

Chemokine release from human rhinovirus–infected airway epithelial cells promotes fibroblast migration

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Background: Thickening of the lamina reticularis, a feature of remodeling in the asthmatic airways, is now known to be present in young children who wheeze. Human rhinovirus (HRV) infection is a common trigger for childhood wheezing, which is a risk factor for subsequent asthma development. We hypothesized that HRV-infected epithelial cells release chemoattractants to recruit fibroblasts that could potentially contribute to thickening of the lamina reticularis.

Objective: We sought to investigate whether conditioned medium from HRV-infected epithelial cells can trigger directed migration of fibroblasts.

Methods: Human bronchial epithelial cells were exposed to medium alone or infected with HRV-16. Conditioned medium from both conditions were tested as chemoattractants for human bronchial fibroblasts in the xCELLigence cell migration apparatus.

Results: HRV-conditioned medium was chemotactic for fibroblasts. Treatment of fibroblasts with pertussis toxin, an inhibitor of G α i-coupled receptors, prevented their migration. Production of epithelial chemoattractants required HRV replication. Multiplex analysis of epithelial supernatants identified CXCL10, CXCL8, and CCL5 as G α i-coupled receptor agonists of potential interest. Subsequent analysis confirmed that fibroblasts express CXCR3 and CXCR1 receptors and that CXCL10 and, to a lesser extent, CXCL8, but not CCL5, are major contributors to fibroblast migration caused by HRV-conditioned medium.

Conclusion: CXCL10 and CXCL8 produced from HRV-infected epithelial cells are chemotactic for fibroblasts. This raises the possibility that repeated HRV infections in childhood could contribute to the initiation and progression of airway remodeling in asthmatic patients by recruiting fibroblasts that produce matrix proteins and thicken the lamina reticularis. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: Airway epithelium, chemoattractants, fibroblasts, human rhinovirus, migration, airway remodeling, inflammation

Airway remodeling is a characteristic feature of asthma that includes thickening of the lamina reticularis, increased smooth muscle mass, angiogenesis, increased numbers of fibroblasts/myofibroblasts, an altered epithelial phenotype, and goblet cell hyperplasia/metaplasia, leading to increased mucus production.¹ Collectively, these changes result in a thickened airway wall and have been suggested to underlie the development and persistence of airways hyperresponsiveness.^{2,3}

Although asthma can manifest at any age, it is usually evident in childhood in the majority of patients.⁴ Therefore it is notable that recent bronchial biopsy studies have demonstrated that several major components of airway remodeling are already present in preschool children with symptoms or airflow limitation, in some instances even before a formal clinical diagnosis of asthma is made.⁵⁻⁷ Interestingly, although thickening of the lamina reticularis was observed in these studies, it was not observed in infants with airflow limitation,⁸ suggesting that this feature of remodeling is induced as a consequence of some initiating stimuli experienced in early childhood.

Human rhinovirus (HRV) infections in early life are a common trigger for childhood wheezing illnesses,⁹ and HRV-induced wheezing illnesses in the first 3 years of life are a major risk factor for subsequent asthma.^{10,11} Longitudinal analysis has shown that preschool children have about 6 HRV infections per year¹² and that serial viral infections can lead to recurrent wheezing episodes.¹³ Because airway remodeling begins to appear in this same early-life timeframe, this raises the concept that HRV infections also can play a role in initiation and progression of airway remodeling.

Thickening of the lamina reticularis results from increased deposition of extracellular matrix proteins believed to be deposited by fibroblasts, myofibroblasts, or both, which are present in increased numbers in the subepithelial region of the airway.¹⁴ Because the airway epithelium is the primary site of HRV infection and replication in the airways, we hypothesized that infected epithelial cells release chemoattractants that could cause migration of human airway fibroblasts toward the epithelial layer.

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Abbreviations used

BEEM: Bronchial epithelial basal medium
 BEGM: Bronchial epithelial growth medium
 DMEM: Dulbecco modified Eagle medium
 GPCR: G protein-coupled receptor
 HBE: Human bronchial epithelial
 HBF: Human bronchial fibroblast
 HRV: Human rhinovirus
 NGS: Normal goat serum
 PDGF: Platelet-derived growth factor
 PTX: Pertussis toxin

METHODS**Materials**

The following reagents were purchased from the indicated suppliers: bronchial epithelial basal medium (BEEM) and additives to create serum-free bronchial epithelial growth medium (BEGM) were from Lonza (Walkersville, Md); HBSS, Dulbecco modified Eagle medium (DMEM), 0.25% Trypsin-EDTA 1X, Tryple Select enzyme solution, and FBS were from Life Technologies (Burlington, Ontario, Canada); WI-38 cells and HRV type 16 were from American Type Culture Collection (Manassas, Va); recombinant CXCL8, CXCL10, and CCL5, antibody for CXCL10 ELISA and neutralization experiments, as well as flow cytometric antibodies for CXCR1, CXCR2, CXCR3, and isotype controls were all from R&D Systems (Minneapolis, Minn); SCH 527123 and SCH 546738 were from MedChem Express (Princeton, NJ); and all other chemicals were purchased from Sigma-Aldrich (Oakville, Ontario, Canada).

Human lungs

Nontransplanted human lungs were obtained from a tissue retrieval service (International Institute for the Advancement of Medicine, Edison, NJ). Ethics approval to receive and use lung tissues was obtained from both the Conjoint Health Research Ethics Board of the University of Calgary and the Internal Ethics Board of Institute for the Advancement of Medicine. No personal identifying information was provided for any of the donors.

Epithelial cell culture

Primary human bronchial epithelial (HBE) cells were obtained by means of protease digestion of dissected airways (main stem bronchus to the fourth generation), as previously described.¹⁵ These airways all contain a morphologically similar pseudostratified epithelium. For the current studies, cells were derived from 6 individual lung donors (5 male donors; age range, 13-63 years). All 6 donors died from head trauma or stroke, and none had any inflammatory lung disease. HBE cells were cultured until 80% to 90% confluence in BEGM at 37°C in 5% CO₂. Before experimentation, cells were cultured for 24 hours in BEGM from which hydrocortisone was removed. HBE cells were then treated with BEEM for 2 hours before stimulation.

Fibroblast cell culture

Human bronchial fibroblasts (HBFs) were also obtained from normal nontransplanted human lungs. For these studies, cells from 17 individual donors (10 male donors; age range, 13-63 years) were used. None of the donors had inflammatory lung disease. One donor died of a central nervous system tumor, and all others died of head trauma or stroke. Main stem bronchi were dissected into small pieces and suspended in DMEM supplemented with 10% FBS. Tissue plugs were placed in 24-well plates, and cells were allowed to grow out to confluence. Tissue plugs were removed and fibroblasts were suspended with Tryple Select and transferred to T-175 flasks (Corning, Corning, NY). This was designated as passage 1. Cells were suspended with Tryple Select and stored at passage 2 in liquid nitrogen. For experimentation, fibroblasts were thawed, placed in a T-175 flask, and grown in 10% FBS

DMEM until approximately 90% to 100% confluence. Once confluence was reached, the cells were again suspended with trypsin and transferred into a cell migration apparatus. Cells were used up to passage 7. Fibroblasts used for migration experiments show typical fibroblast morphology and are negative for α -smooth muscle actin, which is robustly expressed in smooth muscle cells from the same lungs (see Fig E1 in this article's Online Repository at www.jacionline.org).

