

Lung Homing of Endothelial Progenitor Cells in Humans with Asthma after Allergen Challenge

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Rationale: Increased bronchial vascularity is a feature of asthma that can contribute to airflow obstruction and progressive decline in lung function. Angiogenesis is associated with the lung homing and *in situ* differentiation of endothelial progenitor cells (EPC) in mouse models of asthma. We have previously shown that inhibiting allergen (Ag)-induced recruitment of EPC in sensitized mice attenuated increased bronchial vascularity and development of airway hyperresponsiveness. **Objectives:** We investigated the accumulation of EPC and formation of new blood vessels in the lungs of human subjects with asthma after Ag inhalation challenge.

Methods: Consenting patients with mild atopic asthma ($n = 13$) with $FEV_1 \geq 70\%$, methacholine $PC_{20} \leq 16$ mg/ml, and a dual response to Ag were recruited. Sputum levels of EPC were determined by multigating flow cytometry, and lung vascularity was enumerated by immunostaining with von Willebrand factor.

Measurements and Main Results: Sputum levels of EPC were determined by multigating flow cytometry and lung vascularity was enumerated by immunostaining with von Willebrand factor. There was a significant increase in sputum EPC levels 24 hours post Ag but not diluent challenge. Similarly, a significant increase in the number and diameter of blood vessels in lung biopsy tissue 24 hours post Ag was observed. *In vitro* culture of EPC demonstrated the capacity of these cells to differentiate into mature endothelial cells and form tubelike vessel structures. In sputum supernatants, there was a significant increase in CXCR2 agonists, IL-8, and Gro- α 24 hours post Ag. Only Gro- α stimulated a significant EPC migrational response *in vitro*. **Conclusions:** Our data suggest that increased lung homing of EPC may promote bronchial vascularity in allergic asthmatic responses and that the recruitment of these progenitors maybe orchestrated by CXCR2 chemokines.

Keywords: endothelial progenitor cells; angiogenesis; asthma remodeling

Tissue remodeling and persistent inflammation are key features of the airways in asthma (1, 2). The functional sequelae of airway remodeling in patients with asthma are the development of non-specific airway hyperresponsiveness (AHR), accelerated decline in lung function, and loss of post-bronchodilator response to β_2 -adrenoreceptor agonists (3). Airway wall thickening has been

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Increased levels of endothelial progenitor cells (EPC) have been detected in the blood of patients with asthma showing increased clonogenic potential compared with normal subjects. It remains unclear as to whether increases in EPC occur within the lungs of subjects with asthma post-allergen challenge and how this relates to changes in lung vascularity and airway function.

What This Study Adds to the Field

The novel findings of this study are that there is a significant influx of EPC to the airways in response to allergen but not diluent inhalation challenge in dual-responder patients with asthma. Associated with this was an increase in the level of vascularization of lung tissue detected post-allergen challenge in dual-responder patients with asthma. That EPC can contribute to increase lung vascularity is supported by the angiogenic potential of these cells in culture and the expression of receptors reminiscent of mature endothelial cells. Furthermore, generation of CXCR2 chemokines may stimulate the lung homing of these cells in asthmatic responses.

positively correlated with clinical asthma severity and AHR and correlates inversely with airflow obstruction (4).

Airway wall neovascularization, seen as increases in both the size and number of bronchial blood vessels, is a prominent feature of fatal and nonfatal asthma (5–7) and has been shown to correlate with reticular basement membrane thickening (8), extent of airway obstruction (7, 9), and disease severity (4, 10–12). Capillary engorgement, leakage, and vasodilatation can directly increase airway wall thickness, increase airway luminal narrowing, and facilitate inflammatory cell trafficking and are believed to account for the decrease in forced expiratory flow rates in asthmatic responses (13, 14). Thus, increases in subepithelial vascularity are important remodeling events in airway narrowing and airflow obstruction in asthma.

It has been proposed that postnatal angiogenesis, a complex process whereby new blood vessels sprout from extant microvasculature, can arise either from the proliferation of resident mature vascular endothelial cells and/or as a result of the lung homing of endothelial progenitor cells (EPC) from the bone marrow (BM) (15). Although the exact role of EPC remains unclear, recent reports point to progenitors as either having the potential to produce growth factors that stimulate local angiogenesis in a paracrine fashion or incorporating into existing microvessels and acting as building blocks to form new vasculature (10). Increased mobilization of EPC has been detected in several inflammatory lung conditions, including patients with bacterial pneumonia, acute lung injury, and atopic asthma (16–18). In patients with

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asthma, increased numbers of EPC with increased proliferative potential compared with normal asymptomatic subjects has been reported (19). The lung homing of EPC in an allergen (Ag)-induced human asthmatic response and the correlation with angiogenesis and airway function have not been investigated to date, and thus the role of these cells in disease pathology remains unresolved.

The novel findings of this study are that there is a significant influx of EPC to the airways in response to Ag but not diluent inhalation challenge in dual-responder patients with asthma. Associated with this was an increase in the level of vascularization of lung tissue detected post-Ag challenge in dual-responder patients with asthma. That EPC can contribute to increase lung vascularity is supported by the angiogenic potential of these cells in culture and the expression of receptors reminiscent of mature endothelial cells. Furthermore, generation of CXCR2 chemokines may stimulate the lung homing of these cells in asthmatic responses.

METHODS

Subjects

Nonsmoking subjects with mild stable asthma ($n = 13$ for EPC and $n = 10$ for endobronchial biopsy sampling) were recruited. Subjects (aged 19–42 yr) were skin-prick test positive, had FEV₁ greater than or equal to 70% predicted, and had baseline provocative concentration of methacholine causing a 20% drop in FEV₁ (PC₂₀) less than or equal to 16 mg/ml. Subjects were steroid naive, infrequently used inhaled β_2 -agonist, and provided informed written consent, and the study was approved by the local ethics committees.

Study Design

Allergen/diluent (Ag/dil) challenges were randomized with a 2-week washout period. Subjects attended the clinic: At visits 1 and 4, medical history, skin-prick test (visit 1), spirometry, methacholine inhalation, and sputum induction were performed; at visits 2 and 5, Ag/dil inhalation challenge and sputum induction (7 h post-challenge) were performed; at visits 3 and 6, methacholine inhalation and sputum induction were performed. Endobronchial biopsies were sampled at pre- and 24 hours post-Ag challenge.

Methacholine Inhalation

Methacholine inhalation challenge was performed as previously described (20).

Ag Inhalation Challenge

Asthma was required to be stable (FEV₁ within 10% of baseline) to proceed with the Ag challenge. Ag inhalation was performed as previously described (21, 22). Subject characteristics are summarized in Tables 1 and 2.

