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CXCL1 Inhibits Airway Smooth Muscle Cell Migration through the Decoy Receptor Duffy Antigen Receptor for Chemokines

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Airway smooth muscle cell (ASMC) migration is an important mechanism postulated to play a role in airway remodeling in asthma. CXCL1 chemokine has been linked to tissue growth and metastasis. In this study, we present a detailed examination of the inhibitory effect of CXCL1 on human primary ASMC migration and the role of the decoy receptor, Duffy AgR for chemokines (DARC), in this inhibition. Western blots and pathway inhibitors showed that this phenomenon was mediated by activation of the ERK-1/2 MAPK pathway, but not p38 MAPK or PI3K, suggesting a biased selection in the signaling mechanism. Despite being known as a non-signaling receptor, small interference RNA knockdown of DARC showed that ERK-1/2 MAPK activation was significantly dependent on DARC functionality, which, in turn, was dependent on the presence of heat shock protein 90 subunit α. Interestingly, DARC- or heat shock protein 90 subunit α-deficient ASMCs responded to CXCL1 stimulation by enhancing p38 MAPK activation and ASMC migration through the CXCR2 receptor. In conclusion, we demonstrated DARC’s ability to facilitate CXCL1 inhibition of ASMC migration through modulation of the ERK-1/2 MAPK–signaling pathway.

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binds with the highest affinity (27). The expression of KC (the mouse ortholog of human CXCL1) and DARC was found to follow parallel temporal patterns and tissue localization during mouse development (28), suggesting that a relationship may exist between the two. In general, DARC was found to maintain chemokine homeostasis in the blood (29) and to facilitate chemokine transcytosis and presentation across endothelium (30, 31).

In this study, we investigated the effect of CXCL1 on ASM migration and the role of CXCR1, CXCR2, and DARC in mediating this effect. Our results establish that CXCL1 inhibits ASM migration through activation of the ERK-1/2 MAPK pathway and that this effect is mediated via DARC but not the CXCR1 or CXCR2 receptors. In addition, we observed a regulatory effect of the heat shock protein (HSP)90 on DARC protein expression in ASCMs. Finally, we found that, in the absence of DARC, CXCL1 engaged CXCR2 and enhanced ASM migration through p38 MAPK/HSP27 pathway activation.

Materials and Methods

Culture of human ASCMs

Human ASCMs were isolated from lung transplant donors and prepared as described previously (32). The protocol was approved by institutional research ethics boards of the McGill University Health Centre and the Universite de Montre´ al, Montreal, QC, Canada and informed consent for ASMC harvesting was obtained at surgery or at the time of lung transplantation. Cultured cells were identified as smooth muscle cells by immunohistochemical staining for smooth muscle specific α-actin, myosin H chain, and calponin by Western blotting and flow cytometry. ASCMs were cultured in DMEM/F12 (1:1; Life Technologies/Invitrogen, Grand Island, NY) supplemented with 10% FBS and 100 μM ofgent between passages 4 and 8. Prior to each experiment, ASMCs were starved for 24 h in DMEM/F12 culture medium supplemented with 0.1% FBS and 100 μM penicillin/streptomycin. The primary ASCMs from asthmatic subjects used in our protocols were purchased from Lonza (Basel, Switzerland).

Migration assay

ASMC migration was assessed as previously described (33) using a 48-well microchemotaxis Boyden chamber (Neuro Probe, Cabin John, MD). Briefly, different concentrations of recombinant human (rh)CXCL1 alone or in combination with CXCL2 and/or CXCL3, were added to the lower chamber, and 1 × 105 cells/ml normal (or asthmatic, where applicable) ASCMs were added to the upper chamber. A 10-μm-pore polycarbonate membrane (Neuro Probe) treated with 0.1% FBS and 100 μM penicillin/streptomycin. The primary ASCMs from asthmatic subjects used in our protocols were purchased from Lonza (Basel, Switzerland).

The role of CXCR1 and/or CXCR2 receptors or other G(a)i-coupled receptors in CXCL1 inhibition of ASM migration

ASMCs were treated for 1 h at 37°C with 10 μg/ml anti-CXCR1 and/or anti-CXCR2 neutralizing Abs (R&D Systems, Minneapolis, MN) or with 10, 50, or 100 ng/ml pertussis toxin (Ptx; TOCRIS Bioscience, Bristol, UK) prior to the addition of CXCL1 to the Boyden chamber. Species-specific isotype controls were used as controls for the neutralizing Abs. Ptx was reconstituted in sterile distilled water.

