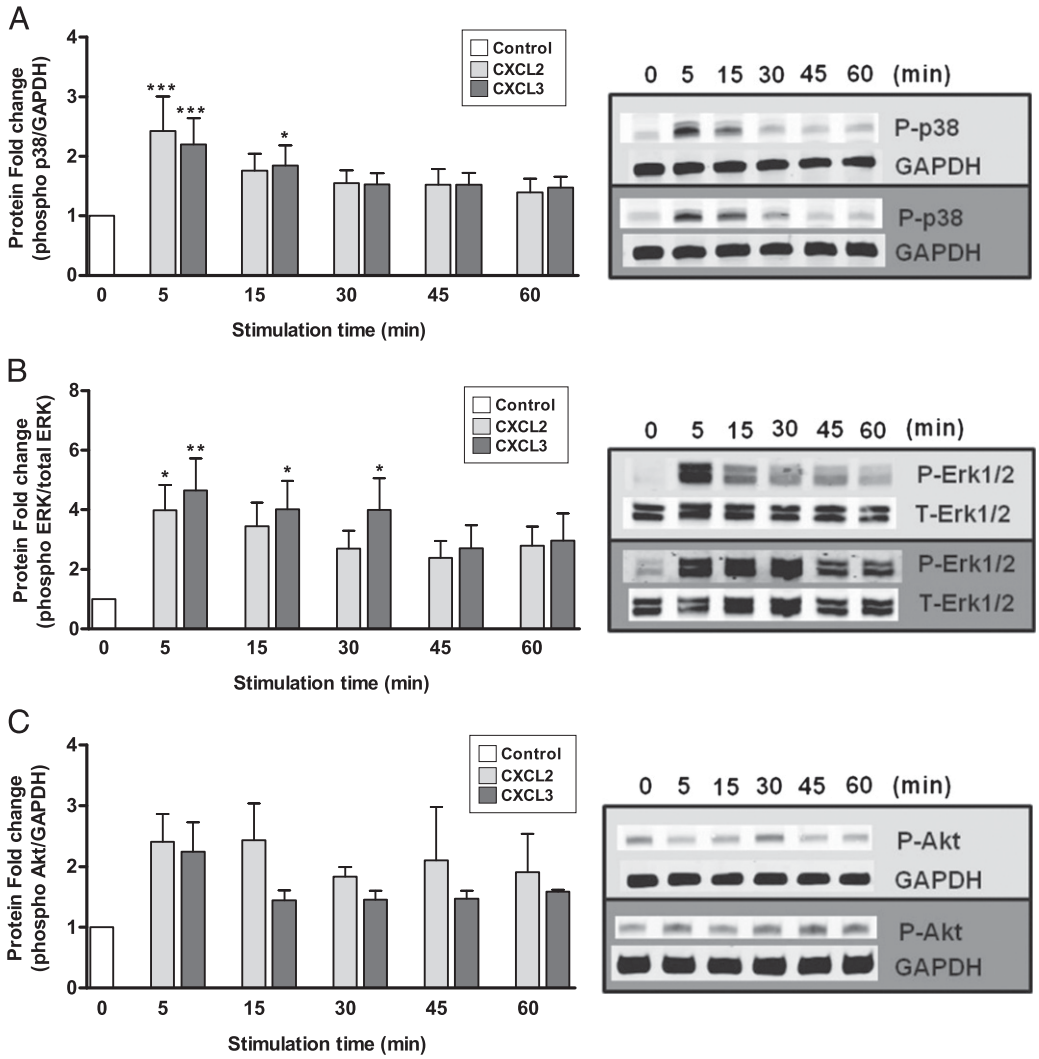

FIGURE 4. CXCL2 and CXCL3 treatment activates ERK1/2 and p38 MAPK, but not PI3K, pathways in ASMCs. Whole-cell lysates from ASMCs stimulated with 4 ng/ml CXCL2 or CXCL3 for 0, 5, 15, 30, 45, and 60 min were used in Western blot. The activation of (**A**) p38 MAPK and (**B**) ERK1/2 MAPK pathways in ASMCs was strongly detected after 5 min of CXCL2 and CXCL3 stimulation. However, the significant activation of MAPKs was longer after CXCL3 stimulation. In contrast, (**C**) the PI3K pathway was not significantly activated by either chemokine. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with nonstimulated control. Studies included five to six independent experiments using five to six subjects. Results are expressed as means ± SEM.

either chemokine (Fig. 4C). These results indicate that CXCL2 and CXCL3 induce comparable p38 and ERK1/2 MAPK activation; however, only CXCL3 utilizes both pathways to mediate ASMC migration.

CXCR1 and CXCR2 play differential roles in CXCL2- and CXCL3-induced p38 and ERK1/2 MAPK activation

To investigate the role of receptors in p38 and ERK1/2 MAPK activation in response to CXCL2 and CXCL3, we measured their phosphorylation levels after knocking down CXCR1 and CXCR2 expression by siRNA transfection. We found significant inhibition of CXCL2-induced p38 MAPK activation after CXCR2, but not CXCR1, knockdown (scrambled siRNA, 1.74 ± 0.15; CXCR2 siRNA, 0.99 ± 0.15, $p < 0.01$, Fig. 5A). Alternatively, ERK1/2 MAPK activation by CXCL2 was not reduced significantly after knockdown of either receptor (Figs. 5B). In contrast, the knockdown of either receptor was sufficient to reduce CXCL3-induced p38 MAPK activation (scrambled siRNA, 2.35 ± 0.26; CXCR1 siRNA, 1.53 ± 0.15, $p < 0.01$; and CXCR2 siRNA, 1.21 ± 0.18, $p < 0.001$, Fig. 5C) as well as ERK1/2 MAPK activation (scrambled siRNA, 3.77 ± 0.81; CXCR1 siRNA, 1.85 ± 0.58; and CXCR2 siRNA, 1.78 ± 0.51, $p < 0.05$, Fig. 5D). These results suggest that CXCR2 is responsible for CXCL2-induced p38 MAPK signaling activity, whereas both CXCR1 and CXCR2 are involved in CXCL3-induced p38 and ERK1/2 MAPK activation.

