

**Monocyte-derived fibrocytes induce an inflammatory phenotype in airway smooth muscle cells**

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**Running title:** Fibrocytes promote synthetic phenotype of airway smooth muscle cells

## **Abstract**

**Background:** Infiltration of fibrocytes (FC) in the airway smooth muscle is a feature of asthma but the pathological significance is unknown.

**Objective:** We sought to explore if FC modulate the phenotype of airway smooth muscle cells (ASMC) in asthmatic vs. control subjects.

**Methods:** FC were isolated from CD14+monocytes from asthmatic and normal subjects. Proliferation of ASMC of asthmatic or normal subjects were analyzed by <sup>3</sup>H-thymidine incorporation, cell number counting and Ki-67 expression after treatment of ASMC with FC-conditioned medium (FCCM) or co-culture with FC. ASMC-associated cytokines/chemokines implicated in asthma (TGF- $\beta$ 1, eotaxin, IL-6 and IL-8) were measured in co-culture or transwell culture of ASMC+FC by ELISA. Immunofluorescence staining was performed to localize these cytokines in ASMC. Cytokine secretion was measured in the transwell culture of ASMC+FC where NF- $\kappa$ B-p65 or ERK1/2 in ASMC was silenced by siRNA. Contractile phenotype of ASMC in transwell culture was assessed by immunoblotting of  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and myosin light chain kinase (MLCK).

**Results:** FC did not affect ASMC expression of TGF- $\beta$ 1, eotaxin,  $\alpha$ -SMA and MLCK; however, ASMC production of IL-8 and IL-6 was increased in the co-culture and transwell culture by FC. ASMC treated with FCCM were immunopositive for IL-8 / IL-6 and produced more IL-8 / IL-6. Furthermore, siRNA silencing of NF- $\kappa$ B-p65 or ERK1/2 in transwell cultures of asthmatic ASMC with normal subject FC decreased IL-8 and IL-6 production.

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**Conclusion:** FC promoted IL-8 and IL-6 production by ASMC, demonstrating a proinflammatory role for FC and a possible mechanism of the inflammatory phenotype in asthma.

## Introduction

Asthma is a chronic inflammatory disease of the airways characterized by variable airflow obstruction and airway remodeling. Structural cells, like airway smooth muscle cells (ASMC), play an important role in the pathogenesis of inflammation and remodeling in asthma [1, 2]. An increase in contractility and airway smooth muscle (ASM) mass can contribute to airway hyperresponsiveness, airflow obstruction and disease severity in asthma [3, 4]. Indeed, by secreting a variety of inflammatory mediators and extracellular matrix (ECM) proteins, ASMC can participate in immunomodulation and airway remodeling in asthma [5].

It has been shown in the asthmatic airway that stimulation or interaction with immune cells affects ASMC contractile, proliferative and biosynthetic functions[5, 6]. Evidence shows that the asthmatic ASM layer is infiltrated with increased number of mast cells [5, 7], and increased release of inflammatory mediators such as cysteinyl leukotrienes, histamine and tryptase by mast cells can contribute to increases in the contractile properties of ASM [8]. Interaction between ASMC and mast cells has been shown to increase alpha-smooth muscle actin ( $\alpha$ -SMA) expression of ASMC and promote ASMC differentiation into contractile phenotype under tryptase stimulation [9]. Microlocalization of CD4+T cells within the ASM layer is a feature of asthma [5, 10] and direct interaction with T cells promotes ASMC proliferation [10, 11]. Numerous studies reveal ASMC as a source of inflammatory mediators that perpetuate chronic inflammation in asthma [5, 6]. Mechanisms for converting ASMC into a synthetic phenotype in asthma are a subject of much investigation. ASMC can release interleukin 6 (IL-6) or IL-8 upon stimulation by tumor necrotic factor  $\alpha$  (TNF- $\alpha$ ), IL-1 $\beta$ , IL-

17 or supernatant from mast cells through various signaling pathways [12-15]. It has been shown that the level of IL-6 and IL-8 is increased in asthmatic airway and peripheral circulation [16] and is associated with persistent asthma [17] and neutrophilic asthma [18].

The monocyte-derived circulating progenitor cell of fibroblasts, which has been named 'fibrocytes' (FC) [19], have been shown to migrate toward ASMC and infiltrate into the ASM compartment in asthmatic subjects [20]. FC have been isolated from CD14+ monocytes and co-express hematopoietic marker CD45, stromal marker (e.g. collagen-1), and bone marrow marker CD34, the last of which is down-regulated during recruitment to tissues or during culture in vitro [21-26]. Subsequent studies demonstrated that FC express chemokine receptors, including CXCR4, and traffic to wound and express ECM protein and fibrotic mediators which promote wound healing and tissue remodeling [27, 28]. Previous studies have shown that increased FC in airway and peripheral blood is associated with asthma severity [20, 29], and FC number in bronchial biopsy of asthmatic patients is positively correlated with thickness of basement membranes [30]. Furthermore, FC isolated from asthmatics produce proinflammatory and profibrotic mediators when stimulated in vitro with IL-4/IL-13 and IL-17 [31]. Although these studies suggest a role for FC in asthma, the pathological significance of FC infiltration in the asthmatic ASM is unknown. As ASMC is well known for its role in inflammation and airway remodeling in asthma, the aim of the present study was to explore if asthmatic FC (ASFC) or normal subject FC (NSFC) modulate the proliferative or contractile phenotype and/or secretion of immunomodulatory factors and ECM expression of asthmatic ASMC (ASASMC) or normal subject ASMC (NSASMC) and the potential signaling mechanisms involved in these processes.

## Methods

### Subjects

Fourteen asthmatic patients and 14 normal subjects were recruited after providing informed consent and protocol approval by the institutional ethics committee (Table 1). Asthmatic patients have a consistent history and one or more of the following: 1. >12% improvement in forced expiratory volume in 1 second (FEV1) after administration of bronchodilator; 2. PC20 of methacholine test <8 mg/mL; 3. Diurnal variation of peak expiratory flow >20%. Control level of asthma was defined by using the current Global Initiative for Asthma (GINA) guidelines. Subjects were non-smokers and free from respiratory or systemic infections in the previous 2 months.

### Isolation of FC

FC were isolated according to previously published methods[22, 24, 32]. Briefly, peripheral blood mononuclear cells (PBMC) were separated from human blood by Ficoll-hypaque density gradient centrifugation. CD14<sup>+</sup> monocytes were isolated from PBMC by immunomagnetic selection (Miltenyi Biotec, Auburn, CA) and purity (>90%) was confirmed by flow cytometry and then cultured on fibronectin-coated culture (BD Biosciences, Bedford, MA) plates with Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). Non-adherent cells were removed at day 3. Cell morphology was checked during day 10 to day 14 of culture. Expression of collagen-1, CD45, CXCR4, CD1C, CD303 and PM-2K was judged by flow cytometry. (Details in the Online supplement data Methods). Mature FC were used in all experiments.

## **Culture of ASMC**

Airway smooth muscle cells from asthmatic (ASASC) and normal (NSASC) subjects were purchased from Lonza. ASMC were maintained in DMEM with 10% FBS at 37° C with 5% CO<sub>2</sub>. ASMC from different lots at the fourth to seventh passage were utilized in subsequent experiments.

## **ASMC incubation with conditioned medium of FC (FCCM)**

Fibrocytes cultured in 12-well plates were grown to 80% confluency with complete medium (DMEM+10% FBS), washed and then subjected to serum starvation (DMEM+0.5% FBS) for 48 h. After 48 h, conditioned medium was collected, centrifuged at 1500 rpm (4° C) for 10 minutes and filtered with 0.2 µm filter and labeled as FCCM. Eighty percent confluent ASMC were serum-starved in a 12 well plate overnight and then incubated with starvation medium alone or 25% or 50% (diluted in DMEM+0.5% FBS) FCCM for 48 hours. ASMC treated with PDGF (10ng/ml) was positive control.

## **Co-culture of ASMC and FC**

ASMC and FC from normal subjects and asthmatics were starved overnight and FC were labeled with 5 µM Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE; Molecular probes, Carlsbad, CA) before co-culture with ASMC. A preliminary experiment of ASMC and CFSE-labeled FC cultured separately for 48 hours revealed no significant overlap in CFSE expression between ASMC and FC as assessed by flow cytometry, suggesting the feasibility to distinguish ASMC from CFSE-labeled FC (Online supplement data Figure S1). FC alone, ASMC alone and ASMC with CFSE-labeled FC in the ratio of cell number (ASMC:FC) 2:1 and 4:1 were cultured in a 6 well plate at a density of  $4.68 \times 10^4/\text{cm}^2$  in starvation medium for 48 hours.

### **Transwell culture of ASMC and FC**

ASMC and FC were serum-starved overnight. ASMC or FC alone were cultured in the lower well of a 12 well transwell plate (0.4  $\mu\text{m}$  pore size, Corning, Lowell, MA) at a density of  $4.68 \times 10^4/\text{cm}^2$  while upper well was cultured with half the cell number. ASMC in lower well at a density of  $4.68 \times 10^4/\text{cm}^2$  and FC in upper well were cultured in the ratio of cell number (ASMC:FC) 2:1 and 4:1 in starvation medium for 48 hours.

### **Flow cytometry**

FCCM-treated ASMC were stained with mouse Phycoerythrin (PE)-conjugated anti-Ki-67 (BD Biosciences, San Diego, CA). Cells from co-culture of ASMC and CFSE-labeled FC were stained with mouse Allophycocyanin (APC)-conjugated anti-Ki-67 (Biolegend, San Diego, CA) or mouse anti-collagen-I antibody (Millipore, Temecula, CA), followed by Alexa Fluor 647 conjugated anti-mouse antibodies (Invitrogen, Burlington, ONT). ASMC were then gated from CFSE-negative population and Ki-67 or collagen-I expression was analyzed. The cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) using CellQuest Pro software (BD Biosciences). (Details in the Online supplement data Methods)

### **ASMC proliferation: $^3\text{H}$ -thymidine incorporation assay and cell counting**

ASMC proliferation in response to FCCM was evaluated by a  $^3\text{H}$ -thymidine incorporation assay. ASMC were seeded at 15,000 cells/well in 24-well plates. Cells were cultured for 2 days, starved for 48 hours in 0.1% FBS and then treated for 48 hours with FCCM at various concentration (0, 25, 50 or 100%). Twenty four hours prior to harvesting onto glass fiber

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filters (Wallac), cells were labeled with 5  $\mu$ Ci/well of  $^3$ H-methyl thymidine (Perkin-Elmer). Thymidine incorporation was then quantified in counts per minute (cpm) by liquid scintillation counting (Wallac). A similar protocol of FCCM treatment was used for ASMC cell number counting (Details in the Online supplement data Methods).

### **Enzyme-linked immunosorbent assay (ELISA)**

Cell culture supernatants from co-culture and transwell experiments were assayed for IL-8 (Biolegend), IL-6, activated and total TGF- $\beta$ 1 (R&D Systems) and eotaxin (Peprotech) levels using commercially available ELISA kits. A similar protocol was used in experiments in which ASMC were treated with FCCM or starvation medium for 48 hours.

### **Immunofluorescence staining**

ASMC and CFSE-labeled FC were cultured together (2:1 co-culture) in 8-chamber culture slide (BD Biosciences, Bedford, MA) for 48 hours, and Golgistop (BD Biosciences, San Diego, CA) was added 4 hours before staining. Cells were stained with mouse anti-IL-8 (R&D systems, Minneapolis, MN) or rabbit anti-IL-6 (Abcam, Cambridge, MA) antibody followed by goat anti-mouse or donkey anti-rabbit Alexa Fluor 555 antibody (Invitrogen, Burlington, ONT), respectively. Cell nucleus was stained with Hoechst 33342 (AnaSpec, Fremont, CA) and images taken with an Olympus BX-51 microscope (Olympus Optical Co., Melville, NY) equipped with reflected ultraviolet light illumination. (Details in the Online supplement data Methods). A similar staining protocol was used in experiments in which ASMC were treated with FCCM or starvation medium for 48 hours.

### **Short-interference RNA (siRNA)**

ASMC were transfected with siRNA targeting NF- $\kappa$ B p65 (Santa Cruz Biotechnology, Santa Cruz, CA) or ERK1/2 (Cell Signaling Technology, Danvers, MA) using the siRNA Reagent System (Santa Cruz Biotechnology, Santa Cruz, CA). The scramble siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a negative control. After starvation overnight, ASMCs were transferred to transwell plates and cultured with FC in cell number ratio 2:1 in staving medium for 48 hours. Supernatant was collected for ELISA and cell layer (ASMC) was harvested for protein extraction to determine gene knockdown efficiency by western blotting.

### **Western blot analysis**

Electrophoresed proteins were transferred to nitrocellulose membranes and then incubated with primary antibodies specific for NF- $\kappa$ B-p65 (Santa Cruz Biotechnology), ERK1/2 (Cell Signaling Technology),  $\alpha$ -SMA (R&D systems), myosin light chain kinase (MLCK; Abcam) and GAPDH (Millipore). Membranes were stained with IRDye 680 goat anti-mouse IgG and IRDye 800 goat anti-rabbit IgG (Rockland Philadelphia, PA) secondary antibodies. Relevant band intensities were quantified and analyzed with an Odyssey IR scanner by using Odyssey imaging software 3.0 (LI-COR Biosciences). (Details in the Online supplement data Methods)

### **Statistical analysis**

Data were expressed as means  $\pm$  SE. An unpaired two-tailed Student's t-test was used for single comparisons.  $\chi^2$  Test was used to compare categorical data. One-way analysis of variance (ANOVA) with Dunnett's test was used to compare the effect of co-culture or transwell culture with FC to control group of ASMC. Wilcoxon Signed Rank test was used to

compare fold change of specific protein in transwell culture with FC comparing to ASMC alone in western blotting. Probability values of  $<0.05$  were considered significant.

