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Nascent Endothelium Initiates Th2 Polarization of Asthma

Kewal Asosingh,* Georgiana Cheng,* Weiling Xu,* Benjamin M. Savasky,*
Mark A. Aronica,*[†] Xiaoxia Li,[‡] and Serpil C. Erzurum*[†]

Asthma airway remodeling is linked to Th2 inflammation. Angiogenesis is a consistent feature of airway remodeling, but its contribution to pathophysiology remains unclear. We hypothesized that nascent endothelial cells in newly forming vessels are sufficient to initiate Th2-inflammation. Vascular endothelial (VE)-cadherin is a constitutively expressed endothelial cell adhesion molecule that is exposed in its monomer form on endothelial tip cells prior to adherens junction formation. Abs targeted to VE-cadherin monomers inhibit angiogenesis by blocking this adherens junction formation. In this study, VE-cadherin monomer Ab reduced angiogenesis in the lungs of the allergen-induced murine asthma model. Strikingly, Th2 responses including, IgE production, eosinophil infiltration of the airway, subepithelial fibrosis, mucus metaplasia, and airway-hyperreactivity were also attenuated by VE-cadherin blockade, via mechanisms that blunted endothelial IL-25 and proangiogenic progenitor cell thymic stromal lymphopoietin production. The results identify angiogenic responses in the origins of atopic inflammation. *The Journal of Immunology*, 2013, 190: 3458–3465.

The origins of atopic inflammation in asthma are linked to epithelial and stromal cell derived cytokine products such as thymic stromal lymphopoietin (TSLP) and IL-25, and downstream Th2 type lymphocyte and B cell IgE responses. Th2-activated signal transducer and activation transcription factor-6 (STAT6) is the central intracellular pathway for mucus hypersecretion of the epithelium, smooth muscle hyperplasia and hyperreactivity during initial stages of asthma (1–8). In chronic stages, however, STAT6-independent pathways can also be involved (1–8). It is increasingly recognized that innate host responses by structural cells, such as the epithelium, are critically important in the origin of Th2 cytokine-driven inflammation (9). Despite the fact that vascular endothelium is robustly able to activate T lymphocytes (10), little is known regarding the contribution of lung vascular endothelium to the genesis of Th2 inflammation. In support of endothelial mechanisms in asthma, neovascularization of the airway is a consistent histopathologic feature of asthma related to disease severity (7, 11–13). In the acute OVA murine allergic airway inflammation model of asthma, lung angiogenesis occurs before onset of airway reactivity, and far in advance of the development of Th2 inflammation in the lung (12). Bone marrow-derived SCA-1⁺C-kit⁺VEGFR2⁺ proangiogenic progenitor cells home to the lungs within hours after allergen challenge and initiate an angiogenic switch. The lung neovascularization is followed

temporally by an influx of eosinophils (14, 15), suggesting that angiogenesis is a first step in the onset of allergic inflammation. Although angiogenesis is well recognized as a participant in the host inflammatory response, it is unknown whether angiogenesis or disruption of stable vessels triggers inflammation. The endothelial junction protein vascular endothelial (VE)-cadherin is essential to the assembly of endothelial cells into mature stabilized vascular tubes (16). Blocking of VE-cadherin during development does not inhibit the initial alignment of endothelial cells, but it induces rapid disassembly of nascent blood vessels (17). Genetic deletion provides conclusive evidence for an indispensable role for VE-cadherin; VE-cadherin-deficient mice die in utero because of severely impaired vasculature (16). A biologic approach using a highly sensitive and specific VE-cadherin blocking Ab, E4G10, has been informative in investigating pathologic neovascularization in the adult animal (18–20). In quiescent vessels, VE-cadherin dimers are responsible for the tight endothelial homotypic adhesion, and the monomer form is not present on endothelial surfaces. Consistent with this finding, E4G10 only binds to nascent vascular endothelium that form during angiogenesis, when VE-cadherin is not yet engaged in the dimers of adherens junctions (19). Likewise, E4G10 is unable to bind or disrupt quiescent vascular endothelium in which VE-cadherin monomers are unexposed (18, 19). Indeed, E4G10 administration in vivo specifically inhibits nascent endothelium and blocks new vessel formation (18–20). In this study, we used E4G10 to test the hypothesis that neovascularization is an initiating mechanistic step in the genesis of the Th2 allergic airway inflammation in a murine model of asthma.