CXCL2 and CXCL3 induce higher migration in asthmatic ASMCs versus normal ASMCs

To address whether CXCL2 and CXCL3 may play roles in increasing ASM mass in asthma through induction of ASMC migration, we used ASMCs obtained from asthmatic patients (Table I) and observed several differences in the responses of asthmatic and normal ASMCs to different concentrations of CXCL2 and CXCL3. First, we found that CXCL2 and CXCL3 could induce asthmatic ASMC migration across a broader range of concentrations compared with normal ASMCs. CXCL2 was able to induce significant migration of asthmatic ASMCs at higher concentrations than it did to normal cells (at 8 ng/ml, 2.28 ± 0.17, $p < 0.05$, 1.52 ± 0.09, respectively, Fig. 6A). The opposite was observed with CXCL3 where the peak effect shifted toward lower concentrations (at 0.125 ng/ml, normal, 1.09 ± 0.08, asthmatic, 2.12 ± 0.10, $p < 0.001$, Fig. 6B). This alteration of the effects of CXCL2 and CXCL3

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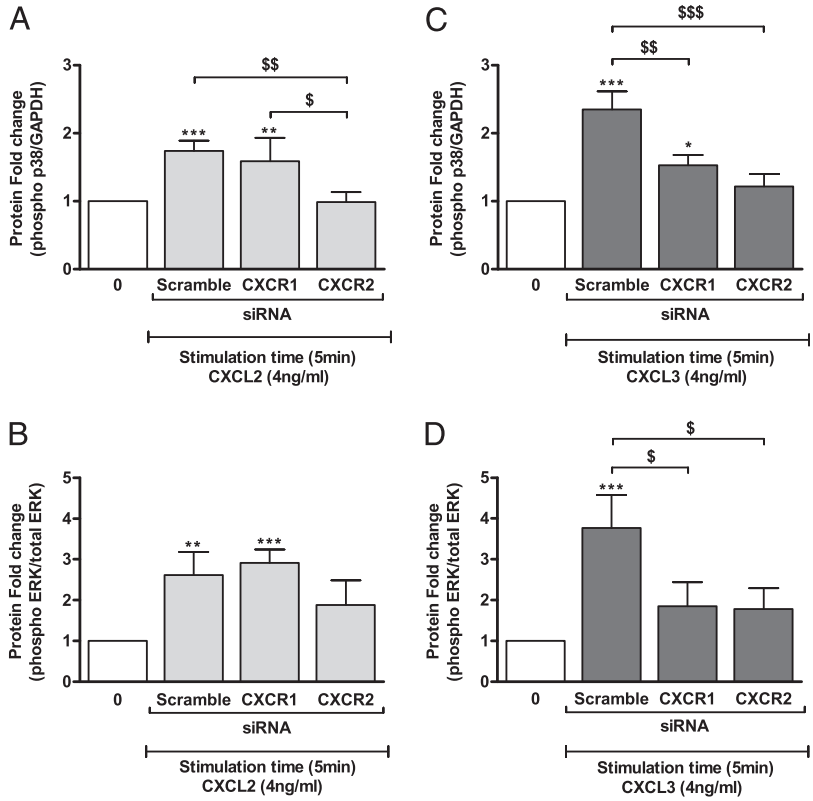

FIGURE 5. CXCR1 and CXCR2 play different roles in CXCL2- and CXCL3-induced p38 and ERK1/2 MAPK activation. ASMCs were transfected with siRNA to knock down CXCR1 or CXCR2 expression, after which they were serum-starved and stimulated with 4 ng/ml (0.5 nM) CXCL2 or CXCL3 for 0, 5, 15, 30, 45, and 60 min. Whole-cell lysates were then collected and used in Western blot for detection of p38 and ERK1/2 MAPK pathways. CXCR2, but not CXCR1, siRNA knockdown efficiently inhibited CXCL2- induced (**A**) p38 and (**B**) ERK1/2 MAPK pathway activation. In contrast, CXCL3-induced (**C**) p38 and (**D**) ERK1/2 MAPK pathway activation significantly decreased after siRNA knockdown of either CXCR1 or CXCR2. Studies included four independent experiments using four subjects. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with nonstimulated controls; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ compared with scrambled siRNA. Results are expressed as means + SEM.

was not due to differences in receptor expression between asthmatic and nonasthmatic ASMCs (Supplemental Fig. 3). Second, the chemotactic functions of CXCL2 and CXCL3 appear to segregate into two distinct curves with asthmatic ASMCs (Fig. 6C) compared with normal ASMCs (Fig. 6D). Moreover, migration of asthmatic ASMCs induced by CXCL3 was significantly higher than that induced by CXCL2 at lower concentrations (Fig. 6C), starting at 1 ng/ml (CXCL2, 1.98 ± 0.14, to CXCL3, 2.60 ± 0.16, $p < 0.05$) down to 0.125 ng/ml (CXCL2, 1.14 ± 0.09, to CXCL3, 2.12 ± 0.10, $p < 0.001$), an effect that was not seen with normal ASMCs (Fig. 6D). The effect of CXCL1 on asthmatic ASMC migration was similar to that observed on normal ASMCs (data not shown). These results suggest that asthmatic ASMCs responsiveness toward different effectors may be increased compared with normal ASMCs.

CXCL2- and CXCL3-induced asthmatic ASMCs is mainly mediated through CXCR1 receptor and PI3K signaling pathway

Most notably, the deviation of asthmatic ASMCs responsiveness toward CXCL2 and CXCL3 from what we observed with normal ASMCs was accompanied with a distinct shift in the receptor mediating this response. For example, at 4 ng/ml, CXCL2-induced asthmatic ASMC migration was CXCR2-dependent (Fig. 7A) similar to normal ASMCs. However, at 8 ng/ml, this effect became CXCR1-dependent (IgG2a, 1.71 ± 0.07; anti-CXCR1, 1.01 ± 0.06, $p < 0.05$, Fig. 7B). In contrast, CXCL3-induced asthmatic ASMC migration was CXCR1-dependent at both 0.125 (Fig. 7C) and 4 ng/ml (Fig. 7D). Quite surprisingly, regardless of the receptor mediating their function, CXCL2 and CXCL3 shifted their dependence from p38 and/or ERK1/2 MAPK pathways to the PI3K pathway when mediating asthmatic ASMC migration (Fig. 7E–H). These results indicate that CXCL2 and CXCL3 are versatile chemokines, and depending on the physiological condition, they may engage differently with cells to induce their migration.