Sputum Induction and Cell Isolation

Sputum was induced and processed as previously described (23). (See online supplement for details.) Supernatants were separated by centrifugation and stored at -70°C until required for ELISA.

Immunofluorescence Staining and Flow Cytometric Enumeration of EPC in Sputum Samples

Sputum-extracted cells were simultaneously immunostained with CD34-phycoerythrin, CD133-peridinin chlorophyll protein, and VEGFR2-allophycocyanin (see online supplement for details).

Endobronchial Bronchoscopy

Endobronchial biopsies were sampled as previously described in detail (24), and tissue processing was performed as previously described (25).

Immunohistochemistry of Factor VIII in Bronchial Biopsies

Lung vascularity was identified in 6- μm sections of human endobronchial biopsies prefixed in paraformaldehyde and stained with a polyclonal rabbit anti-human von Willebrand factor (see online supplement for details).

Endothelial Progenitor Colony Assays

Mononuclear cells isolated from blood of patients with asthma drawn by venipuncture into ethylenediaminetetraacetic acid were seeded in 12-well fibronectin-coated plates and cultured for 21 days (see online supplement for details). Late outgrowth colonies termed endothelial colony-forming cells (ECFC) were harvested, and expression of stem cell and endothelial cell lineage markers was confirmed by immunostaining.

Angiogenesis Assay

The angiogenic potential of EPC was assessed in an *in vitro* angiogenesis assay (see online supplement).

Chemokine and Cytokine ELISA Assays

The levels of vascular endothelial growth factor (VEGF), IL-8, Gro- α , and eotaxin, hepatocyte growth factor, fibroblast growth factor and stromal cell derived factor (SDF)-1 were measured using human cytokine/chemokine MILLIPLEX MAP kit (Millipore, Mississauga, ON, Canada). (See online supplement.)

Progenitor Cell Transmigration Assays

The migrational response of progenitors was assessed using Transwell chambers as previously described (26) (see online supplement).

Statistical Analysis

Results were expressed as mean $\pm$ SEM. The Ag-induced changes from baseline in total sputum cell numbers, sputum differential numbers, CD34⁺ cell numbers, CD34⁺133⁺VEGFR2⁺ cell numbers, and cytokine protein levels were analyzed using a repeated measures analysis of variance (see online supplement for details). The number and size of von Willebrand factor-positive blood vessels were analyzed using a Wilcoxon signed rank test. Alpha was set at 0.05. (see online supplement for details).

RESULTS

Sputum Cells (Total and Differential Cells)

After inhaled Ag challenge of dual-responder patients with asthma, there was a significant increase in the total cell count from induced sputum when pre-Ag and 7-hour post-Ag levels were compared with 24-hour post-Ag levels (pre-Ag, 3.16 ± 0.47 ; 7-h post-Ag, $4.49 \pm 0.79 \times 10^6/\text{ml}$ vs. 24-h post-Ag, $7.62 \pm 1.73 \times 10^6/\text{ml}$; $P < 0.05$). Differential cell counts showed an increase in only eosinophils when pre-Ag levels were compared with 7-hour post-Ag (0.062 ± 0.022 vs. $0.64 \pm 0.20 \times 10^6/\text{ml}$, $P < 0.05$) and between 7-hour and 24-hour post-Ag ($1.12 \pm 0.33 \times 10^6/\text{ml}$, $P < 0.05$) (Figure 1). Significantly greater numbers of total cells and eosinophils were detected in sputum samples taken 24 hours post-Ag compared with diluent challenge (7.62 ± 1.73 and 3.18 ± 0.80 vs. 1.12 ± 0.33 and $0.06 \pm 0.01 \times 10^6/\text{ml}$, respectively; $P < 0.05$). At 7 hours post-challenge, only eosinophils were increased in the Ag compared with diluent challenge group (0.64 ± 0.20 vs. $0.04 \pm 0.13 \times 10^6/\text{ml}$, $P < 0.05$) (Figure 1).

Sputum Progenitor Cells: Ag-Induced Lung Homing

Progenitor cell assessments in sputum were performed by sequential multigating flow cytometric analyses (Figure 2). In response to Ag, but not diluent, inhalation challenge there was a significant increase in number of CD34⁺ progenitors in the

TABLE 1. SUBJECT CHARACTERISTICS

Patient No.	Sex	Age (yr)	FEV ₁ (% predicted)	Allergen	Baseline PC ₂₀	24-h PC ₂₀	EAR (% decrease in FEV ₁)	LAR (% decrease in FEV ₁)
1	F	20	74.1	Cat	8.3	4.72	23.66	16.03
2	F	42	84.4	Cat	0.17	0.11	41.28	17.02
3	F	28	95.2	HDMDP	3.41	0.79	41.67	19.87
4	M	30	92.8	Alternaria	2.23	1.52	24.57	16.9
5	M	19	77.8	HDMDP	1.19	0.44	28.33	45.83
6	M	19	98.3	Grass	16	6.63	26.19	19.05
7	F	21	98.1	Cat	4.96	1.31	24.64	24.64
8	M	33	90.4	HDMDP	11.02	1.34	43.75	18.75
9	M	19	93	RGW	2.89	0.62	40.48	23.81
10	M	21	71.6	Cat	1.86	0.61	30.19	33.44
11	F	21	86.1	HDMDP	11.95	1.19	49.09	24.36
12	M	28	109.7	HDMDP	7.94	7.66	30.87	30.43
13	F	24	92.2	HDMDP	11.62	0.44	53.57	25

Definition of abbreviations: EAR = early airway response; HDMDP = house dust mite *Dermatophagoides farinae*; HDMDP = house dust mite *Dermatophagoides pteronyssinus*; LAR = late airway response; PC₂₀ = provocative concentration of methacholine causing a 20% drop in FEV₁; RGW = ragweed.

sputum when baseline levels were compared with levels 24 hours post-challenge (10.98 ± 2.88 vs. $38.24 \pm 12.85 \times 10^4/\text{ml}$, $P < 0.05$) (Figure 3). In addition, there were significantly more CD34⁺ cells in the Ag- compared with diluent-challenge group at 24 hours post-challenge (38.24 ± 12.85 vs. $8.47 \pm 2.05 \times 10^4/\text{ml}$, $P < 0.05$).

Similarly, there was a significant increase in the absolute number of EPC identified as CD34⁺133⁺VEGFR2⁺ cells in induced sputum when baseline and 7-hour post-Ag levels were compared with 24-hour post-Ag levels (1.59 ± 0.38 and 2.25 ± 0.63 vs. $6.57 \pm 2.57 \times 10^2/\text{ml}$, $P < 0.05$) (Figure 3). In addition, there were significantly more sputum EPC in the Ag- compared with diluent-challenge group at 24 hours post-Ag (6.57 ± 2.57 vs. $1.35 \pm 0.49 \times 10^2/\text{ml}$, $P < 0.05$). Further analyses of our data showed that there were no significant correlations between either 24-h post-Ag total progenitor cell levels (CD34⁺ cells) or EPC levels (CD34⁺45⁺VEGFR2⁺ cells) in the sputum and PC₂₀ dose of methacholine after Ag.