Materials and Methods

Animals

Immunocompetent and nonirradiated female BALB/c mice (6–8 wk old) were purchased from the Jackson Laboratory (Bar Harbor, ME) and used for ova or saline (vehicle) sensitization and challenge as described previously (12, 15). All experiments were approved by the local Institutional Animal Care and Use Committee (IACUC).

OVA sensitization, E4G10 treatment, and methacholine challenge

Standard induction of allergic airways disease was performed as reported previously (12, 15, 21, 22). For this purpose, BALB/c mice were first immunized by i.p. injection with OVA (Sigma Chemicals; 10 µg, adsorbed in Al(OH)₃). After 2 wk, mice were challenged with aerosolized OVA in

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Abbreviations used in this article: AHR, airway hyperreactivity; BALF, bronchoalveolar lavage fluid; Dil-AcLDL, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-acetylated low-density lipoprotein; PAS, periodic acid-Schiff; STAT6, signal transducer and activation transcription factor-6; TSLP, thymic stromal lymphopoietin; VE, vascular endothelial.

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a chamber that was kept saturated with nebulized OVA solution (1% w/v in sterile PBS). Animals were allowed to inhale the allergen for 40 min each day for 6 days, unless indicated differently. Intraperitoneal injections with E4G10 Abs (donated by Dr. Seema Iyer, ImClone Systems) or control IgG were performed according to the schedule in Fig. 2. To analyze if E4G10 treatment directly affected eosinophil recruitment, naive mice were treated with Abs 24 h prior to intranasal eotaxin-I instillation (600 ng per 50 μ l saline). The number of eosinophils in the lung tissue was analyzed by staining for eosinophilic major basic protein 24 h after eotaxin instillation. Twenty-four hours after the last OVA exposure, animals were anesthetized by i.p. injection with pentobarbital and placed on a rodent ventilator inside a body plethysmography chamber. Measurement of lung mechanics was done using the FlexiVent ventilator (FlexiVent, Scireq, Montreal, Canada). Airway hyperreactivity (AHR) and lung mechanics were measured on mice in response to increasing doses of inhaled methacholine as described previously (23, 24).

Bronchoalveolar lavage fluid collection and characterization

Bronchoalveolar lavage fluid (BALF) was collected after instilling 700 μ l sterile saline and then withdrawing the fluid with gentle aspiration via the syringe. Typical 400–600 μ l BALF was collected per mouse. The number of leukocytes was counted using a hemocytometer, after which cytologic examination was performed on cytospin preparations fixed and stained using Diff Quick (American Scientific Products, McGaw Park, IL). Differential counts were performed based on counts of 200 cells using standard morphologic criteria to classify inflammatory cells as eosinophils, lymphocytes, neutrophils, or alveolar macrophages. All counts were performed by a single observer blinded to the study groups.

Quantification of microvessel density

Lung microvessel density was quantified as described previously after staining tissue sections with polyclonal rabbit anti-Von Willebrand Factor Abs (Dako Cytomation, Glostrup, Denmark) (12, 15).

Goblet cell metaplasia

Standard periodic acid–Schiff (PAS) staining of paraffin-embedded lung tissue sections was used to visualize goblet cells. Goblet cell metaplasia was measured by quantification of percentage of goblet cell per bronchiole (25–27).