CXCL2- and CXCL3-induced PI3K signaling pathway activation in asthmatic ASMCs is mainly regulated by CXCR1

To examine the role of CXCR1 and CXCR2 in CXCL2- and CXCL3-induced PI3K pathway activation in asthmatic ASMCs, we first measured PI3K pathway activation after treatment with CXCL2 and CXCL3. We found significant activation of the PI3K pathway after 5 min stimulation with CXCL2 at 4 ng/ml (1.81 ± 0.34, $p < 0.05$, Fig. 8A) and 8 ng/ml (2.2 ± 0.3, $p < 0.01$, Fig. 8B) and

Table I. Medical history of asthmatic subjects

Batch No.	Medications[a]	Duration of Disease	Cause of Death?[a]	Smoking History	Smoking Duration	No. of Packs per Day	Additional Comments
0000187731	Proventil, albuterol	Diagnosed at age 7	No	No	N/A	N/A	None
0000195154	Albuterol	Unknown	No	Yes	30 y	2	None
0F3518	Unknown pulmonary medications/none noted	Unknown	Unknown	No	N/A	N/A	None
0000208192	Unknown pulmonary medications/none noted	Diagnosed at age 18 mo	Yes	No	N/A	N/A	Two prior admittances for severe asthma attacks prior to demise

[a]Related to respiratory disease only.

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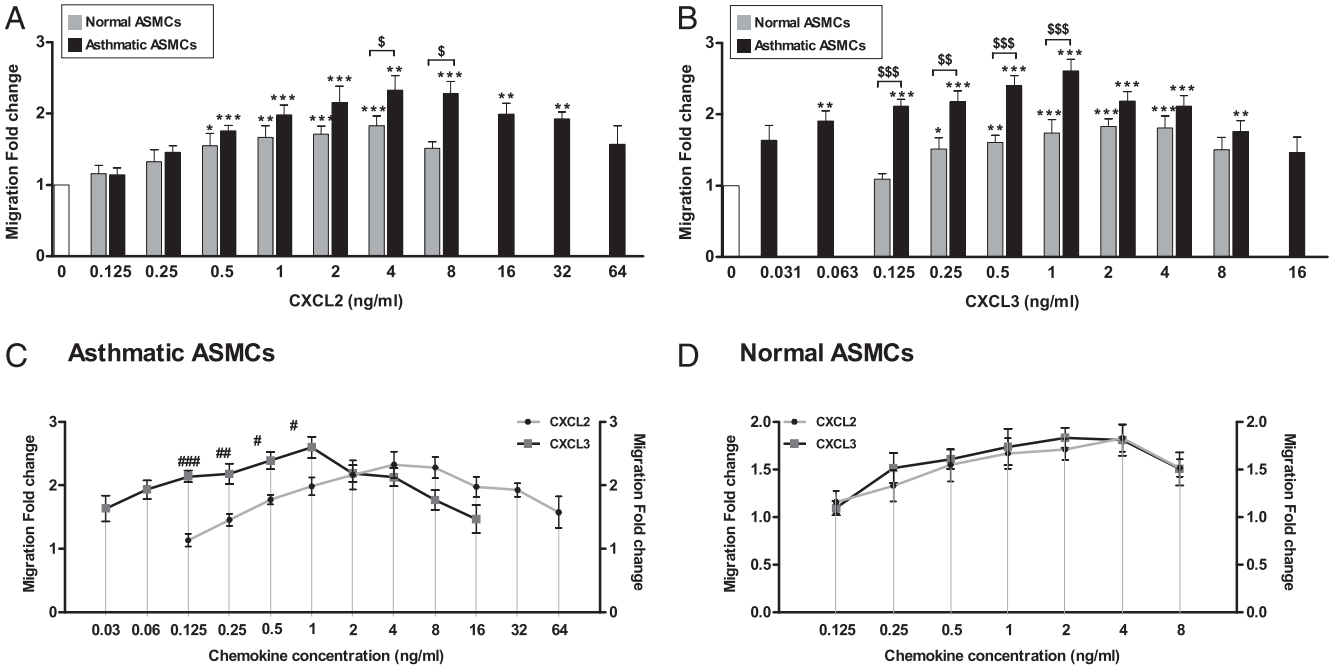

FIGURE 6. CXCL2 and CXCL3 induce higher migratory response in asthmatic ASMCs. Serum-starved asthmatic ASMC migration was investigated by Boyden chamber. Higher concentrations (8 ng/ml) of (**A**) CXCL2 were able to induce significant migration of asthmatic ASMCs compared with normal ASMCs. In contrast, lower concentrations (0.125 ng/ml) of (**B**) CXCL3 were able to induce significant migration of asthmatic ASMCs compared with normal ASMCs. CXCL2- versus CXCL3-induced migration exhibits (**C**) dissociated curves of asthmatic ASMCs compared with (**D**) semi-superimposed curves of normal ASMCs. Studies included three to six independent experiments using three to six asthmatic subjects. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with nonstimulated control; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ between asthmatics and nonasthmatics under similar concentrations; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#}p < 0.001$ between CXCL2 and CXCL3 under similar concentrations. Results are expressed as means ± SEM.

CXCL3 at 0.125 ng/ml (2.16 ± 0.33, $p < 0.01$, Fig. 8C) and 4 ng/ml (2.09 ± 0.4, $p < 0.01$, Fig. 8D). We then used neutralizing Abs to examine the effect of CXCR1 or CXCR2 blockade on CXCL2- and CXCL3-induced PI3K activation. In agreement with the results of migration, we found that blockade of CXCR2, but not CXCR1, significantly inhibited CXCL2-induced PI3K activation at 4 ng/ml (IgG2a, 1.82 ± 0.17-fold change; anti-CXCR2, 0.89 ± 0.21-fold change, $p < 0.01$, Fig. 8E). In contrast, blockade of CXCR1, but not CXCR2, was sufficient to reduce the PI3K pathway activation induced by CXCL2 at 8 ng/ml (IgG2a, 2.34 ± 0.3-fold change; anti-CXCR1, 1.21 ± 0.09-fold change, $p < 0.01$, Fig. 8F) and CXCL3 at 0.125 ng/ml (IgG2a, 1.95 ± 0.12-fold change; anti-CXCR1, 1.17 ± 0.20-fold change, $p < 0.01$, Fig. 8G) and at 4 ng/ml (IgG2a, 2.29 ± 0.09-fold change; anti-CXCR1, 1.52 ± 0.02-fold change, $p < 0.05$, Fig. 8H). These results indicate that, in asthmatic ASMCs, CXCL2 activates the PI3K pathway through different receptors at different concentrations, whereas CXCL3-induced PI3K activation is mainly dependent on CXCR1 at the concentrations tested.