## **Results**

### **Purification of FC**

During 10 to 14 days of culture, cells differentiated into spindle shape (Fig. 1, A) and  $>90\%$  expressed FC specific markers collagen-1, CD45, CXCR4 (Fig 1. B). Because the common markers for dendritic cells (DC), CD11b and CD11c and macrophage marker CD68 are also expressed on FC, we checked the expression of CD1C (myeloid DC), CD303 (plasmacytoid DC) and PM-2K (macrophage) on FC[33] (Online supplement data Figure S2). No expression of these markers was noted in our FC population.

### **FC do not affect proliferation and collagen-I expression of ASMC**

We examined thymidine incorporation of ASMC, cell number and the proliferative marker Ki-67 of ASMC after incubation with FCCM for 48 hours.. Treatment with FCCM did not affect thymidine incorporation and cell number of ASMC (online supplement data Figure S3). There was no difference in Ki-67 expression by ASMC treated with starvation medium, 25% and 50% FCCM, irrespective of whether ASMC or FC were derived from asthmatic or normal subjects (Online supplement data Figure S3, B). We further examined if direct cell contact by FC has an effect on ASMC proliferation. Ki- 67 expression was evaluated when ASMC were cultured alone or with CFSE-labeled FC for 48 hours. Cell populations of ASMC and CFSE-labeled FC were distinguished by CFSE expression using flow cytometry (Online supplement data Figure S4, C). There was no difference in Ki-67 expression between

ASMC cultured alone or with FC (Online supplement data Figure S4, D). We also measured collagen-I expression by ASMC in the co-culture experiments. There was no difference in collagen-I expression between ASMC cultured alone or with FC (Online supplement data Figure S5).

### **Co-culture of ASMC and FC increases IL-8 and IL-6 production**

Culture supernatant from FC, ASMC or co-culture of ASMC+FC in cell number ratio 2:1 and 4:1 was analyzed for IL-8, IL-6, eotaxin and TGF- $\beta$ 1 production by ELISA. Compared to ASMC cultured alone, when ASMC were cultured with FC at a ratio of 2:1 (ASMC:FC) the levels of IL-8 (Fig 2, A) and IL-6 (Fig 2, B) were significantly increased in both asthmatic and normal subjects. IL-8 levels were also increased in co-culture of NSASMC:NSFC 4:1. However, IL-6 levels did not significantly change in co-culture of ASASMC+NSFC ( $p=0.07$ , Fig. 2, B). There was no increase in eotaxin, total and active TGF- $\beta$ 1 levels in the co-culture of ASMC+FC compared to ASMC alone (Online supplement data Figure S6).

### **ASMC co-cultured with FC produce IL-8 and IL-6**

Immunofluorescence staining of IL-8 (Fig 3, A to D) and IL-6 (Fig 3, E to H) was performed in co-culture of unlabeled ASMC+CFSE-labeled FC to identify the cellular source of these cytokines. ASMC were distinguished from FC by the absence of green color staining (no CFSE labeling) and larger and elongated nuclei (Fig 3, A and E, enlarged pictures). ASMC (marked by arrows) were stained positive for IL-8 (Fig. 3, D) and IL-6 (Fig 3, H), indicating that a major portion of IL-8 and IL-6 were derived from ASMC in co-culture; however, a few FC were also positive for IL-8 and IL-6 (cells that show both green and red fluorescence in colocalization).

## **FC and ASMC interaction via paracrine mechanism to promote IL-8 and IL-6 production of ASMC**

We further performed transwell cell culture experiments with the same cell density as co-culture to evaluate if paracrine mechanisms could also promote IL-8 and IL-6 production in the absence of direct cell-cell contact. There was a significant increase in IL-8 and IL-6 production in the transwell culture of ASMC:FC 2:1 (Fig 4) when compared with ASMC alone. IL-6 was also increased in transwell culture of ASASC:ASFC 4:1 (Fig 4, G) in comparison to ASASC alone. These data suggest that ASMC interact with FC to produce IL-8 and IL-6 through a paracrine mechanism. In order to determine whether IL-8 and IL-6 production of ASMC can be influenced by the paracrine effect from FC, ASMC were treated with FCCM or starvation medium for 48 hours. We used ELISA to measure ASMC production of IL-6 and IL-8 in the supernatant after 48 hour culture with FCCM or starvation medium. Immunofluorescence was used to study cell surface staining of the above cytokines. Compared to ASMC cultured in starvation medium, ASMC treated with FCCM produced higher level of IL-8 and IL-6 in the supernatant (Online supplement data Figure S7) and were more immunopositive for IL-8 and IL-6 (Online supplement data Figure S8) and supporting our notion that paracrine mechanism (s) mediated by factor (s) released by the FC can induce IL-8 and IL-6 production by ASMC.

## **Signaling pathways involved in ASMC production of IL-8 and IL-6 in transwell culture with FC**

In order to evaluate the signaling pathways involved in the IL-8 and IL-6 production by ASMC in transwell culture, knockdown of NF- $\kappa$ B-p65 or ERK1/2 in ASMC was performed by siRNA before transwell culture of ASMC:FC 2:1 (Fig 5). No significant cellular toxicity was observed in our siRNA knockdown experiments. In the transwell culture of ASASC+NSFC, we found decreased level of IL-8 when NF- $\kappa$ B-p65 was knocked down in

ASMC (Fig 5, E) while IL-6 was decreased when NF- $\kappa$ B-p65 and ERK 1/2 were knocked down in ASMC (Fig 5, G and H). However, there was no statistical difference in cytokine production in the transwell culture of NSASMC+NSFC, ASASMC+ASFC and ASASMC+NSFC post knockdown compared to scramble (Online supplement data Figure S9).

#### **$\alpha$ -SMA and MLCK expression of ASMC in the transwell culture with FC**

To evaluate if FC affected the contractile phenotype of ASMC, we examined the contractile protein,  $\alpha$ -SMA and MLCK, which phosphorylates myosin light chain and promotes contraction, in the transwell culture of ASMC:FC 2:1 using western blotting. There was no significant difference in the expression of  $\alpha$ -SMA and MLCK from ASMC+FC in the transwell culture when compared to ASMC alone (Fig 6).

#### **Discussion**

Infiltration into the ASM compartment by mast cells, T cells and FC is a classic feature of asthma. Although interactions between ASMC and mast cells or ASMC and T cells in the pathogenesis of asthma have been studied extensively, the pathological implication of FC infiltration in ASM bundles is unknown. The present work is the first study to investigate the effects of FC on ASMC phenotype. Our study reveals a significant increase in IL-8 and IL-6 cytokine production from co-cultures of ASMC and FC, with ASMC as the source of IL-8 and IL-6. FC interact with ASMC to produce IL-8 and IL-6 through a paracrine mechanism, although direct cell contact may also play a role. Interestingly, cytokine production was decreased by siRNA silencing of NF- $\kappa$ B and ERK1/2 in asthmatic ASMC cultured with normal subject FC in the transwell experiments. Our data do not support a role for FC in affecting the proliferation or expression of contractile proteins of ASMC.

IL-8 is a potent chemoattractant for neutrophils and our present findings offer a potential mechanism for airway neutrophilia in asthma. The sputum level of IL-8 is increased in neutrophilic asthma[18] and is correlated with sputum neutrophil numbers[17]. A recent study reported sputum neutrophilia in 21% of persistent non-eosinophilic asthma, which is associated with poor response to anti-inflammatory therapy. A cluster characterized by late onset asthma, high sputum neutrophils and low lung function has also been identified in stable and severe asthma[34] [35]. IL-6 can perpetuate inflammation and remodeling of asthmatic airway through activation of mast cells, recruitment of T cells, stimulation of mucus production and promotion of Th17 cell differentiation[5, 36]. Sputum IL-8 and IL-6 levels in asthma are also associated with a lower pre and post-bronchodilator FEV1[18, 37].

Our approach is based on published evidence describing the origin of fibrocytes. [21-24, 26, 38, 39]. Abe et al. reported that ex vivo cultured human fibrocytes can differentiate from a CD14-enriched mononuclear cell population and there was no fibrocyte differentiation when the CD14+cells were depleted [21]. Curnow et al [40] and Yang et al [22] also demonstrated similar results in humans. Niedermeier et al. described that murine fibrocytes develop from a fraction of monocytes (GR1+), and depleted Ly6C (which is part of the epitope of GR1) monocytes could decrease fibrocytes from the kidney [23, 41]. These papers demonstrate that fibrocytes develop in vitro from a subpopulation of CD14<sup>+</sup> monocytes, similar to our findings. This has been described in a recent review on fibrocytes [39], although according to this review CD14 expression can be equivocal. In the study reported by Abe et al [21] fibrocytes were obtained from a CD14<sup>+</sup> population of PBMC that was depleted of T and B cells. Although this group did not test a pure population of CD14<sup>+</sup> cells, in the current paper we show that fibrocytes can be obtained from ex-vivo cultured CD14<sup>+</sup> monocytes, and these differentiated fibrocytes can secrete cytokines, chemokines and extracellular matrix protein and also interact with airway smooth muscle cells. We purified fibrocytes following the method of Garcí'a-de-Alba et al and Varcoe et al. with slight modification[24, 32]. No

granulocyte-macrophage colony stimulating factor or lipopolysaccharide was added, so as to prevent the development of heterogeneous cell populations during FC culture and there was no expression of markers of DC or macrophage in the FC (Online supplement data Figure S2). Further, we did not observe spindle-shaped cell differentiation from the CD14-depleted cell population (data not shown). These monocyte-derived, spindle-shaped and collagen-I positive cells represent a kind of fibroblast-like cell coming from the circulation, differentiate into spindle-shaped cells in local tissue and are able to interact with residential cells, all of which fit the original definition of “fibrocytes” proposed by Bucala and colleagues[19, 39].

Co-culture or transwell culture studies appear to be a suitable model for evaluation of the FC and ASMC interaction. Initially, using a co-culture system of FC with ASMC we measured ASMC-associated cytokine and chemokine (TGF- $\beta$ 1, eotaxin, IL-8 and IL-6) production and collagen-I expression that have been shown to be implicated in inflammation and remodeling of the airways in asthma. Co-culture of FC with ASMC did not significantly influence the levels of TGF- $\beta$ 1, eotaxin and collagen-I, but significantly increased the levels of IL-8 and IL-6, indicating that cell-cell interaction is involved in cytokine production. To determine if this cytokine/chemokine production was also mediated by soluble fibrocyte-derived mediators, we measured IL-8 and IL-6 levels in a transwell culture system or in ASMC treated with FCCM. As shown for co-cultures, increased levels of IL-8 and IL-6 were also found in transwell culture experiments in which ASMC were cultured separate from the FC or treated with FCCM. Together, these findings demonstrate that cytokine/chemokine production occurs through direct contact between ASMC and FC, as well as through a soluble factor released by FC that does not require cell-cell interaction. This is similar to data in mast cells, where elevated levels of IL-8 and IL-6 cytokines have been reported when ASMC were treated with conditioned medium from mast cells[15] Our in vitro findings are indicative of what is seen pathologically in asthmatic airways. The co-culture results mimic FC infiltration into ASM bundles and FC are also found in the subepithelium of the asthmatic airway [30].

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The levels of IL-8 and IL-6 in transwell cultures are increased as was noted in co-cultures, indicating that even in the absence of direct contact with ASMC, distantly located FC are capable of influencing ASMC to produce more inflammatory cytokines.

Growing evidence shows an immunomodulatory function of FC in chronic inflammation[39]. It has been shown that FC can secrete factors such as TNF- $\alpha$  or IL-1 $\beta$ [42], which could also stimulate ASMC to produce IL-8 and IL-6[5]. As demonstrated in this study, fibrocytes from both normal and asthmatic subjects promote IL-8 and IL-6 production in co-culture with ASMC. However, infiltration of FC in ASM bundles is observed only in asthmatic subjects[20], implying that FC with immunomodulatory capacity is recruited to ASMC significantly in the clinical situation. How cell-cell contact plays a role in the cytokine production in coculture requires further study. It has been shown that ASMC interact with mast cells via CD44 (hyaluronate receptor) and collagen-1[43] and with T cells via adhesion molecule VCAM-1[10]. Since FC and ASMC both express abundant adhesion molecules e.g. ICAM-1, VCAM-1, CD44, integrins and ECM proteins e.g. collagen and hyaluronate[27, 31, 39, 44], it is reasonable to hypothesize that direct cell contact may play a role in cytokine production and in cell interaction in co-culture. Further studies are needed to address these mechanisms in the asthmatic airway.

