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*J Immunol* 2007; 178:6482-6494; doi: 10.4049/jimmunol.178.10.6482

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Th1- and Th2-Dependent Endothelial Progenitor Cell Recruitment and Angiogenic Switch in Asthma

Kewal Asosingh, Shadi Swaidani, Mark Aronica, and Serpil C. Erzurum

Increased numbers of submucosal vessels are a consistent pathologic component of asthmatic airway remodeling. However, the relationship between new vessel formation and asthmatic inflammatory response is unknown. We hypothesized that angiogenesis is a primary event during the initiation of airway inflammation and is linked to the recruitment of bone marrow-derived endothelial progenitor cells (EPC). To test this hypothesis, circulating EPC and EPC-derived endothelial cell colony formation of individuals with asthma or allergic rhinitis and health controls was evaluated. Circulating EPC were increased in asthma, highly proliferative, and exhibited enhanced incorporation into endothelial cell tubes as compared with controls. In an acute allergen challenge murine asthma model, EPC mobilization occurred within hours of challenge and mobilized EPC were selectively recruited into the challenged lungs of sensitized animals, but not into other organs. EPC recruitment was Th1 and Th2 dependent and was temporally associated with an increased microvessel density that was noted within 48 h of allergen challenge, indicating an early switch to an angiogenic lung environment. A chronic allergen challenge model provided evidence that EPC recruitment to the lung persisted and was associated with increasing microvessel density over time. Thus, a Th1- and Th2-dependent angiogenic switch with EPC mobilization, recruitment, and increased lung vessel formation occurs early but becomes a sustained and cumulative component of the allergen-induced asthmatic response. The Journal of Immunology, 2007, 178: 6482–6494.

Angiogenesis is a complex regulated process of new blood vessel formation from existing blood vessels (1). Tissue resident endothelial cells (EC) participate in the process, but endothelial progenitor cells (EPC) have been recently shown to play an essential role in the formation of new blood vessels (2–6). In adults, EPC are defined as a specific subset of bone marrow-derived cells with characteristics similar to those of embryonal angioblasts, i.e., the common hemopoietic and endothelial stem cells. The majority of EPC are believed to be bone marrow resident cells, of which only a minor fraction escape to circulate through the peripheral blood and contribute to the formation of new blood vessels and to vascular homeostasis (2–6). EPC are easily identified by the coexpression of the stem cell marker CD133 on the subpopulation of the cells expressing CD34, the common cell surface marker for hemopoietic stem cells and mature EC. The fully committed EC lineage progenitor cells also express vascular EC growth factor (VEGF) receptor-2 (VEGFR2) and represent a smaller fraction (~1%) of the circulating CD133CD34 cells. During the process of terminal differentiation into mature EC, the expression of CD133 and, for some EC types, the expression of CD34 are gradually down-regulated together with the up-regulation of other EC-specific markers such as CD31 and vascular endothelial (VE)-cadherin as well as VEGFR2 (8). In addition to these cell surface markers, the ability to form endothelial progenitor cell colonies in vitro is used to functionally identify EPC (7, 9). Recent studies show that EPC are incorporated into new and existing blood vessels in many pathological conditions, including tumor vascularization (10–12) and myocardial ischemia (9, 13–15) and that circulating EPC are elevated and serve as biomarkers for inflammatory diseases such as atherosclerosis (16), cerebrovascular disease (17), rheumatoid arthritis (18), and acute lung injury (19).

In this context, studies of resected lung and bronchial biopsies describe increased vascularity in asthmatic lungs (20, 21). In general asthma is characterized by remodeling of the airway structure, including damage to airway epithelium, eosinophil infiltration, smooth muscle hyperplasia, and basement membrane thickening (22, 23). An increase in the number and size of vessels in the airway wall is a long-recognized occurrence and one of the most consistent features of asthma remodeling, occurring in mild, moderate, and severe asthmatic lungs (24–27). Recent studies suggest that angiogenesis occurs in chronic asthma (24, 25) and indicate a relation between the numbers of blood vessels in the bronchial wall and the severity of asthma (25–27). Although an understanding of new vessel formation and its genesis in asthma is still in its early stages, it has been suggested that vascular remodeling may be a critical component in the pathophysiology of asthma (28). We hypothesized that angiogenesis is an early event with onset during the initiation of airway inflammation and is linked to the mobilization of bone marrow-derived EPC. In this study we show that asthmatic individuals have increased levels of...
circulating EPC that are highly proliferative and exhibit enhanced incorporation into tubes in an angiogenesis assay. In the experimental allergen challenge mouse model of asthma, EPC mobilization is an early and selective event during allergen challenge of sensitized animals, which is followed by an increase in microvessel density (MVD) that progresses over time with chronic allergen exposure. Altogether, the data support the concept that EPC recruitment and an angiogenic switch are early events that occur during the derivation of allergic airway inflammation.

Materials and Methods

Study population

Nonsmoking healthy individuals and individuals with asthma were recruited to participate in the study. Exclusion criteria for the two groups included an age younger than 18 years or older than 65 years, pregnancy, HIV infection, a history of respiratory infection in the previous 6 wk, prolonged exposure to second-hand smoke at home or work, and exposure to dusty environments or known pulmonary disease-producing agents. In addition, the exclusion criteria for healthy controls included any history or symptoms of chronic lung disease or allergies. None of the volunteers were allowed to have had a recent asthma exacerbation, hospitalization, or change in medications for 6 wk before the study. Asthma was defined based on the National Asthma Education Prevention Program Guidelines, including episodic respiratory symptoms, reversible airway obstruction by the documentation of a variability of forced expiratory volume in 1 s (FEV1) and/or forced vital capacity (FVC) by 12% and 200 ml either spontaneously or after two puffs of inhaled albuterol, and/or a positive methacholine challenge (29). For methacholine challenge testing, increasing concentrations of methacholine were delivered until FEV1 fell at least 20% when compared with a control level. The measure used to compare the sensitivity of one individual to another was the first provocative concentration that caused a 20% fall in FEV1 (PC20). Allergic rhinitis patients were recruited from control level. The measure used to compare the sensitivity of one individual to another was the first provocative concentration that caused a 20% fall in FEV1 (PC20).