Discussion

Structural cell migration is an important biological phenomenon that may be involved in many biological processes and diseases. Migration of ASMCs has been proposed as one of the mechanisms leading to increase in ASM mass and consequently airway remodeling in asthma (42). We have previously demonstrated that CXCL1, CXCL2, and CXCL3 have the potential to be important mediators of ASMC migration and thus we set out to establish their capacity to mediate normal and asthmatic ASMC migration and to explore the molecular mechanism by which this migration occurs. Our findings suggest that CXCL2 and CXCL3 differentially regulate asthmatic and nonasthmatic ASMC migration by acting through different receptors and different signaling pathways.

We started by testing the effects of GROs using 10-fold concentrations following a widely established practice (43–45). Our results indicated that CXCL2 and CXCL3, but not CXCL1, were able to induce significant migration of ASMCs. However, we reasoned that the ability of the chemotactic mediators to promote efficient cell migration along the migration path with very minimal concentrations would be essential. Accordingly, we established the ability of CXCL2 and CXCL3 to induce ASMC migration with as low as picomolar concentrations. Interestingly, we found the effect of CXCL2 and CXCL3 on ASMC migration to be comparable to that of other known mediators of ASMC migration such as PDGF-BB, CXCL8, and CCL11. These results indicate that CXCL2 and CXCL3 are potent mediators of ASMC migration at a wide range of concentrations (high and low).

Although CXCR1 and CXCR2 share ~78% homology in amino acid sequence, they have been shown to bind to the ELR+ CXC family of chemokines with different affinities (17, 46). Ahuja et al. (17) found that CXCL2 and CXCL3 were equally potent in their binding to CXCR2, based on calculating their mean EC_{50} values, and were able to induce a biological response in either stably CXCR2-transfected HEK cells or neutrophils. On the contrary, using CXCR1-transfected HEK cells, they found the EC_{50} values of CXCL2 (>100 nM) and CXCL3 (65 nM) to be significantly higher than that of CXCL8 (4 nM), a selective agonist of CXCR1, suggesting that CXCL2 and CXCL3 bind to CXCR1 with very low affinity. Since then, CXCR2, but not CXCR1, was established as the receptor recognized to mediate CXCL2 and CXCL3 biological functions (47). In contrast, we found that the migratory effects of CXCL2 and CXCL3 on ASMCs were differentially mediated by the CXCR1 and CXCR2 receptors in that, unlike CXCL2, CXCL3-induced ASMC migration was dependent on both CXCR1 and CXCR2. Of interest, the concentration of CXCL3 used to induce ASMC migration was ≤0.5 nM. These findings

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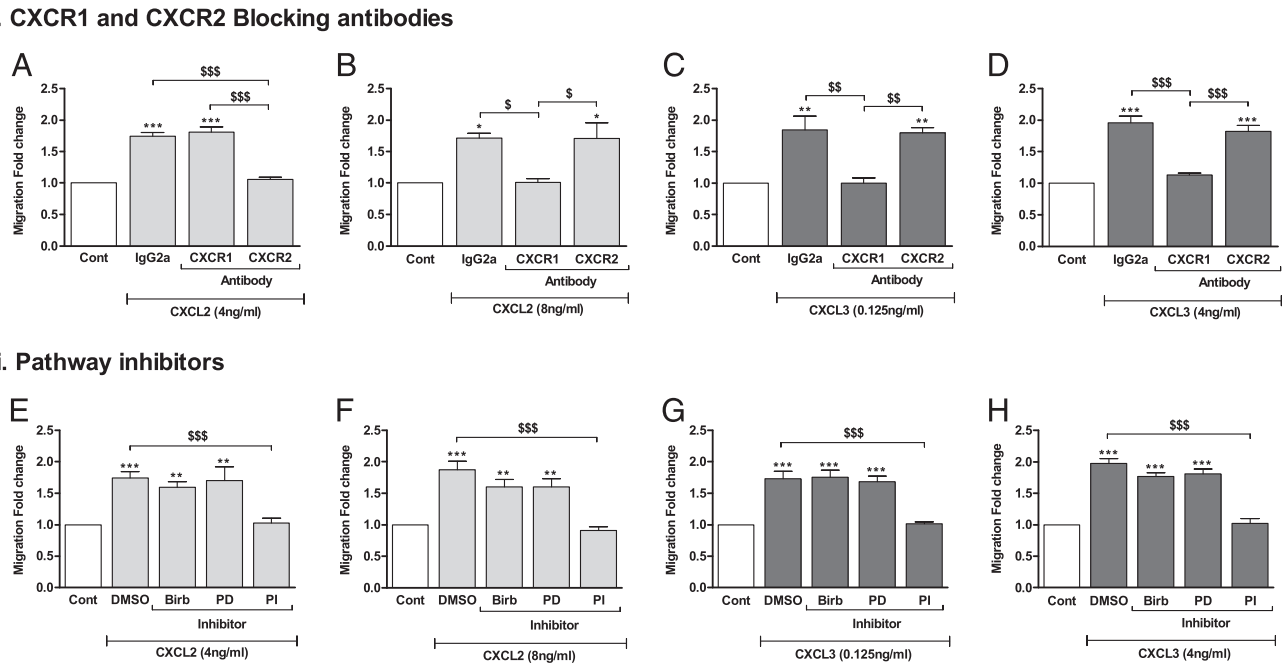

FIGURE 7. CXCL2- and CXCL3-induced asthmatic ASMC migration is mainly CXCR1- and PI3K pathway–dependent. To assess the involvement of receptors and signaling pathways in asthmatic ASMC migration, serum-starved ASMCs were incubated with Abs against CXCR1 or CXCR2 for 1 h prior to migration experiments. ASMC migration toward (**A**) 4 ng/ml CXCL2 was completely abolished following treatment with CXCR2, but not CXCR1, neutralizing Ab. In contrast, neutralizing CXCR1, but not CXCR2, was effective in reducing ASMC migration toward higher concentrations (**B**) 8 ng/ml CXCL2. ASMC migration toward either (**C**) 4 ng/ml or (**D**) 0.125 ng/ml CXCL3 was significantly inhibited following blockade of CXCR1, but not CXCR2, in asthmatic ASMCs. ASMC migration induced by 4 and 8 ng/ml of CXCL2 [(**E**) and (**F**), respectively] and 0.125 and 4 ng/ml of CXCL3 [(**G**) and (**H**), respectively] was significantly reduced after inhibition of the PI3K pathway using PI103 (PI), but not after inhibition of p38 or ERK1/2 MAPK pathways (BIRB0796 [BIRB] and PD184352 [PD], respectively). Studies included three independent experiments using three subjects. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with nonstimulated controls; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ compared with isotype or DMSO control. Results are expressed as means ± SEM.

were further supported by use of siRNA knockdown of CXCR1 or CXCR2 in ASMCs. The discrepancy in our results from those of others may be due to using primary cells instead of a transfected cell system. However, it is possible that the interaction between CXCL3 and CXCR1 may be conceivable only with ASMCs. Nonetheless, to the best of our knowledge, our results provide the first evidence that CXCR1 has an essential role in CXCL3-induced ASMC migration.