The role of ERK-1/2 MAPK-signaling pathway and HSP90 in CXCL1 inhibition of ASM migration

ASMCs were treated for 1 h at 37°C with either ERK-1/2 MAPK inhibitor PD184352 (2 μM; US Biological, Swampscott, MA) (34) or with different concentrations (1, 10, or 100 nM) of 17-N-allylamino-17-demethoxygal-danamycin (17-AAG), a low-toxicity profile inhibitor of HSP90 (35) (AG Scientific, San Diego, CA) prior to the addition of CXCL1 to the Boyden chamber. Controls were exposed to the same concentration of DMSO.

Small interference RNA transfection

ASMCs were grown to 60–80% confluency in medium containing 10% FBS. The cells were transfected with small interference RNA (siRNA) targeting CXCR1 and/or CXCR2, DARC, or HSP90α using the siRNA Reagent System (all from Santa Cruz Biotechnology, Santa Cruz, CA). Scrambled siRNAs (Santa Cruz Biotechnology) were used as a negative control. After transfection, ASMCs were either used directly for migration experiments or stimulated with CXCL1 and prepared for Western blotting.

Protein lysates from transfected, nonstimulated ASMCs were saved to evaluate the efficiency of siRNA knockdown by Western blotting using anti-CXCR2 (Abcam, Cambridge, MA), anti-CXCR1 (R&D Systems, Minneapolis, MN), anti-DARC (Abcam), or anti-HSP90α (Abcam) Abs. GAPDH (Millipore, Billerica, MA) was used as a loading control.

Immunofluorescence staining

ASMCs were seeded on a Lab-Tek Chamber Slide System (Thermo Fisher Scientific, Rochester, NY) and left to adhere overnight at 37°C. After which they were treated with 4 ng/ml rhCXCL1 for 30 min, washed 1× PBS, and fixed with 4% paraformaldehyde. To eliminate nonspecific binding, cells were incubated with Protein Block (Dako Canada, Burlington, ON, Canada) for 1 h at room temperature (RT). Subsequently, cells were treated with 1:250 of rabbit monoclonal anti-DARC (Abcam) and mouse monoclonal anti-HSP90α (Abcam) Abs diluted in Ab Diluent (Dako Canada) at 4°C overnight. Cells were washed with 0.1% Tween 20/ PBS and incubated with 1:300 Alexa Fluor 555–conjugated donkey anti-rabbit IgG (highly cross-adsorbed) and Alexa Fluor 488–conjugated goat anti-mouse IgG (Invitrogen, Carlsbad, CA) for 1 h at RT. After washing, cells were stained with the nuclear staining Hoechst 33342 (AnaSpec, Fremont, CA) for 10 min. Using laser scanning confocal microscopy (LSM780; Carl Zeiss Microscopy, Jena, Germany), stained ASMCs were viewed using a 63×/1.40 oil DIC “Plan Achromat” objective, and images were analyzed using ImageJ (36) (National Institutes of Health, Bethesda, MD). Negative controls were performed using 1:250 of species-specific isotype Ab.

Western blot for signaling pathway analysis

Normal ASMCs, or transfected ASMCs where applicable, were treated with 4 ng/ml rhCXCL1 for 0, 5, 15, 30, 45, or 60 min, washed twice with cold 1× PBS, and lysed. Whole-cell lysates (10–15 μg) were loaded on 10% acrylamide SDS-PAGE NEXT GEL (AMRESCO, Solon, OH), followed by transfer to nitrocellulose membranes (Bio-Rad, Hercules, CA). The blots were blocked for 1 h at RT and incubated for 24 h at 4°C with Abs specific to total ERK1/2 MAPK and phosphorylated ERK1/2 MAPK (42 and 44 kDa; both from Cell Signaling), phosphorylated p38 MAPK (42 kDa; Cell Signaling), GADDH (38 kDa; Millipore), phospho-HSP27 (25 kDa; Cell Signaling), phospho-aSkt (~60 kDa; Cell Signaling) for PI3K pathway activation, and β-actin (poly 20 kDa; Santa Cruz Biotechnology). After washing (0.1% Tween 20/PBS), the membranes were incubated with a 1:15,000 dilution of IRDye 680 goat anti-mouse IgG and IRDye 680 goat anti-rabbit IgG (Rockland, Philadelphia, PA) in blocking buffer and analyzed with an Odyssey IR scanner using Odyssey imaging software 3.0 (LI-COR Biosiences).