Animals

Immunocompetent, nonirradiated, 6- to 8-wk-old female BALB/c mice from The Jackson Laboratory were used for OVA sensitization and challenge and adoptive transfer experiments. DO-11.10 mice (BALB/c) transgenic for a TCR recognizing the OVA peptide 323–339 (30) were bred in the National Asthma Education Prevention Program Guidelines, including episodic respiratory symptoms, reversible airway obstruction by the documentation of a variability of forced expiratory volume in 1 s (FEV1) and/or forced vital capacity (FVC) by 12% and 200 ml either spontaneously or after two puffs of inhaled albuterol, and/or a positive methacholine challenge (29). For methacholine challenge testing, increasing concentrations of methacholine were delivered until FEV1 fell at least 20% when compared with a control level. The measure used to compare the sensitivity of one individual to another was the first provocative concentration that caused a 20% fall in FEV1 (PC20). Allergic rhinitis patients were recruited from control level. The measure used to compare the sensitivity of one individual to another was the first provocative concentration that caused a 20% fall in FEV1 (PC20).

VEGF ELISA

VEGF concentration in bronchoalveolar lavage (BAL) fluid was measured by a human VEGF quantikine ELISA kit (R&D Systems). Total protein concentration in BAL fluid was measured using a Coomassie (Bradford) protein assay kit (Pierce).

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OVA sensitization, lung analyses, and methacholine challenge

Standard induction of allergic airway disease was performed as previously described (31, 32). In brief, BALB/c mice were immunized i.p. with 10 μg of OVA (precipitated in Al(OH)3; Sigma-Aldrich). Two weeks later, a series of eight daily inhalations (40 min per day) were started with mice placed as a group in a chamber kept saturated with nebulized OVA solution (1% (w/v) in sterile PBS). For chronic exposure, animals were challenged three times per week (Monday, Wednesday, and Friday) for 6 wk. At the indicated times, animals were anesthetized by i.p. injection with pentobarbital and placed on a rodent ventilator inside a body plethysmography chamber. After a stable baseline pressure was established, mice were given three serially increasing doses of methacholine (45–411 μg/kg in a volume of 40–55 μl per dose). Methacholine doses were administered 5 min apart and only after the transpulmonary pressure and volume had returned to baseline. Resistance and compliance were determined using Bioelectronics and Biosystems XA software. Methacholine dose-response curves were obtained by calculating the mean ± SE for individual animals at each methacholine dose. BAL was performed by instilling 700 μl of RPMI 1640 and then withdrawing the fluid with gentle suction via the syringe. The typical BAL fluid return was 400–600 μl. White blood cells were counted on a hemocytometer whereas cytologic examination was performed on cytospin preparations fixed and stained using Diff-Quick (American Scientific Products). Differential counts were based on counts of 200 cells using standard morphologic criteria to classify the cells as eosinophils, lymphocytes, or other mononuclear leukocytes (alveolar macrophages and monocytes). Counts were performed by a single observer who was blinded to the study group.

In parallel experiments, mice were sensitized and challenged as described above and, at the indicated time points, anesthetized by i.p. injection with pentobarbital. Blood was drawn in EDTA syringes by cardiac puncture and lungs were dissected. Right lung lobes were fixed in Histo-Choice (Amresco) and processed for immunohistochemistry. Left lungs were gently squeezed through 100-μm pore size nylon mesh in DMEM medium with 10% FBS. Mononuclear cells were obtained from blood and lungs by centrifugation on Lympholyte gradient (Cedarlane) and the number of cells was counted. In some experiments, mononuclear cells were also isolated from the spleen in the same manner as that described for the lungs.

Differentiated CD4+ effector T cell populations and adoptive transfers

CD4+ T cells from the spleen and lymph nodes of DO11.10 TCR transgenic mice were isolated by negative selection using anti-CD8 and anti-MHC II microbeads (Miltenyi Biotec) according to the manufacturer’s instructions and as previously described (31, 32). Naive (CD42Lhigh) cells were gently squeezed through 100-μm pore size nylon mesh in DMEM medium with 10% FBS. Mononuclear cells were obtained from blood and lungs by centrifugation on Lympholyte gradient (Cedarlane) and the number of cells was counted. In some experiments, mononuclear cells were also isolated from the spleen in the same manner as that described for the lungs.

Endothelial progenitor colony assays

Mononuclear cells were isolated from human EDTA-blood by centrifugation on Ficoll-Paque Plus gradient (Amersham Biosciences). The formation of endothelial progenitor cell colonies was analyzed by using an established in vitro CFU-EC assay (9). We used the commercially standardized form of this assay shown as the EndoCult liquid medium kit (Stem Cell Technologies). A quantity of 4.75 × 105 mononuclear cells were incubated in 1 ml of EndoCult medium per well in fibronectin-coated 12-well plates (day 0) (BD Biosciences) at 37°C with 5% CO2 and 95% humidity. On day 2, nonadherent cells were harvested by gentle pipetting, counted, and seeded in fibronectin-coated 12-well plates at a density of 1.9 × 106 cells per well. Five days later, colonies were stained with the dye 4′,6-diamidino-2-phenylindole (DAPI) and then withdrawing the fluid with gentle suction via the syringe. The typical BAL fluid return was 400–600 μl. White blood cells were counted on a hemocytometer whereas cytologic examination was performed on cytospin preparations fixed and stained using Diff-Quick (American Scientific Products). Differential counts were based on counts of 200 cells using standard morphologic criteria to classify the cells as eosinophils, lymphocytes, or other mononuclear leukocytes (alveolar macrophages and monocytes). Counts were performed by a single observer who was blinded to the study group.