After knocking down NF- $\kappa$ B and ERK 1/2 in ASMC, decreased IL-8 and IL-6 level was only observed in the transwell culture of ASASC+NSFC (Fig 5), but not in NSASC+NSFC, ASASC+ASFC or NSASC+ASFC (Online supplement data Figure S9). FC and ASMC are both well known to secrete a variety of immunomodulatory factors[5, 39]. Complex interactions through these factors from both cell types of asthmatic or normal subjects with the involvement of multiple signaling pathways such as phosphatidylinositol 3-kinase and p38 MAPK in ASMC maybe the possible explanation for a lack of effect on cytokine

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production by single pathway knockdown in ASMC. Another explanation is FC are also able to produce IL-8 and IL-6[42] and indeed our results showed some immunopositivity by FC for IL-6 and IL-8 (Fig. 3), however, the contribution of cytokines from each cell type is unknown.

Similar to mast cells[45], our data did not show any effect of FC on ASMC proliferation. FC are considered to produce some mitogenic factors for ASMC, e.g. PDGF, VEGF and MIP-1[42] and IL-8 is also mitogenic to ASMC[46]. It also has been shown that CD4+T cells induce ASMC proliferation through direct intercellular contact via VCAM-1[10]. However, neither conditioned medium nor direct intercellular contact by FC modulated proliferation of ASMC in our study. Although FC did not modulate ASMC proliferation, other mechanisms, which can affect cell number, like apoptosis, need to be addressed in future studies.

There are many aspects of ASMC abnormalities in asthma, including increasing ASM mass (hyperplasia or hypertrophy), increasing contractile protein expression and contractility. How fibrocytes affect these processes is still unknown. We have shown that fibrocytes did not affect the proliferation of ASMC, implying a lack of effect on ASMC hyperplasia. The expression of  $\alpha$ -SMA and MLCK in ASMC was not affected by FC. The actin-myosin cross-bridge is the contractile apparatus of ASM. After activation by calcium-calmodulin complex, MLCK phosphorylates myosin light chain (MLC), which is responsible for cross-bridge activation and promotes contraction[3]. We chose  $\alpha$ -SMA and MLCK as markers of contractile phenotype of ASMC because levels of these markers are increased in asthmatic ASMC and associated with increased contractility and/or increased velocity of contraction[9, 47, 48]. Ma et al showed that asthmatic ASMC from bronchial biopsy express higher mRNA of MLCK than the ASMC from normal subjects[47]. Benayoun et al demonstrated increasing MLCK in the ASMC from severe asthmatics by immunohistochemistry[48]. Woodman et al

showed that mast cells increase ASMC alpha-smooth muscle actin expression[9]. It has also been shown that mast cells secrete  $\beta$ -tryptase and promote  $\alpha$ -SMA expression in ASMC by autocrine TGF- $\beta$  [9]. Although we did not check the level of TGF- $\beta$  in the supernatant of transwell culture, there was no increase in the level of TGF- $\beta$  in the co-culture (Fig. S6). This may account in part for the observation that the expression of contractile protein was unchanged.

There are some limitations in the present study. Firstly, our data were based on fibrocytes obtained from normal and asthmatic subjects, whereas ASMC were purchased commercially. However, it should be noted that these ASMC were primary cells and these cells, whether derived from normal subjects or asthmatics, behaved similarly to that of ASMC isolated in the laboratory and responded well to inflammatory cytokine and gene knockdown treatments[49]. Our results on the effect of FC on ASMC cytokine production in the co-culture and transwell culture are consistent between experiments from different subjects and different lots of ASMC; ie our findings were reproducible. Secondly, though we studied ASMC contractile phenotype by measuring contractile protein expression, another measure of ASMC contraction, e.g. a true measurement of the contractility of ASM or phosphorylation of MLC should be performed in future studies.

In conclusion, FC promoted IL-8 and IL-6 production by ASMC. FC did not affect the proliferation and the expression of  $\alpha$ -SMA and MLCK of ASMC. Our findings provide a potential pathological effect of infiltration of FC in ASM in asthmatic subjects and a possible mechanism to explain the inflammatory phenotype and remodeling observed in the asthmatic airway.

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## References

1. Halwani R, Al-Muhsen S, Hamid Q, Airway remodeling in asthma. *Curr Opin Pharmacol* 2010;10: 236-45.
2. Fixman ED, Stewart A, Martin JG, Basic mechanisms of development of airway structural changes in asthma. *The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology* 2007;29: 379-89.
3. Leguillette R, Lauzon AM, Molecular mechanics of smooth muscle contractile proteins in airway hyperresponsiveness and asthma. *Proc Am Thorac Soc* 2008;5: 40-6.
4. Pepe C, Foley S, Shannon J, Lemiere C, Olivenstein R, Ernst P, Ludwig MS, Martin JG, Hamid Q, Differences in airway remodeling between subjects with severe and moderate asthma. *J Allergy Clin Immunol* 2005;116: 544-9.
5. Xia YC, Redhu NS, Moir LM, Koziol-White C, Ammit AJ, Al-Alwan L, Camoretti-Mercado B, Clifford RL, Pro-inflammatory and immunomodulatory functions of airway smooth muscle: emerging concepts. *Pulm Pharmacol Ther* 2013;26: 64-74.
6. Chung KF, Airway smooth muscle cells: contributing to and regulating airway mucosal inflammation? *The European respiratory journal* 2000;15: 961-8.
7. Brightling CE, Bradding P, Symon FA, Holgate ST, Wardlaw AJ, Pavord ID, Mast-cell infiltration of airway smooth muscle in asthma. *N Engl J Med* 2002;346: 1699-705.

- Accepted Article
8. Bradding P, Walls AF, Holgate ST, The role of the mast cell in the pathophysiology of asthma. *J Allergy Clin Immunol* 2006;117: 1277-84.
  9. Woodman L, Siddiqui S, Cruse G, Sutcliffe A, Saunders R, Kaur D, Bradding P, Brightling C, Mast cells promote airway smooth muscle cell differentiation via autocrine up-regulation of TGF-beta 1. *J Immunol* 2008;181: 5001-7.
  10. Ramos-Barbon D, Fraga-Iriso R, Brienza NS, Montero-Martinez C, Vereza-Hernando H, Olivenstein R, Lemiere C, Ernst P, Hamid QA, Martin JG, T Cells localize with proliferating smooth muscle alpha-actin+ cell compartments in asthma. *Am J Respir Crit Care Med* 2010;182: 317-24.
  11. Ramos-Barbon D, Presley JF, Hamid QA, Fixman ED, Martin JG, Antigen-specific CD4+ T cells drive airway smooth muscle remodeling in experimental asthma. *J Clin Invest* 2005;115: 1580-9.
  12. Ammit AJ, Lazaar AL, Irani C, O'Neill GM, Gordon ND, Amrani Y, Penn RB, Panettieri RA, Jr., Tumor necrosis factor-alpha-induced secretion of RANTES and interleukin-6 from human airway smooth muscle cells: modulation by glucocorticoids and beta-agonists. *Am J Respir Cell Mol Biol* 2002;26: 465-74.
  13. Wuyts WA, Vanaudenaerde BM, Dupont LJ, Demedts MG, Verleden GM, Involvement of p38 MAPK, JNK, p42/p44 ERK and NF-kappaB in IL-1beta-induced chemokine release in human airway smooth muscle cells. *Respir Med* 2003;97: 811-7.
  14. Henness S, van Thoor E, Ge Q, Armour CL, Hughes JM, Ammit AJ, IL-17A acts via p38 MAPK to increase stability of TNF-alpha-induced IL-8 mRNA in human ASM. *Am J Physiol Lung Cell Mol Physiol* 2006;290: L1283-90.

- Accepted Article
15. Xia YC, Harris T, Stewart AG, Mackay GA, Secreted factors from human mast cells trigger inflammatory cytokine production by human airway smooth muscle cells. *Int Arch Allergy Immunol* 2013;160: 75-85.
  16. Broide DH, Lotz M, Cuomo AJ, Coburn DA, Federman EC, Wasserman SI, Cytokines in symptomatic asthma airways. *J Allergy Clin Immunol* 1992;89: 958-67.
  17. Gibson PG, Simpson JL, Saltos N, Heterogeneity of airway inflammation in persistent asthma : evidence of neutrophilic inflammation and increased sputum interleukin-8. *Chest* 2001;119: 1329-36.
  18. Wood LG, Baines KJ, Fu J, Scott HA, Gibson PG, The neutrophilic inflammatory phenotype is associated with systemic inflammation in asthma. *Chest* 2012;142: 86-93.
  19. Bucala R, Spiegel LA, Chesney J, Hogan M, Cerami A, Circulating fibrocytes define a new leukocyte subpopulation that mediates tissue repair. *Mol Med* 1994;1: 71-81.
  20. Saunders R, Siddiqui S, Kaur D, Doe C, Sutcliffe A, Hollins F, Bradding P, Wardlaw A, Brightling CE, Fibrocyte localization to the airway smooth muscle is a feature of asthma. *J Allergy Clin Immunol* 2009;123: 376-84.
  21. Abe R, Donnelly SC, Peng T, Bucala R, Metz CN, Peripheral blood fibrocytes: differentiation pathway and migration to wound sites. *J Immunol* 2001;166: 7556-62.
  22. Yang L, Scott PG, Giuffre J, Shankowsky HA, Ghahary A, Tredget EE, Peripheral blood fibrocytes from burn patients: identification and quantification of fibrocytes in adherent cells cultured from peripheral blood mononuclear cells. *Laboratory investigation*, 2002;82: 1183-92.

- Accepted Article
23. Niedermeier M, Reich B, Rodriguez Gomez M, Denzel A, Schmidbauer K, Gobel N, Talke Y, Schweda F, Mack M, CD4+ T cells control the differentiation of Gr1+ monocytes into fibrocytes. *Proc Natl Acad Sci U S A* 2009;106: 17892-7.
  24. Garcia-de-Alba C, Becerril C, Ruiz V, Gonzalez Y, Reyes S, Garcia-Alvarez J, Selman M, Pardo A, Expression of Matrix Metalloproteases by Fibrocytes. Possible Role in Migration and Homing. *Am J Respir Crit Care Med* 2010.
  25. Aiba S, Tagami H, Inverse correlation between CD34 expression and proline-4-hydroxylase immunoreactivity on spindle cells noted in hypertrophic scars and keloids. *J Cutan Pathol* 1997;24: 65-9.
  26. Phillips RJ, Burdick MD, Hong K, Lutz MA, Murray LA, Xue YY, Belperio JA, Keane MP, Strieter RM, Circulating fibrocytes traffic to the lungs in response to CXCL12 and mediate fibrosis. *J Clin Invest* 2004;114: 438-46.
  27. Bianchetti L, Barczyk M, Cardoso J, Schmidt M, Bellini A, Mattoli S, Extracellular matrix remodelling properties of human fibrocytes. *J Cell Mol Med* 2012;16: 483-95.
  28. Chesney J, Metz C, Stavitsky AB, Bacher M, Bucala R, Regulated production of type I collagen and inflammatory cytokines by peripheral blood fibrocytes. *J Immunol* 1998;160: 419-25.
  29. Wang CH, Huang CD, Lin HC, Lee KY, Lin SM, Liu CY, Huang KH, Ko YS, Chung KF, Kuo HP, Increased circulating fibrocytes in asthma with chronic airflow obstruction. *Am J Respir Crit Care Med* 2008;178: 583-91.
  30. Nihlberg K, Larsen K, Hultgardh-Nilsson A, Malmstrom A, Bjermer L, Westergren-Thorsson G, Tissue fibrocytes in patients with mild asthma: a possible link to thickness of reticular basement membrane? *Respir Res* 2006;7: 50.

31. Bellini A, Marini MA, Bianchetti L, Barczyk M, Schmidt M, Mattoli S, Interleukin (IL)-4, IL-13, and IL-17A differentially affect the profibrotic and proinflammatory functions of fibrocytes from asthmatic patients. *Mucosal Immunol* 2012;5: 140-9.
32. Varcoe RL, Mikhail M, Guiffre AK, Pennings G, Vicaretti M, Hawthorne WJ, Fletcher JP, Medbury HJ, The role of the fibrocyte in intimal hyperplasia. *J Thromb Haemost* 2006;4: 1125-33.
33. Pilling D, Fan T, Huang D, Kaul B, Gomer RH, Identification of markers that distinguish monocyte-derived fibrocytes from monocytes, macrophages, and fibroblasts. *PLoS One* 2009;4: e7475.
34. Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, D'Agostino R, Jr., Castro M, Curran-Everett D, Fitzpatrick AM, Gaston B, Jarjour NN, Sorkness R, Calhoun WJ, Chung KF, Comhair SA, Dweik RA, Israel E, Peters SP, Busse WW, Erzurum SC, Bleecker ER, Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. *Am J Respir Crit Care Med* 2010;181: 315-23.
35. McGrath KW, Icitovic N, Boushey HA, Lazarus SC, Sutherland ER, Chinchilli VM, Fahy JV, A large subgroup of mild-to-moderate asthma is persistently noneosinophilic. *Am J Respir Crit Care Med* 2012;185: 612-9.
36. Miossec P, Korn T, Kuchroo VK, Interleukin-17 and type 17 helper T cells. *N Engl J Med* 2009;361: 888-98.
37. Shaw DE, Berry MA, Hargadon B, McKenna S, Shelley MJ, Green RH, Brightling CE, Wardlaw AJ, Pavord ID, Association between neutrophilic airway inflammation and airflow limitation in adults with asthma. *Chest* 2007;132: 1871-5.
38. Shao DD, Suresh R, Vakil V, Gomer RH, Pilling D, Pivotal Advance: Th-1 cytokines inhibit, and Th-2 cytokines promote fibrocyte differentiation. *J Leukoc Biol* 2008;83: 1323-33.