Apoptosis assays

Viability of ASMCs after treatment with pathway inhibitors or different concentrations of CXCL1 (0.25–4 ng/ml) or after siRNA transfection was measured using flow cytometry (BD FACSCalibur; BD Biosciences) for annexin V and propidium iodide (PI) staining. Briefly, ASCMs from healthy controls, and asthmatic subjects where applicable, were treated with 1) DMSO or PD184352 for 1 hr at 37°C or 2) different concentrations of CXCL1 (0.25–4 ng/ml) for 4 h at 37°C or 3) transfected with different siRNAs (scrambled siRNA [siScr] controls, siCXCR1, siCXCR2, siDARC or siHSP90α). Subsequently, cells were trypsinized, washed, and stained using an annexin V and PI staining kit (Annexin V:FITC Apoptosis Detection Kit; BD Biosciences) to quantify the percentage of cells undergoing apoptosis, following the manufacturer’s suggested protocol. There was no evidence of cytotoxicity in ASMCs treated with the inhibitors or the siRNAs at the concentrations used (data not shown).

Assessment of HSP90α and HSP90β mRNA by quantitative real-time PCR

Serum-starved ASCMs were treated with 4 ng/ml rhCXCL1 for 0.5, 1, or 3 h, and total RNA was extracted using the RNeasy Mini kit (QIAGEN; Mississauga, ON, Canada). Quality control and RNA concentrations were determined using a spectrophotometer (Nanodrop Products, Wilmington, DE).
A total of 40 μl reaction mix containing 250 ng total RNA, 1 μl reverse transcriptase Superscript II (Invitrogen), 0.5 μl RNase inhibitor, 8 μl 5× first-strand buffer (Invitrogen), 4 μl 0.1 M DTT, 0.5 μM each deoxyribonucleotide triphosphate, and 0.5 mg oligo dT12–18 primers (Invitrogen) was prepared. The reaction program was 37°C for 10 min, 42°C for 50 min, 45°C for 10 min, and then cooling at 4°C. cDNA was amplified using the 7500 Fast Real-Time PCR system thermal cycler, using SYBR Green (both from Applied Biosystems, Foster City, CA) and the following primers: HSP90a (forward 5′-GGC AGA GCC TGA TAA GAA CG-3′ and reverse 5′-CGT GAT TGC TCT TCA TT-3′), HSP90b (forward 5′-GAA CCA TTG CCA AGT CTG GT-3′ and reverse 5′-AGC CAC AGT GAA GGA ACC TC-3′), and GAPDH (forward 5′-AGT CAA GGG ATT TGG TCG TAT T-3′ and reverse 5′-ATG GGA ATC ATA TTG GAA C-3′). The HSP90 mRNA expression was normalized to GAPDH and compared using the ΔΔCt method. All results were expressed as relative quantity compared with nonstimulated controls.

**Statistical analysis**

All data are presented as mean ± SEM. Statistical analysis was performed using ANOVA, followed by the post hoc Dunnett test. The Bonferroni multiple-comparison post hoc test was performed for statistical analysis within and between stimulated conditions. The p values < 0.05 were regarded as statistically significant. Statistical analysis was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA; http://www.graphpad.com).

