

Human rhinovirus infection of epithelial cells modulates airway smooth muscle migration†

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ABSTRACT

Rationale: Airway remodeling, a characteristic feature of asthma, begins in early life. Recurrent human rhinovirus (HRV) infections are a potential inciting stimulus for remodeling. One component of airway remodeling is an increase in airway smooth muscle cell mass with a greater proximity of the airway smooth muscle cells (ASMC) to the airway epithelium. We asked whether human bronchial epithelial cells infected with HRV produced mediators that are chemotactic for ASMC.

Methods: ASMC migration was investigated using the modified Boyden Chamber and the xCELLigence Real-Time Cell Analyzer. Multiplex bead analysis was used to measure HRV-induced epithelial chemokine release. The chemotactic effects of CCL5, CXCL8 and CXCL10 were also examined.

Results: Supernatants from HRV-infected epithelial cells caused ASMC chemotaxis. Pre-treatment of ASMC with pertussis toxin abrogated chemotaxis, as did treatment with formoterol, forskolin, or 8-bromo-cAMP. CCL5, CXCL8, and CXCL10 were most up-regulated chemokines produced by HRV-infected airway epithelial cells. When recombinant CCL5, CXCL8 and CXCL10 were used at levels found in epithelial supernatants they induced ASMC chemotaxis similar to that seen with epithelial cell supernatants. When examined individually, CCL5 was the most effective chemokine in causing ASMC migration and treatment of supernatant from HRV-infected epithelial cells with anti-CCL5 antibodies significantly attenuated ASMC migration.

Conclusion: These findings suggest that HRV-induced CCL5 can induce ASMC chemotaxis, and thus may contribute to the pathogenesis of airway remodeling in asthmatic patients.

INTRODUCTION

The airways of patients with asthma typically display a number of structural changes, including goblet cell metaplasia and excessive mucus production, thickening of the lamina reticularis, angiogenesis, and increased smooth muscle mass with a reduced distance between muscle cells and the airway epithelium as asthma severity increases (1, 2). These changes, collectively termed airway remodeling, lead to a thickening of the airway wall. This has been suggested to contribute to airways hyperresponsiveness, in part by induction of increased airway narrowing for a given degree of smooth muscle shortening (3, 4).

In most patients, asthma initially manifests in childhood (5). It is therefore noteworthy that, while it was traditionally believed that airway remodeling occurred after years of chronic inflammation, a number of bronchial biopsy studies have now established that several major components of airway remodeling, including increased smooth muscle mass and thickening of the lamina reticularis, are already present in pre-school children, often before the clinical diagnosis of asthma has been made (6-8). Interestingly, signs of airway remodeling are not detected in infants with wheezing illnesses (9), implying that exposure to some inciting stimuli in early childhood induces remodeling.

It is now well established that children who experience human rhinovirus (HRV)-induced wheezing illnesses in early life are at increased risk of subsequently developing asthma (10, 11), particularly if they are also sensitized to inhaled allergens (12). Pre-school age children have been shown to experience approximately six HRV infections each year (13), and serial HRV infections can cause recurrent wheezing episodes (14). Given the concomitant time frame of recurrent HRV-induced wheezing episodes and the development of airway remodeling, it is feasible that HRV infections could contribute to the initiation and progression of airway

remodeling in asthma. In the current studies we tested the hypothesis that HRV infection of airway epithelial cells induces release of chemotactic agents that could cause directional migration of airway smooth muscle cells (ASMC) towards the epithelium to contribute to the process of remodeling.

METHODS

Details regarding all methods are provided in the Online Data Supplement.

Cell Cultures: Primary human bronchial epithelial (HBE) cells were obtained via protease digestion of non-transplanted normal human bronchi as described (15), and grown in bronchial epithelial growth medium (BEGM). For these studies, cells were derived from 6 lung donors (5 male; age range 13-63 years). All 6 donors died from non-respiratory causes and none of them had any underlying lung disease. Human airway smooth muscle cells (ASMC) were obtained by micro-dissection and protease digestion and cultured in DMEM containing 10% FBS and antibiotics as described (16). A total of 10 individual ASMC donors were used (8 male; age range 13-63 years). Protocols for obtaining cells were approved by the University of Calgary Conjoint Health Ethics Board.