HRV-16 and viral infection of epithelial cells

HRV-16 viral stocks were propagated in WI-38 cells. Each viral stock was purified to remove soluble products from WI-38 cells by means of sucrose density centrifugation, as previously described.¹⁶ Replication-deficient HRV-16 was produced by exposing aliquots of the same purified stock used for live virus experiments for 5 minutes to a Spectroline Model XX-15F high-intensity short-wavelength (254 nm) UV lamp (Spectronics Corp, Westbury, NY) at a distance of 5 cm. Inactivation was confirmed by the inability of stocks to replicate and cause cytopathic effects in WI-38 cells.

HBE cells from 6 individual tissue donors were exposed to 10⁵ 50% tissue culture-infective dose/mL of HRV-16, UV-treated HRV-16, or control medium (BEEM) for various lengths of time up to 24 hours at 34°C in 5% CO₂. Incubations were performed at 34°C because this is the optimal temperature for HRV replication and is routinely used in the field. After the treatment period had elapsed, conditioned medium from each of the 6 donors was removed and pooled into respective conditions to provide a single consistent stock of material from each condition for migration experiments. These conditions included the following: HRV-conditioned medium (HRV-infected HBEs), UV-HRV-conditioned medium (UV-infected HBEs), and control medium (BEEM-treated HBEs). These media were then used as chemoattractants for HBFs from the indicated number of individual tissue donors in each experiment (n = number of fibroblast donors).

Pharmacologic treatment of fibroblasts

Before treatment with pertussis toxin (PTX), HBFs were seeded at 10⁶ cells per T-75 flask. Once confluence was reached, HBFs were treated with FBS-free DMEM or the same medium containing 3 ng/mL PTX for 12 hours before use for experimentation. The chemokine receptor antagonists SCH 546738 (CXCR3) and SCH 527123 (CXCR1/CXCR2) were used at final concentrations of 20 and 75 nmol/L, respectively, based on preliminary concentration-response experiments against recombinant cytokines.

Neutralizing antibody treatment of conditioned medium

Neutralizing antibodies to CXCL10 (R&D Systems) and CXCL8¹⁷ or appropriate control antibodies at concentrations of 150 and 240 ng/mL, respectively, were added to HRV-16 condition media and incubated at room temperature for 2 hours before use as chemoattractants for HBFs.

Migration assays

Briefly, HBFs were suspended with trypsin and placed into the top wells of the migration assay chamber. Chemoattractants were placed in the bottom wells of the migration apparatus. Stimuli used were as follows: DMEM (negative control), HRV-16 alone at the same dose used to infect HBE, platelet-derived growth factor (PDGF) AB (positive control), and conditioned media removed from HBEs. Both the Boyden chamber (Neuroprobe, Gaithersburg, Md) and xCELLigence apparatus (ACEA Biosciences, San Diego, Calif) were used to complete these experiments. Detailed methods for both systems are provided in the **Methods** section in this article's Online Repository at www.jacionline.org.

Flow cytometry

Fibroblast expression of CXCR3, CXCR2, and CXCR1 was assessed by using flow cytometry after incubation of HBFs with specific antibodies or

appropriate isotype controls. Experiments were performed with HBFs from 5 different donors. Detailed methods are provided in the [Methods](#) section in this article's Online Repository.

Statistical analysis

All data were normally distributed and are presented as means $\pm$ SEMs. Data were analyzed by using 1-way ANOVA with *post hoc* testing with the Newman-Keuls multiple comparison test to determine sources of significant differences. A 2-tailed *P* value of less than .05 was considered significant.

RESULTS

Fibroblasts migrate to conditioned medium from HRV-infected HBE cells

Because the xCELLigence system provides real-time data, we used this apparatus to establish that HBF migration was maximal by 6 hours (see [Fig E2](#) in this article's Online Repository at www.jacionline.org) and thus used 6 hours as the end point time for all subsequent experiments. Because the xCELLigence system is relatively new technology, we initially compared results from this system with those from the traditional modified Boyden chamber. Both systems generated comparable data ([Fig 1, A and B](#)) in which PDGF-AB (positive control) and HRV-conditioned medium induced a significantly greater HBF migratory response compared with control medium. Control medium-driven migration was not significantly different from the HRV-only control in either system. Given these comparable data, the xCELLigence apparatus was used for all subsequent HBF migration experimentation because it has the advantage of generating quantitative real-time data. To determine whether HBF migration induced by epithelial supernatants was chemotactic (directional movement) or chemokinetic (random movement), we abolished chemotactic gradients by adding stimuli to both the bottom and top wells of the xCELLigence apparatus. Under these conditions, migration was completely abrogated ([Fig 1, C](#)), indicating that fibroblast migration to HRV-conditioned medium was chemotactic in nature.

Chemoattractant release from HRV-infected HBE cells is dependent on time and viral replication

To examine the time course of chemoattractant production, we collected conditioned media at 3, 9, 15, and 24 hours after HRV treatment. The chemotactic response of fibroblasts increased with time after HRV infection ([Fig 2, A](#)). Cells were exposed to either replication-competent HRV or to HRV that had been rendered replication deficient by UV light exposure to determine whether this increase was dependent on HRV replication. Cells exposed to intact virus capable of replication generated a chemotactic response, as already seen, but medium from cells exposed to UV treatment did not induce significant fibroblast migration ([Fig 2, B](#)), indicating that viral replication within HBE cells is required to promote chemoattractant release.

Treatment with PTX inhibits HBF migration

Many chemotactic agents, particularly chemokines, exert their effects through G protein-coupled receptors (GPCRs) often linked to the G protein $G_{\alpha i}$.¹⁸ To determine whether the chemoattractant or chemoattractants contained in the HRV-conditioned medium act through a $G_{\alpha i}$ -coupled GPCR, we treated fibroblasts

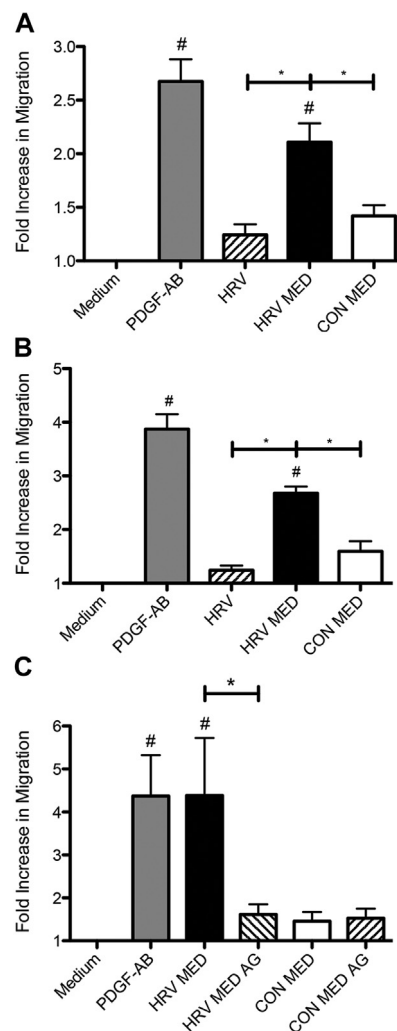


FIG 1. Conditioned medium from epithelial cells infected with HRV (HRV MED) is chemotactic for fibroblasts. **A** and **B**, HRV MED significantly increased migration of HBFs relative to medium alone, conditioned medium from uninfected cells (CON MED), or HRV alone in the Boyden chamber ($n = 4$; [Fig 1, A](#)) or the xCELLigence system ($n = 5$; [Fig 1, B](#)). **C**, Abolition of the chemotactic gradient abrogated migration to HRV MED. # $P < .05$ versus medium alone. * $P < .05$ versus other treatments. Conditions with abolished gradient are indicated by AG.