**Results**

**CXCL1 is a negative regulator of ASMC migration**

Recently, we showed that CXCL2 and CXCL3 induced significant migration of ASMCs over a range of concentrations (37). Using similar approaches, we sought to investigate the effect of CXCL1 on ASMC migration and found that, over a range of 10-fold increasing concentrations, CXCL1 did not appear to affect normal or asthmatic ASMC migration (Supplemental Fig. 1A, 1B, respectively). However, by exploring a narrower range of concentrations (0.25, 0.5, 1, 2, and 4 ng/ml), it was possible to demonstrate that CXCL1 could significantly inhibit both normal and asthmatic ASMC migration at 2 ng/ml (normal: 0.52-fold ± 0.07, p < 0.01; asthmatic: 0.52-fold ± 0.11, p < 0.01) and 4 ng/ml (normal: 0.48-fold ± 0.11, p < 0.001; asthmatic: 0.52-fold ± 0.10, p < 0.01) (Fig. 1A, 1B, respectively). The inhibitory effect of CXCL1 was not attributable to toxicity (Supplemental Fig. 1C, 1D). These results indicate that CXCL1 inhibits ASMC migration over a very narrow range of concentrations, without affecting cell viability.

We previously established that the effect of CXCL2 and CXCL3 on ASMC migration was not significantly different from platelet-derived growth factor (PDGF)-BB (39), one of the known potent mediators of ASMC migration (38). Therefore, we sought to investigate the extent of the inhibitory effect of CXCL1 on ASMC migration by adding CXCL1 to CXCL2 and/or CXCL3 in equal concentrations, using normal ASMCs. We found that CXCL1 inhibits CXCL2-induced ASMC migration at all concentrations tested, with the exception of 0.25 ng/ml (Fig. 1C). It also inhibits CXCL3- and CXCL2/CXCL3-induced ASMC migration at final concentrations of 2 and 4 ng/ml (Fig. 1D, 1E, respectively). Interestingly, however, the addition of 4 ng/ml of CXCL1 to 5 ng/ml of PDGF-BB significantly enhanced PDGF-BB–induced ASMC migration (Fig. 1F). These results indicate that CXCL1 may act as an inhibitor of CXCL2- and/or CXCL3-enhanced ASMC migration but not PDGF-induced ASMC migration.

**CXCL1 inhibition of ASMC migration is mediated through DARC but not CXCR1, CXCR2, or other Gai–protein-coupled receptors**

Because CXCL1 was shown to bind to both CXCR1 and CXCR2 (39), we investigated the role of both receptors in CXCL1-mediated inhibition of ASMC migration. We found that CXCL1 inhibition of ASMC migration was not sensitive to the effect of neutralizing Abs (Fig. 2A) or siRNA knockdown (Fig. 2B) of CXCR1 and CXCR2 receptors, or to the effect of Ptx, which inhibits Gai signaling (40) (Fig. 2C), suggesting that it is unlikely for CXCR1, CXCR2, and other Gai–protein-coupled receptors (Gai-PCRs) to be involved in mediating CXCL1 inhibition of ASMC migration.

DARC, a decoy receptor that binds to CXCL1 with high affinity (27), was shown to be expressed in RBCs (24), endothelial cells (30), brain (41), and epithelial cells (42), but it has never been described in ASMCs. After establishing DARC expression in ASMCs by immunofluorescence staining of cultured normal ASMCs (Fig. 2D), we investigated its involvement in CXCL1-mediated inhibition of ASMC migration using siRNA knockdown (siDARC). Transfection of ASMCs with siDARC eliminated the inhibitory effect of CXCL1 on ASMC migration and actually led to enhanced ASMC migration compared with the siScram control (siScram: 0.64-fold ± 0.06, siDARC: 1.4-fold ± 0.08; p < 0.001, Fig. 2E), indicating that DARC facilitates CXCL1-mediated inhibition of ASMC migration. Moreover, in the absence of DARC, CXCL1 was able to enhance ASMC migration over that of control levels, suggesting the activation of alternate signaling mechanisms, possibly through other receptor(s).

The siRNA knockdown efficiency reached 45% for CXCR1, 49% for CXCR2, and 56% for DARC, as measured by Western blotting (Supplemental Fig. 2A, 2B, 2C, respectively).