While investigating the signaling pathways involved in CXCL2 and CXCL3-induced ASMC migration, we found that CXCL2-induced ASMC migration was dependent only on p38 MAPK pathway, whereas CXCL3-induced migration was dependent on p38 and ERK1/2 MAPK pathways. It is possible that the number of receptors recruited may determine the number of signaling pathways activated. However, when examined, we found that the activation of p38 and ERK1/2 MAPK pathways induced by either CXCL2 or CXCL3 was comparable. Nevertheless, only CXCL3 used ERK1/2 MAPK activity to induce ASMC migration, suggesting CXCR1 as an essential factor to direct ERK1/2 MAPK activity toward migration. The phenomenon of G protein–coupled receptor signaling bias (48) or functional selectivity (49) has been reported. Signaling bias occurs when a ligand selectively binds and favors a specific confirmation of receptors leading to activation of specific signaling pathways (50). Our findings suggest that, unlike CXCL2, CXCL3 may induce ASMC migration by selectively activating ERK1/2 MAPK signaling through CXCR1 recruitment. To further examine the relationship between receptors and signaling pathways, we used the siRNA transfection technology to knock down the expression of CXCR1 or CXCR2 prior to CXCL2 or CXCL3 stimulation. Consistent with the above results, we found that CXCL2-induced p38 and ERK1/2 MAPK activation was mediated through CXCR2 only, whereas CXCL3-induced MAPK activation was dependent on both CXCR1 and CXCR2. These results emphasize that CXCL3 requires two receptors and, consequently, two signaling pathways to induce ASMC migration.

Although CXCL2 and CXCL3 expression has been shown to be increased in corticosteroid resistant asthma (14), their role in asthma severity in terms of ASMC migration and airway remodeling was rarely investigated (7). Therefore, we sought to establish the chemotactic effect of CXCL2 and CXCL3 on asthmatic ASMCs and to examine the receptors and signaling pathways mediating their effects. We chose to test CXCL2- and CXCL3-induced asthmatic ASMC migration using 2-fold increases in picomolar concentrations and observed a shift in the migration response curve toward higher concentrations of CXCL2 and lower concentrations of CXCL3. The shift was accompanied by significant increase in the maximal degree of migration of asthmatic ASMCs compared with normal cells. One way to explain this phenomenon is probably through increase in receptor expression in asthmatic ASMCs compared with their normal counterparts. However, we, as well as others (51), did not find any difference in receptor expression between asthmatic and nonasthmatic ASMCs, suggesting that this shift may be due to increase in receptor sensitivity, binding capacity, or recycling in asthmatic ASMCs. Of interest, An et al. (52) observed an increase in the contractile abilities, the cytoskeleton remodeling, and the temperature of the cytoskeleton matrix in ASMCs obtained from Fisher rats, a model of asthma hyperresponsiveness, upon agonist stimulation. The increase in cytoskeleton turnover seen in this model suggests that parallel changes

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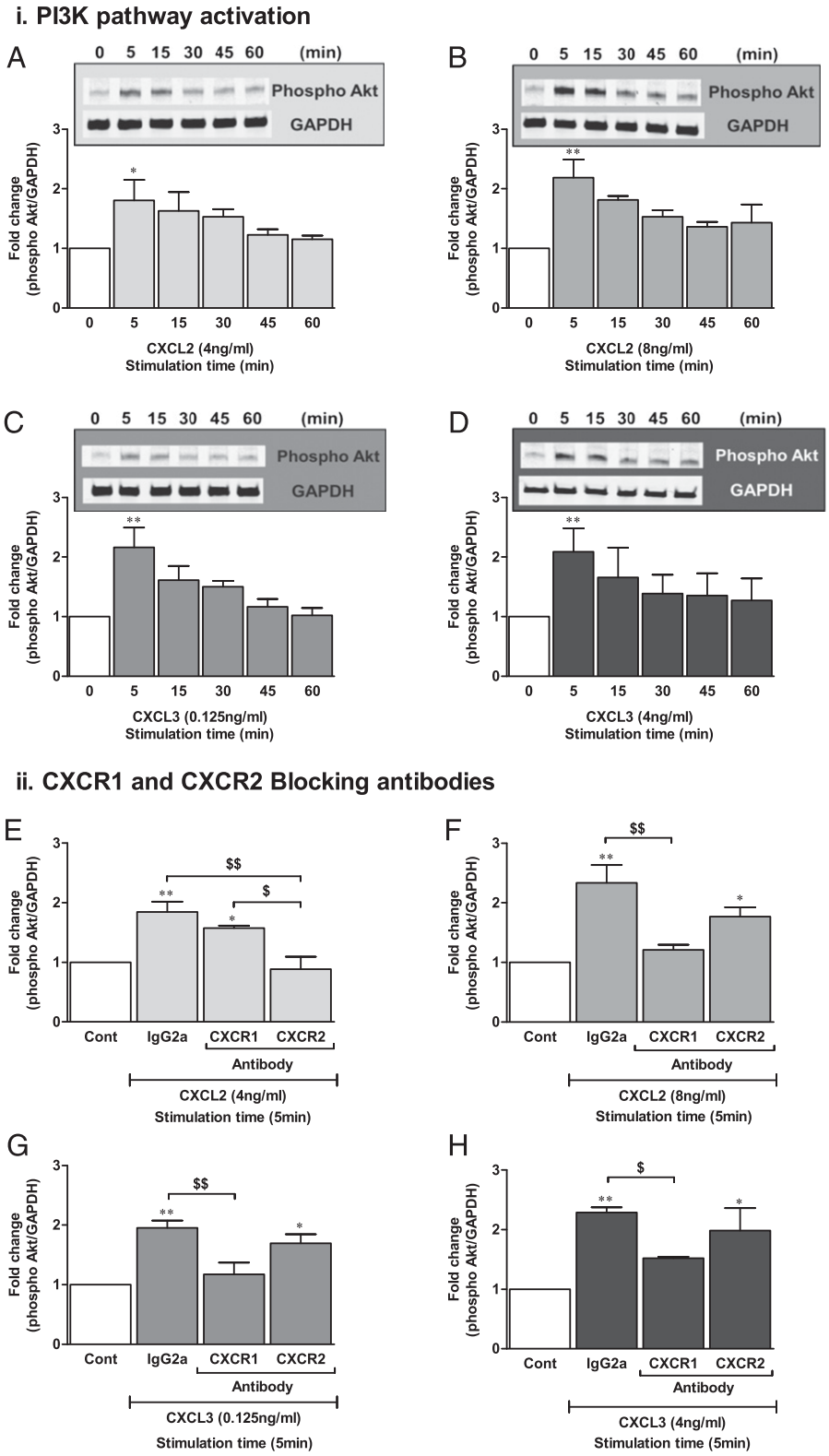