39. Reilkoff RA, Bucala R, Herzog EL, Fibrocytes: emerging effector cells in chronic inflammation. *Nat Rev Immunol* 2011;11: 427-35.
40. Curnow SJ, Fairclough M, Schmutz C, Kissane S, Denniston AK, Nash K, Buckley CD, Lord JM, Salmon M, Distinct types of fibrocyte can differentiate from mononuclear cells in the presence and absence of serum. *PLoS One* 2010;5: e9730.
41. Reich B, Schmidbauer K, Rodriguez Gomez M, Johannes Hermann F, Gobel N, Bruhl H, Ketelsen I, Talke Y, Mack M, Fibrocytes develop outside the kidney but contribute to renal fibrosis in a mouse model. *Kidney Int* 2013;84: 78-89.
42. Quan TE, Cowper S, Wu SP, Bockenstedt LK, Bucala R, Circulating fibrocytes: collagen-secreting cells of the peripheral blood. *The international journal of biochemistry & cell biology* 2004;36: 598-606.
43. Girodet PO, Ozier A, Trian T, Begueret H, Ousova O, Vernejoux JM, Chanez P, Marthan R, Berger P, Tunon de Lara JM, Mast cell adhesion to bronchial smooth muscle in asthma specifically depends on CD51 and CD44 variant 6. *Allergy* 2010;65: 1004-12.
44. Ozier A, Allard B, Bara I, Girodet PO, Trian T, Marthan R, Berger P, The pivotal role of airway smooth muscle in asthma pathophysiology. *J Allergy (Cairo)* 2011;2011: 742710.
45. Kaur D, Hollins F, Saunders R, Woodman L, Sutcliffe A, Cruse G, Bradding P, Brightling C, Airway smooth muscle proliferation and survival is not modulated by mast cells. *Clin Exp Allergy* 2010;40: 279-88.
46. Halwani R, Al-Abri J, Beland M, Al-Jahdali H, Halayko AJ, Lee TH, Al-Muhsen S, Hamid Q, CC and CXC chemokines induce airway smooth muscle proliferation and survival. *J Immunol* 2011;186: 4156-63.

47. Ma X, Cheng Z, Kong H, Wang Y, Unruh H, Stephens NL, Laviolette M, Changes in biophysical and biochemical properties of single bronchial smooth muscle cells from asthmatic subjects. *Am J Physiol Lung Cell Mol Physiol* 2002;283: L1181-9.
48. Benayoun L, Druilhe A, Dombret MC, Aubier M, Pretolani M, Airway structural alterations selectively associated with severe asthma. *Am J Respir Crit Care Med* 2003;167: 1360-8.
49. Al-Alwan LA, Chang Y, Mogas A, Halayko AJ, Bagloli CJ, Martin JG, Rousseau S, Eidelman DH, Hamid Q, Differential roles of CXCL2 and CXCL3 and their receptors in regulating normal and asthmatic airway smooth muscle cell migration. *J Immunol* 2013;191: 2731-41.

**Table 1. Clinical characteristics of subjects**

|                     | <b>Normal subjects<br/>(n=14)</b> | <b>Asthmatic patients (n=14;<br/>GINA I=4, III=6, IV=2,<br/>V=2)</b> |
|---------------------|-----------------------------------|----------------------------------------------------------------------|
| Age, year           | 42.9±3.0                          | 51.6±3.9                                                             |
| Gender, male/female | 7/7                               | 7/7                                                                  |
| Atopy, n            | 2                                 | 9*                                                                   |
| FVC, liter          | 3.2±0.2                           | 2.9±0.2                                                              |

|                                                                          |          |             |
|--------------------------------------------------------------------------|----------|-------------|
| FVC, % of prediction                                                     | 90.8±1.2 | 78.2±5.0*   |
| FEV1, liter                                                              | 2.6±0.2  | 2.0±0.2     |
| FEV1, % of prediction                                                    | 90.2±1.1 | 65.5±6.9**  |
| FEV1/FVC, %                                                              | 81.5±1.0 | 66.5±4.0*** |
| % change of Post bronchodilator FEV1                                     | 4.3±4.3  | 8.9±2.4     |
| Treatments with albuterol as needed, n                                   | 0        | 8           |
| Treatment with inhaled corticosteroid and long-acting bronchodilators, n | 0        | 8           |
| Treatment with oral corticosteroid, n                                    | 0        | 2           |

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Data are expressed as means ± SEM or number of subjects. (\*p<.05, \*\*p<.01, \*\*\*p<.001)

Definition of abbreviations: GINA=Global initiative for asthma; FVC= forced vital capacity;

FEV1= forced expiratory volume in one second

### Figure Legends

Figure 1. Purification of fibrocytes (FC) after 10 to 14 days of cell culture. (A) Cells differentiated into spindle shape. These cells were positive staining for CD45, collagen-I and CXCR4 by flow cytometry: (B) Differentiated FC expressed CD45 and collagen-I. Cross line was defined by isotype staining. The dot plot gating from collagen-I and CD45 double positive population revealed more than 90% expressed CXCR4.

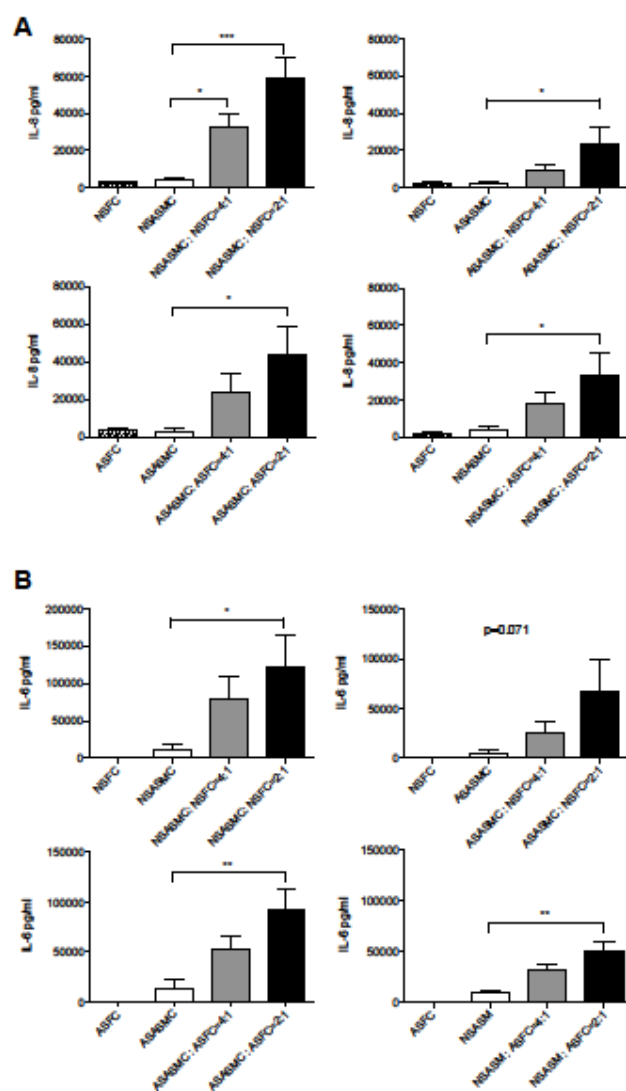
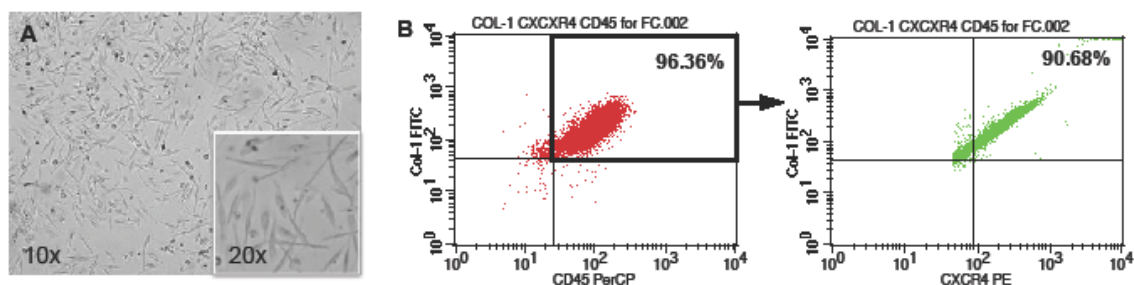
Figure 2. Increasing IL-8 and IL-6 secretion in the co-culture of ASMC and FC. Levels of IL-8 (A) and IL-6 (B) in the supernatant from FC alone, ASMC alone, co-culture of ASMC and FC in the ratio of cell number 4:1 (ASMC:FC) and 2:1. Cells were cultured at the same density in each well for 48 hours and cytokine levels of supernatant were detected by ELISA. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , One-way ANOVA with Dunnett analysis compared to ASMC. AS: asthmatic; NS: normal subject ( $n = 3 \sim 5$ , each experiment had equal subject numbers).

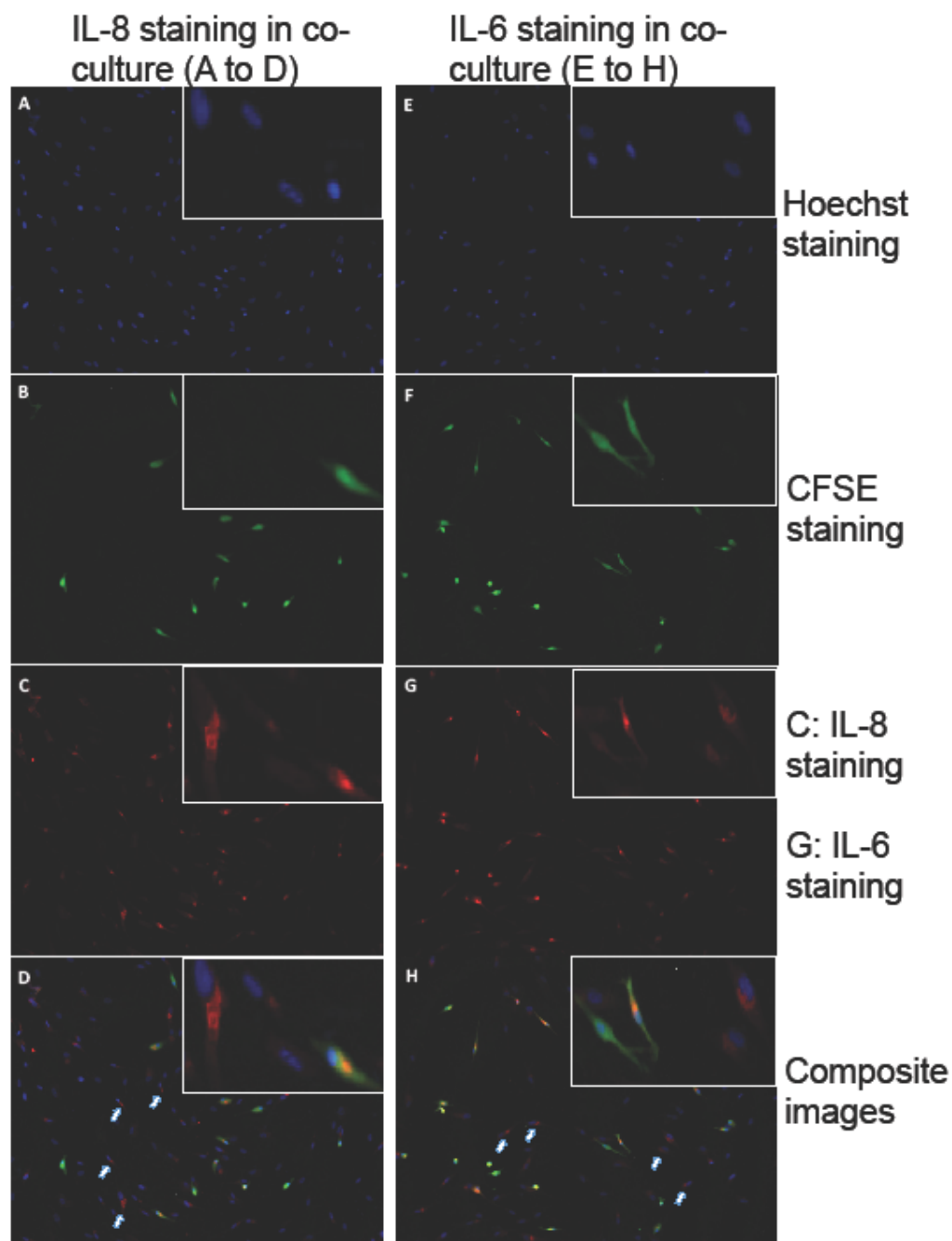
Figure 3. ASMC produce IL-8 and IL-6 in the co-culture with FC. Immunofluorescence staining of IL-8 (A to D) and IL-6 (E to H) in the co-culture. FC were labeled with CFSE before co-culture with ASMC. (A and E) Hoechst staining (blue). (B and F) CFSE-labeled FC (green). (C) Anti-human IL-8 and (G) IL-6 antibody following 2<sup>nd</sup> antibody of Alexa Fluor 555 (red). (D and H) Composite image of A+B+C and E+F+G, respectively. ASMC could be distinguished from FC by no green color staining (no CFSE labeling) and larger and elongated nuclei (Enlarged pictures of A and E). ASMC stained with anti-IL-8 and IL-6 antibody (cells without green color stained with red color) were marked by arrows.