**CXCL1 inhibits ASMC migration through selective activation of ERK1/2 MAPK, but not p38 or PI3K, pathways**

DARC, which lacks the ability to recruit small G proteins, has never been shown to participate in signaling cascades (23). However, in light of our observation that DARC mediates the inhibitory effect of CXCL1 on ASMC migration, we sought to test whether treatment with CXCL1 activated any of the signaling pathway(s) (ERK1/2, p38, or PI3K) in ASMCs. We found that, although CXCL1 induced significant activation of ERK1/2 MAPK starting at 5 min (10.22-fold ± 1.7; p < 0.001) and continuing for up to 60 min (4.34-fold ± 0.89; p < 0.05) after stimulation (Fig. 3A), the p38 MAPK and PI3K pathways were not activated (Fig. 3B, 3C, respectively). We used the pharmacological inhibitor PD184352 to further establish a possible role for the ERK1/2 MAPK pathway in CXCL1-mediated inhibition of ASMC migration. Similar to the effect of siDARC, inhibition of the ERK1/2 MAPK pathway reversed the effect of CXCL1 on ASMC migration from inhibitory to stimulatory (DMSO: 0.74-fold ± 0.05, PD184352: 1.334-fold ± 0.08, p < 0.001, Fig. 3D). These results indicate that CXCL1 inhibits ASMC migration through activation of the ERK1/2 MAPK pathway, but not the p38 MAPK or PI3K pathway, suggesting a biased selection in the activation of signaling cascades.

**siDARC inhibits ERK1/2 MAPK while enhancing p38 MAPK and HSP27 activation in ASMCs in response to CXCL1**

Administration of siDARC led to a significant reduction in ERK1/2 MAPK activation compared with siScram, beginning at 5 min (siScram: 9.36-fold ± 0.53, siDARC: 5.33-fold ± 0.36; p < 0.001) and persisting for up to 60 min (siScram: 6.06-fold ± 0.66, siDARC: 3.37-fold ± 0.36; p < 0.01) after stimulation (Fig. 4A). We used siCXCR1 and siCXCR2 to exclude the possibility that CXCR1 and CXCR2 play roles in ERK1/2 MAPK pathway activation and found no significant difference compared with siScram (Supplemental Fig. 3). These findings suggest that DARC, but not CXCR1 and/or CXCR2, mediates CXCL1-induced ERK1/2 MAPK activation.

To investigate the signaling pathway(s) responsible for CXCL1-enhanced ASMC migration in the absence of DARC, we sought to
examine the activation of the PI3K and p38 MAPK pathways in CXCL1-stimulated ASMCs after siDARC. We found a significant increase in p38 MAPK activation in ASMCs at 5 min (siScram: 1.32-fold ± 0.17, siDARC: 2.32-fold ± 0.40; p < 0.01) and 15 min (siScram: 0.98-fold ± 0.18, siDARC: 2-fold ± 0.28; p < 0.01) after stimulation (Fig. 4B). In contrast, the PI3K pathway was not activated significantly (Fig. 4C). Activation of HSP27 has been established as a regulator of cell migration downstream of the p38 MAPK pathway in both endothelial cells (43) and ASMCs (44). Therefore, we examined HSP27 in response to CXCL1 after siDARC and found that it also was activated at 5 min (siScram: 0.9-fold ± 0.35, siDARC: 1.79-fold ± 0.21; p < 0.05) and 15 min (siScram: 0.81-fold ± 0.25, siDARC: 1.73-fold ± 0.27; p < 0.05) after stimulation (Fig. 4D). These results suggest that, in the absence of DARC, CXCL1-enhanced ASMC migration is likely to be mediated through the p38 MAPK/HSP27 pathway.

In the absence of DARC, CXCR2 mediates CXCL1-enhanced ASMC migration and p38 MAPK pathway activation

As a first step in investigating which receptor(s) mediates CXCL1-enhanced ASMC migration and p38 MAPK activation after siDARC, we examined the protein expression of CXCR1 and CXCR2 by Western blotting. Administration of CXCL1 to siDARC-ASMCs significantly enhanced the protein expression of CXCR2 (Fig. 5B). In contrast, CXCR1 protein expression was not affected (Fig. 5A). We next examined the effect of CXCL1 on ASMC migration and p38 MAPK activation after simultaneous double siRNA knockdown of DARC and CXCR2 (siDARC/CXCR2) and found that it significantly blunted both the inhibitory and the enhancing effects of CXCL1 on ASMC migration compared with siScram and to siDARC, respectively, (siScram: 0.66 ± 0.03, siDARC: 1.45 ± 0.09, siDARC/CXCR2: 0.9 ± 0.04; p < 0.001, Fig. 5C). In addition, it reduced p38 MAPK enhanced activation to control levels (Fig. 5D). These results indicate that, in the absence of DARC, CXCL1 enhances ASMC migration through CXCR2/p38 MAPK pathway activation.