with PTX, which ADP ribosylates and inactivates $G_{\alpha i}$, before using then in migration assays. There was no effect of PTX on HBF viability (data not shown). As expected, PTX did not inhibit migration induced by PDGF-AB, which functions through a receptor tyrosine kinase. By contrast, PTX significantly reduced fibroblast migration to HRV-conditioned medium ([Fig 3](#)).

CXCL10 and CXCL8 are chemotactic for fibroblasts, but CCL5 is not

To identify potential chemoattractants that might contribute to fibroblast migration in response to HRV-conditioned medium from epithelial cells, we performed a multiplex assay for 64 analytes (Luminex, Toronto, Ontario, Canada) on medium obtained from HBE cells exposed either to medium alone, UV-HRV, or intact HRV. A full listing of data for all analytes is included as [Table E1](#) in this article's Online Repository at

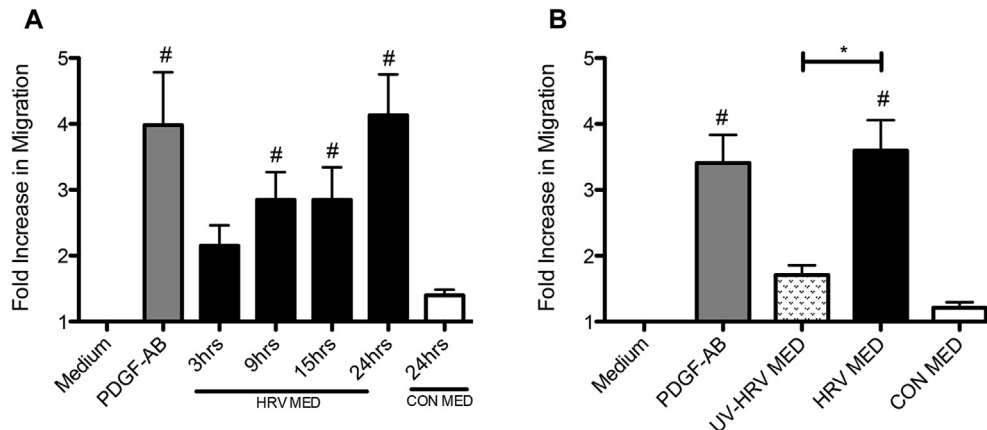


FIG 2. HRV-induced chemoattractant production depends on time after infection and viral replication. **A**, HBF migration to conditioned medium from epithelial cells infected with HRV (HRV MED) increases with time after HRV infection ($n = 5$). **B**, Fibroblast migration to conditioned medium from cells exposed to UV-treated HRV is significantly reduced compared with HRV MED ($n = 6$). CON MED, Conditioned medium from uninfected cells. # $P < .05$ versus medium alone. * $P < .05$ versus other treatments.

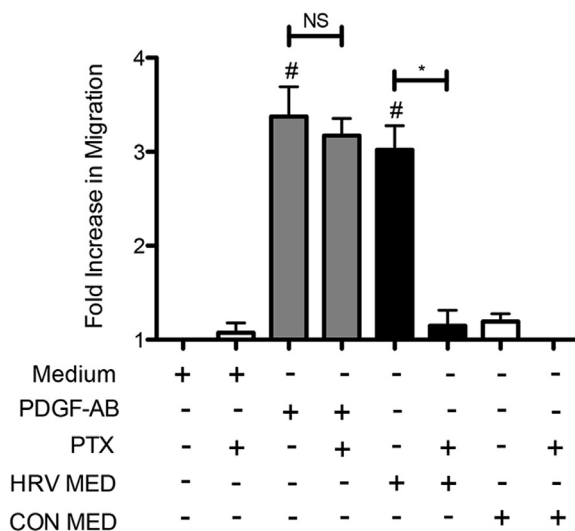


FIG 3. Treatment of fibroblasts with PTX inhibits their migration to conditioned medium from epithelial cells infected with HRV (HRV MED) but not to PDGF. Data are from 6 experiments. # $P < .05$ versus medium alone. * $P < .05$ versus other treatments. CON MED, Conditioned medium from uninfected cells; NS, not significant.

www.jacionline.org. We were interested in candidates fulfilling the following criteria: (1) minimally present in control medium; (2) present at relevant concentrations in HRV-conditioned medium; (3) present at low concentrations or absent in UV-HRV-treated medium; and (4) ligands known to interact with the G α i-coupled GPCRs. Three chemokines satisfied these criteria, namely CXCL8, CXCL10, and CCL5 (Fig 4, A).

To evaluate the potential roles of these candidate chemokines in fibroblast migration, we tested each chemokine individually as chemoattractants for HBFs at the concentrations in which they were found in the multiplex assay. CXCL10 and CXCL8 each induced HBF migration at levels similar to those observed with HRV-conditioned medium or PDGF, but CCL5 was ineffective (Fig 4, B). Concentration response curves were performed to further characterize the ability of these chemokines to serve as chemoattractants for HBF. Both CXCL10 and CXCL8 showed

concentration-dependent ability to induce chemotaxis of HBF (Fig 5, A and B), but CCL5 did not induce fibroblast migration, even at a concentration 10-fold higher than that detected in HRV-conditioned medium (Fig 5, C).

Given that CXCL10 and CXCL8 were chemoattractants that could play a potential role in the ability of HRV-conditioned medium to cause chemotaxis of HBF, we assessed the level of expression of the receptors for these chemokines on fibroblasts. Representative histograms for CXCR3, CXCR1, and CXCR2 are shown (Fig 6, A-C). Mean data from 5 distinct donors indicated that about 75% of fibroblasts showed expression for CXCR3, whereas approximately 25% of cells showed expression for CXCR1 (Fig 6, D). However, few, if any, expressed CXCR2 (Fig 6, C and D).

HBF migration is mediated by CXCL10 and CXCL8

Although CXCL10 and CXCL8 can cause migration of HBFs, we sought to definitively establish whether they played a role in the migration caused by conditioned medium from HRV-infected epithelial cells. To do this, we used 2 complementary approaches. First, we examined whether preincubation of HRV-conditioned medium with blocking antibodies to either chemokine reduced HBF chemotaxis. Blocking antibodies to either CXCL10 (Fig 7, A) or CXCL8 (Fig 8, A), but not isotype control antibodies, significantly reduced migration of fibroblasts to HRV-conditioned medium. These studies were complemented by experiments examining the effects of pretreating fibroblasts with either the CXCR3 receptor antagonist (SCH 546738) or a dual CXCR1/CXCR2 antagonist (SCH 527123) on migration to each respective chemokine and to HRV-conditioned medium. Consistent with the antibody blockade data, pretreatment of fibroblasts with the CXCR3 antagonist significantly reduced HBF migration to HRV-conditioned medium (Fig 7, B). However, treatment with SCH 527123 caused only a modest reduction in fibroblast migration, which was not statistically significant (Fig 8, B).