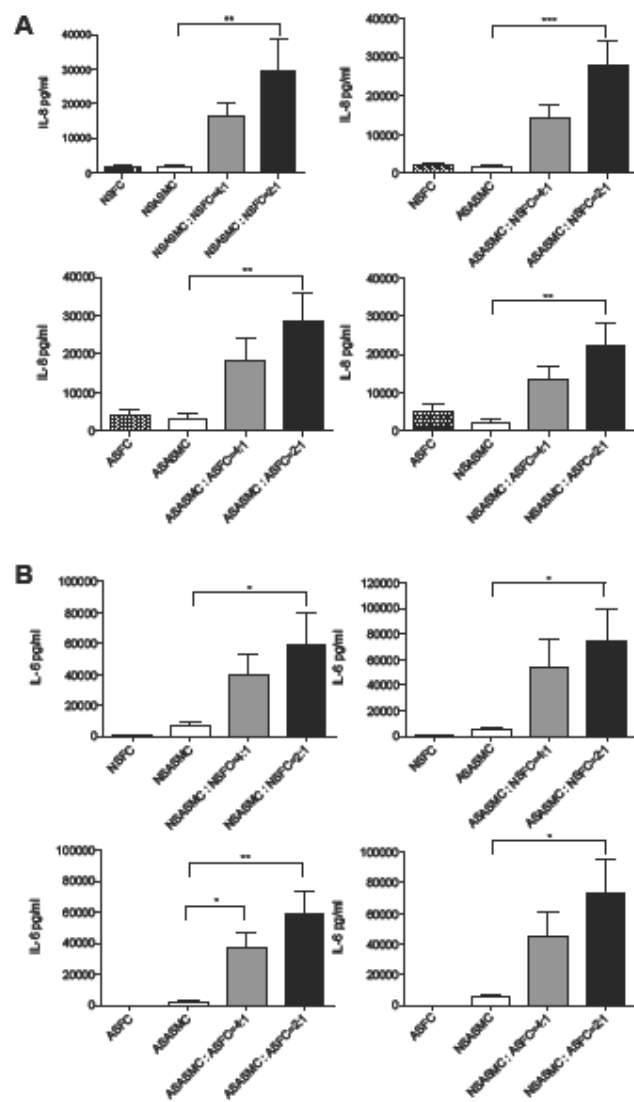
Figure 4. Increasing IL-8 and IL-6 secretion in the transwell culture of ASMC and FC. ASMC in lower well and FC in upper well were cultured in the ratio of cell number 2:1 and 4:1 to evaluate the paracrine effect between ASMC and FC on cytokine production. FC or ASMC alone were cultured in the lower well and half the cell number was cultured in the upper well. Cells in each lower well were cultured at the same cell density as co-culture experiment. Levels of IL-8 (A) and IL-6 (B) in the supernatant after 48 hours of culture were detected by ELISA. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  by One-way ANOVA with Dunnett analysis compared to ASMC. AS: asthmatic; NS: normal subject. ( $n = 3 \sim 4$ , each experiment had equal subject numbers between groups).

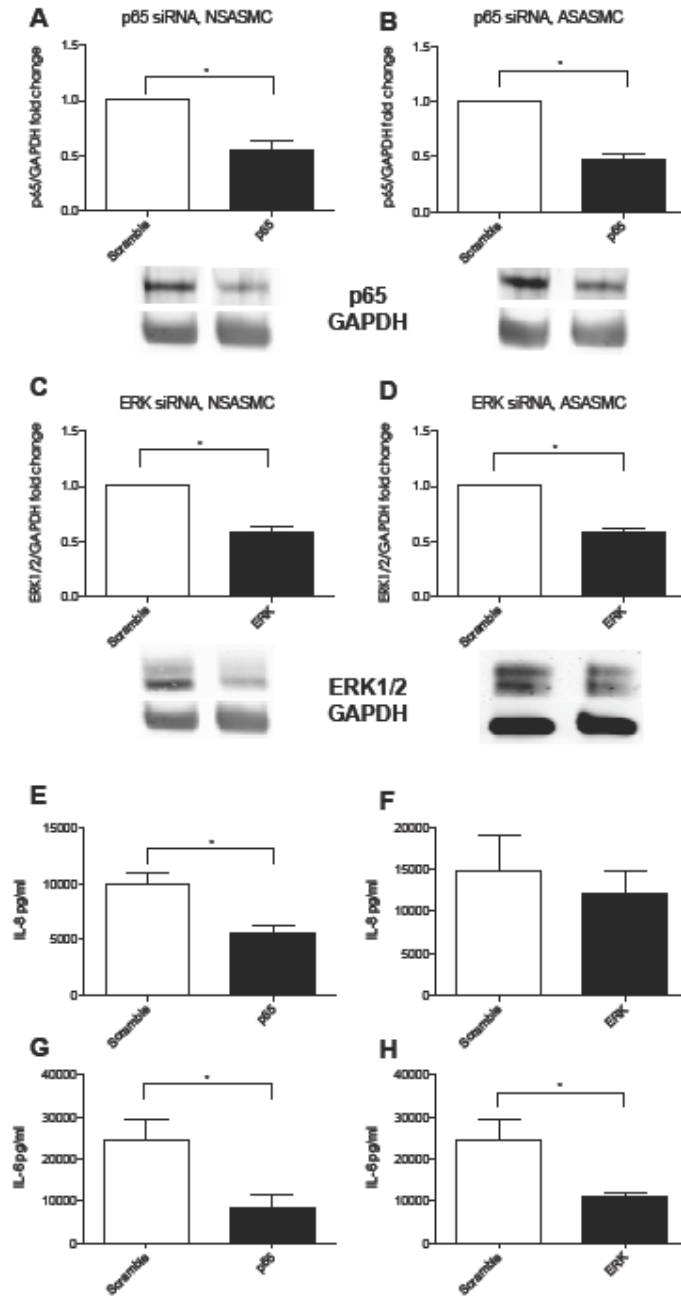
Figure 5. ERK1/2 and NF- $\kappa$ B p65 in ASMC affected IL-8 and IL-6 production partially in the transwell culture with FC. ERK1/2 and NF- $\kappa$ B p65 in ASMC were knocked down by siRNA transfection before transwell culture. Western blot analysis of siRNA-treated p65 in (A) normal subject airway smooth muscle cells (NSASMC) and (B) asthmatic airway smooth muscle cells (ASASMC) and siRNA-treated ERK1/2 in (C) NSASMC and (D) ASASMC was assessed after 48 hours transwell culture. Protein levels were normalized with GAPDH intensity and expressed as fold change from scramble. \* $p < 0.05$  by Wilcoxon Signed Rank Test (  $n = 4 \sim 6$ ). Levels of IL-8 (E) and IL-6 (G) and (H) were decreased in the supernatant from the transwell culture, in which p65 or ERK1/2 in ASASMC were knocked down in lower well and normal subject fibrocytes (NSFC) in upper well. The ratio of cell number ASMC:FC was 2:1 and cytokine levels of supernatant after 48 hours culture were detected by ELISA. \* $p < 0.05$  by unpaired t-test ( $n = 3 \sim 5$ , each experiment had equal subject numbers between groups).

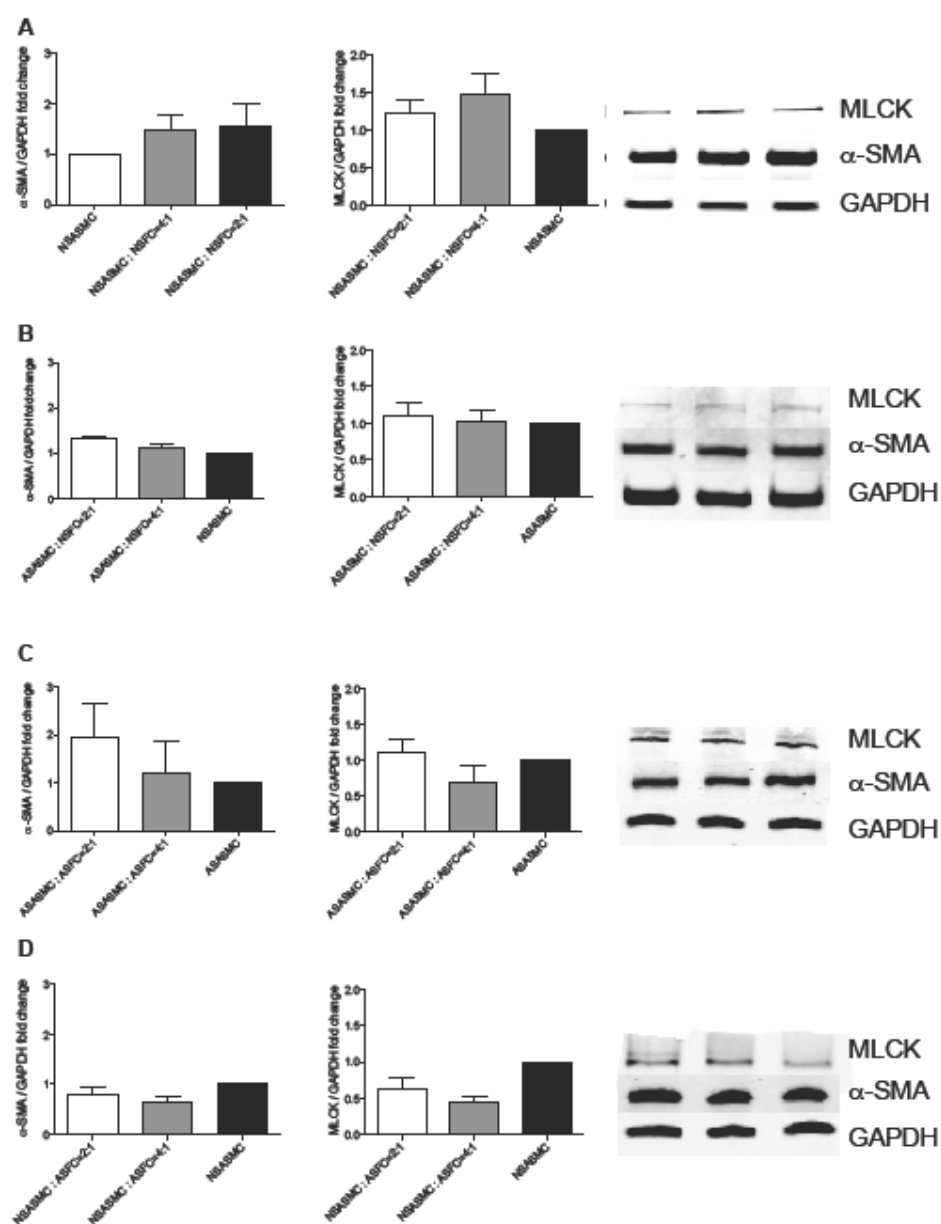
Figure 6.  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) and myosin light chain kinase (MLCK) expression in ASMC after transwell culture with FC. ASMC in lower well and FC in upper well were cultured in the ratio of cell number 4:1 and 2:1 for 48 hours. ASMC alone was cultured in lower and upper well in the cell ratio 2:1 as a control. Expression of  $\alpha$ -SMA and MLCK in ASMC was detected by western blotting. The specific protein was normalized with GAPDH intensity and expressed as fold change to control (ASMC). A: Transwell culture of NSASMC+NSFC, B: ASASMC+NSFC, C: ASASMC+ASFC and D: NSASMC+ASFC. Analysis by Wilcoxon Signed Rank test ( $n = 3 \sim 5$ , each experiment had equal subject numbers).