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Flow cytometry

CD34<sup>+</sup>CD133<sup>+</sup> progenitors in the human peripheral blood circulation were quantified by staining 1 × 10<sup>6</sup> mononuclear cells with 10 μl of anti-human CD34-FITC (BD Biosciences) and 10 μl of anti-human CD133-PE (Miltenyi Biotec) mAbs. Isotype-matched irrelevant Abs were used as controls for nonspecific Ab binding. Cell suspensions were incubated for 30 min on ice in the dark, subsequently washed with 500 μl of PBS with 1% BSA and 0.02% sodium azide and resuspended in 400 μl of FACSFlow fluid (BD Biosciences). Samples were analyzed on a FACSscan (BD Biosciences) flow cytometer. Events (0.5 × 10<sup>5</sup>) were acquired and data analysis was performed using CellQuest 3.3 software (BD Biosciences). Day 5 CFU-EC cells were also stained for CD34 and CD133 expression. The expression of CD31 and VE-cadherin was analyzed by staining with 20 μl of anti-human CD31-FITC (BD Biosciences) and 20 μl of anti-human VE-cadherin-PE (BD Biosciences) mAbs, respectively. Isotype-irrelevant Abs were used as negative controls for nonspecific binding. Samples were analyzed on a FACSscan (BD Biosciences) flow cytometer. Ten thousand events were acquired and analyzed using CellQuest 3.3 software (BD Biosciences).

Immunohistochemistry

To analyze the MVD of mouse lungs and human endobronchial biopsy, paraffin-embedded tissues were cut into 5-μm sections and stained with polyclonal rabbit anti-human von Willebrand factor (VWF), which also cross-reacts with the mouse Ag (DakoCytomation). Samples were pre-treated with proteinase K for 8 min and stained with diaminobenzidine and hydrogen peroxide. All samples were stained by using an automated biotin-avidin peroxidase system (Ventana Medical Systems). The secondary Ab alone was used as a negative control. All sections were counterstained with hematoxylin. To quantify the MVD, the number of blood vessels per field was counted in five random fields in each sample at a final magnification of ×200 using an Olympus BX40 light microscope. To correct for differences in lung inflation, the lung area was also measured. For this purpose, photographs were taken of each field and Image-Pro Plus version 5.1 software was used to process and calculate the area of lung tissue in each image. The software calibrated each image for the specific microscope and objective used. The lung tissue in each image was then selected for quantification using the color cube-based algorithm and the total area occupied by lung tissue per image was quantified.

To analyze eosinophil infiltration, slides were deparaffinized with ClearRite 3 and rehydrated with Flex 100 and Flex 90 (Richard-Allan Scientific), respectively. After blocking with 2% FBS in PBS for 3 h at room temperature, slides were incubated overnight at 4°C with an eosinophil-specific, goat anti-mouse major basic protein (Santa Cruz Biotechnology). After washing with 2% FBS, the sections were incubated for 2h at room temperature with Alexa Fluor 488-conjugated donkey anti-goat IgG (Molecular Probes). Slides were mounted with VECTASHIELD mounting medium with 4',6-diamidino-2-phenylindole H-1220 (Vector Laboratories).

Pictures were a final magnification of ×400 using a Leica DMR fluorescence microscope equipped with a Retiga EX digital camera and by using QImaging software.

In vitro angiogenesis assay

Angiogenic activity of human EPC-derived EC (EPC-EC) were assessed using an in vitro angiogenesis assay from Chemicon International. On day
5 CFU-EC were harvested with trypsin and further matured by incubation in EGM-2 medium (0.7 × 10⁶ cells per fibronectin-coated 24-well plate; Cambrex) for 1–2 wk. The medium was exchanged three times per week. Staining with Dil-AcLDL and Ulex europaeus lectin-FITC confirmed that a pure (>95%) culture of EC was obtained. EPC-EC were harvested by trypsin, counted, and fluorescently labeled with CellTracker Green (Molecular Probes). HUVEC were mixed with control EPC-EC or asthma EPC-EC at a ratio of 9:1 in EGM-2 medium and seeded on the angiogenesis extracellular matrix in 24-well plates. After 8 h, phase-contrast and fluorescence images of the EC in tube formation were acquired using a Leica DM IRB inverted wide-field microscope with a ×10 objective and a MicroMax charge-coupled device camera (Photometrics). To evaluate angiogenic activity, EC networks were manually delineated in the phase-contrast images of each field (three fields per sample) using Adobe

**FIGURE 2.** CFU-EC formation by CD133⁺C34⁺ EPC cells in asthma, allergic rhinitis, and healthy control subjects. CFU-EC assays were performed to analyze the EC commitment of the circulating EPC. Mononuclear cells were seeded on fibronectin-coated wells in EndoCult medium and the number of CFU-EC colonies was quantitated under an inverted microscope. A, Representative CFU-EC from control and asthma samples. B and C, EC commitment of CFU-EC cells and the expression of stem cell markers was confirmed by staining for Dil-AcLDL and lectin-FITC (B) and by single-color FACS staining (C) for CD31 and VE-cadherin (CD144-PE) and by double FACS staining for CD34 and CD133, respectively. Dil-AcLDL and lectin stain are shown in cells obtained from a patient with allergic rhinitis, and FACS profiles of healthy control and asthma CFU-EC are shown. Values represent mean ± SE of five patients. D, Number of CFU-EC in mononuclear cell fraction of control and asthmatic subjects.
with asthma revealed airflow limitation (FVC predicted, 86% ; FEV\(_1\) predicted, 71% ). The study population included 28 nonsmoking healthy individuals and race (age for control was 36; 27 asthma patients who were similar in terms of gender, age, and 2 years, for asthma 38; 3 years; gender (male/female) for control was 12/16, for asthma 11/16; race (African American/Caucasian/Hispanic/Other) for control was 7/15/1/5, for asthma 8/15/3/1; all \( p \) values were NS). Asthma was confirmed by positive methacholine challenge (methacholine \( \mathrm{PC}_{20} \), 4.4 ± 1.6 µg/ml). The lung function evaluated in volunteers with asthma revealed airflow limitation (FVC predicted, 86 ± 3%; \( \mathrm{FEV}_1 \) predicted, 71 ± 5%; percentage of \( \mathrm{FEV}_1/\mathrm{FVC} \), 66 ± 3%). As disease control, 7 patients with allergic rhinitis were enrolled (age, 39 ± 4 years; gender (male/female), 3/4; race (African American/Caucasian/Hispanic/Other): 0/5/0/2). Table I summarizes the characteristics of the study subjects. In a subgroup of asthmatic individuals, a bronchoscopy was performed for endobronchial biopsy of the airway (\( n = 4 \), one male; mean values for lung function were similar to those in Table I).