FIGURE 8. CXCL2- and CXCL3-induced PI3K signaling pathway activation in asthmatic ASMCs is mainly regulated by CXCR1. Whole-cell lysates from asthmatic ASMCs stimulated with CXCL2 (4 and 8 ng/ml) or CXCL3 (0.125 and 4 ng/ml) for 0, 5, 15, 30, 45, and 60 min were used in Western blot. Significant activation of the PI3K pathway was detected 5 min after stimulation with CXCL2 at (**A**) 4 ng/ml and (**B**) 8 ng/ml or with CXCL3 at (**C**) 0.125 ng/ml and (**D**) 4 ng/ml. To evaluate the involvement of receptors in the PI3K activation in asthmatic ASMCs, serum-starved ASMCs were incubated with Abs against CXCR1 or CXCR2 for 1 h prior to collecting cell lysates after stimulation with CXCL2 and CXCL3. Blockade of CXCR2 completely abolished PI3K activation after stimulation with (**E**) 4 ng/ml CXCL2, whereas blockade of CXCR1 was effective in inhibiting PI3K activation following treatment (**F**) with 8 ng/ml CXCL2. Similar to the latter effect, neutralizing CXCR1, but not CXCR2, was effective in reducing PI3K activation after stimulating asthmatic ASMCs with (**G**) 0.125 ng/ml and (**H**) 4 ng/ml CXCL3. Studies included three independent experiments using three subjects. $*p < 0.05$, $**p < 0.01$ compared with nonstimulated controls; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ compared with isotype control. Results are expressed as means ± SEM.

may occur in human asthmatic ASMCs and thus may explain their heightened migration responsiveness toward external agonists.

Most surprisingly, when we examined the effect of receptors on CXCL2- and CXCL3-induced asthmatic ASMC migration, at 8 and 0.125 ng/ml, respectively, we found CXCR1 to be the main receptor involved. However, when comparing the effects of CXCL2 and CXCL3 on asthmatic versus normal ASMCs, using equal concentrations (4 ng/ml), we found CXCL2-induced migration to be CXCR2-dependent, similar to what we observed with normal ASMCs. On the contrary, CXCL3-induced asthmatic ASMC migration was CXCR1-dependent. This lack of effect via CXRC2 was not due to differences in receptor expression because we found that CXCR2 expression in asthmatic ASMCs was significantly higher than CXCR1 expression. However, it is not clear whether the change in receptor recruitment is a phenomenon of the asthma milieu, increased receptor sensitivity, or both. Moreover, whether it was the

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CXCR1 or CXCR2 receptor that mediated CXCL2- or CXCL3-induced asthmatic ASMC migration, PI3K was the sole signaling pathway governing this migration. A similar observation has been reported by Burgess et al. (53) while examining the proliferation of asthmatic and normal ASMCs, where they found that asthmatic ASMC proliferation was mediated by PI3K, whereas normal ASMC proliferation was mediated through the ERK1/2 MAPK pathway. Moreover, we established that the PI3K pathway was activated by CXCL2 and CXCL3 in asthmatic ASMCs but not in normal ASMCs and found that the effects of blocking CXCR1 or CXCR2 on the PI3K pathway activation were consistent with their effect on migration in asthmatic ASMCs. This is not necessarily surprising, because the PI3K pathway has been long associated with allergic asthma (54), corticosteroid-resistant asthma (55), as well as airway remodeling in asthma (56). Of note, the PI3K pathway that we studied is part of class IA PI3Ks, which are known to function as a heterodimer of a catalytic (p110) and a regulatory (p85-like) subunit (57). Depending on the catalytic subunit involved in class IA PI3K activation, PI3K can be divided into α, β, or δ isoforms (57). Although the PI3K pathway has been shown to be involved in mediating SMC migration of different origins (58–62), the isoform of the activated PI3K pathway involved in such studies has seldom been examined. However, among the three isoforms of class IA PI3Ks (α, β, and δ), the PI3K p110α isoform has been repeatedly shown to be involved in migration of structural cells such as endothelial cells (63, 64), keratinocytes (65), and also in metastasis of several cancers (66–70). Although considered as a pan-PI3K inhibitor (71, 72), the pharmacological inhibitor used in our study (PI103) has been reported to have higher selectivity for the p110α subunit compared with other subunits (73, 74), suggesting the possibility that the PI3Kα isoform may be involved in mediating asthmatic ASMC migration as well. These results indicate that the asthmatic milieu may induce intrinsic changes in ASMCs, leading to heightened sensitivity toward exogenous stimuli and especially favoring the PI3K pathway activation for migration and proliferation processes. However, further investigation is warranted for better understanding of the involvement of PI3K isoforms in asthmatic ASMC migration in particular and asthma remodeling in general.

In conclusion, our findings demonstrate that CXCL2 and CXCL3 are potent mediators of normal and asthmatic ASMC migration. They indicate that ASMC responsiveness and intrinsic machinery may change, either due to asthma milieu or due to their plasticity to adjust their function to accommodate this milieu. They also establish GROs/CXCR1/ASMC interaction as a potential mechanism that may play an important role in the pathogenesis of airway remodeling in asthma.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Figure legends

Supplemental FIGURE 1. CXCL2 and CXCL3 induce higher migrating cell numbers than control medium. Migrating cell number of normal ASMCs towards CXCL2 at A) 0.5ng/ml, B) 1ng/ml, C) 2ng/ml, D) 4ng/ml or CXCL3 at E) 0.5ng/ml, F) 1ng/ml, G) 2ng/ml and H) 4ng/ml. Both CXCL2 and CXCL3 were able to induce significant migration of ASMCs at the concentrations tested. N= 6 independent experiments using 6 subjects. A two-tailed paired t-test was used for the statistical analysis. **, $p<0.01$; ***, $p<0.001$ compared with non-stimulated control.