siHSP90α mediates DARC protein expression and CXCL1 inhibition of ASMC migration

HSP90 is a chaperone protein involved in many cellular processes and is known to act as an essential molecular chaperone of GPCR- and non–GPCR-mediated ERK1/2 phosphorylation (45, 46). Therefore, we sought to investigate the possible role of HSP90 in CXCL1/DARC-mediated ERK1/2 MAPK pathway activation.
and ASMC migration inhibition. Using 17-AAG, we found that CXCL1 inhibition of ASMC migration was reversed to control levels at 10 and 100 nM (Fig. 6A), supporting a possible role for HSP90 in CXCL1-mediated ASMC migration inhibition. However, there are two isoforms of HSP90, HSP90α and HSP90β (47, 48), which are known to differentially regulate cellular processes (49). When we examined the mRNA expression of HSP90α and HSP90β in ASMCs in response to 4 ng/ml of CXCL1, we found a significant increase in the mRNA expression of HSP90α, but not HSP90β, after 30 and 60 min of stimulation (Supplemental Fig. 4). Similar to siDARC, administration of siHSP90α changed the effect of CXCL1 on ASMC migration from inhibitory to stimulatory (Fig. 6B), and it significantly reduced ERK1/2 MAPK activation (Fig. 6C) while increasing p38 MAPK activation at 5 and 15 min (Fig. 6D) after treatment. These results suggest that the HSP90α isoform plays a role in CXCL1-induced ERK1/2 MAPK pathway activation and ASMC migration inhibition.

Given that siHSP90α produced similar results to siDARC, we postulated that some relationship may exist between the two proteins. Using confocal microscopy, we found that, in the presence of HSP90α, treatment of ASMCs with CXCL1 increased DARC recruitment at the cell periphery, near the cell surface (Fig. 6E). Conversely, siHSP90α caused near-complete disappearance of DARC expression, which was not rescued by CXCL1 treatment (Fig. 6F). These results indicate that HSP90α may be important for the regulation and/or the stability of DARC protein expression in ASMCs.

The siRNA knockdown efficiency reached 68% for HSP90α, as measured by Western blotting (Supplemental Fig. 2D).

**Discussion**

Although cell migration is a fundamental mechanism involved in many biological processes, when dysregulated or inappropriate in magnitude it could lead to extremely damaging outcomes, such as airway remodeling in asthma (7). Directed cell migration, also known as chemotaxis, is a complex process initiated by a concentration gradient of stimulus sensed by specific receptors, followed by activation of signaling pathways, cellular polarization, recycling of actin filaments and actin binding proteins, and recruitment of cell–cell and cell–matrix adhesion proteins (50, 51). There has been little progress in understanding the underlying mechanisms driving cell migration, and there are few examples of molecules capable of its inhibition (52, 53). In this study, we demonstrate that CXCL1 is a physiologically significant inhibitor of both basal and stimulated ASMC migration. We also show that the DARC receptor is able to modulate this inhibition through its effect on the activity of the ERK-1/2 MAPK–signaling pathway.

Previously, we investigated the effect of chemokines on ASMC migration and found evidence that the range of concentrations in conventional use (0.01–100 ng/ml) may not accurately reflect the capacity of chemokines to induce ASMC migration (37). Moreover, depending on disease status, the concentrations of CXCL1 measured in biological fluids of humans or animal models do not...
exceed 10 ng/ml (18, 54, 55). Therefore, using a narrower range of concentrations of CXCL1 to examine its effect on ASMC migration may better reflect its biological significance in asthmatic airways remodeling. In this article, we show that, over a narrower range of concentrations, CXCL1 exhibited an inhibitory effect on normal and asthmatic ASMC migration, an outcome that was not observed using the conventional concentrations. In support of our finding that CXCL1 is capable of inhibiting ASMC migration in vitro, a very recent study on a murine model of prostate cancer demonstrated that in vivo engraftment of cancer cells capable of producing CXCL1 (TRAMP-G2LG) inhibited tumor growth and invasion, in contrast with cell lines that do not produce CXCL1 (TRAMP-G2) (56). Interestingly, these investigators found that the production of CXCL1 from TRAMP-G2LG cancer cells reached ~4 ng/ml, similar to the concentration of CXCL1 that inhibited ASMC migration in our study. These results suggest that, although known as a mediator of cell chemotaxis (16, 17), at specific concentrations CXCL1 may act as a negative regulator of cell migration/invasion both in vitro and in vivo.