Asthma patients have increased levels of CD133\(^{+}\)CD34\(^{+}\)

The presence of circulating EPC in the peripheral circulation of asthma patients, healthy controls, and patients with allergic rhinitis was analyzed by flow cytometric staining for stem cell marker CD133 and common hemopoietic stem cell EC marker CD34 (Fig. 1A). Individuals with asthma had a ~1.7-fold increase in CD133\(^{+}\)CD34\(^{+}\) progenitor cells in their blood circulation compared with healthy controls and patients with allergic rhinitis (percentage of CD133\(^{+}\)CD34\(^{+}\) cells in blood; healthy controls, 0.06 ± 0.04; allergic rhinitis, 0.05 ± 0.01; asthma, 0.10 ± 0.03; \( p < 0.0002 \)) (Fig. 1B). There was also a significant increase in the total CD34\(^{+}\) and total CD133\(^{+}\) progenitor cells for asthmatics compared with healthy controls (percentage of CD34\(^{+}\) cells in blood: healthy controls, 0.10 ± 0.01 vs asthma, 0.18 ± 0.02, \( p < 0.0015 \); percentage of CD133\(^{+}\) cells in blood: healthy controls, 0.30 ± 0.05 vs asthma, 0.73 ± 0.13, \( p < 0.0059 \)). There was no significant difference in total CD34\(^{+}\) or CD133\(^{+}\) cells between patients with allergic rhinitis (percentage of CD34\(^{+}\) cells in blood: 0.12 ± 0.03; percentage of CD133\(^{+}\) cells in blood: 0.67 ± 0.12) and asthma patients, but compared with healthy controls the CD133 levels were significantly higher in allergic rhinitis (\( p < 0.0001 \)). In allergic rhinitis a significantly lower fraction of CD34\(^{+}\) progenitor cells coexpressed CD133 (\( p < 0.005 \) and \( p < 0.01 \) in comparison to healthy controls and asthmatics, respectively) (percentage of CD34 cells expressing CD133: healthy controls, 65 ± 3%; allergic rhinitis, 44 ± 5%; asthma, 57 ± 3%). These values are in the range of CD133 coexpression on CD34\(^{+}\) cells in nonmobilized adult peripheral blood (35) and suggest that allergic rhinitis patients have more lineage-committed progenitor cells in their circulation. Circulating progenitors were unrelated to the percentages of neutrophils, eosinophils, monocytes and basophils or hemoglobin or hematocrit (all \( p > 0.05 \) but were inversely correlated to the percentage of lymphocytes in circulation (\( R = -0.673, p = 0.004 \)). There was no significant relation of the

**FIGURE 3.** Proliferation capacity of circulating EPC. A, To assess proliferation capacity, the number of cells per CFU-EC from allergic rhinitis, healthy control, and asthmatic individuals was counted under an inverted microscope. B, Distribution of the CFU-EC colony size in terms of cell number per CFU-EC was analyzed as an additional parameter for the proliferation potential. CFU-EC were grouped according to indicated range of cell numbers/CFU-EC on the abscissa and their percentage of the total population of colonies scored was calculated. C, VWF staining of asthmatic and healthy control endobronchial biopsy. Asthmatic samples characteristically have increased basement membrane thickness and smooth muscle and display greater numbers and size of blood vessels in the submucosa. A high power view of the boxed region of the asthmatic tissue shows that most vessels contain inflammatory cells such as eosinophils and neutrophils within their lumen (arrowheads).

Photoshop CS and subsequently skeletonized using Image-Pro Plus 5.0 (Media Cybernetics). Total skeletal length and branch to branch lengths were extracted from the skeletonized images using Fovea 3.0 (Reindeer Graphics). To measure the incorporation efficiency of EPC-EC, binary image skeletons from phase-contrast images were used as masks for the corresponding green fluorescence images in Image-Pro Plus. The resulting “green skeletons” were thresholded to remove the background fluorescence and the green skeletal length was calculated using Image-Pro Plus. For each field, total green skeletal length was divided by the corresponding phase-contrast skeletal length to assess the contribution of EPC-EC to the overall angiogenic tube formation.

**Statistical analysis**

Data were analyzed by using the JMP 5.1 software program. ANOVA or Student’s \( t \) test was used for comparisons of parametric data, and a Wilcoxon test was used for comparisons of nonparametric data as appropriate. \( p < 0.05 \) was considered as significant. Mean ± SD value for each group is shown unless indicated differently.

**Results**

**Clinical characteristics**

The study population included 28 nonsmoking healthy individuals and 27 asthma patients who were similar in terms of gender, age, and race (age for control was 36 ± 2 years, for asthma 38 ± 3 years; gender (male/female) for control was 12/16, for asthma 11/16; race (African American/Caucasian/Hispanic/Other) for control was 7/15/1/5, for asthma 8/15/3/1; all \( p \) values were NS). Asthma was confirmed by positive methacholine challenge (methacholine \( \mathrm{PC}_{20} \), 4.4 ± 1.6 µg/ml). The lung function evaluated in volunteers with asthma revealed airflow limitation (FVC predicted, 86 ± 3%; \( \mathrm{FEV}_1 \) predicted, 71 ± 5%; percentage of \( \mathrm{FEV}_1/\mathrm{FVC} \), 66 ± 3%). As disease control, 7 patients with allergic rhinitis were enrolled (age, 39 ± 4 years; gender (male/female), 3/4; race (African American/Caucasian/Hispanic/Other): 0/5/0/2). Table I summarizes the characteristics of the study subjects. In a subgroup of asthmatic individuals, a bronchoscopy was performed for endobronchial biopsy of the airway (\( n = 4 \), one male; mean values for lung function were similar to those in Table I).
levels of circulating progenitors to airway reactivity, airflow limitation, or the atopy status of asthmatic individuals, i.e., as determined by skin test positivity or IgE levels (all \( p > 0.05 \)). However, VEGF levels in BAL were significantly higher in asthmatics as compared with healthy controls (VEGF ng/mg total protein: asthma, 4.0 ± 0.46 (\( n = 19 \)); controls, 1.8 ± 0.72 (\( n = 9 \)), \( p < 0.01 \)).