HRV Infection: HRV type 16 (HRV-16) was propagated in WI-38 cells and purified as described (17). Viral titers were determined as described (17). HBE cells were exposed to control medium or to $10^{5.5}$ 50% tissue culture infective dose (TCID₅₀) U/ml (multiplicity of infection ~1.0) of HRV-16 for 24 h at 34°C with 5% CO₂. Epithelial cell supernatants from 6 individual donors were pooled for each experimental condition to generate stocks of conditioned medium (CM) and HRV-conditioned medium (HRV CM) for migration experiments.

Migration Assay: ASMC migration was performed in both a AP48 modified Boyden chamber and the xCELLigence Real-Time Cell Analyzer system. ASMC were added to top wells of the AP48 chamber (40,000 cells/well) or the RTCA system (80,000 cells/well), while chemoattractants were added to the bottom chambers. Platelet-derived growth factor-AB (PDGF-

AB) was used as a positive control stimulus for ASMC migration. Plates were incubated (4 h) at 37°C and 5% CO₂. Impedance data for the RTCA system were normalized at the earliest time point to control for differences in well loading and cell settling. Migration experiments used ASMC from the indicated number of individual tissue donors in each experiment (i.e. n = number of ASMC donors).

64-chemokine Multiplex assay: Pooled CM or HRV-CM were used in a 64-chemokine multiplex protein assay performed by Eve Technologies (Calgary, AB, Canada).

Flow Cytometry Analysis: ASMC were suspended using a non-enzymatic cell dissociation buffer. Fluorescently labeled antibody(s) was then added and the cells were allowed to incubate in the dark at 4°C for 2 h with rocking. Cells were fixed and stored at 4°C until analyzed by flow cytometry.

ELISA assay for CXCL8: CXCL8 was assayed as previously described (18).

Neutralizing antibody treatment of conditioned medium: Neutralizing monoclonal antibody to CXCL5, or appropriate isotype control, were added to HRV-CM and incubated at room temperature (2 hours) prior to use as chemoattractants for ASMCs.

Cell viability: Cell viability following experimental interventions, was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Statistical Analysis: Data are presented as means \pm SEM. Statistical analysis was performed by using ANOVA, and the Newman Keuls multiple comparison *post hoc* test. *P* values of less than 0.05 were regarded as statistically significant. Statistical analysis was performed with GraphPad Prism 6 software.

RESULTS

ASMC migrate to conditioned medium from HRV infected HBE cells

Because the xCELLigence system provides real-time data, we used this apparatus to establish that ASMC migration started to plateau by 4 h (**Figure E1** in the Online Supplement). Therefore, we used 4 h as the outcome time point for all subsequent experiments.

Conditioned medium from HBE cells treated with medium control (CM) or HRV-16 (HRV-CM) for 24 hours were used as chemoattractants in both the Boyden Chamber and the xCELLigence system. CM was able to drive a migratory response that was higher than the medium alone; however the HRV-CM condition was able to drive significantly higher migration in both the Boyden chamber (**Figure 1A**) and the xCELLigence system (**Figure 1B**). HRV virion alone, at levels used to stimulate HBE cells, did not induce any migration of ASMC (**Figure 1B**). We next examined whether ASMC migration was directional or random by abolishing the chemotactic gradient in the xCELLigence system by adding the chemoattractant to both the top and bottom wells (**Figure 1C**). While CM and HRV-CM were able to drive ASMC migration normally, migration to both stimuli was significantly attenuated ($p < 0.001$) when there was no gradient present. Given the concordance in data from the modified Boyden chamber and the xCELLigence system, we used the xCELLigence system for all subsequent migration experiments.