Immunohistochemistry of Factor VIII in Bronchial Mucosa

Compared with pre-Ag, there was a significant increase in the number of factor VIII+ blood vessels in endobronchial biopsies from patients with asthma when pre-Ag was compared with 24-hour post-Ag challenge (11.8 ± 1.4 vs. 17.2 ± 1.4 vessels/mm²; $n = 10$; $P < 0.05$) (Figure 4). Similarly, there was a significant increase in the diameter of factor VIII+ blood vessels when prechallenge biopsy tissue was compared with 24-hour post-Ag tissue (2.4 ± 0.37 vs. $5.6 \pm 0.57 \mu\text{m}$, $P < 0.05$) (Figure 4).

Confirmation of Clonogenic Activity and Angiogenic Potential of Blood EPC

In semisolid culture medium, we identified the potential of blood cells to form late outgrowth colonies (Figure 5). 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine acetylated low density lipoprotein uptake and lectin-fluorescein isothiocyanate binding confirmed that the ECFC expressed both stem cell makers and endothelial lineage markers (Figure 5). In addition, as determined by flow cytometry, ECFC expressed progenitor cell receptors CD34, CD133, and VEGFR2 but not CD45⁺, a pan-leukocyte/hemopoietic cell marker, or CD14⁺ monocyte cell marker. Also, the ECFC expressed CD31, CD144 (VE-cadherin), CD146 (MUC18), and CD105 (endoglin), confirming EC lineage commitment (Figure 6).

Angiogenesis Assay

When seeded in an angiogenic matrix, ECFC formed tubelike structures over a period of 8 to 14 hours. This confirmed the angiogenic potential of blood-extracted EPC from subjects with asthma (Figure 7).

Chemokines in Sputum Supernatants

Cytokine/chemokine levels in sputum supernatants were assessed by Luminex Technology (Table 3). When pre-Ag was compared with 24-hour post-Ag levels, a significant increase in IL-8 (306.4 ± 51.8 vs. $680.2 \pm 160.1 \text{ pg/ml}$, $P < 0.05$) was found. Similarly, a significant increase in Gro- α was detected as early as 7 hours post-Ag, maintained at 24 hours post-Ag ($387.8 \pm$

TABLE 2. SUBJECT LUNG FUNCTION DETAILS

	Allergen Challenge	Diluent Challenge
Subjects, No.	13	8
Age, yr	25.0 (1.9)	27.3 (2.8)
Sex, M/F	7/6	4/4
Early-phase % fall in FEV ₁	-35.25 (2.81)*	-4.51 (1.04)
Late-phase % fall in FEV ₁	-24.24 (2.32)*	-4.49 (1.55)
Methacholine PC ₂₀ pre-challenge, mg/ml	4.05 (1.42)	4.45 (1.43)
Methacholine PC ₂₀ 24 h Post-challenge, mg/ml	1.12 (1.39)*	6.69 (1.54)

Definition of abbreviations: PC₂₀ = provocative concentration of methacholine causing a 20% drop in FEV₁.

Values are presented as means $\pm$ SEM except for PC₂₀ values, which are geometric means with % SEM in parentheses. There was a significant difference for both the early- and late-phase % decrease in FEV₁ and methacholine log PC₂₀ post-challenge when allergen versus diluent challenge values were compared.

* $P < 0.05$ for between-group comparisons.

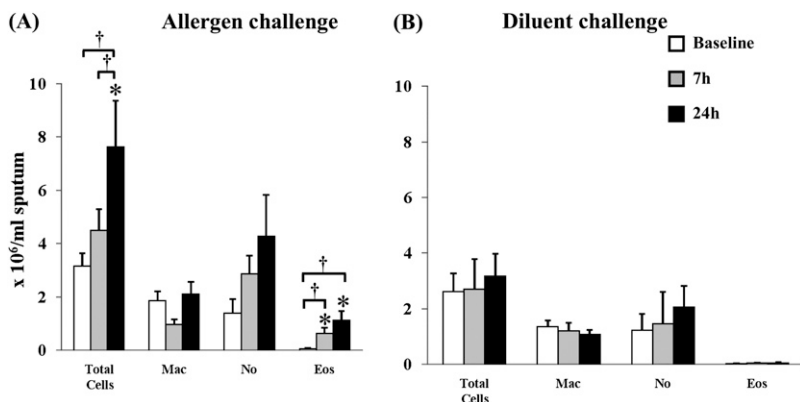


Figure 1. Total sputum differential cell counts from patients with asthma after allergen ($n = 13$) and diluent inhalation challenge ($n = 8$). Compared with diluent challenge, in which no significant change in any cell population was observed, a significant increase in total cells and eosinophils (Eos) was detected at 7 hours and 24 hours post-allergen challenge. Data are presented as mean $\pm$ SEM, ($^{\dagger}P < 0.05$ for within-group comparisons to baseline levels and $^*P < 0.05$ for between-group comparisons). Mac = macrophages; No = neutrophils.

95.7 vs. 956.1 ± 279.1 and 942.0 ± 262.1 pg/ml, $P < 0.05$). Levels of SDF-1 in all sputum supernatants were found to be below the lower limit of detection by the ELISA assay.

Migrational Responses of Progenitor Cells

We found no migrational activity for progenitor cells in sputum supernatants taken pre- and 24 hours post-Ag (data not shown). However, we then assessed the migrational response of progenitors extracted from patients with atopic asthma to the recombinant chemokines at levels that were similar to those detected in the sputum supernatants. Compared with SDF-1, which was used as a positive control, significant migrational response of EPC was elicited by Gro- α (0.5 ng/ml) at concentrations that were comparable with those detected within the sputum supernatants post-Ag challenge (Figure 8).