DISCUSSION

HRV-induced wheezing illnesses in early childhood are strongly associated with an increased risk for subsequent asthma

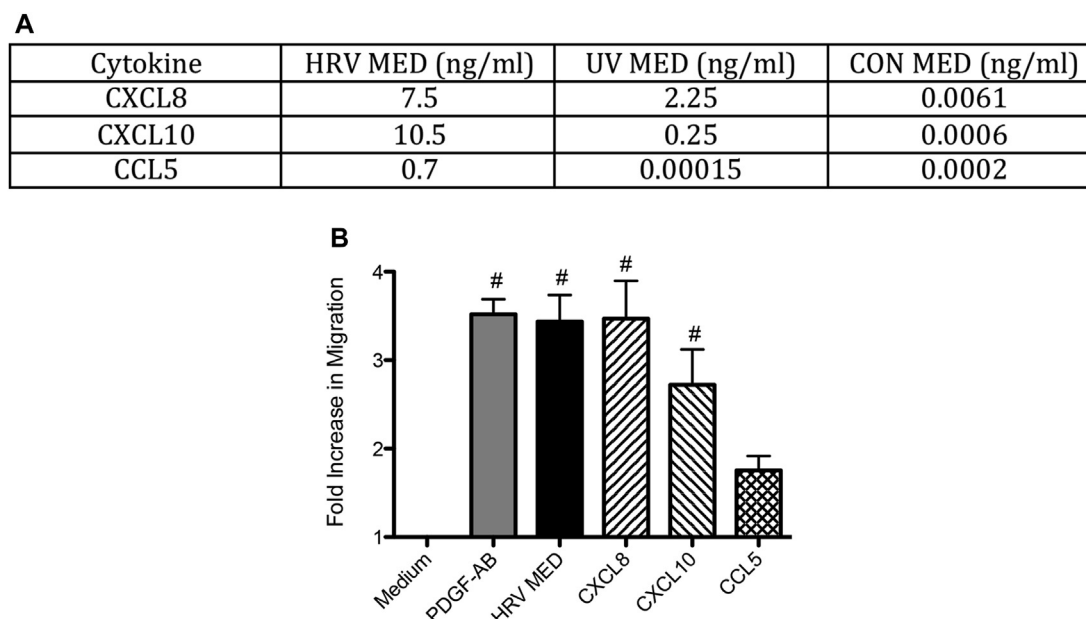


FIG 4. CXCL10 and CXCL8 at levels present in conditioned medium from epithelial cells infected with HRV (HRV MED) induce fibroblast chemotaxis, whereas CCL5 does not. **A**, Levels of each chemokine in epithelial conditioned medium from different treatments (pool of 6 HBE donors). **B**, Fibroblast migration to each chemokine at the level present in HRV MED. Data are from 4 experiments. # $P < .05$ versus medium alone. CON MED, Conditioned medium from uninfected cells.

development,^{10,11} and preschool children who are recurrent wheezers shows signs of airway remodeling, including thickening of the lamina reticularis.^{5,6} Lung fibroblasts play a critical role in maintaining homeostasis of extracellular matrix proteins in the lung, and it is generally believed that thickening of the lamina reticularis is caused by matrix protein deposition by fibroblasts recruited to the subepithelial region of the airway. We now provide evidence that human airway epithelial cells infected with HRV release chemoattractants that can trigger directed migration of fibroblasts toward the epithelial layer. This observation, together with earlier demonstrations that HRV-infected epithelial cells release growth factors linked to structural changes of the airways,^{19,20} provides support for the hypothesis that HRV infections in early childhood might contribute to the initiation and progression of airway remodeling and the subsequent development of asthma.

The demonstration that fibroblast migration to HRV-conditioned medium but not to the receptor tyrosine kinase agonist PDGF could be abrogated by treating fibroblasts with PTX indicated a role for agonists of G α i-coupled GPCRs in the migratory response. This, together with the observation that the generation of epithelial chemoattractants was dependent on HRV replication, allowed us to perform multiplex analysis of supernatants from epithelial cells exposed to medium alone, UV-inactivated HRV, and intact HRV with criteria to identify potential candidates as chemotactic mediators of interest. We identified CXCL10, CXCL8, and CCL5 as 3 mediators that use G α i-coupled GPCRs, which were present in relevant amounts in supernatants from HRV-infected cells and were either absent or present at substantially lower levels in conditioned medium from epithelial cells exposed to medium alone or to UV-inactivated HRV. The generation of CXCL10, CXCL8, and CCL5 from epithelial cells infected with HRV is not surprising because production of all of these chemokines has been reported previously.²¹

The majority of studies of lung fibroblast migration, particularly in murine systems, have emphasized the role of the CC chemokine receptor family and their ligands.²² Therefore it might seem surprising that CCL5, which is a ligand for the CCR1, CCR3, and CCR5 receptors,²² was an ineffective stimulus for fibroblast migration, even at concentrations 10-fold higher than those found in HRV-conditioned epithelial medium. However, there is precedent that CCL11, which is a selective agonist for CCR3, is also ineffective as a direct chemoattractant for human lung fibroblasts.²³ By contrast, our data demonstrate that CXCL10 and CXCL8 both show a concentration-dependent ability to stimulate directed fibroblast migration and, importantly, were effective in concentrations found in HRV-conditioned medium. The expression of CXC chemokine receptor family members on lung fibroblasts has not been extensively studied. The expression of CXCR3 on murine fibroblasts is controversial.^{24,25} To our knowledge, our data are the first to identify CXCR3 expression on normal human fibroblasts, although positive staining of fibroblasts was detected by using immunohistochemistry in biopsy specimens from patients with idiopathic pulmonary fibrosis.²⁵ We also provide clear data that CXCR1 is expressed on a subset of fibroblasts, whereas CXCR2 was minimally expressed, implying that fibroblast chemotaxis to CXCL8 is mediated primarily through interactions with CXCR1.