Trichrome staining and quantification

Trichrome staining was used to visualize and measure airway fibrosis in mouse lung tissue sections (25–27). To measure total fibrosis in the samples, digital mosaic images were collected for each section on a Leica DM 5000B upright microscope (Leica Microsystems CMS, Wetzlar, Germany) with a Prior H101 motorized stage (Prior Scientific, Rockland, MA) and a QImaging Retiga-SRV CCD camera (QImaging, Surrey, Canada) using an HC Plan APO 10 $\times$ /0.40 objective. Image-Pro Plus version 6.1 (Media Cybernetics, Bethesda, MD) with the Pro-Series Turboscan plug-in (Objective Imaging, Cambridge, U.K.) was used to control the Prior stage and perform tile scanning of the entire section for each sample to generate a mosaic image. The area of fibrosis stained blue by trichrome (collagen deposition) and total tissue area of the mosaic images were quantified using Image-Pro Plus v.6.1. Fibrosis was quantified across all sections by first establishing the threshold criteria for the blue staining using a test set of images in Image-Pro Plus version 6.1. The same selection criteria for fibrosis were then applied to all the mosaic images to quantify the area of fibrosis. Area of fibrosis per total tissue area was then calculated.

Isolation of proangiogenic hematopoietic progenitor cells

Proangiogenic hematopoietic progenitors were isolated as described previously (28–31). In short, bone marrow mononuclear cells were seeded on fibronectin-coated (1 mg/cm² fibronectin; Sigma-Aldrich, Milwaukee, WI) 12-well plates at a concentration of 8×10^6 cells/ml in 20% FBS, 1% penicillin–streptomycin in EBM-2 medium supplemented with 20 ng/ml vascular endothelial cell growth factor (VEGF; Invitrogen, Carlsbad, CA) added. Colonies of adherent cells were trypsinized at day 7 and characterized by flow cytometric staining using anti-mouse CD45-FITC (eBioscience, San Diego, CA), SCA-1-APC, C-kit-FITC (eBioscience) and VEGFR2-PE (BD Bioscience, San Jose, CA). Isotype controls were used as negative staining. In some experiments proangiogenic progenitor cells were directly detected among bone marrow mononuclear cells as described previously (12, 15).

Isolation of lung endothelial cells

Lung endothelial cells were isolated as described previously (15). Lungs were perfused with warm PBS via the heart to remove all blood cells,

minced, and dissociated in 0.1% Collagenase A (Roche Applied Science, Indianapolis, IN), 0.04% DNase I (Sigma-Aldrich), 0.5 mM CaCl₂ (Sigma-Aldrich) in 1 ml Dispase II (Roche Applied Science). Dead cells were removed by using MACS Dead Cell Removal Kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). Viable cells were labeled with anti-mouse CD31 (eBioscience) and sorted by magnetic activated cell sorting. Obtained endothelial cells were seeded on rat-tail collagen I coated plates in MCDB-131 complete medium (VEC Technologies, Rensselaer, NY). Purity of CD31⁺ fraction was assessed by flow cytometry analysis and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-acetylated low-density lipoprotein (Dil-AcLDL; Molecular Probes) uptake. Where indicated, single-cell suspensions after lung digestions were used for flow cytometric analysis of endothelial cells proliferation. Cells were stained for cell surface expression of anti-mouse CD45-APC and CD31-PE-Cy7 (eBioscience), followed by intracellular staining for proliferation marker Ki-67-FITC (BD Biosciences). Dead cells were excluded by staining with ultraviolet Live/Dead dye (Invitrogen). All samples were analyzed on LSRII flow cytometer and data were analyzed using FlowJo software version 9.0.2 (TreeStar).

Secretion of Th2 cytokines and plasma IgE

Lung endothelial cells (0.5×10^6) or bone marrow–derived proangiogenic progenitors (100×10^3) were incubated in MCDB-131 medium. At day 5, supernatants were collected and stored at -80°C until analysis. Levels of IL-25, TSLP, and IL-33 were measured using BioLegend (IL-25) or R&D Quantikine ELISA Kits (TSLP and IL-33). Values from different experiments were normalized against the PBS/PBS group. Eotaxin-2 and IL-13 levels in BALF were also measured using R&D quantikine ELISA kits. Values were normalized against total protein concentration. OVA-specific plasma IgE levels were analyzed using OVA-IgE ELISA kit (MD Bio-products).