DISCUSSION

A considerable body of evidence shows that activation of hemopoietic events within the BM and increased accumulation of

hemopoietic progenitor cells in the lung is associated with the development of proinflammatory responses in the airways in allergic asthma. Understanding the role of BM-derived progenitors in tissue remodeling changes within the asthmatic lung still remains to be defined. In recent studies in which the traffic of vascular endothelial progenitor cells has been investigated in a mouse model of asthma, we and others have shown a direct correlation between the rapid influx of EPC to the lungs and the development of methacholine AHR as well as the level of microvessel density in the lungs (19). To support a direct role of BM-derived EPC in Ag-induced airway responses, we showed that inhibition of the Ag-induced lung homing of EPC attenuated lung angiogenesis, airway eosinophilia, and increased methacholine airway responsiveness (27). In this study, mice were pretreated intranasally during the Ag sensitization period with a receptor blocking antibody (AMD3100; CXCR4 blocker) to a pan-progenitor cell chemoattractant, SDF-1. This drug not only prevented the Ag-induced lung homing of EPC but also attenuated the associated inflammatory and lung function changes as well as lung angiogenesis. In human studies,

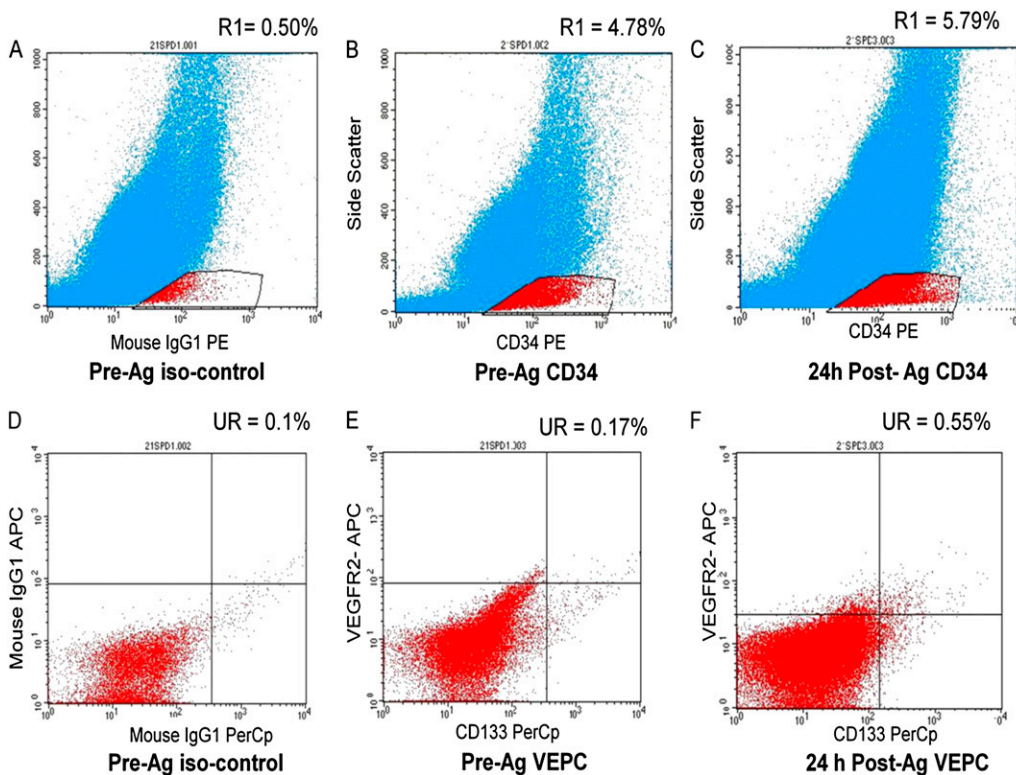


Figure 2. Flow cytometric enumeration of vascular endothelial cells in sputum. Endothelial progenitor cells ($CD34^{+}133^{+}VEGFR2^{+}$ cells) were enumerated by sequential multigating analyses using the Cell Quest Program on FACSCalibur. (A) Compared with isotype control, a gate was drawn on $SSC^{low}CD34-PE^{hi}$ region with a 99.5% confidence limit (B) pre-allergen (Ag) challenge compared with (C) 24 hours post-Ag challenge. (D) Region R1 was then back-gated on a dot-plot of PerCp versus APC to calculate $CD133-PerCp+VEGFR2-APC^{+}$ specifically stained events (E) pre- versus (F) post-Ag challenge. APC = allophycocyanin; VEPC = vascular endothelial progenitor cells.

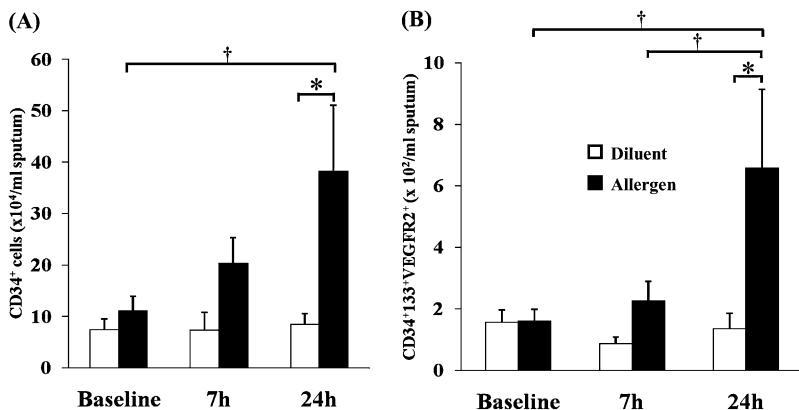


Figure 3. Fluctuations in sputum (A) total progenitor cells (CD34⁺ cells) and (B) endothelial progenitor cells (EPC) (CD34⁺133⁺VEGFR2⁺ cells) after diluent (open bars) and Ag (solid bars) inhalation challenge. In sputum, CD34⁺ cells and EPC significantly increased at 24 hours post-allergen inhalation compared with pre-allergen inhalation. Data are presented as mean $\pm$ SEM, ($^{\dagger}P < 0.05$ for within-group comparisons to baseline levels and $^*P < 0.05$ for between-group comparisons).

several cross-sectional studies have reported increased levels of vascularization of lung tissue from subjects with asthma and correlated this with disease severity (6, 7, 9–11). In addition, treatment with steroid for 6 months showed that there was a reversal in the level of vascularization, which was associated with an improvement in airway responsiveness (20). The kinetics of the angiogenic response have not been studied in human patients with asthma, particularly in response to controlled Ag inhalation challenge. In the current study, we report for the first time that there is a rapid and Ag-specific accumulation of EPC in the airways of patients with asthma. In addition, associated with these changes we show for the first time that there is an increase in the number and size of the blood vessels in endobronchial biopsies taken from dual-responder patients with asthma pre- compared with 24 hours post-Ag inhalation challenge. A significant increase in levels of Gro- α in sputum supernatants post-Ag and the significant migrational response of EPC by this chemokine highlights a potential critical role that CXCR2 agonists may play in the lung homing of EPC and angiogenic responses in human asthma.