To definitively quantify and evaluate the function of EPC in asthma, we identified the formation of EPC colonies by using CFU-EC assay (Fig. 2A) (7, 9). Dil-AcLDL uptake and lectin binding confirmed that the CFU-EC differentiate to the EC lineage (Fig. 2B) (7, 36). In addition, CFU-EC expressed the EC markers CD31 and CD144 (VE-cadherin), confirming that the cells within the CFU-EC were committed to the EC lineage (Fig. 2C). Moreover, the cells were intermediate positive for CD133 and CD34, which is in accordance with the down-regulation of these Ags during EPC differentiation into mature EC (2, 7).

Endothelial cells derived from asthma EPC have a higher incorporation into angiogenic tubes

According to current concepts, EPC-derived EC contribute to new vessel formation in vivo by paracrine activation of tissue resident EC and by incorporation in the new blood vessels (6, 37, 38). We investigated the capacity of asthma EPC to participate in angiogenesis using an in vitro tube formation assay. For this purpose EPC-derived EC were used. Endothelial cell lineage was again confirmed by analysis of the EC for Dil-AcLDL and for the binding of lectin (Fig. 4A). Fluorescently labeled EPC-derived EC from asthma (EPC-EC) or control subjects (control EPC-EC) were mixed with HUVEC and incubated on an angiogenic extracellular matrix. Quantitation of incorporation showed that asthma EPC-EC had a 12-fold higher incorporation into tubes as compared with control EPC-EC (\( p < 0.002 \)) (Fig. 4, B and C). These data suggest that EPC from asthma patients are highly proangiogenic.
FIGURE 5. SCA-1<sup>+</sup>c-Kit<sup>+</sup>VEGFR2<sup>+</sup> cells in a murine allergic asthma model. Mice were sensitized to OVA or exposed to PBS by a single i.p. injection and subsequently subjected to a series of allergen or PBS inhalations. Following day 8 challenge, mononuclear cells were isolated from blood, lungs, and spleen, counted and stained for SCA-1, c-Kit, and VEGFR2 for FACS analysis. A, Quantification of SCA-1<sup>+</sup>c-Kit<sup>+</sup>VEGFR2<sup>+</sup> cells. Dot plots from a control lung are used as an example. First, the percentage of SCA-1<sup>+</sup>c-Kit<sup>+</sup> cells were analyzed on a FL1/FL2 dot plot. Quadrants were placed based on isotype-matched control stainings. Double-positive cells were defined in gate R2. Absolute numbers of SCA-1<sup>+</sup>c-Kit<sup>+</sup> cells were calculated using this percentage and cell count was obtained after Ficoll separation. Second, the expression of VEGFR2 on the gated SCA-1<sup>+</sup>c-Kit<sup>+</sup> cells was analyzed on FL1/FL3 dot plots. Quadrants were on placed isotype-matched control stainings. SCA-1<sup>+</sup>c-Kit<sup>+</sup> cells expressing VEGFR2 were defined in gate R3. Absolute numbers of SCA-1<sup>+</sup>c-Kit<sup>+</sup>VEGFR2<sup>+</sup> cells were calculated with this percentage and the absolute numbers of SCA-1<sup>+</sup>c-Kit<sup>+</sup> cells was analyzed.

Recruitment of EPC into the lung is an early and ongoing process during airway allergy challenge

The observation of selective EPC recruitment into the allergic lung prompted us to determine at which stage the recruitment was initiated and how it progressed over the course of an acute and chronic allergen challenge. A time course experiment was performed during which animals were challenged daily during the first week (acute challenge) and 3 times a week during the subsequent 6 wk (chronic challenge). Eosinophils in the BAL reached a peak at 8 days after the initiation of challenge as previously reported (43) (Fig. 6A), while the levels of lymphocytes, macrophages, and neutrophils did not change significantly over time. Airway hypersensitivity as measured by methacholine challenge also increased during the time of acute challenge, increasing at day 2 of challenge and remaining elevated during chronic challenge in sensitized animals ($p = 0.018$) (Fig. 6B), but there was no significant change compared with day 0 lung resistance in control mice groups (PBS/OVA, PBS/PBS, OVA/PBS). Progenitor cells (SCA-1<sup>+</sup>c-Kit<sup>+</sup> cells) also reached maximum levels at the end of the acute challenge (day 8) (Fig. 6C). Notably, EPC numbers increased in the lung as early as 24 h after the first challenge (Fig. 6D). Although eosinophil and SCA-1<sup>+</sup>c-Kit<sup>+</sup> cells decreased after day 8 of challenge, SCA-1<sup>+</sup>c-Kit<sup>+</sup>VEGFR2<sup>+</sup> EPC remained elevated in the lung during the time of chronic allergen challenge (Fig. 6E). Overall, rapid EPC recruitment to the lung occurred during the acute challenge and continued to increase and peak during the chronic challenge phase (Fig. 6E). The percentage of progenitor cells followed a similar pattern as the absolute numbers, while there was no significant alteration in the proportion of progenitor cells expressing VEGFR2 (data not shown). Although there was a significant correlation between eosinophil levels and EPC recruitment during the acute phase, eosinophils appeared to increase in BAL only after EPC reached a level of $\sim$1000 cells/lung as indicated by the eosinophils varying directly in proportion to the OVA-sensitized and OVA-challenged (OVA/OVA) mice, but not in the control groups (PBS/PBS, OVA/PBS, or PBS/OVA) (Fig. 5B). The increased levels of EPC in the blood circulation paralleled the recruitment of EPC to the challenged lung (Fig. 5C). The percentage of EPC in the blood followed a similar pattern (data not shown). The lungs were intensively perfused via the heart with PBS to remove all blood to diminish the possibility that the increased numbers of EPC were attributable to the retention of blood in the lung vascular bed. Furthermore, the levels of EPC were similar in the spleen among the groups (Fig. 5D). These experiments indicate that mobilization and recruitment of EPC specifically into the lung was not due to sensitization or challenge alone but occurred only when sensitization was followed by challenge (OVA/OVA group).