We confirmed that CXCL10 and CXCL8 are contributors to the chemotactic activity of HRV-conditioned epithelial medium for fibroblasts through use of both specific blocking antibodies and selective chemokine receptor antagonists. Antibodies to CXCL10 and CXCL8 significantly reduced chemotaxis to their respective recombinant cytokines. Although antibody to CXCL10 significantly reduced chemotaxis of fibroblasts to HRV-conditioned epithelial medium, inhibition was not complete. This would be expected given that the conditioned medium also contained other chemoattractants, such as CXCL8, but could also reflect that the level of antibody used did not neutralize all CXCL10 in the

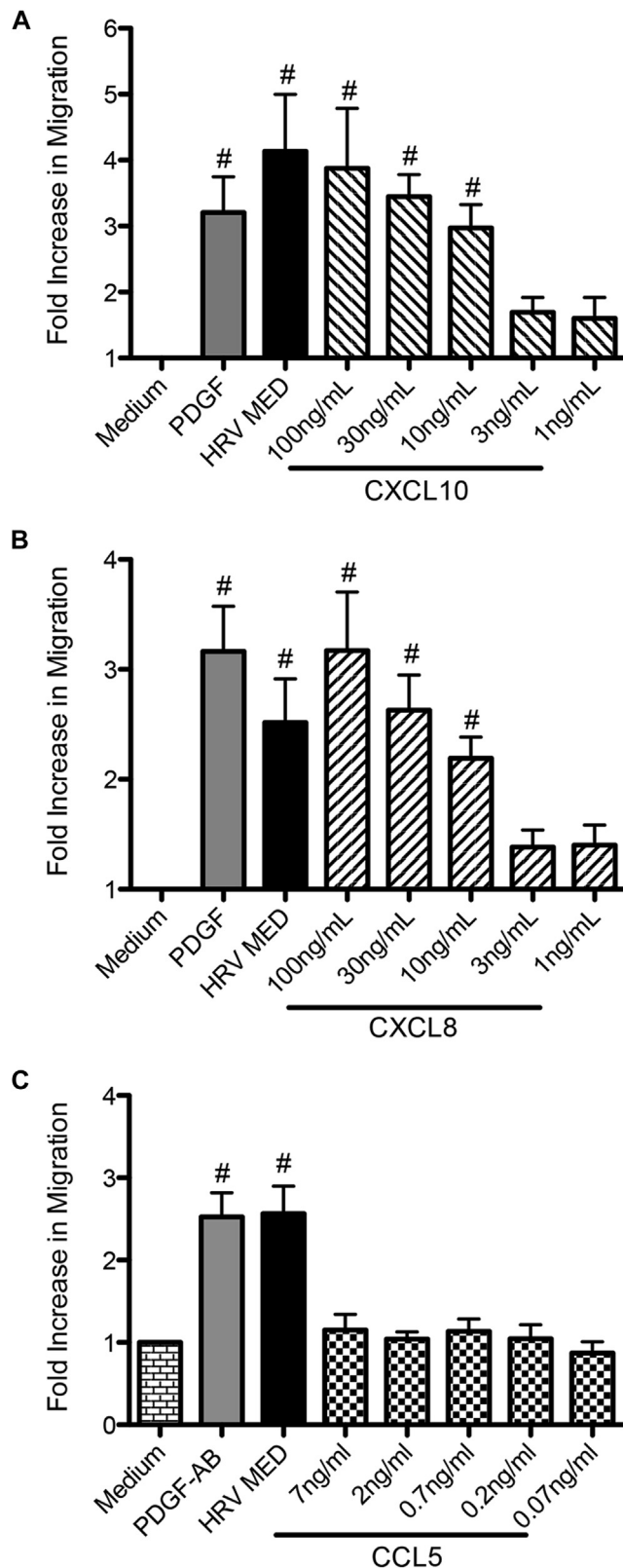


FIG 5. Recombinant CXCL10 and CXCL8, but not CCL5, induce concentration-dependent fibroblast migration. **A**, Response to CXCL10 ($n = 6$). **B**, Response to CXCL8 ($n = 6$). **C**, Response to CCL5 ($n = 3$). Data are shown as means $\pm$ SEMs. # $P < .05$ versus medium alone. HRV MED, Conditioned medium from epithelial cells infected with HRV.

epithelial supernatant. The same concepts would also explain the significant but partial inhibition of chemotaxis to conditioned medium by antibody to CXCL8. Interestingly, data obtained with selective receptor antagonists provide additional insights into the relative role of CXCL10, CXCL8, and their respective receptors in chemotaxis induced by HRV-conditioned epithelial medium. SCH 546738 is a selective and potent antagonist of CXCR3 that has been shown to be effective in several model systems.²⁶ We confirmed its effectiveness in blocking fibroblast chemotaxis to recombinant CXCL10 in our experiments. When this compound was used with HRV-conditioned epithelial medium, a significant and virtually complete abrogation of chemotaxis was observed. A selective antagonist to CXCR1 was not available, and therefore we used the combined CXCR1/2 antagonist SCH 527123, which is actually more potent in blocking CXCR2 receptors than CXCR1 receptors.²⁷ Because we found little expression of CXCR2 on HBFs, we were concerned about the effectiveness of this antagonist but found that it significantly inhibited chemotaxis induced by recombinant CXCL8. In contrast to SCH 546738, however, SCH 527123 did not significantly inhibit chemotaxis to HRV-conditioned epithelial medium.

Taken together, these data would suggest that CXCL10 exerts the dominant chemotactic response in HRV-conditioned epithelial medium through interactions with CXCR3, which is expressed on most fibroblasts. When this is blocked, CXCL8 plays a relatively minor role. By contrast, when CXCR1/2 is antagonized, the minor contribution of CXCL8 is largely blocked, but the major chemoattractant effect of CXCL10 is not affected and maintains fibroblast chemotaxis.

Interestingly, generation of CXCL10 is known to be entirely dependent on viral replication,²⁸ whereas CXCL8 expression is increased both in response to replication intermediates and HRV-16 binding to its receptor, intercellular adhesion molecule 1.²⁹ This is reflected by the presence of substantial levels of CXCL8 in conditioned medium from epithelial cells exposed to UV-treated HRV, and this likely explains the residual chemoattractant effect of UV-HRV-conditioned medium in causing fibroblast migration.

CXCL10 and CXCL8 play well-characterized roles in leukocyte recruitment and inflammation during HRV infections.³⁰ The demonstration that these chemokines can also serve as chemoattractants for fibroblasts broadens their potential role to suggest they might also contribute to aspects of airway remodeling. These data indicate that CXCL10 might be a major contributor to fibroblast recruitment and remodeling in human subjects. Interestingly, however, CXCR3-deficient mice have been reported to show reduced collagen deposition and airway remodeling in response to cigarette smoke.³¹

Our studies have a number of limitations. First, we used cells from adolescent and adult donors, and studies are needed to examine whether epithelial cells from infants would show enhanced responses to HRV infection. In addition, both epithelial cells and fibroblasts used for the current work were obtained from healthy nonasthmatic donors, in whom airway remodeling would not be present. Even in the airways of healthy subjects responding to an HRV infection, however, there is likely turnover of matrix proteins in the airways and accompanying recruitment of fibroblasts. We hypothesize that the responses we show here would be exaggerated if cells from asthmatic donors were used. Additional studies are needed to determine whether epithelial