The striking correlation between EPC levels and level of tissue vascularization in studies using animal tumor models,

corneal neovascularization assays, and matrigel plug perfusion assay (28, 29) provide confidence that assessment of EPC levels serves as a valid biomarker for ongoing angiogenesis *in vivo*. Despite this, the identification criteria for EPC remain contentious, particularly in light of unsuccessful clinical trials in which EPC for adoptive transfer were enumerated solely on the basis of either surface receptor expression or cell culture (30–32). The general current consensus is that a combination of these methods provides the best estimate of EPC. In support of these criteria, we reproduced in the current study the late outgrowth colonies assay from mononuclear cell cultures, which at Day 21 had the capacity to form tubelike structure in angiogenesis assays and express the markers unique to mature vascular endothelium (30, 33). Although we did not enumerate changes in ECFC post-Ag challenge, the current data support the identification of the CD34⁺133⁺VEGFR2⁺ cells as precursor cells for vascular endothelium based on the coexpression of endothelial surface markers shown by flow cytometry and angiogenic potential *in vitro*.

Angiogenesis is proposed to be controlled by the relative balance between proangiogenic and antiangiogenic factors within the local milieu of the tissue (10). In this regard, significant

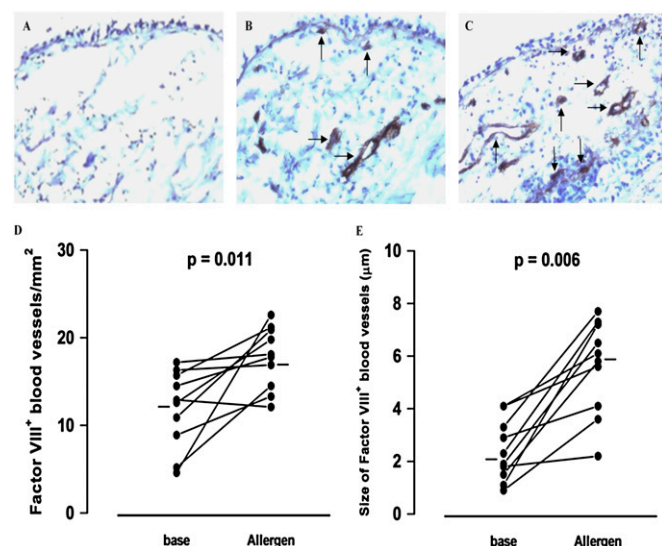


Figure 4. Immunohistochemistry (IHC) of factor VIII in bronchial mucosa. (A–C) show IHC with antibody control (A) and immunostaining of factor VIII⁺ blood vessels (arrows) in sections of bronchial biopsies from a patient with asthma (B) before and (C) 24 hours after allergen inhalation challenge. (D) Numbers and (E) sizes of factor VIII⁺ blood vessels are presented.

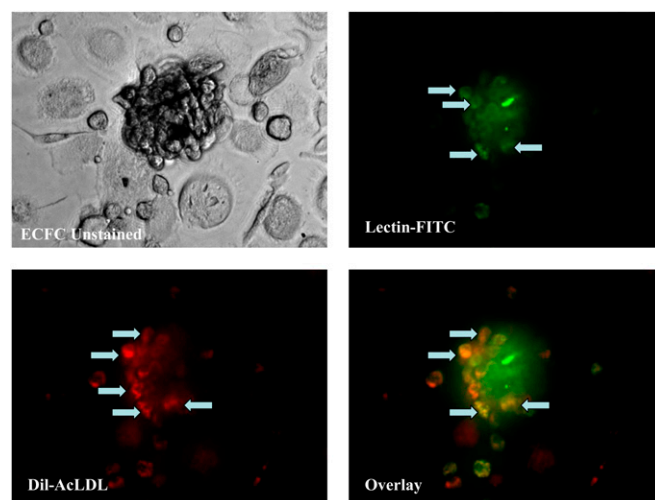


Figure 5. Endothelial progenitor colony assay using blood of subjects with asthma. Blood mononuclear cells from a subject with asthma were seeded on fibronectin-coated plates. Late outgrowth colonies collected after 21 days in culture were assessed for expression of stem cell markers by staining of endothelial colony-forming cells (ECFC) with lectin-fluorescein isothiocyanate (FITC) and 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine acetylated low density lipoprotein (DiI-AcLDL).