The increased recruitment of EPC to the lung during the time of chronic allergen challenge prompted us to determine at which stage the recruitment was initiated and how it progressed over the course of an acute and chronic allergen challenge. A time course experiment was performed during which animals were challenged daily during the first week (acute challenge) and 3 times a week during the subsequent 6 wk (chronic challenge). Eosinophils in the BAL reached a peak at 8 days after the initiation of challenge as previously reported (43) (Fig. 6A), while the levels of lymphocytes, macrophages, and neutrophils did not change significantly over time. Airway hypersensitivity as measured by methacholine challenge also increased during the time of acute challenge, increasing at day 2 of challenge and remaining elevated during chronic challenge in sensitized animals ($p = 0.018$) (Fig. 6B), but there was no significant change compared with day 0 lung resistance in control mice groups (PBS/OVA, PBS/PBS, OVA/PBS). Progenitor cells (SCA-1<sup>+</sup>c-Kit<sup>+</sup> cells) also reached maximum levels at the end of the acute challenge (day 8) (Fig. 6C). Notably, EPC numbers increased in the lung as early as 24 h after the first challenge (Fig. 6D). Although eosinophil and SCA-1<sup>+</sup>c-Kit<sup>+</sup> cells decreased after day 8 of challenge, SCA-1<sup>+</sup>c-Kit<sup>+</sup>VEGFR2<sup>+</sup> EPC remained elevated in the lung during the time of chronic allergen challenge (Fig. 6E). Overall, rapid EPC recruitment to the lung occurred during the acute challenge and continued to increase and peak during the chronic challenge phase (Fig. 6E). The percentage of progenitor cells followed a similar pattern as the absolute numbers, while there was no significant alteration in the proportion of progenitor cells expressing VEGFR2 (data not shown). Although there was a significant correlation between eosinophil levels and EPC recruitment during the acute phase, eosinophils appeared to increase in BAL only after EPC reached a level of $\sim$1000 cells/lung as indicated by the eosinophils varying directly in proportion to the OVA-sensitized and OVA-challenged (OVA/OVA) mice, but not in the control groups (PBS/PBS, OVA/PBS, or PBS/OVA) (Fig. 5B). The increased levels of EPC in the blood circulation paralleled the recruitment of EPC to the challenged lung (Fig. 5C). The percentage of EPC in the blood followed a similar pattern (data not shown). The lungs were intensively perfused via the heart with PBS to remove all blood to diminish the possibility that the increased numbers of EPC were attributable to the retention of blood in the lung vascular bed. Furthermore, the levels of EPC were similar in the spleen among the groups (Fig. 5D). These experiments indicate that mobilization and recruitment of EPC specifically into the lung was not due to sensitization or challenge alone but occurred only when sensitization was followed by challenge (OVA/OVA group).

Recruitment of EPC into the lung is an early and ongoing process during airway allergy challenge

The observation of selective EPC recruitment into the allergic lung prompted us to determine at which stage the recruitment was initiated and how it progressed over the course of an acute and chronic allergen challenge. A time course experiment was performed during which animals were challenged daily during the first week (acute challenge) and 3 times a week during the subsequent 6 wk (chronic challenge). Eosinophils in the BAL reached a peak at 8 days after the initiation of challenge as previously reported (43) (Fig. 6A), while the levels of lymphocytes, macrophages, and neutrophils did not change significantly over time. Airway hypersensitivity as measured by methacholine challenge also increased during the time of acute challenge, increasing at day 2 of challenge and remaining elevated during chronic challenge in sensitized animals ($p = 0.018$) (Fig. 6B), but there was no significant change compared with day 0 lung resistance in control mice groups (PBS/OVA, PBS/PBS, OVA/PBS). Progenitor cells (SCA-1<sup>+</sup>c-Kit<sup>+</sup> cells) also reached maximum levels at the end of the acute challenge (day 8) (Fig. 6C). Notably, EPC numbers increased in the lung as early as 24 h after the first challenge (Fig. 6D). Although eosinophil and SCA-1<sup>+</sup>c-Kit<sup>+</sup> cells decreased after day 8 of challenge, SCA-1<sup>+</sup>c-Kit<sup>+</sup>VEGFR2<sup>+</sup> EPC remained elevated in the lung during the time of chronic allergen challenge (Fig. 6E). Overall, rapid EPC recruitment to the lung occurred during the acute challenge and continued to increase and peak during the chronic challenge phase (Fig. 6E). The percentage of progenitor cells followed a similar pattern as the absolute numbers, while there was no significant alteration in the proportion of progenitor cells expressing VEGFR2 (data not shown). Although there was a significant correlation between eosinophil levels and EPC recruitment during the acute phase, eosinophils appeared to increase in BAL only after EPC reached a level of $\sim$1000 cells/lung as indicated by the eosinophils varying directly in proportion to the OVA-sensitized and OVA-challenged (OVA/OVA) mice, but not in the control groups (PBS/PBS, OVA/PBS, or PBS/OVA) (Fig. 5B). The increased levels of EPC in the blood circulation paralleled the recruitment of EPC to the challenged lung (Fig. 5C). The percentage of EPC in the blood followed a similar pattern (data not shown). The lungs were intensively perfused via the heart with PBS to remove all blood to diminish the possibility that the increased numbers of EPC were attributable to the retention of blood in the lung vascular bed. Furthermore, the levels of EPC were similar in the spleen among the groups (Fig. 5D). These experiments indicate that mobilization and recruitment of EPC specifically into the lung was not due to sensitization or challenge alone but occurred only when sensitization was followed by challenge (OVA/OVA group).
To the exponential of EPC numbers (Fig. 6F). Thus, EPC recruitment to the lung is an early event during acute challenge and is ongoing during the chronic phase of allergen exposure. EPC recruitment paralleled airway resistance even over the chronic challenge phase, a time period during which eosinophil influx resolved but EPC and airway resistance remained high. These findings suggest that airway resistance may be more closely related to EPC than eosinophilic infiltration.