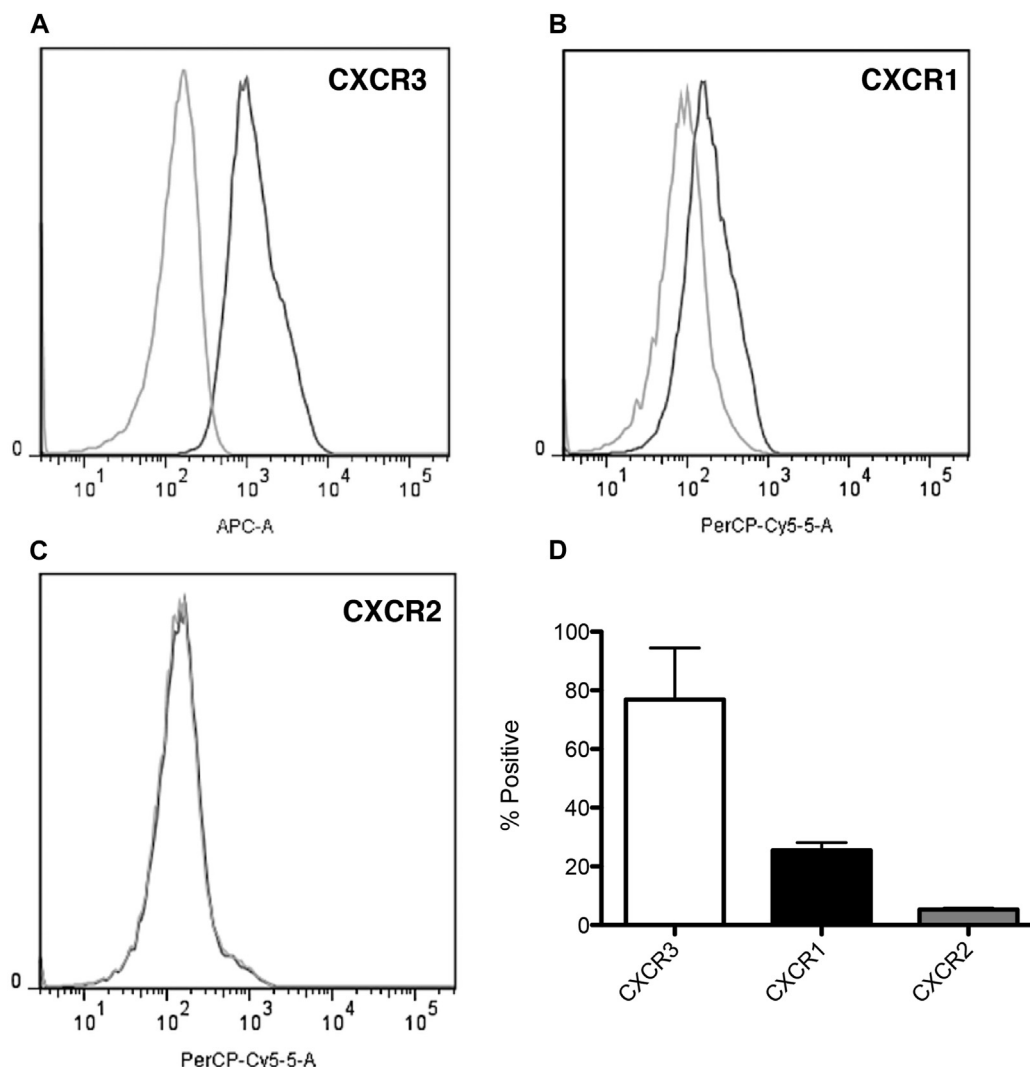


FIG 6. Fibroblasts express CXCR3 and CXCR1 but little CXCR2. **A–C,** Representative histograms are shown for CXCR3 (Fig 6, A), CXCR1 (Fig 6, B), and CXCR2 (Fig 6, C). In each case the light peak is the isotype control, and the heavy peak is the specific chemokine receptor antibody. **D,** Means $\pm$ SEMs of percentage of fibroblasts positive for each receptor ($n = 5$). APC, Allophycocyanin; PerCP, peridinin-chlorophyll-protein complex.

cells from asthmatic donors produce greater levels of chemotactic cytokines in response to HRV infection, whether fibroblasts from asthmatic donors are more responsive to the effects of chemoattractants, or both. In this context, it is of interest that CCL11 (eotaxin), which is induced in patients with allergic airway inflammation, is not chemotactic for fibroblasts but enhances fibroblast migration to chemotactic stimuli.²³

In summary, we demonstrate that HRV-infected epithelial cells are able to induce fibroblast chemotaxis. CXCL10 appears to be the dominant chemoattractant for fibroblasts, acting through the CXCR3 receptor, but CXCL8 is also a contributor. These observations, together with earlier data that HRV-infected epithelial cells also release growth factors linked to airway remodeling, support the possibility that recurrent HRV-induced wheezing illnesses in early childhood can

contribute to the initiation and progression of airway remodeling. Additional studies are needed to extend the current observations to cells from asthmatic patients and to use experimental HRV infections to confirm these observations *in vivo*.

Key messages

- Rhinovirus-infected airway epithelial cells produce chemoattractants for fibroblasts.
- CXCL10 and CXCL8 are the major drivers of fibroblast migration.
- The data support the concept that recurrent HRV infections can contribute to aspects of airway remodeling in asthmatic patients.

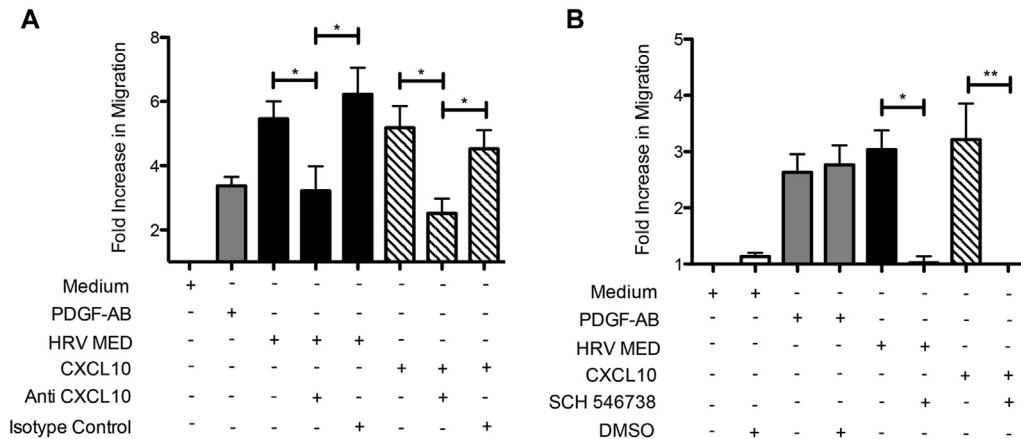


FIG 7. CXCL10 contributes to fibroblast migration induced by conditioned medium from epithelial cells infected with HRV (*HRV MED*). **A**, Neutralizing antibody to CXCL10, but not isotype control, inhibits chemotaxis induced by HRV MED ($n = 7$). **B**, A selective CXCR3 receptor antagonist abrogates fibroblast migration to HRV MED ($n = 6$). * $P < .05$ versus other treatments. ** $P < .01$ versus other treatments. *DMSO*, Dimethyl sulfoxide.