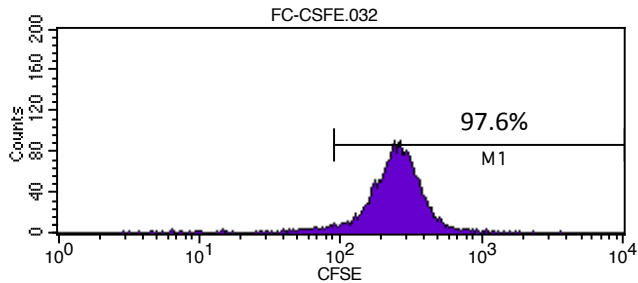
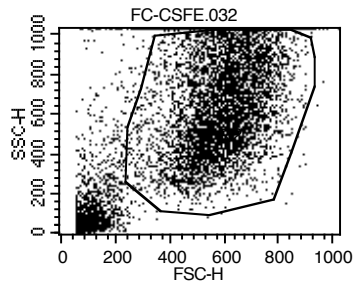
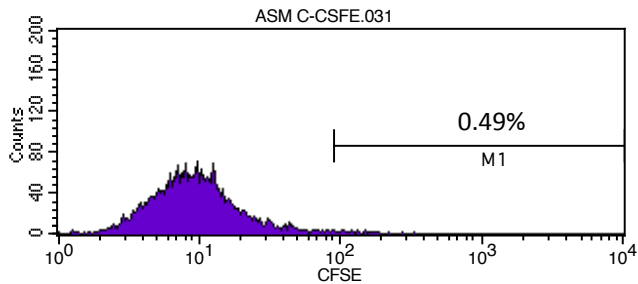
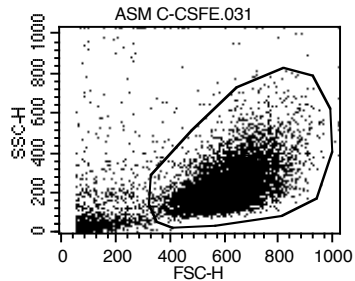


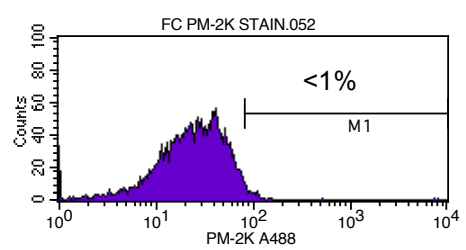
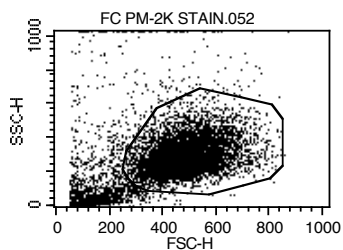
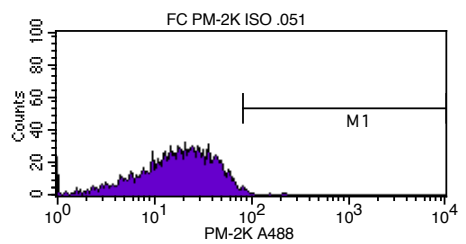
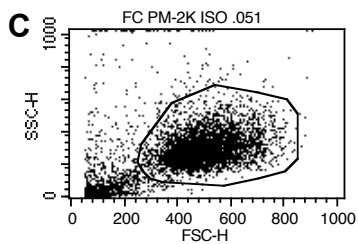
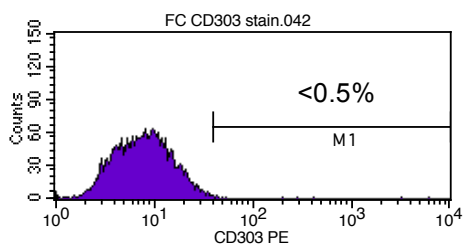
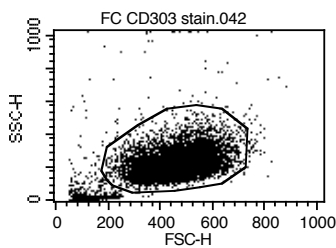
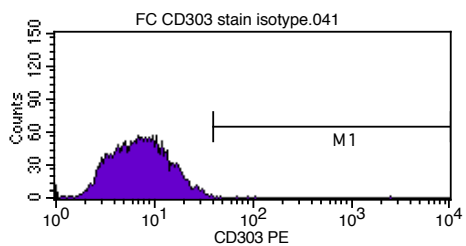
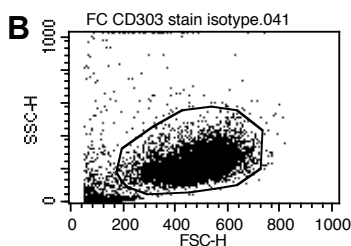
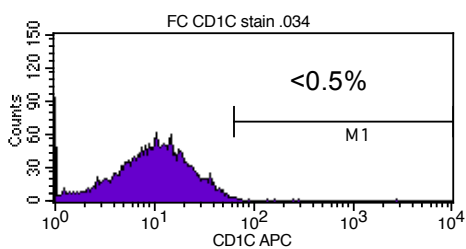
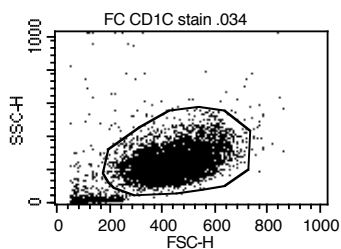
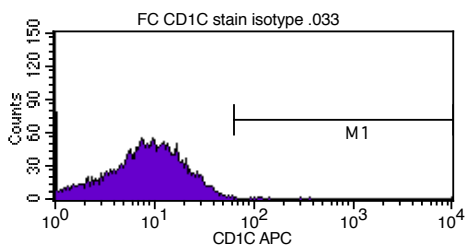
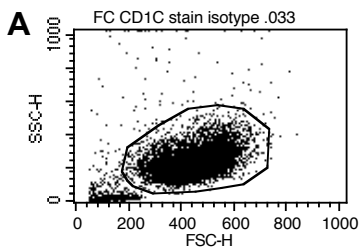




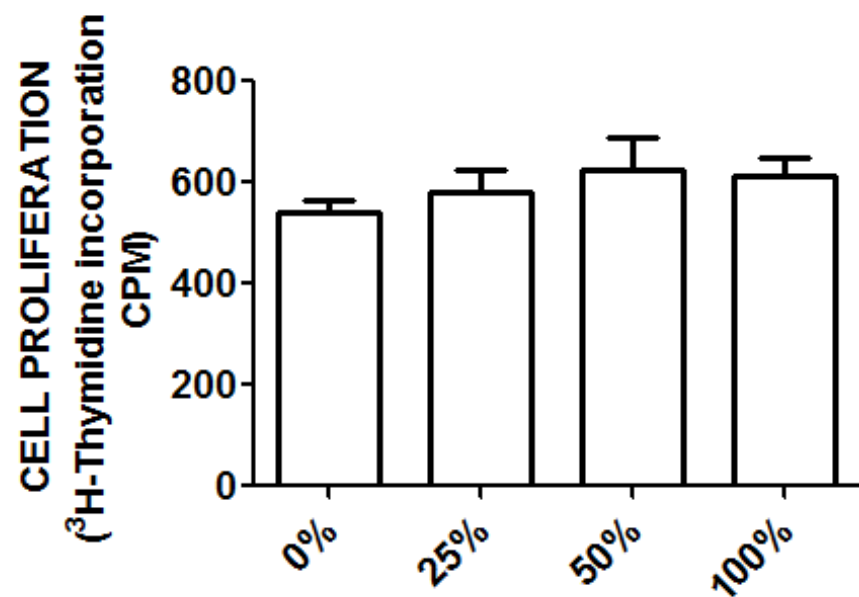




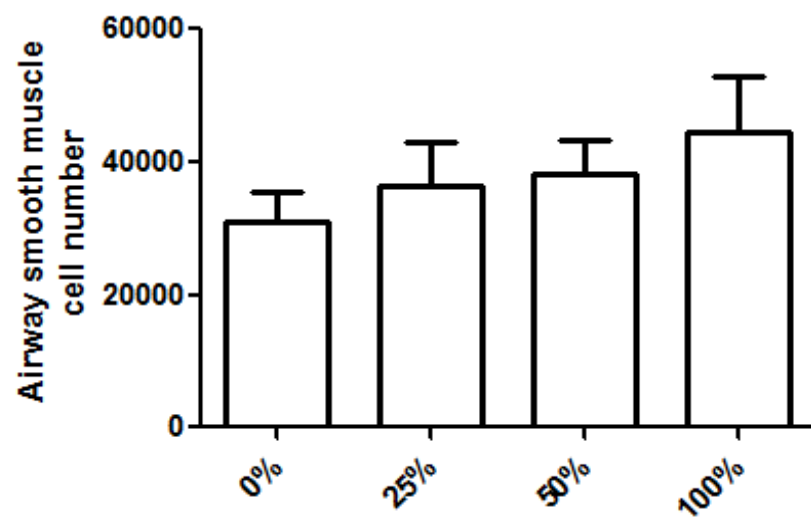


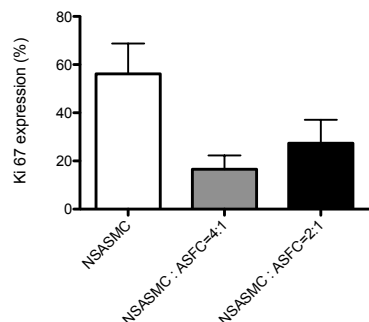
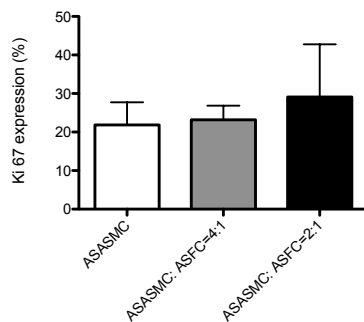
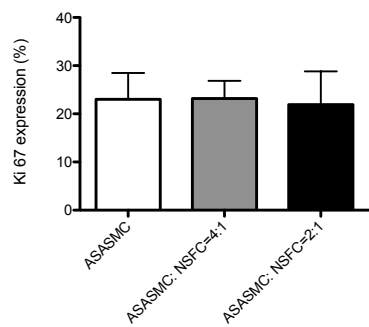
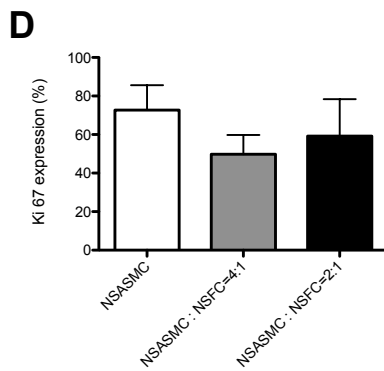
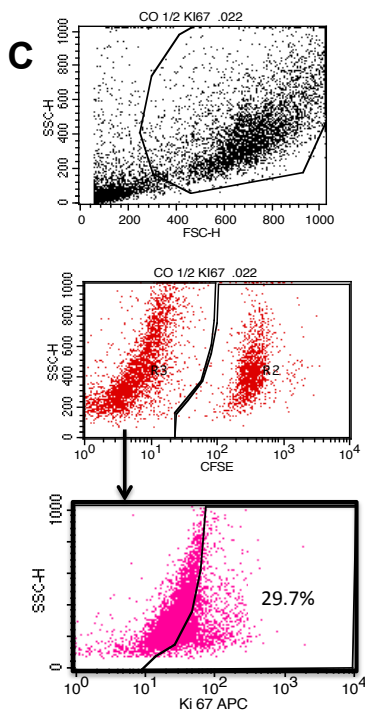
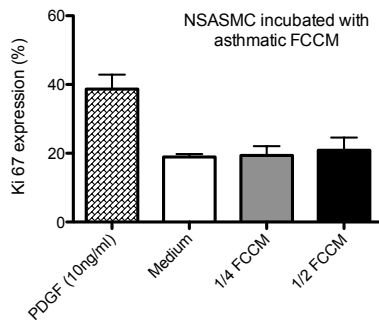
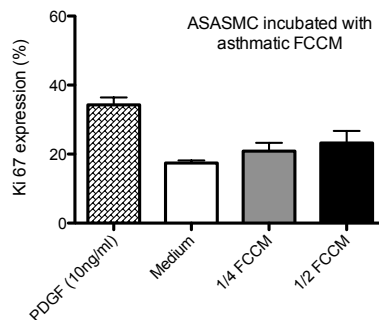
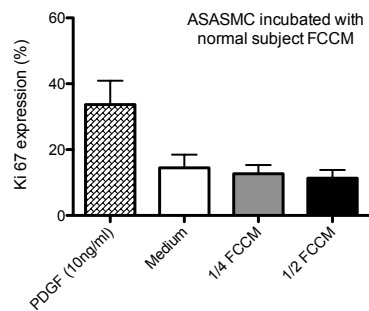
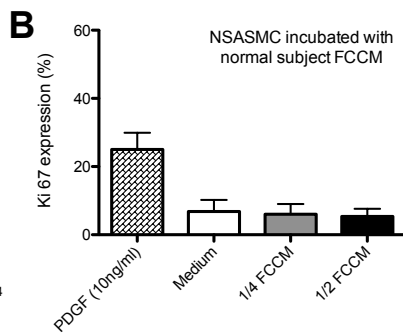
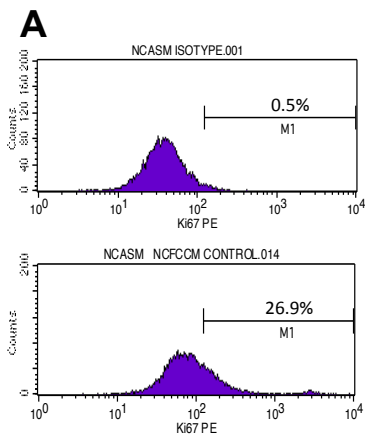