**Angiogenic switch in the lung is an early event during acute allergen challenge and is associated with EPC recruitment**

To determine whether EPC recruitment was associated with new vessel formation in the allergen-challenge murine model of asthma, as it is in other systems (44, 45), we enumerated MVD (number of blood vessels per lung tissue area) on lung tissue sections stained for the VWF. VWF Ag was chosen to stain lung vessels because, in contrast to other EC markers such as CD31 and CD34, VWF is not expressed, or poorly expressed, on alveolar capillaries but is strongly expressed on other lung EC, allowing the identification of nonalveolar blood vessels (46–48) and alveolar space. MVD increased rapidly during the acute phase and gradually increased further during the chronic challenge in OVA/OVA mice. In contrast, MVD did not change over time in control mice (Fig. 7, A and B). Analysis at early time points during the acute challenge phase indicated a significant increase in the MVD already occurring within 24–48 h, suggesting an early angiogenic switch that paralleled the EPC recruitment to the lung after challenge (Fig. 7C). Taken together, these data suggest that the angiogenic switch is an early event during allergen challenge of the lung and is associated with the recruitment of EPC.

**Correlations between EPC recruitment, lung neovascularization, and airway resistance during acute allergen challenge**

There was a significant correlation between EPC recruitment to the lungs, neovascularization of the lung, and the airway resistance...
These correlations indicate that EPC recruitment and increased lung microvessel density are associated with airway hyperreactivity. Moreover, although cellular inflammation decreased during the chronic allergen challenge (Fig. 6A), EPC recruitment to the lung did not diminish and continued to correlate with airway resistance and microvessel density (all $R^2 \geq 0.20$ and all $p \leq 0.03$ for correlations).

**Both Th1 and Th2 cells are needed to induce angiogenic switch**

In the next set of experiments, we analyzed whether angiogenesis in the allergen-challenged lung is Th1 and/or Th2 dependent. Naive T cells or OVA-specific Th1 and Th2 cells were generated in vitro from DO11.10 TCR transgenic mice and transferred into naive, nonirradiated BALB/c mice. Subsequently, these mice were challenged daily and on day 8 their lungs were analyzed for EPC recruitment and angiogenesis. To confirm Th2 and Th1 response in vivo, the lungs of the allergen-challenged animals that received OVA-specific Th1 or Th2 cells were analyzed for inflammation and eosinophil infiltration. Similarly as in previous reports (49, 50), animals receiving Th2 cells typically developed lung inflammation with eosinophil infiltration, while Th1 cells induced a noneosinophilic inflammation (Fig. 9A). Lungs from OVA-sensitized and -challenged mice and from naive mice were stained in parallel as positive control and negative control, respectively. Adoptive transfer of OVA-specific Th1 cells, Th2 cells, or both together were able to recruit EPC in the allergen-challenged lungs (Fig. 9B), although this was lower in the mice that received only Th1 cells. However, MVD analysis indicated that neovascularization occurred only in allergen-challenged lungs when both Th1 and Th2 OVA-specific cells were present (Fig. 9C). These data suggest that although Th1 cells or Th2 cells alone are sufficient to recruit EPC into the lungs, both Th types are needed to switch into a microenvironment that is permissive for angiogenesis.
Discussion

One of the most striking features reported in the early detailed histopathologic studies of asthmatic lungs was the increased amount and size of submucosal vessels (20), and this has been repeatedly confirmed in other, more recent, reports (21, 24–27). It has been speculated that the enlarged airway vascular bed contributes to the airflow limitation of asthma either through the vascular tissue itself, increasing the airway wall thickness, and/or through edema formation. However, whether asthma-associated angiogenesis is an epiphenomenon secondary to chronic inflammation and/or reparative processes or an important early step during the genesis of asthma is unknown. Furthermore, how and when the endothelial progenitor, a recently discovered cell type (7) essential to the formation of new blood vessels in other physiological and pathological conditions (2–6), is involved in the increased vessel density in asthma is unknown.

In this study, individuals with stable asthma are shown to have striking and consistently increased levels of circulating EPC in their blood, levels of which are unrelated to the severity of the asthma or to indicators of atopy. The EPC maintain a highly proliferative phenotype ex vivo and exhibit an enhanced angiogenesis capacity in tube formation assays using unrelated EC, suggesting that the intrinsic capacity to incorporate into vessels and promote angiogenesis is greater for asthmatic EPC as compared with non-asthmatic control cells. Although angiogenesis has been described in allergic rhinitis (51, 52), circulating EPC levels and CFU-EC were not increased. Steady-state levels of EPC are apparently sufficient to sustain angiogenesis restricted to the small surface area of the upper airway in the nasal mucosa. However, as inflammation and angiogenesis are interrelated (53), it is likely that increased EPC mobilization occurs during other extensive inflammatory processes. The OVA murine model of allergic asthma recapitulates the human asthma condition in that there are increased numbers of circulating EPC and increased lung vessels following allergen exposure in sensitized animals, allowing this model to be used to investigate the angiogenesis process in asthma.