Supplemental FIGURE 2. Validation of siRNA knock-down of CXCR1 and CXCR2. 60-70% confluent ASMCs were transfected with or without siRNA to knock down expression of either CXCR1 or CXCR2 or with scrambled siRNA. Cell lysates were collected and used to assess the knock-down efficiency of A) CXCR1 which reached 46% and B) CXCR2 which reached 43%. N= 5 independent experiments using 5 subjects. **, $p<0.01$; ***, $p<0.001$ compared with non-stimulated control. $$, $p<0.01$; $$$, $p<0.001$ compared to scrambled siRNA control. Results are expressed as mean + S.E.M.

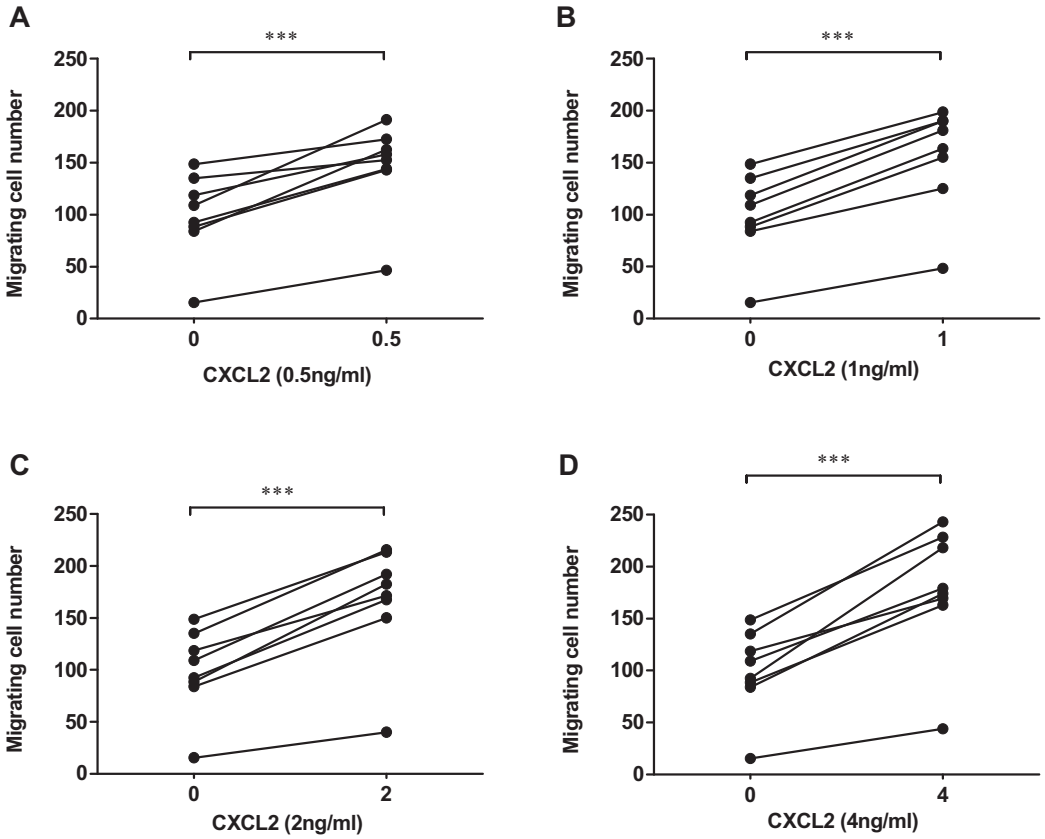
Supplemental FIGURE 3. CXCR1 and CXCR2 expression in normal and asthmatic ASMCs. Starved ASMCs were treated with CXCR1 or CXCR2 antibodies or isotype control and CXCR1 and CXCR2 expression levels were assessed by FACS. There was a significant difference between CXCR1 and CXCR2 expression levels in A) normal ASMCs and B) asthmatic ASMCs. However, no significant difference has been observed

22 in percentage of positive cells expressing C) CXCR1 or D) CXCR2 between normal and
23 asthmatic ASMCs. E), F), G) and H) are representative histograms of CXCR1 or CXCR2
24 expression in normal or asthmatic ASMCs. N= 4-6 independent experiments using 4-6
25 subjects. Results are expressed as means + SEM.