Migration to HRV-CM is mediated by molecules less than 30 kDa and chemoattractant production depends upon viral replication

To gain insight into the molecule(s) responsible for ASMC migration to HRV-CM, centrifugal filtration devices were used to remove all materials >30 kDa (including HRV virions) from the

HBE cell supernatants. We validated that filtration did not retain low molecular weight molecules by demonstrating minimal loss of CXCL8 levels in the filtrate (**Figure E2**). Filtration did not alter the ability of HRV-CM to drive ASMC migration but significantly attenuated the ability of CM alone to drive ASMC migration (**Figure 2A**). To study the time course of chemoattractant generation from HBE cells, we collected conditioned medium at 3, 9, 15, and 24h post HRV treatment. The chemotactic ability of HBE-CM for ASMC increased with time following HRV infection (**Figure 2B**). To determine if this increase was dependent upon the replication of HRV, cells were exposed to either intact HRV, capable of replication or to UV-light treated, replication deficient HRV. Medium from cells exposed to replication competent virus generated a chemotactic response as described, whereas medium from cells exposed to replication deficient HRV did not induce significant ASMC chemotaxis (**Figure 2C**), confirming that viral replication within HBE cells is necessary to promote chemoattractant release.

Exposure to pertussis toxin inhibits ASMC migration to HRV-CM

A number of low-molecular weight chemoattractants, including chemokines, exert their effects via G-protein coupled receptors (GPCRs) which are often linked to the G-protein, G α i (19). We therefore studied whether the chemoattractant(s) contained in HRV-CM act through a G α i-coupled GPCR by exposing ASMC to pertussis toxin (PTX), which ADP-ribosylates and inactivates G α i, prior to performing migration assays. There was no deleterious effect of PTX on ASMC viability (data not shown). As anticipated, PTX did not attenuate migration to PDGF-AB, which functions via a receptor tyrosine kinase. In contrast, PTX significantly reduced ASMC migration to HRV-CM (**Figure 3**).

ASMC migration is sensitive to intracellular cAMP

Given that G α i-coupled GPCR can lower intracellular cAMP levels, we next examined the effects of elevating intracellular cAMP levels on ASMC migration. Activation of its G α _s-coupled GPCR by the β ₂-adrenergic agonist formoterol completely abolished migration to both CM and HRV-CM (**Figure 4A**). Similarly, direct activation of adenylyl cyclase in ASMC using the forskolin analogue NKH-477, or pretreatment with the cell-permeable cAMP analogue, 8-bromoadenosine 3', 5'-cyclic monophosphate (8-Br-cAMP), 15 min prior to use in the migration assay also both abrogated the migratory capacity of the ASMC (**Figure 4B & 4C**). Given that migration to HRV-CM and the receptor tyrosine kinase linked positive control, PDGF-AB, were both inhibited by agents that elevate intracellular cAMP, this suggests that this is a broad ranging regulatory pathway and not simply a competitor to G α i-coupled GPCR-mediated reduction in intracellular cAMP.

Identification of chemokines that facilitate HRV-induced ASMC migration

To identify potential chemoattractants that may contribute to ASMC migration in response to HRV-CM, we performed a multiplex assay for 64 analytes (Luminex, Toronto, ON, Canada) on media obtained from a pool of 6 HBE cell donors exposed either to medium alone or to HRV. The 4 most highly up-regulated chemokines are shown in **Figure 5**.

Flow cytometry data indicate the presence of several chemokine receptors

Flow cytometry was performed to examine the expression of potential cell surface receptors for the chemokines identified above at the protein level. These experiments used ASMC obtained

from 5 donors. A representative series of histograms showing surface expression of CXCR1, CXCR2, CXCR3, CCR1, CCR3 and CCR5 on ASMC from a single individual is shown (**Figure 6A**). Mean percentage of cells expressing each receptor using cells from all 5 donors is shown in **Figure 6B**.

HRV-induced ASMC migration is mediated, at least in part, via the chemokine CCL5

To determine the potential of CCL5, CXCL8, and CXCL10 to induce ASMC migration using concentrations similar to those found in HRV-CM, we initially used the recombinant chemokines in concentrations of 500 pg/mL, 5000 pg/mL and 10000 pg/mL, alone and in combination. The combination of the three chemokines induced ASMC migration that was significantly greater than that seen with CM and that was comparable to that observed for HRV-CM (**Figure 7A**). When examined individually, CXCL10 caused no significant migration, despite the high expression of CXCR3 on ASMC cells. Moreover, CXCL8 alone induced only a modest chemotactic response, presumably mediated via interactions with CXCR1. By contrast, CCL5 alone induced migration similar to that seen with HRV-CM, suggesting that CCL5 is the major chemoattractant for ASMC in HRV-CM. To further evaluate the role CCL5 in the ASMC migratory response, we incubated HRV-CM with a neutralizing antibody to CCL5 or with an isotype control. While the isotype control did not alter ASMC migration, the antibody to CCL5 significantly abrogated ASMC migration to HRV-CM (**Figure 7B**).