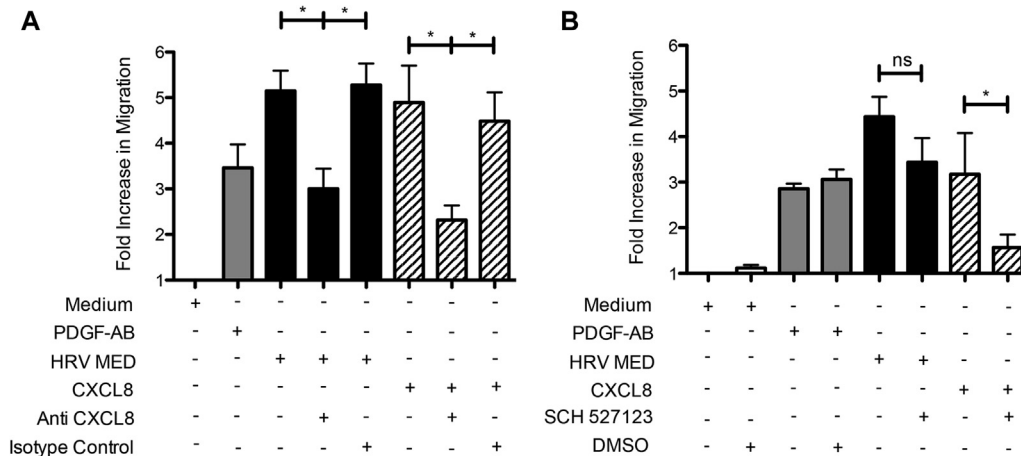


FIG 8. CXCL8 contributes to fibroblast migration induced by conditioned medium from epithelial cells infected with HRV (*HRV MED*). **A**, Neutralizing antibody to CXCL8, but not isotype control, inhibits chemotaxis induced by HRV MED ($n = 6$). **B**, A CXCR1/2 receptor antagonist significantly inhibits fibroblast migration to CXCL8 but not to HRV MED ($n = 6$). * $P < .05$ versus other treatments. *DMSO*, Dimethyl sulfoxide.

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METHODS

Fibroblast morphology

Fibroblasts and airway smooth muscle cells were grown in 4-well chamber slides (BD Falcon, Pittsburgh, Pa) to examine fibroblast morphology and phenotype. On achieving confluence, growth medium was removed, and cells were washed with 1 mL of PBS. Slides were then fixed in a 75%/25% acetone/ethanol mixture at -20°C for 5 minutes. All subsequent steps were performed at room temperature. Cells were then washed in PBS 3 times for 3 to 5 minutes, and 0.03% H_2O_2 in PBS was added for 3 minutes to block endogenous peroxidases. Slides were washed in PBS and treated with 2% normal goat serum (NGS) in PBS for 20 minutes. Cells were then washed with PBS and treated with 100 μL of antibody to α -smooth muscle actin (Sigma, Oakville, Ontario, Canada) diluted 1:1000 in 2% NGS/PBS for 2 hours. Cells were washed with PBS and treated with goat anti-mouse secondary antibody diluted 1:200 in 2% NGS for 60 minutes. After washing, peroxidase-labeled avidin biotin complex (Vector Laboratories, Burlington, Ontario, Canada) was applied for 30 minutes, and cells were washed with PBS and then distilled water. Fast DAB chromogen solution (Sigma) was applied to cells for 5 minutes, and cells were counterstained with hematoxylin. Slides were dehydrated with 1 change of 95% and 2 changes of 100% ethanol, treated with 2 changes of xylene, and subsequently mounted with a coverslip using Permount (Fisher Scientific, Ottawa, Ontario, Canada). Slides were then visualized at $\times 4$ magnification.

Flow cytometry

HBFs were suspended with a nonenzymatic cell dissociation solution (Life Technologies). The HBFs were centrifuged (at 331g for 8 minutes) before being resuspended in 1500 μL of flow cytometry buffer (PBS containing 0.5% BSA and 2 mmol/L EDTA, pH 7.2). Aliquots (100 μL) of the HBF suspension containing 10^6 HBFs were added to fresh round-bottom test tubes. Fluorescently labeled antibodies or appropriate isotype control was added at the concentration recommended by the manufacturer, and cells were incubated in the dark at 4°C for 2 hours. Antibodies used were anti-CXCR3–allophycocyanin (5 μL per 10^6 cells), anti-CXCR2–peridinin-chlorophyll-protein complex (10 μL per 10^6 cells), and anti-CXCR1–phycoerythrin-Cy5 (10 μL per 10^6 cells). HBFs were then washed with 1 mL of flow cytometry buffer, followed by centrifugation (600g for

10 minutes). Pelleted cells were then resuspended in 200 μL of flow cytometry buffer, and samples were analyzed with the LSR II Flow Cytometer (Becton Dickinson, Mississauga, Ontario, Canada). Experiments were performed with HBFs from 5 different donors. Data analysis was performed with FlowJo 7 software (TreeStar, Ashland, Ore).

Chemotaxis assays

A 48-well modified Boyden chamber (Neuroprobe) was initially used to investigate HBF migration. The bottom chambers were filled with relevant chemoattractants, and an 8- μm polycarbonate membrane coated with a 0.1% Collagen I (rat tail) solution (Life Technologies, Burlington, Ontario, Canada) for 30 minutes was placed on top. The apparatus was assembled and allowed to incubate for 15 minutes. At this time, HBFs that had been suspended with trypsin were placed in the top wells of the chamber by using 40,000 cells per well. The chambers were then placed into a 37°C incubator containing 5% CO_2 for the duration of the migratory period. At the conclusion of the experiment, nonmigratory cells are scraped off the upper surface of the filter membrane, and the migrated cells are fixed and stained with Diff-Quik stain (VWR International, Mississauga, Ontario, Canada) and counted at $\times 200$ magnification. Values from 6 different fields of view per condition were counted, and the average was taken for data analysis.

Migration experiments were also performed with the xCELLigence System (ACEA Biosciences). This apparatus uses cellular invasion and migration plates that resemble a modified Boyden chamber, except that the bottom of the permeable membrane is coated with microelectrodes. Cells that migrate through the filter bind to the microelectrodes, resulting in an increase in impedance, which is measured in real time. The upper surface of the polyethylene terephthalate porous membrane was coated with 0.1% Collagen I (rat tail) solution for 30 minutes and washed twice with sterile PBS. Chemoattractants were placed into the bottom wells, and serum-free DMEM is placed into the top wells (to moisten the filter and allow incubation). The chamber was assembled and allowed to preincubate in an incubator at 37°C and 5% CO_2 for 1 hour. A background scan of the apparatus was then performed wherein the impedance value for each well was artificially reduced to zero. HBFs suspended by means of trypsinization were then added to the top wells (at 40,000 cells per well), and the apparatus was activated. Migration impedance data were normalized at the 20-minute time point to account for differences in well loading and cell settling.