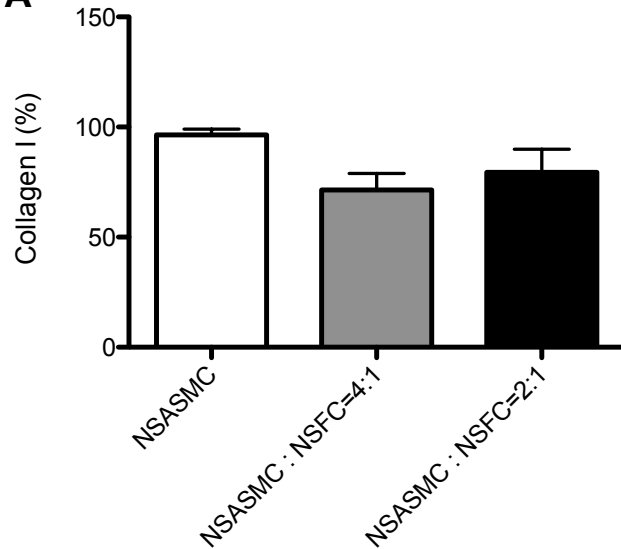
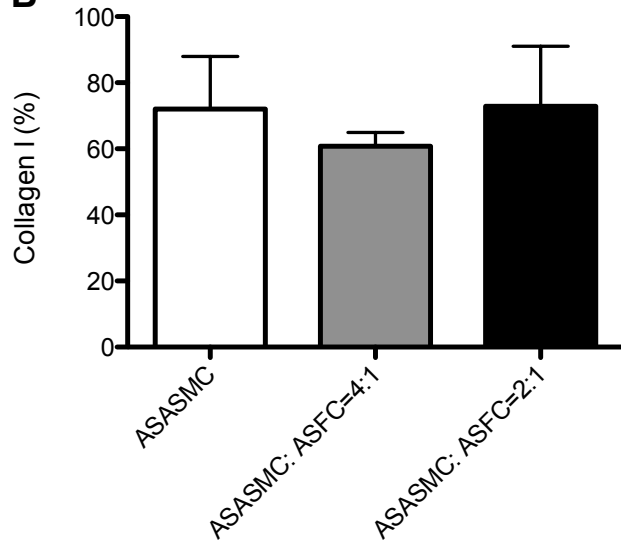
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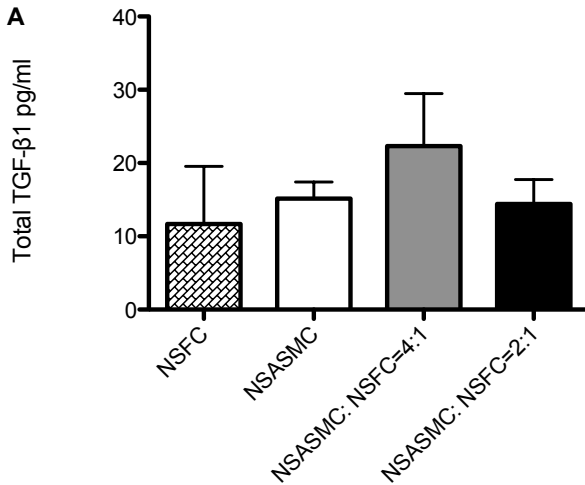
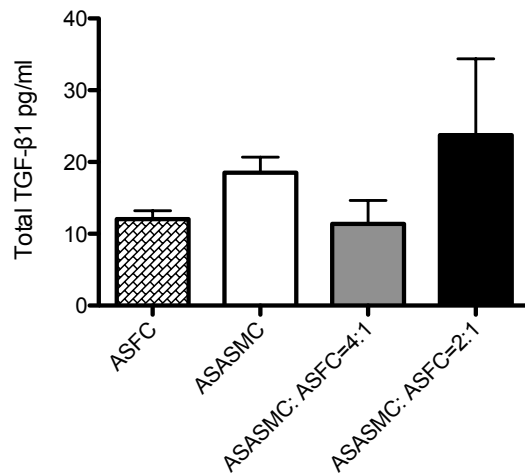
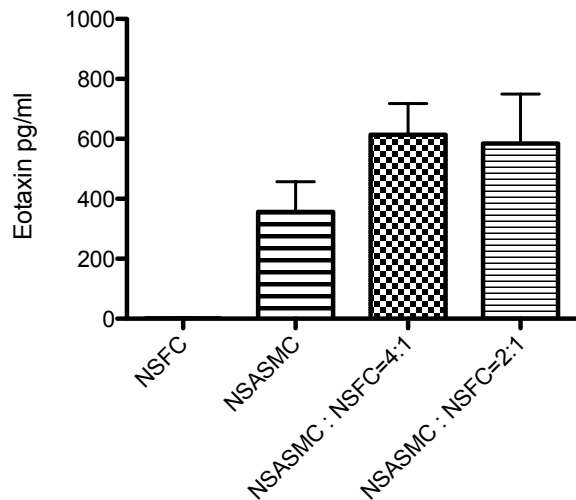
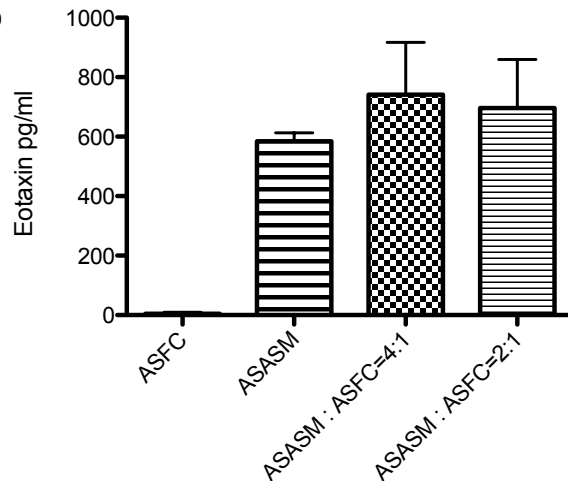


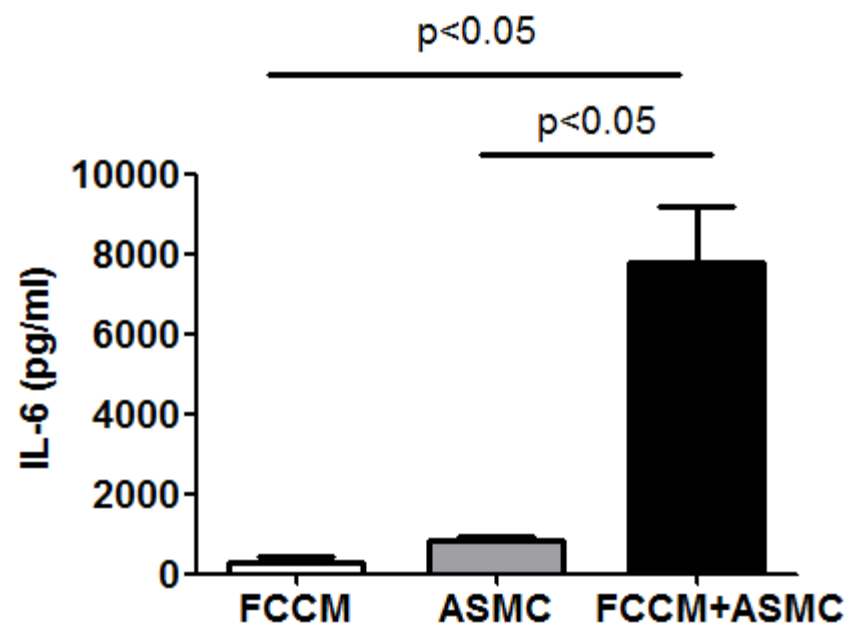
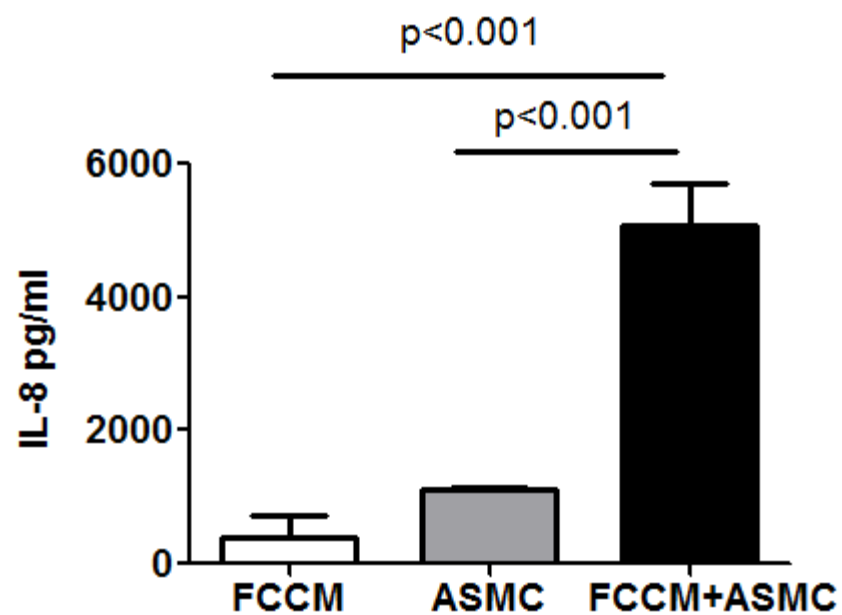
Effect of NSFCCM on NSASM C proliferation

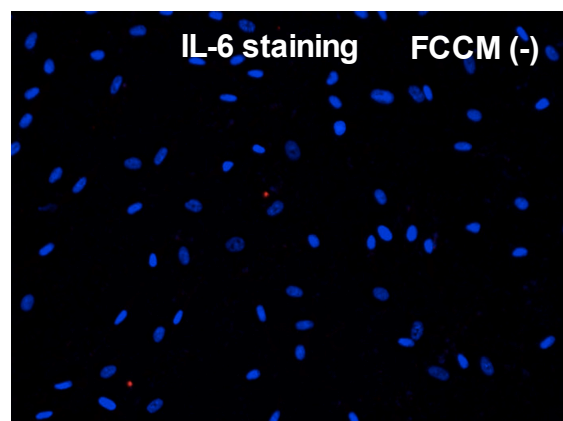
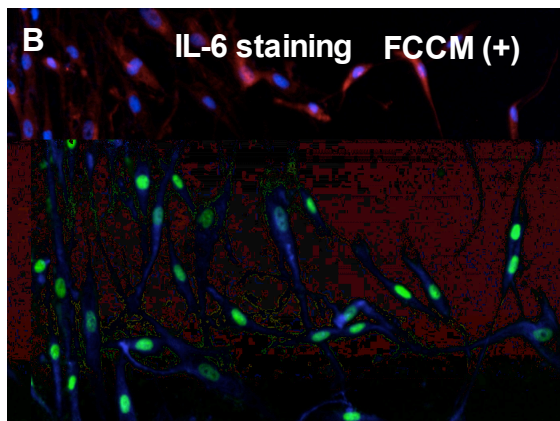
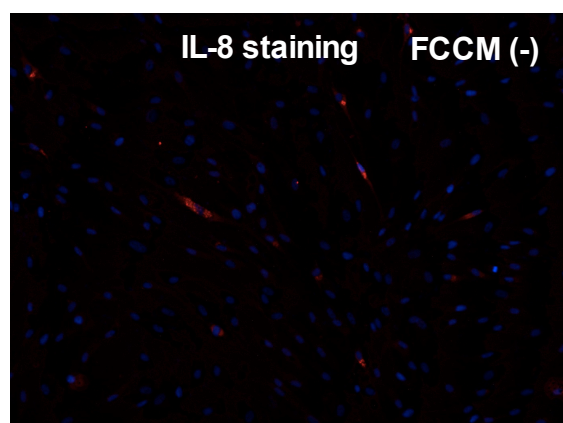
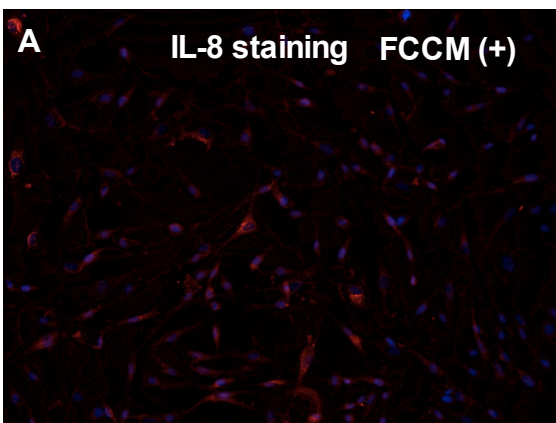


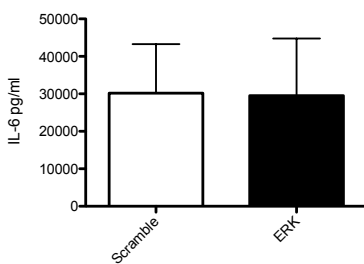
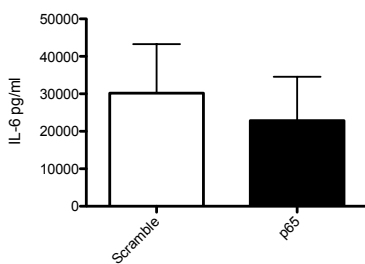
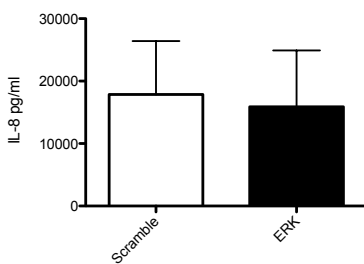
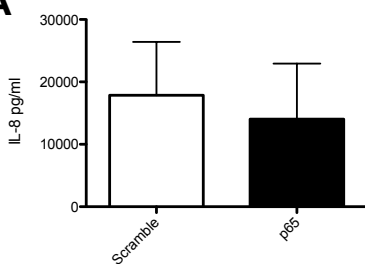
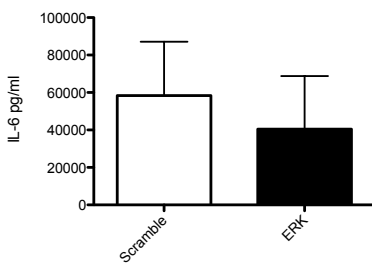
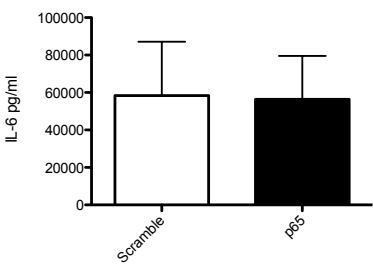
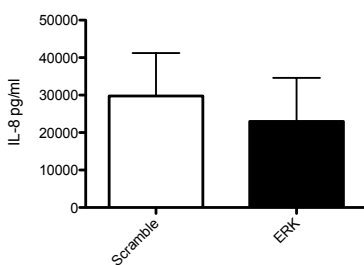
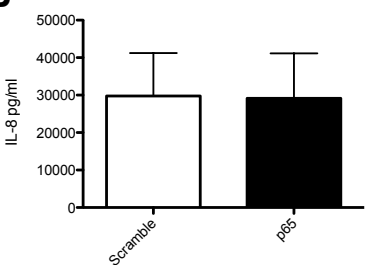
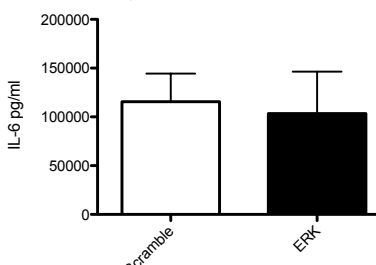
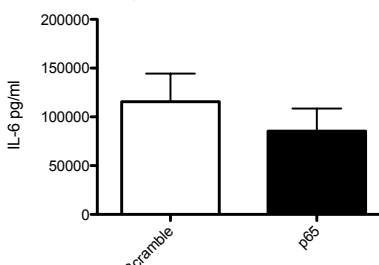
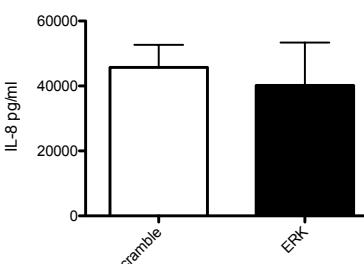
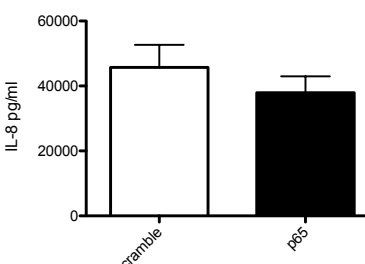