DISCUSSION

Pre-school children who experience recurrent HRV-induced wheezing illnesses are not only at increased risk of subsequently developing asthma (10, 11), but already shows signs of airway remodeling, including increased airway smooth muscle mass (8). The relative contribution of hyperplasia versus hypertrophy to the increased smooth muscle mass in asthma is still somewhat controversial (20, 21), and may vary depending on the size of airways examined (22). The observation that airway smooth muscle cells are found closer to the epithelium in asthma implies that ASMC migration also occurs in asthma (2). Indeed, a number of studies have indicated that ASMC are capable of migrating to a variety of growth factors, including PDGF (23), as well as to selected chemokines (24-27). In this study, we show that human airway epithelial cells infected with HRV release chemotactic agents that can cause directed migration of ASMC towards the epithelial layer. The production of these chemoattractants depends upon viral replication within HBE cells, as conditioned medium from HBE cells exposed to UV-treated, replication deficient HRV did not induce significant ASMC migration.

To gain insights into the nature of the chemoattractants in HRV-CM we first demonstrated that chemotactic activity was retained in the filtrate after passage through a 30,000 Dalton filter, indicating that chemotactic agents were of relatively low molecular weight. The observation that treatment of ASMC with pertussis toxin abrogated ASMC migration to HRV-CM indicated that chemoattractant agents in HRV-CM act via interactions with G α i-coupled GPCR(s). Although a number of mediators satisfy these characteristics, the prior evidence for a role of chemokines in inducing ASMC migration in other systems (24-27) led us to perform multiplex analysis of chemokines in supernatants from epithelial cells exposed to medium or HRV. Induction of CCL5, CXCL8, and CXCL10 identified these chemokines as potential candidates to cause

ASMC chemotaxis, analysis, and this paradigm was further supported by detection of low level expression of receptors for each chemokine on ASMC by flow cytometry. Subsequent functional studies revealed that while CXCL10 was ineffective as a chemotactic agent for ASMC, despite the expression of the CXCR3 receptor on significant percentage of ASMC. Although this seems paradoxical, these observations are consistent with those reported by Saunders et al (28).

Presumably the inability of CXCL10 to function as a chemoattractant for ASMC relates to a lack of signaling downstream of the receptor. This is quite distinct from our prior data showing that generation of this chemokine from HRV-infected HBE cells plays an important role in causing chemotaxis of fibroblasts (29). We also found that CXCL8 caused only modest ASMC migration, and that CCL5 was the most effective chemoattractant for ASMC. We confirmed that CCL5 was a major contributor to the chemotactic activity of HRV-CM for ASMC by demonstrating that incubation of HRV-CM with a specific blocking antibody to CCL5 largely abolished ASMC chemotaxis. Taken together our data are consistent with earlier reports that CCL5 derived from TNF α -stimulated epithelial cells was chemotactic for ASMC (30), and that CXCL8 can also stimulate ASMC migration (25, 30).

We also demonstrated in this study that elevation of cAMP in ASMC inhibits migration of these cells to a variety of stimuli, including HRV-CM and PDGF. This was the case whether cAMP was induced via activation of the β_2 -adrenergic receptor, via direct activation of adenylyl cyclase, or via use of a cell permeable cAMP analogue. This data are consistent with an earlier report of a modulatory role of cAMP in regulating ASMC migration (31), but differ somewhat in that we found all mechanisms of cAMP elevation to be equally effective while Goncharova and colleagues found that inhibition of migration varied depending on the means of elevating cAMP (31). Our observation that a β_2 -adrenergic receptor agonist inhibits ASMC migration to a variety

of stimuli raises the possibility that the use of β_2 -adrenergic receptor agonists may help to attenuate some aspects of remodeling in asthma. This would be consistent with a recent study showing that combination therapy with an inhaled corticosteroid and long-acting β_2 -adrenergic receptor agonist, but not an inhaled corticosteroid alone, inhibited antigen induced alterations in airway smooth muscle and myofibroblast numbers in asthmatic subjects (32).