We also found that combining CXCL1 with CXCL2 and/or CXCL3 in equal concentrations effectively inhibited CXCL2- and/or CXCL3-induced ASMC migration. These results are supported by a recent report demonstrating that both endogenously produced and the exogenous addition of CXCL1 equally inhibited CXCL8- and CXCL10-induced mast cell migration (57). Conversely, combining CXCL1 with PDGF-BB significantly enhanced the latter’s effect on ASMC migration (data not shown). The discrepancy in the combinatorial effect of CXCL1 on ASMC migration also was shown with primary oligodendrocyte progenitor cells (58), albeit using different chemotactic mediators, namely PDGF-A and fibroblast growth factor-2. Using these mediators, Vora et al. (58) showed that CXCL1 was able to inhibit PDGF-induced, but not fibroblast growth factor-2-induced, oligodendrocyte progenitor cell migration, and that the effect of CXCL1 was concentration dependent. Taken together, these findings indicate that CXCL1 is capable of inhibiting cell migration alone or in the presence of other chemotactic mediators and that its inhibitory effect may not be restricted to ASMCs. Moreover, our results and those of other investigators demonstrate the complexity of the interaction between CXCL1 and other chemotactic mediators in different cell types in which the inhibitory effect of CXCL1 is likely to be governed by many factors, including, but not limited to, cell type, the chemotactic mediators used, and the concentrations of CXCL1 that were used in these combinations.

Although CXCL1 is known to specifically signal through CXCR2 and, in some instances, CXCR1 (20), we were able to demonstrate, using neutralizing Abs, siRNA transfection, and inhibition by Ptx, that the inhibitory effect of CXCL1 on ASMC migration was not mediated through CXCR1, CXCR2, or any other G(αi)-PCR. Instead, our findings indicate that CXCL1 inhibited ASMC migration via DARC. This finding is of particular interest given that a recent study of three populations of African
descent found a relationship between a functional polymorphism in the DARC gene and an increased susceptibility to asthma (59). Moreover, in several types of cancer, increased DARC expression was associated with decreased tumor metastasis and tumor size, as well as higher survival rates (60, 61). Our results indicate that the inhibitory effect of CXCL1 is mediated through DARC and suggest that DARC may play a protective role in migration-dependent diseases, such as asthma and cancer.

It is widely accepted that, because of its lack of the DRYLAIV motif, DARC does not induce canonical GPCR signaling (23). Nevertheless, our finding that CXCL1's inhibition of ASMC migration was mediated through DARC suggested the possibility that DARC contributes to signaling initiation. Indeed, treating ASMCs with CXCL1 significantly induced ERK-1/2 MAPK pathway activation, which mediated CXCL1 inhibition of ASMC migration. However, administering CXCL1 to ASMCs after siDARC resulted in a significant reduction in ERK-1/2 MAPK activity; conversely, we observed enhanced activity of p38 MAPK and downstream HSP27, a signaling pathway that has long been established as a modulator of cell migration (44). Interestingly, we found this enhancing effect to be mediated through CXCR2 in DARC-deficient ASMCs. Of interest, binding studies using a mutant form of CXCL1, named E6A, demonstrated that it was able to bind to DARC on RBCs and inhibit malaria parasite invasion as efficiently as the wild-type CXCL1. However, it was not able to bind to CXCR2 on neutrophils (62), suggesting the possibility that