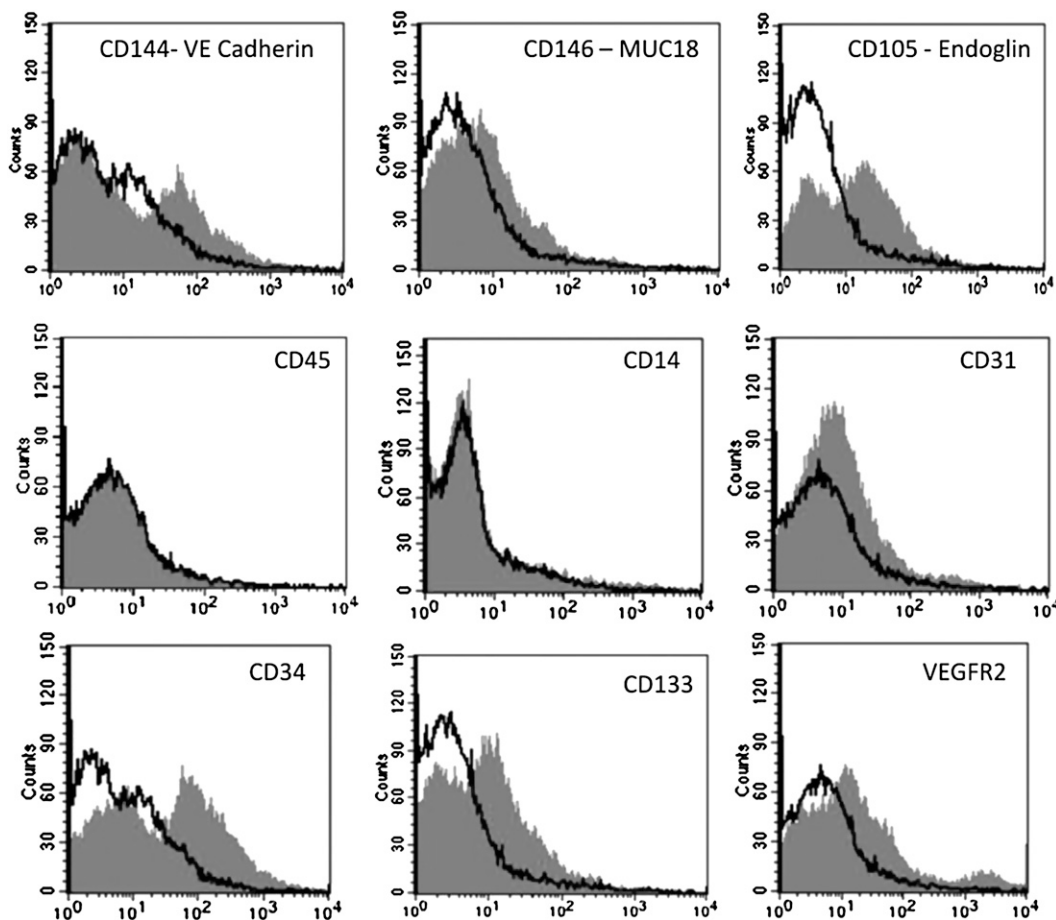


Figure 6. Phenotypic analyses of endothelial progenitor cell-derived endothelial cells. Endothelial colony-forming cells (ECFC) express CD34 ($90 \pm 4\%$), CD133 ($85 \pm 3\%$), VEGFR2 ($87 \pm 6\%$), CD31 ($90 \pm 4\%$), CD144 ($78 \pm 4\%$), CD146 ($82 \pm 5\%$), and CD105 ($85 \pm 4\%$), but not CD45 or CD14. Graphs shown are representative of data from six independent experiments using blood from patients with atopic asthma. Data in parenthesis are mean $\pm$ SEM % expression. Isotype control results are overlaid in a black line on each histogram for each surface antigen tested.

increases in the bioactive levels of VEGF, which promotes vessel formation, has been detected in the bronchoalveolar lavage (BAL) fluid of patients with asthma compared with normal healthy control subjects (34). In a transgenic mouse model with lung-targeted regulatable VEGF expression, local overexpression of VEGF induced an angiogenic switch and airway remodeling predisposed to an asthmatic phenotype by enhancing the Th2-mediated sensitization and inflammation (35). Most striking was the rapidity of the outgrowth of new blood vessels occurring within 1 day of VEGF expression in the lung. Although it is known that BM-derived EPC express the receptor for VEGF (19), it is not clear as to whether the effect of this factor is to stimulate terminal differentiation into vasculature or to act as a chemoattractant regulating the homing of EPC to the lungs in asthma. Other receptors expressed by EPC are CXCR2 reported in both humans and rodents (36, 37). There are eight CXCR2 ligands, of which some, including CXCL1 (Gro- α), CXCL2 (MIP-2), and CXCL8 (IL-8), promote angiogenesis *in vivo* and *in vitro* (38–40) and have been shown in contrast to VEGF to be essential for EPC recruitment after arterial injury (36). As such, controlling the CXCR2 ligands/CXCR2 biological axis is a prime focus of antiangiogenic therapeutics (41). In the context of asthma, CXCR2 knockout mice were shown to have diminished Ag-induced mast cell progenitor recruitment to the lungs (42). More recently, it has been shown that increased levels of CXCL1/2 are detected in the BAL of mice post-Ag challenge and that these levels correlated with the influx of EPC to the lungs (43). In mice pretreated with a CXCR2 antagonist there was a diminished accumulation of EPC, lung angiogenesis, and AHR without affecting other aspects of airway inflammation or BAL VEGF levels after Ag challenge (43).

In the current study, we investigated the CXCR2 chemokine levels in sputum supernatants taken from patients with asthma pre-Ag challenge and 7 hours and 24 hours post-Ag challenge.

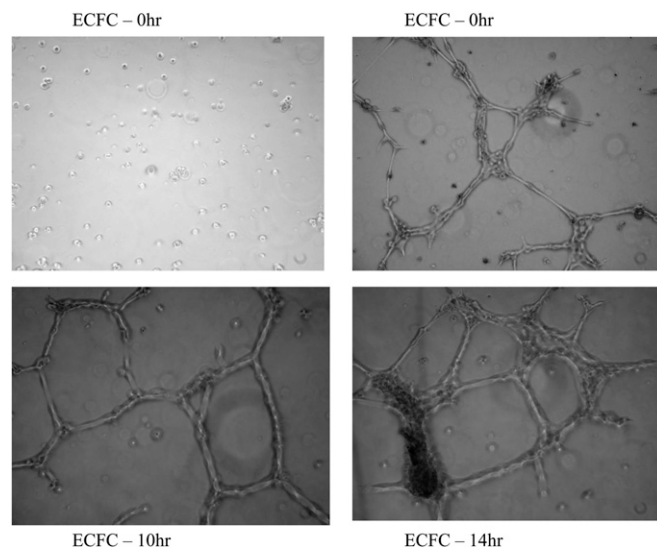


Figure 7. Angiogenic tube formation capacity of endothelial colony-forming cells (ECFC). To analyze the angiogenic capacity, ECFC were seeded on an angiogenic matrix and cocultured with endothelial growth medium-2 (EGM-2) for up to 18 hours. The development of a network of angiogenic tubes was visualized between 8 and 14 hours of culture. A representative time course of pictographs from four separate experiments performed is shown.

TABLE 3. SPUTUM SUPERNATANT ELISA

		Baseline	7 h Post Challenge	24 h Post Challenge
IL-8, pg/ml	Dil Challenge	793.1 ± 367.5	447.0 ± 103.7	622.3 ± 241.8
	Ag Challenge	306.4 ± 51.8	462.5 ± 119.0	680.2 ± 160.1*
Gro- α , pg/ml	Dil Challenge	1,273.42 ± 539.7	676.5 ± 361.6	790.7 ± 271.5
	Ag Challenge	387.8 ± 95.7	956.1 ± 279.1*	942.0 ± 262.1*
HGF, pg/ml	Dil Challenge	203.0 ± 131.7	117.8 ± 59.0	166.5 ± 68.3
	Ag Challenge	113.2 ± 24.8	217.9 ± 58.3	183.3 ± 44.0
VEGF, pg/ml	Dil Challenge	600.2 ± 116.3	791.0 ± 233.9	726.3 ± 158.9
	Ag Challenge	572.7 ± 101.4	768.3 ± 163.6	767.8 ± 152.1
Eotaxin, pg/ml	Dil Challenge	49.4 ± 10.6	56.2 ± 10.4	42.9 ± 11.5
	Ag Challenge	44.4 ± 10.8	58.3 ± 13.8	63.9 ± 6.7

Definition of abbreviations: Ag = allergen; Dil = diluent; Gro- α = growth regulated oncogene- α ; HGF = hepatocyte growth factor; VEGF = vascular endothelial growth factor.

Chemokine levels in sputum supernatants were assessed by Luminex Technology (n = 7 and n = 10 for diluent and allergen challenge arms, respectively). Values are presented as mean ± SEM.

*P < 0.05 represents within-group comparisons to baseline levels.