Our study has several limitations. We used cells from adolescent and adult donors, and future studies will need to examine whether epithelial cells from young children will show similar chemotactic responses to HRV infection. In addition, both epithelial cells and ASMC used in the current study were procured from healthy, non-asthmatic donors, in whom airway remodeling is unlikely to have been present. However, even in the airways of normal subjects infected with HRV, there is likely to be some effect and alteration in ASMC numbers and this may be what is reflected in our current model in the setting of low chemokine receptor expression on ASMC. We speculate that the chemotactic responses demonstrated in our present study, would be exaggerated if cells from asthmatic donors were used. Further studies will need to determine whether epithelial cells from subjects with asthma generate higher levels of chemokines following HRV infection, and if so, whether ASMC from these asthmatic subjects show a greater chemotactic response to these chemotactic cytokines. In that context, given the modest level of expression of receptors for CCL5 on our normal cells, it is of interest that ASMC from asthmatic subjects show increased expression of the CCR3 chemokine receptor (24), for which CCL5 is a known ligand. This would be expected to result in an increased number of ASMC cells from asthmatic subjects migrating to this chemokine.

In summary, we show that HRV infected epithelial cells release chemoattractants that can stimulate directed migration of ASMC. CCL5 is the dominant chemoattractant present in HRV-

CM, although CXCL8 may also contribute. These data, together with earlier reports that HRV infected epithelial cells show expression of genes related to airway remodeling (33), and release a number of growth factors and matrix proteins linked to airway remodeling (34-36), further support the concept that recurrent HRV infections may contribute to the initiation and progression of airway remodeling in asthma. Future studies will need to determine if the current observations are relevant to cells obtained from asthmatic subjects (both children and adults), while clinical studies of either naturally-acquired or experimental HRV infections will confirm whether these observations occur *in vivo*.

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Figure Legends

Figure 1: Conditioned Medium from HRV infected HBE cells (HRV-CM) drives ASMC chemotaxis. ASMC migration was assessed to conditioned medium from cells exposed to medium only (CM) or HRV-CM. **A)** Data using the modified Boyden chamber (n=4). **B)** Data using the xCELLigence system (n=3). Platelet derived growth factor-AB (PDGF-AB, 300 ng/mL) was used as a positive control. **C)** Migration is abrogated when the chemotactic gradient is abolished (n=3). Asterisk indicates $p < 0.05$ for comparisons shown.

Figure 2: HRV-induced induction of chemoattractants from HBE cells depends upon viral replication and chemoattractants are less than 30 kDa. **A)** HRV-CM and CM were passed over a 30 kDa filter and migration to the filtrates (white bars) were compared to those of unfiltered conditioned medium (grey bars), (n=3); **B)** HRV-CM was harvested at multiple time points (3, 9, 15, 24h) after HRV infection and used in chemotaxis assays (n=4); **C)** Conditioned medium from UV-inactivated HRV-16 treated epithelial cells (UV-HRV-CM) is ineffective as a chom attractant for ASMC (n=3). Asterisk indicates $p < 0.05$ for comparisons shown.

Figure 3: Treatment of ASMC with pertussis toxin (PTX) inhibits their migration to HRV-CM but not to PDGF. Data are from 3 experiments. Asterisk indicates $p < 0.05$ for comparison shown.

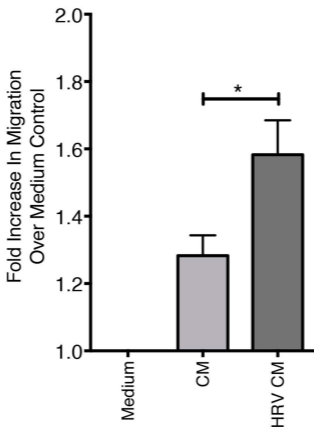
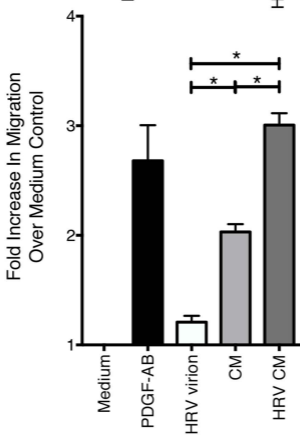
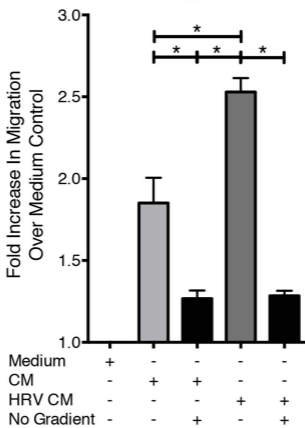
Figure 4: Agents that elevate intracellular cAMP in ASMC inhibit migration to both HRV-CM and PDGF. Migration of ASMC in response to PDGF-AB, CM, or HRV-CM was assessed in the xCELLigence system following pre-treatment (15 min) with: **A)** Formoterol (n=3); **B)**