FIGURE 4. siDARC inhibits ERK-1/2 MAPK while enhancing p38 MAPK and HSP27 activation in ASMCs in response to CXCL1. Whole-cell lysates from siDARC ASMCs treated with 4 ng/ml of CXCL1 for 0, 5, 15, 30, 45, or 60 min were used for detection of pathway activation by Western blotting. siDARC significantly reduced CXCL1-induced ERK-1/2 MAPK pathway activation (A) while enhancing p38 MAPK pathway (B) and downstream HSP27 (D) activation. (C) In contrast, there was no change in PI3K pathway activation before or after siDARC. Representative blots of ERK-1/2 MAPK, p38 MAPK, and HSP27 activation in response to CXCL1 after siScram (E) or siDARC (F). Results are expressed as mean ± SEM (n = 4–5 subjects). *p < 0.05, **p < 0.01, ***p < 0.001 versus nonstimulated controls; $p < 0.05, $$$p < 0.001 versus siScram.
DARC may compete with CXCR2 for the binding of CXCL1. In addition, CCX-CKR, another decoy receptor, was reported to inhibit CXCR3-mediated chemotaxis of cotransfected HEK293 cells by forming heterodimeric complexes with CXCR3 and subsequently affecting its binding properties (63). Accordingly, it is reasonable to speculate that DARC may also form complexes with the CXCR2 receptor and, consequently, prevents its binding to CXCL1. However, in the aforementioned study, the investigators did not find any difference in CXCR3 expression in HEK293 cells, whether or not they were cotransfected with CCX-CKR (63). In contrast, we observed an increase in CXCR2 protein expression in DARC-deficient ASMCs upon treatment with CXCL1, suggesting that the presence of DARC may have a regulatory influence on CXCR2 expression. Moreover, CCX-CKR was only able to inhibit CXCR3/CXCL9- and CXCR3/CXCL10-enhanced chemotaxis. In contrast, DARC/CXCL1 inhibited both basal and enhanced ASMC migration, suggesting that this inhibition is probably regulated through a different mechanism. Taken together, our results establish that ERK1/2 MAPK is the sole pathway mediating CXCL1 inhibition of ASMC migration and that, in the absence of DARC, CXCL1 enhances ASMC migration through the CXCR2/p38MAPK/HSP27-signaling pathway. Moreover, they suggest that the presence of DARC in ASMCs may negatively regulate CXCR2 function, either through competition or by influencing its expression, conformation, or binding capacity to CXCL1.

There has been recent interest in the ability of GPCRs to signal through both G protein and arrestin pathways in a ligand-specific manner. The latter mechanism, termed “bias signaling,” primarily activates the ERK1/2 MAPK pathway and is specifically regulated by β-arrestin 2, independent of G protein coupling (64–66). Using Western blotting, we could not detect β-arrestin 2 protein expression in ASMCs with or without CXCL1 stimulation (data not shown), suggesting that β-arrestin 2 is unlikely to mediate CXCL1/DARC-induced ERK-1/2 MAPK pathway activation. Moreover, neither inhibition of src nor blockade of epidermal growth factor receptor, two pathways known to mediate ERK-1/2 MAPK pathway activation (67) using the pharmacological inhibitor PP2 (68) and PD153035 (69), was able to inhibit CXCL1/DARC-mediated ERK-1/2 MAPK pathway activation (data not shown), suggesting that they may not be involved in this activation. Instead, we chose to examine the effect of HSP90, a chaperone protein that was found to be involved in the activation of ERK1/2 MAPK pathway in vascular smooth muscle cells through direct association with phospho-ERK1/2 (46). We found that inhibition of HSP90, specifically HSP90α, gave similar results to those obtained with siDARC. Interestingly, however, the observed
effect of siHSP90α was not directly related to ERK-1/2 activation per se; rather, it appears to be due to the effect that HSP90α has on DARC protein expression. Taken together, our results suggest that HSP90α may have a regulatory effect on DARC protein expression and function.

In conclusion, we found that, over a specific range of concentrations, CXCL1 acts as a negative regulator of ASMC migration and that this effect is mediated by activation of the ERK-1/2 MAPK pathway. In addition, our results provide a demonstration of the ability of the decoy receptor DARC to mediate CXCL1 signaling and function. They also establish the CXCL1/DARC/ASMC interaction as a pathway that may play a role in the pathology of asthma remodeling, meriting further study to establish its role in ASMC migration and as a therapeutic objective in airway remodeling in asthma.

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Disclosures
The authors have no financial conflicts of interest.

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