The murine asthma model provides clear evidence that the mobilized EPC are selectively recruited into the allergic lungs. Kinetic experiments show that EPC mobilization occurs as one of the early responses to allergen, i.e., within 24 h after challenge, and that the
mobilization is followed by a switch to an angiogenic lung environment as demonstrated by the accelerated increase in lung vascularization. Adoptive transfer experiments indicated that both Th1 and Th2 responses together are necessary to induce angiogenesis in the allergen-challenged lungs and are in line with the observation that although there is a Th2-skewed response in this disease, a full asthmatic phenotype, including eosinophilic airway inflammation and airway hyperreactivity, is dependent on the presence both Th1 and Th2 cells and not on Th1 or Th2 alone (50, 54, 55). It is noteworthy that the inflammation peak, as monitored by eosinophil count in the BAL fluid and airway hyperresponsiveness, occurs during but does not precede the stage of accelerated angiogenesis. Further evidence that angiogenesis is not a response to the acute inflammatory cell influx is that during chronic allergen exposure the EPC levels remain at an elevated plateau level and new vessels do not recede but rather continue to increase at a moderate level, although acute inflammation resolves significantly. The moderate but ongoing increase in angiogenesis during the chronic allergen exposure suggests that the neovascularization in asthma is not dependent solely on inflammation, although this does not exclude the possibility that the initiation of EPC mobilization and recruitment into the lungs is triggered by the acute inflammation. Using this same murine asthma model, it has been reported that eosinophil numbers increase significantly in the BAL fluid within hours after allergen challenge (56) and that eosinophils or other inflammatory cells may contribute to the initiation of EPC mobilization through the release of a myriad of angiogenic factors (53, 57). Activated lung resident mast cells, another rich source of angiogenic factors such as VEGF (53), may also contribute to the EPC recruitment. In this context, the mobilization of EPC has been reported recently in other inflammatory lung conditions. Increased endothelial progenitors have been observed in the circulation of patients with bacterial pneumonia (58), and circulating EPC in patients with acute lung injury is associated with improved survival (19). These reports suggest that EPC recruitment in the lung is necessary for repair following injury (19, 58, 59). In this study, however, the mobilization and recruitment of EPC appears as an early event, suggesting participation in the pathogenesis. In support of this concept, the mobilization of other progenitors plays an active role in the pathogenesis of asthma. For example, circulating fibrocyte precursors contribute to the genesis of subepithelial fibrosis in asthma patients (44). In the allergen challenge murine model, early and ongoing eosinophil progenitor mobilization and recruitment into the lung contributes to ongoing allergic inflammation (56). Regardless of whether angiogenesis is a reparative response and/or participatory in the pathogenesis of asthma, the direct correlation between vessel density in the lung and the recruitment of EPC in this study is consistent with other recent studies showing a striking correlation between EPC levels and angiogenesis by using animal tumor models, corneal neovascularization assays, and Matrigel plug perfusion assays (45, 60). Collectively these studies provide confidence that EPC levels serve as a valid independent biomarker for ongoing angiogenesis in vivo (45, 60). Given the fact that CD34<sup>+</sup>CD133<sup>+</sup> progenitors are pluripotent, we cannot exclude the possibility that these precursors may also give rise to other cells that participate in the genesis of asthma, in particular mast cells and fibrocytes (61).

Although there is strong evidence from this and other studies (2–6) that EPC are essential to angiogenesis, the exact mechanisms remain controversial (60). According to current concepts, EPC can contribute to neovascularization by serving as building blocks for the newly formed blood vessels and/or by the activation of a tissue-resident endothelium in a paracrine manner (6, 37, 38). The relative state of balance between proangiogenic and antiangiogenic molecules regulates new vessel formation and regression, in part by effects on EPC. The term angiogenic switch has been proposed to refer to the tissue environment when the balance of angiogenic inhibitors and activators is shifted toward an overall proangiogenic state (62, 63). Our data suggest that this proangiogenic milieu in allergic lungs is only reached in the presence of both Th1 and Th2 cells. Indeed, both Th1 and Th2 (64, 65) cytokines can modulate neovascularization by controlling EC function, including migration, invasion, proliferation, and apoptosis, all of which are crucial for the formation of new blood vessels.

VEGF is one of the key proangiogenic factors in physiological and pathological neovascularization and very likely plays a role in neovascularization in asthma (66, 67). VEGF, one of the major angiogenic factors secreted by EPC (68), is increased in the spum (69–71) and BAL fluid of individuals with asthma as compared with healthy controls (72). In this study, VEGF is also increased in the BAL of asthmatics as compared with healthy controls, supporting the concept of an angiogenic environment in the asthmatic lung. In the BALB/c murine model of asthma, the treatment of animals with antiangiogenic agents results in reduced inflammation (73, 74), decreased airway hyperresponsiveness, and the reduction of OVA-specific IgE (74). Definitive evidence of a role for angiogenesis, and likely VEGF, in asthma pathogenesis is provided by a transgenic mouse model with lung-targeted, regulatable VEGF expression. In this model, the overexpression of VEGF induced an angiogenic switch and airway remodeling and also predisposed to an asthmatic phenotype by enhancing Th2-mediated sensitization and inducing inflammation in the lung (75). Perhaps most striking is the rapidity of new vessel formation in this model, with the outgrowth of new blood vessels occurring within 1 day after the onset of VEGF expression in the lung (76). Angiogenesis in asthma may play multiple roles in disease progression, including the recruitment of inflammatory cells to the airways, the contribution to altered airway physiology, and the secretion of a variety of mediators that determine or regulate the inflammatory response (27, 28, 77). Interestingly, increased vascularization of the asthmatic lung appears to be the only component of airway remodeling that is fully reversible with treatment using glucocorticosteroids (78). Taken together, these data suggest that neovascularization in asthma contributes to the disease process and may serve as a therapeutic target.

At the time of diagnosis, expansion of submucosal vessels is already well established in all asthma patients, including mild to severe phenotypes (24). In this study, using an acute allergen challenge model of asthma, EPC mobilization and recruitment to the lungs are shown to be very early events before the genesis of inflammation and are followed by an Th1- and Th2-dependent angiogenic switch in the lung. Importantly, analogous to the findings in human asthma, EPC mobilization/recruitment and angiogenesis, as well as airway hyperreactivity, continue over the course of the chronic allergen exposure murine model, which suggest that the model system is relevant for understanding the relation of new vessel formation to asthma in the human. Thus, together these data suggest that angiogenesis is not an epiphenomenon of chronic asthma but an early step in the onset of the disease in which highly proliferating and angiogenic potent EPC play an active role.

**Acknowledgments**

We thank L. Vargo, J. Drazba, A. Vasanji, and J. Peterson in the Lerner Research Institute Digital Imaging Core, J. Pyle and M. Baaklini for assistance in volunteer recruitment, S. Comhair for the clinical samples database, C. Shemo and S. O’Bryant in the Lerner Research Institute Flow Cytometry Core, S. Dannenmiller in the Lerner Research Institute Biological Resources Unit, and Lauren Licina for excellent technical assistance.
Disclosures
The authors have no financial conflict of interest.

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