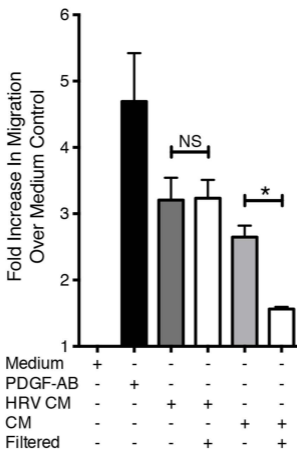
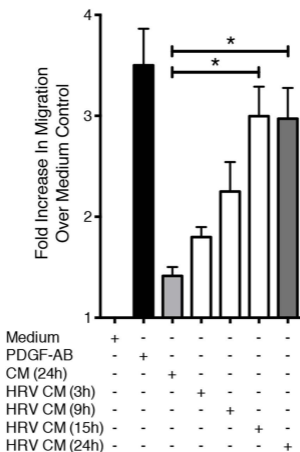
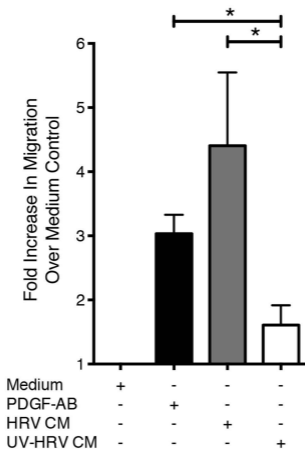
NKH-477 (n=6); C) 8-Br-cAMP (n=3) or appropriate vehicle control for each agent. Asterisk indicates $p < 0.05$ for comparisons shown.

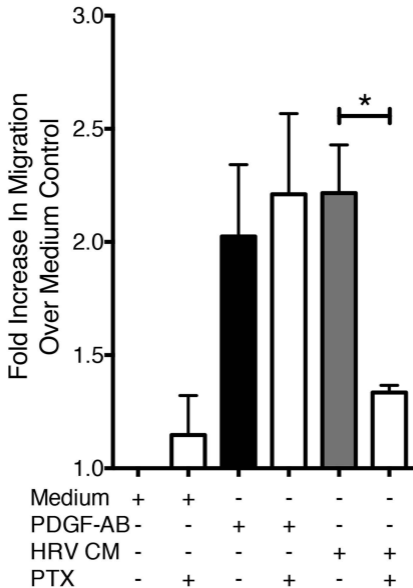
Figure 5: Identification of CCL5, CXCL8 and CXCL10 as possible chemotactic mediators of ASMC. Levels of the 4 chemokines most strongly induced in response to HRV infection as assessed using a multiplex assay of combined supernatant from six HBE donors. Values are shown for cells exposed to medium alone, intact HRV-16 and UV-treated (replication deficient) HRV-15.