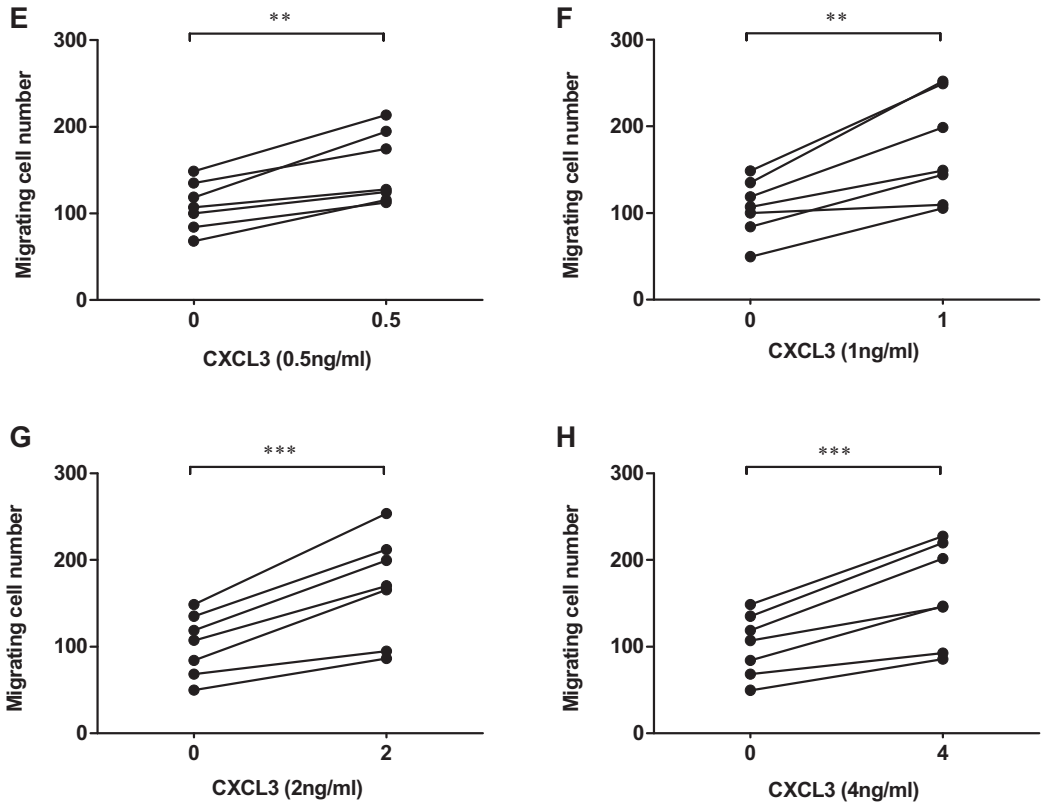
26

Supplemental Figure 1

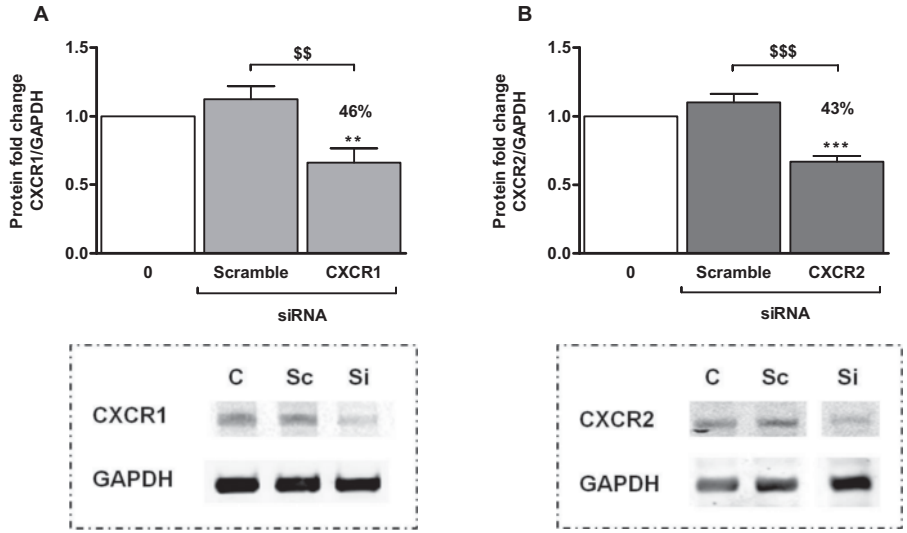
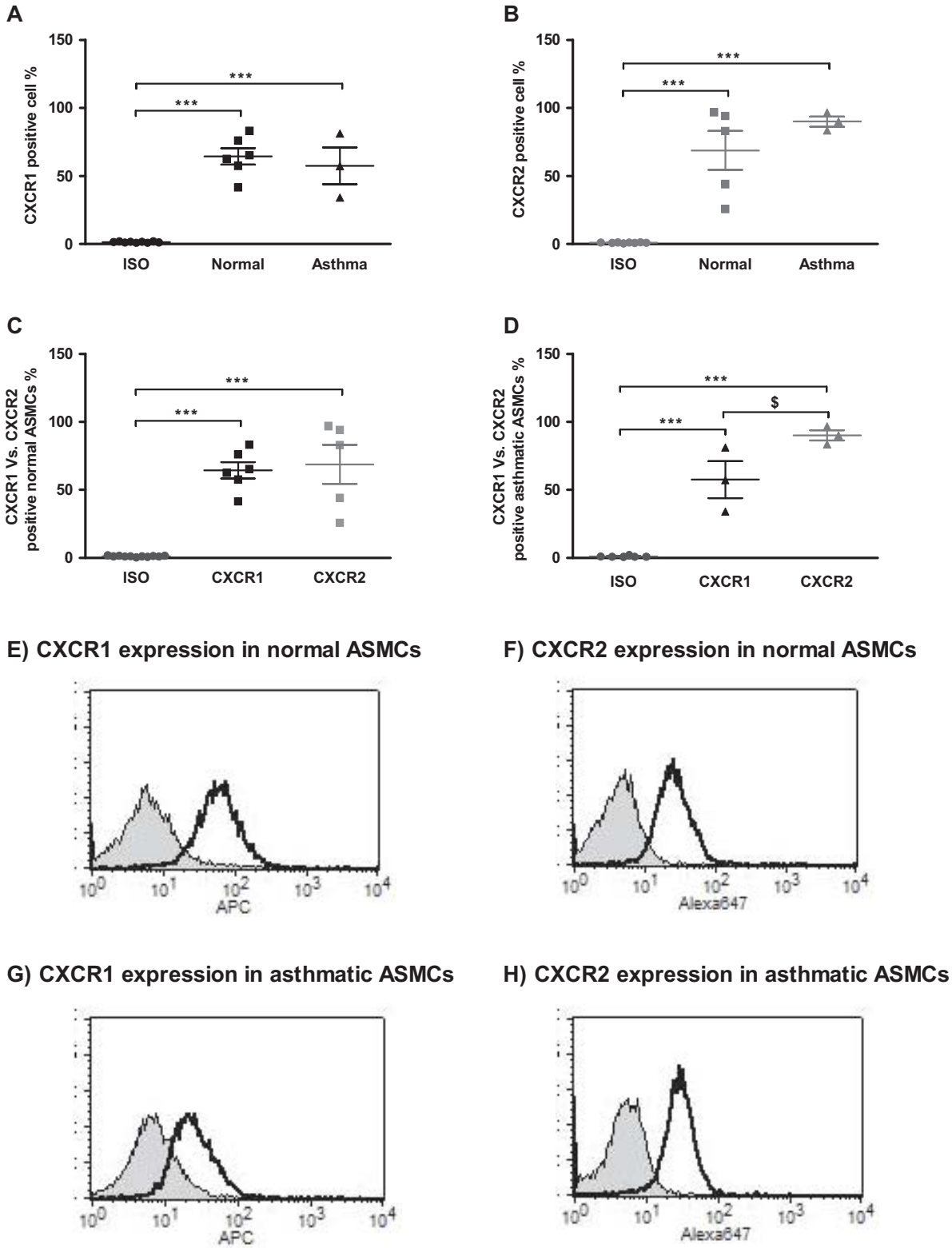
i) CXCL2



ii) CXCL3



Supplemental Figure 2



Supplemental Figure 3