Significant increases in levels of IL-8 and Gro- α were detected after Ag but not after diluent challenge. When the sputum supernatants were directly tested in migration assays there was no detectable chemotactic activity for progenitor cells. This may have been due to inactivation of the biological activity of the chemokines by agents that are used to dissociate the clumps of cells found within the sputum, for example dithiothreitol (23). When recombinant human cytokines were tested at physiologically relevant concentrations, Gro- α but not IL-8 stimulated

a significant and preferential migrational response by EPC. In contrast, eotaxin, a hemopoietic progenitor cell chemoattractant (44) produced by EPC (45), and VEGF did not stimulate the migrational response of EPC. Further investigation into factors that stimulate the lung homing of EPC to the lungs could help to define the role of these precursor cells in lung angiogenic responses in human asthma and the effect on lung function.

In summary, our data show that after Ag challenge, but not diluent inhalation challenge, in dual-responder patients with asthma, there is a rapid and significant increase in sputum levels of EPC. In addition, in patients with asthma, after Ag challenge there is an increase in both the number and size of the blood vessels within the bronchial mucosa. The lung homing of the progenitor cells with the capacity to form tubulelike structures in angiogenic assays may be orchestrated by CXCR2 agonists such as Gro- α .

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References

- Davies DE, Wicks J, Powell M, Puddicombe SM, Holgate ST. Airway remodeling in asthma: new insights. *J Allergy Clin Immunol* 2003;111:215–225.
- Bousquet J, Vignola AM, Chanez P, Campbell A, Bonsignore G, Michel FB. Airways remodelling in asthma: no doubt, no more? *Int Arch Allergy Immunol* 1995;107:211–214.
- Peat JK, Woolcock AJ, Cullen K. Rate of decline of lung function in subjects with asthma. *Eur J Respir Dis* 1987;70:171–179.
- Chetta A, Zanini A, Torre O, Olivieri D. Vascular remodelling and angiogenesis in asthma: morphological aspects and pharmacological modulation. *Inflamm Allergy Drug Targets* 2006;6:41–45.
- Dunnill MS, Massarella GR, Anderson JA. A comparison of the quantitative anatomy of the bronchi in normal subjects, in status asthmaticus, in chronic bronchitis, and in emphysema. *Thorax* 1969;24:176–179.

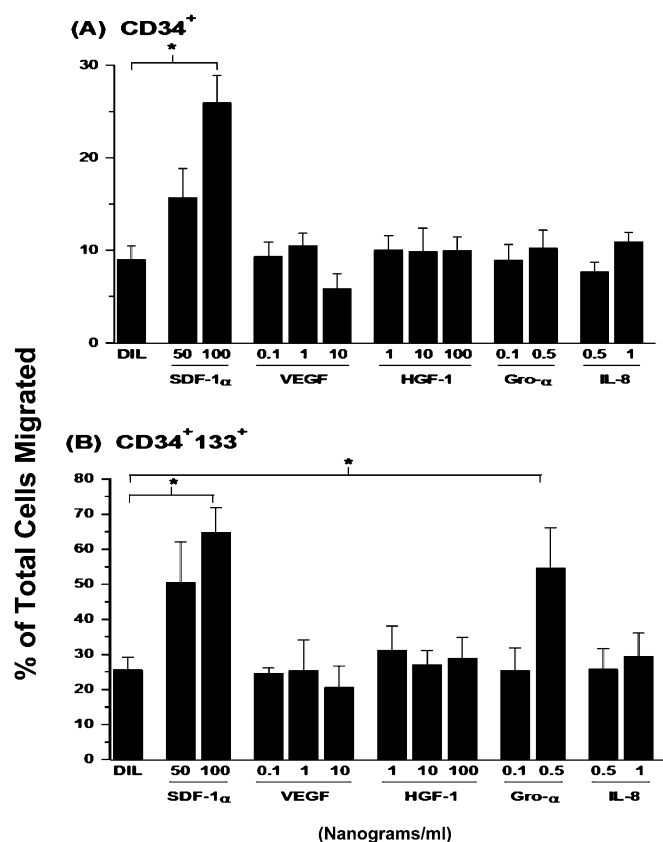


Figure 8. Migrational responsiveness of blood-extracted progenitor cells. Stromal cell-derived factor-1 (SDF-1) was used as a positive control. Tested at physiologically relevant concentrations, only Gro- α stimulated a significant migrational response by endothelial progenitor cells but not other CD34⁺ cell populations *in vitro*. Gro- α = growth regulated oncogene- α ; HGF = hepatocyte growth factor; VEGF = vascular endothelial growth factor.