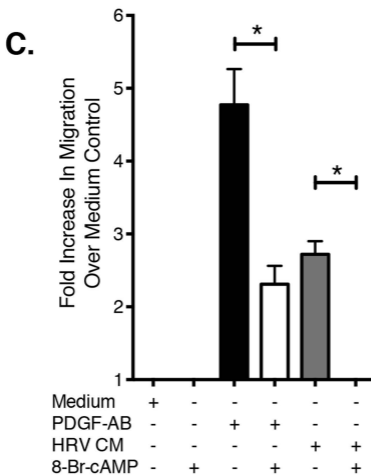
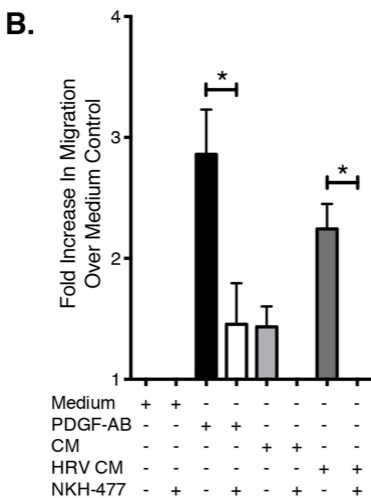
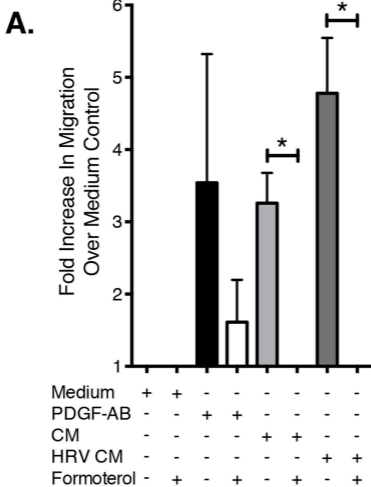
Figure 6: Flow cytometry analysis of ASMC donor cells indicates the presence of several different chemokine receptors on ASMC membrane surfaces. A) Histograms showing expression of chemokine receptors relative to appropriate isotype control antibodies on ASMC from an individual donor. B) Mean \pm SEM percentage of ASMC cells from 5 donors expressing each chemokine receptor.

Figure 7: CCL5 is the major chemoattractant for ASMC in HRV-CM. A) Migration of ASMC was assessed to CXCL10 (10,000 pg/mL), CXCL8 (5,000 pg/mL), CCL5 (500 pg/mL), CM, or HRV-CM (n=3); B) ASMC migration was significantly inhibited after 1 h pre-treatment of HRV-CM with anti-CCL5 but not with isotype control (n=3). Asterisk indicates $p < 0.05$ for comparisons shown.

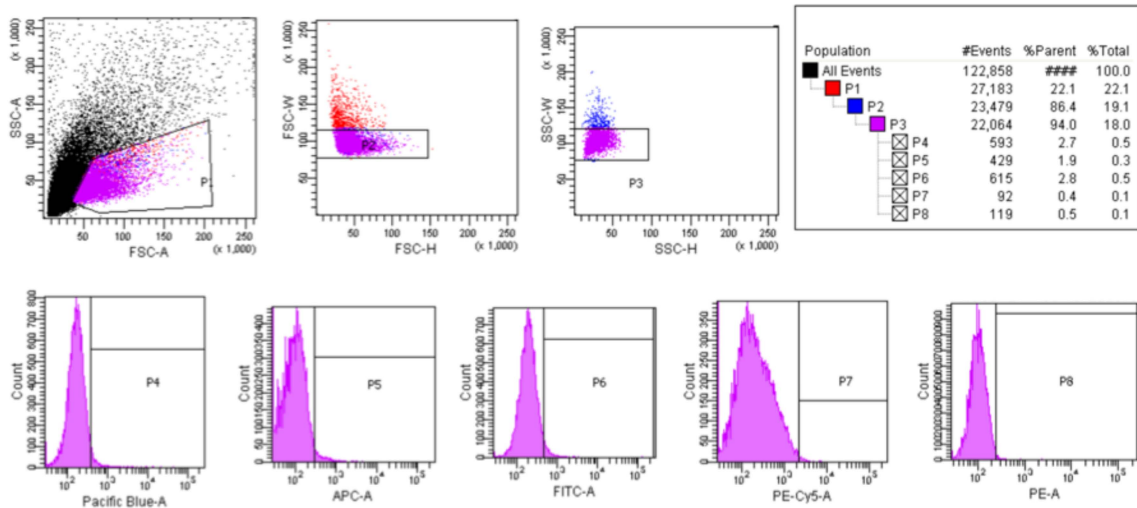
A.**B.****C.**

A.**B.****C.**





A.



B.

Gate	Chemokine Receptor	% Positive
P4	CCR3	1.8 ± 0.5
P5	CXCR3	0.7 ± 0.6
P6	CCR1	2.1 ± 0.5
P7	CXCR1	0.7 ± 0.4
P8	CCR5	0.9 ± 0.2