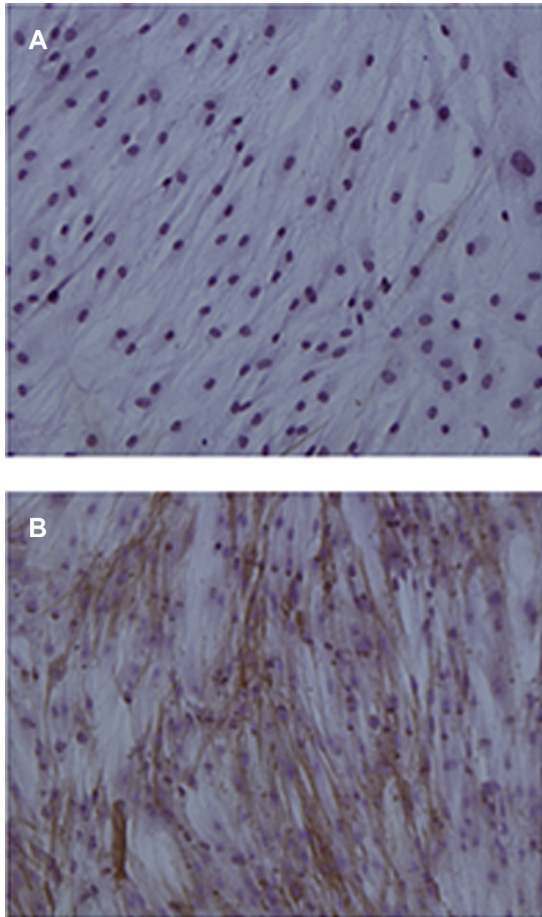


FIG E1. Fibroblast morphology. Fibroblasts (**A**) showed typical morphology and did not express α -smooth muscle actin, whereas robust expression of α -smooth muscle actin (brown staining) was seen in airway smooth muscle cells (**B**).

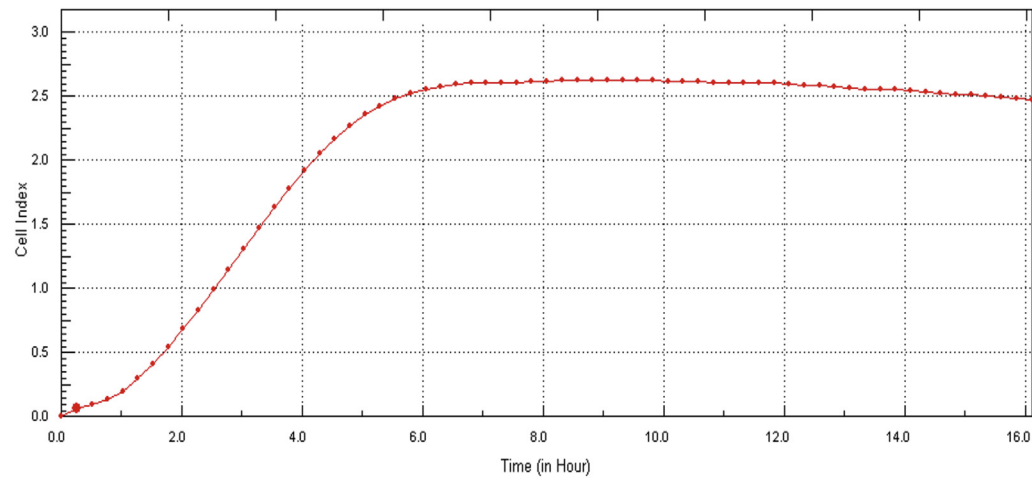


FIG E2. Time course of HBF migration in the xCELLigence apparatus. These representative data show the “cell index” values (changing impedance value) representing increasing numbers of fibroblasts interacting with the microelectrodes in the cellular invasion and migration plates. HBF migration plateaus at 6 hours. This was used as the standard time in both the xCELLigence system and the modified Boyden chamber.

TABLE E1. Results of a 64-chemokine multiplex assay of conditioned media

Chemokine	CON MED treatment (pg/mL)	UV HRV infection (pg/mL)	HRV infection (pg/mL)
CXCL10	6.41	247.44	10,437.195
CXCL8	61.46	2,155.88	7,503.345
Pan GRO	118.27	2,242.3	4,099.54
CCL5	2.04	11.56	674.95
VEGF	61.16	403.64	476.36
IL-1RA	25.28	105.75	320.14
CX3CL1	42.78	50.16	174.51
PDGF-AA	42.56	145.43	158.45
CCL2	4.07	30.67	105.82
CCL22	23.75	29.38	91.74
FGF-2	21.88	31.01	74.73
GM-CSF	2.71	3.62	35.06
IL-1 α	3.93	11.07	32.81
CCL11	7.04	19.08	31.16
IL-6	0.42	8.12	27.66
CXCL5	0.01	13.24	25.29
IFN- α 2	4.18	11.2	24.96
G-CSF	2.15	3.5	19.60
PDGF-BB	2.49	6.04	13.86
CCL7	4.34	5.83	12.80
IL-12p40	1.89	1.5	10.99
CCL-13	0.01	3.54	10.34
TGF- α	1.98	11.13	9.66
TNF- α	0.01	0.83	9.41
Flt-3L	4.20	3.65	5.84
CCL4	1.16	1.98	5.84
TRAIL	0.15	1.12	5.60
IL-7	0.01	2.85	5.42
CCL8	0.01	0.01	4.83
IFN- γ	0.41	1.75	4.49
CCL21	0.01	0.01	4.26
CCL3	0.01	0.01	4.19
IL-20	5.33	10.32	4.12
IL-1 β	0.22	0.6	3.10
EGF	1.41	2.47	2.74
sCD40L	3.20	3.98	2.66
IL-23	0.01	0.01	2.54
IL-28A	0.01	0.01	2.19
CCL24	0.13	2.14	2.12
SCF	0.01	0.01	1.97
IL-4	0.01	0.01	1.96
IL-15	0.22	0.63	1.80
TNF- β	0.90	1.54	1.68
IL-12p70	1.11	1.03	1.59
IL-13	0.01	0.15	1.48
IL-21	0.01	0.82	0.87
IL-10	0.49	0.3	0.79
BCA-1	0.16	0.41	0.67
IL-3	0.10	0.32	0.64
CCL1	0.05	0.21	0.52
CCL26	0.01	0.01	0.48
IL-17A	0.09	0.65	0.40
IL-9	0.11	0.06	0.31
IL-5	0.14	0.12	0.16
CCL17	0.01	0.01	0.05
IL-16	0.01	0.01	0.01
LIF	0.01	0.01	0.01
TSLP	0.01	0.01	0.01
CCL15	0.01	0.01	0.01
IL-2	0.01	0.01	0.01

(Continued)

TABLE E1. (Continued)

Chemokine	CON MED treatment (pg/mL)	UV HRV infection (pg/mL)	HRV infection (pg/mL)
TPO	0.01	0.01	0.01
IL-33	0.01	0.01	0.01
CCL27	0.01	0.01	0.01
SDF-1 α + β	0.01	0.01	0.01

BCA-1, B cell-attracting chemokine 1; CON MED, Conditioned medium from uninfected cells; EGF, Epidermal growth factor; FGF-2, fibroblast growth factor 2; G-CSF, granulocyte colony-stimulating factor; GRO, growth-related oncogene; IL-1RA, IL-1 receptor antagonist; LIF, leukemia inhibitory factor; OOR, out of range of the assay (ie, less than the threshold of detection); sCD40L, soluble CD40 ligand; SCF, stem cell factor; SDF-1 α + β , stromal cell-derived factor 1 α + β ; TPO, thrombopoietin; TRAIL, tumor necrosis factor related apoptosis inducing ligand; TSLP, thymic stromal lymphopoietin; VEGF, vascular endothelial growth factor.