6. Li X, Wilson JW. Increased vascularity of the bronchial mucosa in mild asthma. *Am J Respir Crit Care Med* 1997;156:229–233.
7. Hoshino M, Takahashi M, Aoike N. Expression of vascular endothelial growth factor, basic fibroblast growth factor, and angiogenin immunoreactivity in asthmatic airways and its relationship to angiogenesis. *J Allergy Clin Immunol* 2001;107:295–301.
8. Chetta A, Zanini A, Foresi A, D'Ippolito R, Tipa A, Castagnaro A, Baraldo S, Neri M, Saetta M, Olivieri D. Vascular endothelial growth factor up-regulation and bronchial wall remodeling in asthma. *Clin Exp Allergy* 2005;35:1437–1442.
9. Asai K, Kanazawa H, Kamoi H, Shiraishi S, Hirata K, Yoshikawa J. Increased levels of vascular endothelial growth factor in induced sputum in asthmatic patients. *Clin Exp Allergy* 2003;33:595–599.
10. Wilson JW, Hii S. The importance of the airway microvasculature in asthma. *Curr Opin Allergy Clin Immunol* 2006;6:51–55.
11. Wilson JW, Kotsimbos TC. Airway vascular remodeling in asthma. *Curr Allergy Asthma Rep* 2003;3:153–158.
12. Townley RG, Horiba M. Airway hyperresponsiveness: a story of mice and men and cytokines. *Clin Rev Allergy Immunol* 2003;24:85–109.
13. McFadden ER. Hypothesis: exercise-induced asthma as a vascular phenomenon. *Lancet* 1990;335:880–883.
14. Hogg JC, Pare PD, Moreno R. The effect of submucosal edema on airways resistance. *Am Rev Respir Dis* 1987;135:S54–S56.
15. Pandya NM, Dhalla NS, Santani DD. Angiogenesis - a new target for future therapy. *Vascul Pharmacol* 2006;44:265–274.
16. Yamada H, Kubo H, Ishikawa K, Kobayashi S, Shinkawa M, Sasaki H. Increased circulating endothelial progenitor cells in patients with bacterial pneumonia: evidence that bone marrow derived cells contribute to lung repair. *Thorax* 2005;60:410–413.
17. Burnham EL, Taylor WR, Quyyumi AA, Rojas M, Brigham KL, Moss M. Increased circulating endothelial progenitor cells are associated with survival in acute lung injury. *Am J Respir Crit Care Med* 2005;172:854–860.
18. Bergeron C, Al-Ramli W, Hamid Q. Remodeling in asthma. *Proc Am Thorac Soc* 2009;6:301–305.
19. Asosingh K, Swaidani S, Aronica M, Erzurum SC. Th1- and Th2-dependent endothelial progenitor cell recruitment and angiogenic switch in asthma. *J Immunol* 2007;178:6482–6494.
20. Cockcroft D.W. Measurement of airway responsiveness to inhaled histamine or methacholine; method of continuous aerosol generation and tidal breathing inhalation. In Hargreave FE, Woolcock AJ, editors. *Airway responsiveness: measurement and interpretation*. Mississauga, ON: Astra Pharmaceutical, Canada, Limited; 1985. pp. 22–28.
21. O'Byrne PM, Dolovich J, Hargreave FE. Late asthmatic response. *Am Rev Respir Dis* 1987;136:740–756.
22. Cockcroft DW, Murdock KY, Kirby J, Hargreave FE. Prediction of airway responsiveness to allergen from skin sensitivity to allergen and airway responsiveness to histamine. *Am Rev Respir Dis* 1987;135:264–267.
23. Pizzichini E, Pizzichini MMM, Efthimiadis A, Evans S, Morris MM, Squillace D, Gleich G, Dolovich J, Hargreave FE. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am J Respir Crit Care Med* 1996;154:308–317.
24. Kariyawasam HH, Pegorier S, Barkans J, Xanthou G, Aizen M, Ying S, Kay AB, Lloyd CM, Robinson DS. Activin and transforming growth factor-beta signaling pathways are activated after allergen challenge in mild asthma. *J Allergy Clin Immunol* 2009;124:454–462.
25. Hamid Q, Azzawi M, Ying S, Moqbel R, Wardlaw AJ, Corrigan CJ, Bradley B, Durham SR, Collins JV, Jeffery PK, et al. Expression of mRNA for interleukin-5 in mucosal bronchial biopsies from asthma. *J Clin Invest* 1991;87:1541–1546.
26. Dorman SC, Babirad I, Post J, Watson R, Foley R, Jones GL, O'Byrne PM, Sehmi R. Role of SDF-1 α and Eotaxin in modulating the egress of hemopoietic progenitor cells from the bone marrow in asthmatics following allergen challenge. *J Allergy Clin Immunol* 2005;115:501–507.
27. Doyle TM, Ellis R, Park HJ, Peng K, Inman MD, Sehmi R. Modulating progenitor cell accumulation attenuates lung angiogenesis in a mouse model of asthma. *Eur Respir J* (In press).
28. Rohan RM, Fernandez A, Yuan T, D'Amato RJ. Genetic heterogeneity of angiogenesis in mice. *FASEB J* 2000;14:871–876.
29. Shaked Y, Bertolini F, Man S, Rogers MS, Cervi D, Foutz T, Rawn K, Voskas D, Dumont DJ, Ben-David Y, et al. Genetic heterogeneity of the vasculogenic phenotype parallels angiogenesis: implications for cellular surrogate marker analysis of antiangiogenesis. *Cancer Cells* 2005;7:101–111.
30. Yoder MC. Defining human endothelial progenitor cells. *J Thromb Haemost* 2009;7:49–52.
31. Luttun A, Verfaillie CM. Will the real EPC please stand up? *Blood* 2007; 109:1795–1796.
32. Hristov M, Erl W, Weber PC. Endothelial progenitor cells: isolation and characterization. *Trends Cardiovasc Med* 2009;13:201–206.
33. Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, Krasich R, Temm CJ, Prchal JT, Ingram DA. Redefining endothelial progenitor cells via clonal analyses and hematopoietic stem/progenitor cell principals. *Blood* 2007;109:1801–1809.
34. Simcock DE, Kanabar V, Clarke GW, O'Connor B, Lee TH, Hirst SJ. Proangiogenic activity in bronchoalveolar lavage fluid from patients with asthma. *Am J Respir Crit Care Med* 2007;176:146–153.
35. Lee CG, Link H, Baluk P, Homer RJ, Chapoval S, Bhandari V, Kang MJ, Cohn L, Kim YK, McDonald DM, et al. Vascular endothelial growth factor (VEGF) induces remodeling and enhances TH2-mediated sensitization and inflammation in the lung. *Nat Med* 2004;10:1095–1103.
36. Hristov M, Zernecke A, Bidzhekov K, Liehn EA, Shagdarsuren E, Ludwig A, Weber C. Importance of CXCR2 chemokine receptor 2 in the homing of human peripheral blood endothelial progenitor cells to sites of arterial injury. *Circ Res* 2007;100:590–597.
37. Liehn EA, Schober A, Weber C. Blockade of keratinocyte-derived chemokine inhibits endothelial recovery and enhances plaque formation after arterial injury in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* 2004;24:1891–1896.
38. Addison CL, Daniel TO, Burdick MD, Liu H, Ehlert JE, Xue YY, Buechi L, Walz A, Richmond A, Strieter RM. The CXCR2 chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXCR2 chemokine-induced angiogenic activity. *J Immunol* 2000;165:5277.
39. Sukkar A, Jenkins A, Sanchez J, Wagner EM. Inhibition of CXCR2 attenuates bronchial angiogenesis in the ischemic rat lung. *J Appl Physiol* 2008;104:1470–1475.
40. Keane MP, Belperio JA, Xue YY, Burdick MD, Strieter RM. Depletion of CXCR2 inhibits tumor growth and angiogenesis in a murine model of lung cancer. *J Immunol* 2004;172:2860.
41. Teicher BA. Antiangiogenic agents and targets: A perspective. *Biochem Pharmacol* 2011;81:6–12.
42. Hallgren J, Jones TG, Abonia JP, Xing W, Humbles A, Austen F, Gurish MF. Pulmonary CXCR2 regulates VCAM-1 and antigen-induced recruitment of mast cell progenitors. *Proc Natl Acad Sci USA* 2007;104:20478–20483.
43. Jones CP, Pitchford SC, Lloyd CM, Rankin SM. CXCR2 mediates the recruitment of endothelial progenitor cells during allergic airways remodeling. *Stem Cells* 2009;27:3074–3081.
44. Sehmi R, Dorman S, Baatjes A, Watson R, Foley R, Ying S, Robinson DS, Kay AB, O'Byrne PM, Denburg JA. Allergen-induced fluctuations in CCR3 expression on bone marrow CD34⁺ cells from asthmatics subjects: significance for mobilization of hemopoietic progenitor cells in allergic inflammation. *Immunology* 2003;109:536–546.
45. Asosingh K, Hanson J, Cheng G, Aronica M, Erzurum SC. Allergen-induced eotaxin-rich, angiogenic bone marrow progenitors: a blood-borne cellular envoy for lung eosinophilia. *J Allergy Clin Immunol* 2010;125:918–925.

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