**A****B**

**A****B****C****D**





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

## **Supporting information**

### **Methods**

#### **Determination of ASMC proliferation and cell viability**

The effect of FCCM on ASMC proliferation was also evaluated by direct cell counting using Scepter 2.0 Handheld Automated Cell Counter with 40 and/or 60  $\mu\text{m}$  Scepter Sensors (EMD Millipore). Cell counts were performed according to the manufacturer's instructions, using the same cell starting suspension and identical dilutions. Cell viability was determined by the trypan blue exclusion test.

#### **Flow cytometry assessment for FC purity**

Cells in CD14<sup>+</sup> monocyte culture 10 to 14 days were fixed and permeabilized with BD Cytofix/Cytoperm™ Solution Kit (BD Biosciences, San Diego, CA) prior to staining with mouse anti-collagen-I antibody (Millipore, Temecula, CA), followed by Alexa Fluor 488 conjugated anti-mouse antibodies (Invitrogen, Burlington, ONT); Cells were then stained with Phycoerythrin (PE)-conjugated mouse anti-CXCR4 and peridinin chlorophyll protein (PerCP)–conjugated anti-CD45 antibodies (BD Biosciences, San Diego, CA). Mouse IgG1 $\kappa$ , PE-mouse IgG2 $\alpha$  and PerCP-Mouse IgG1 $\kappa$  (BD Biosciences, San Diego, CA) were used as isotype, respectively. For expression of dendritic cells markers, cells were stained with Allophycocyanin (APC)-conjugated goat anti-CD1C (R&D systems, Minneapolis, MN) , PE-conjugated mouse anti-CD303 (Miltenyi Biotec, Auburn, CA). APC-conjugated goat IgG and PE-conjugated mouse IgG1 (R&D systems, Minneapolis, MN) were used as matched isotypes, respectively. Cells were also stained with mouse anti-PM-2K (Abcam, Cambridge, MA) or mouse IgG1 isotype (R&D systems, Minneapolis, MN) followed by goat anti-mouse Alexafluor 488 antibody (Invitrogen, Burlington, ONT), respectively. Cells were analyzed using a flow cytometer BD FACSCalibur with CellQuest Pro software (BD Biosciences).

#### **Immunofluorescence staining for IL-8 and IL-6 expression in ASMC**

After overnight starvation,  $1 \times 10^4$  ASMC and  $5 \times 10^3$  FC were cultured in one well of a 8-chamber culture slide (BD Biosciences, Bedford, MA) for 48 hours. Golgistop (BD Biosciences, San Diego, CA) was added 4 hours before staining and cells were then washed with PBS and fixed with 4% paraformaldehyde and permeabilized with 0.1% Tween 20 in PBS (PBST). Cells were blocked with 1% BSA in PBST and stained with mouse anti-IL-8 (R&D systems, Minneapolis, MN) or rabbit anti-IL-6 (Abcam, Cambridge, MA) antibody followed by goat anti-mouse or donkey anti-rabbit Alexafluor 555 antibody (Invitrogen, Burlington, ONT), respectively. Diluent without primary antibodies was used as control. After washing with PBST, cell nucleus was stained with Hoechst 33342 (AnaSpec, Fremont, CA), mounted with PermaFluor (Thermo scientific, Fremont, CA). Cells were viewed with an Olympus BX-51 microscope (Olympus Optical Co., Melville, NY) equipped with reflected ultraviolet light illumination.

### **Short-interference RNA (siRNA)**

ASMC were plated in 6-well plates and grown to 70–80% confluency in medium containing 10% FBS. Cells were then transfected with siRNA targeting NF- $\kappa$ B p65 (Santa Cruz Biotechnology, Santa Cruz, CA) or ERK1/2 (Cell Signaling, Danvers, MA) using the siRNA Reagent System (Santa Cruz Biotechnology, Santa Cruz, CA). The scramble siRNA (Santa Cruz Biotechnology) was used as a negative control. After overnight starvation, ASMC were transferred to the lower well of transwell plate and cultured with FC in the upper well with cell number ratio 2:1 in starvation medium for 48 hours. Supernatant was collected for ELISA of IL-8 and IL-6, while protein lysates of ASMC were harvested to examine gene knockdown efficiency by western blotting.

### **Western blot analysis**

Assessment of knockdown efficiency: ASMC post knockdown in transwell culture with FC for 48 hours were washed twice with cold PBS and lysed. Whole-cell lysate was loaded onto a 10% acrylamide SDS-PAGE NEXT GEL (Amresco, Solon, Ohio), followed by transfer to nitrocellulose membranes

(Bio-Rad Laboratories, Hercules, Calif). The blots were then blocked for 1 hour at room temperature and incubated overnight at 4°C with antibodies specific for rabbit anti-NFκB (Santa Cruz Biotechnology) and mouse anti-GAPDH (Millipore). After washing (0.1% Tween-20/PBS), membranes were incubated with IRDye 680 goat anti-mouse IgG and IRDye 800 goat anti-rabbit IgG (Rockland, Philadelphia, PA) and analyzed with an Odyssey IR scanner by using Odyssey imaging software 3.0 (LI-COR Biosciences). Membranes were then reprobed with rabbit anti-total ERK1/2 (Cell Signaling) and IRDye 680 goat anti-rabbit IgG (Rockland, Philadelphia, Pa).

Assessment of α-smooth muscle actin (α-SMA) and myosin light chain kinase (MLCK): ASMC in transwell culture with FC for 48 hours were washed twice with cold PBS and lysed. Whole-cell lysate was loaded onto a 10% acrylamide SDS-PAGE NEXT GEL (Amresco, Solon, Ohio), followed by transfer to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, Calif). The blots were then blocked for 1 hour at room temperature and incubated overnight at 4°C with antibodies specific for rabbit anti-MLCK (Abcam) and mouse anti-α-SMA (R&D Systems, MN, USA). After washing (0.1% Tween-20/PBS), membranes were incubated with IRDye 680 goat anti-mouse IgG and IRDye 680 goat anti-rabbit IgG (Rockland, Philadelphia, Pa) and analyzed with an Odyssey IR scanner by using Odyssey imaging software 3.0 (LI-COR Biosciences). The membranes were then reprobed with mouse anti-GAPDH (Millipore) and IRDye 680 goat anti-mouse IgG (Rockland, Philadelphia, Pa).

## Supplement figure legends

**Figure S1.** Airway smooth muscle cells (ASMC) and Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE)-labeled (5  $\mu$ M) fibrocytes (FC) were cultured separately for 48 hours. CFSE expression was tested by flow cytometry. Histograms of CFSE expression of ASMC (upper panel) and CFSE-labeled FC (lower panel) revealed no overlap of ASMC and FC by CFSE expression.

**Figure S2.** Purified FC do not express markers of myeloid dendritic cells (CD1C), plasmacytoid dendritic cells (CD303) or macrophage (PM-2K). Purified FC after culture 10 to 14 days were assessed the expression of (A) CD1C and (B) CD303 (C) PM-2K by flow cytometry. Histograms of matched isotypes (upper panels) and CD1C/CD303/PM-2K staining FC (lower panels) revealed no expression of these markers in FC.

**Figure S3.** Thymidine incorporation and cell number of ASMC treated with Fibrocytes (FC) conditioned medium (FCCM). ASMC were treated for 48 hours with FCCM at various concentration (0, 25, 50 or 100%). 24 hours prior harvesting onto glass fiber filters (Wallac), cells were labeled with 5  $\mu$ Ci/well of  $^3$ H-methyl thymidine and thymidine incorporation was then quantified. ASMC were also detached and cell number was counted.

**Figure S4.** Fibrocytes (FC) conditioned medium (FCCM) or co-culture with FC did not affect Ki-67 expression of airway smooth muscle cells (ASMC). (A) and (B): FC were starved for 48 hours and FCCM was harvested and mixed with starvation medium in the ratio of 1:4 or 1:2. PDGF treatment (10 ng/ml) was used as positive control. (A) Example of Ki-67 expression in ASMC post-PDGF treatment assessed by flow cytometry. ASMC stained with isotype (upper histogram) and anti-Ki-67-PE (lower histogram). Positive expression of Ki-67 was defined by isotype. (B) Ki-67 expression in ASMC incubated with

FCCM from asthmatic or normal subjects (n=3~5, each experiment had equal subject numbers between groups). (C) and (D): Ki-67 expression in ASMC after 48 hours co-culture with CFSE-labeled FC in the ratio of cell number 2:1 (ASMC:FC), 4:1 or ASMC alone at the same cell density in the lower well. (C) ASMC and CFSE-labeled FC were distinguished by CFSE expression. Ki-67 expression in ASMC was analyzed from CFSE negative population. (D) Ki-67 expression in ASMC alone or ASMC co-cultured with FC from asthmatic or normal subjects. AS: asthmatic; NS: normal subject (n=3).

**Figure S5.** Collagen-I expression of airway smooth muscle cells (ASMC) after 48 hours co-culture with fibrocytes (FC) in the ratio of cell number 2:1 (ASMC:FC), 4:1 or ASMC alone. Collagen-I of ASMC was tested by flow cytometry. (A) Co-culture of NSASMC+NSFC. (B) Co-culture of ASASMC+ASFC. AS: asthmatic; NS: normal subject. (n=3)

**Figure S6.** Levels of total TGF- $\beta$  and eotaxin in the supernatant from fibrocytes (FC), airway smooth muscle cells (ASMC), co-culture of ASMC and FC in the ratio of cell number 4:1 (ASMC:FC) and 2:1. Cells were cultured at a density of  $4.68 \times 10^4/\text{cm}^2$  for 48 hours and cytokines levels of supernatant were detected by ELISA. (A and B) Level of total TGF- $\beta$  and eotaxin (C and D) in the co-culture of NSASMC+NSFC and ASASMC+ASFC. Level of active TGF-beta in all experiments was below the detection limit of ELISA kit. AS: asthmatic; NS: normal subject. (n=3).

**Figure S7.** Effect of fibrocyte conditioned medium (FCCM) on IL-8 and IL-6 production by airway smooth muscle cells (ASMC). ASMC were treated with conditioned medium collected from fibrocytes (FCCM) or starving medium and ELISA of IL-8 and IL-6 of FCCM or in the supernatant was performed after 48 hours culture with starving medium (ASMC) or FCCM (FCCM+ASMC)(n=3).

**Figure S8.** ASMC produce IL-8 and IL-6 after treatment of fibrocytes conditioned medium (FCCM). FC were starved for 48 hours and FCCM harvested. ASMC were cultured in the chamber slide with FCCM or starving medium for 48 hours. Production of IL-8 and IL-6 of ASMC was assessed by immunofluorescence staining. (A) Anti-human IL-8 and (B) IL-6 antibody followed by 2<sup>nd</sup> antibody of Alexa Fluor 555 (red) and Hoechst 33342 for nuclei (blue) were stained on ASMC that were treated with FCCM (FCCM+) or medium only (FCCM-). Composite images revealed higher expression of IL-8 and IL-6 on ASMC treated with FCCM than ASMC in medium only.

**Figure S9.** Levels of IL-8 and IL-6 in the supernatant from transwell of (A) NSASMC+NSFC, (B) ASASMC+ASFC and (C) NSASMC+ASFC. P65 and ERK1/2 of ASMC were knocked down by siRNA before transwell experiment. ASMC in lower well and fibrocytes (FC) in upper well were cultured in the ratio of cell number 2:1. IL-8 and IL-6 levels of supernatant after 48 hours transwell culture were detected by ELISA. \* $<0.05$  by unpaired t-test. (n=3~5)