STAT6 EMSA

Whole cell extracts from mice lungs were prepared as described previously (32). The duplex oligonucleotide high-affinity sis-inducible element (hSIE) (5'-AGCTTCATTTCCCGTAAATCCCTAAAGCTA-3') and duplex oligonucleotide (5'-GATCGCTCTTCTTCCAGGAAGTCAATG-3') specific for STAT6 binding (33), with the consensus sequences underlined sequences used in EMSA, were end-labeled with [γ -³²P] ATP by T4 polynucleotide kinase (Invitrogen, Carlsbad, CA). To specifically identify DNA-binding-factor in binding complexes, rabbit polyclonal anti-STAT6 Ab (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the binding reaction mix.

Statistical analysis

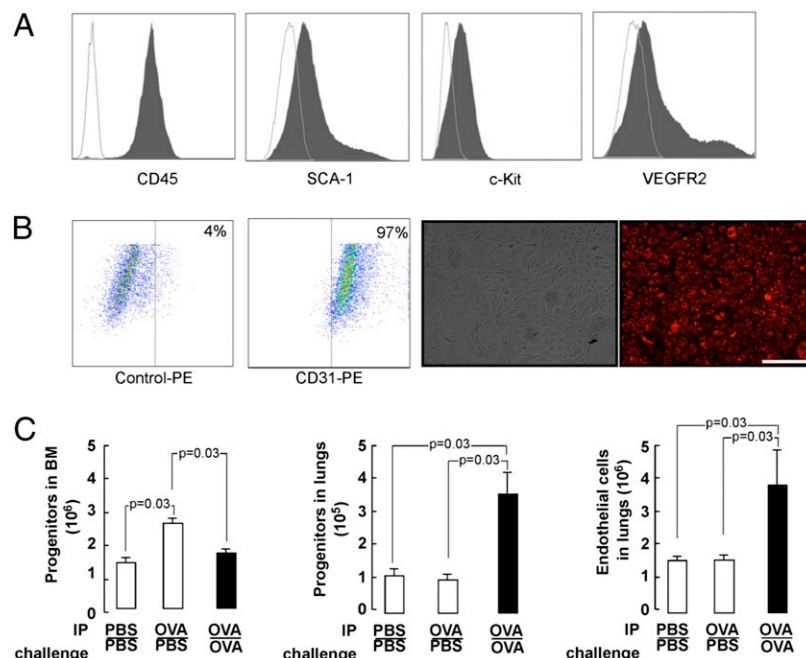
Data were analyzed by using JMP 5.1 software. ANOVA or Student *t* test were used for comparisons of parametric data, and Wilcoxon or median test were used for comparison of nonparametric data, as appropriate; *p* < 0.05 was considered significant. Mean $\pm$ SEM value for each group is shown.

Results

Bone marrow hematopoietic proangiogenic progenitor cell and lung endothelial cell proliferation are initial events after allergen exposure

Twenty-four hours after the first airway allergen exposure, numbers of proangiogenic progenitors in bone marrow and lungs and numbers of endothelial cells in the lung were analyzed (Fig. 1). Hematopoietic proangiogenic progenitors isolated from the bone marrow were CD45, SCA-1, c-Kit, and VEGFR2 positive, consistent with previous reports (12, 15) (Fig. 1A). Endothelial cells isolated from the lungs were ascertained to be endothelial phenotype by positivity for CD31, a prototypical endothelial cell surface marker, and by the uptake of Dil-AcLDL (Fig. 1B). The number of proangiogenic progenitor cells in the bone marrow was increased after allergen OVA, but not after sham PBS, sensitization (proangiogenic progenitor cells $\times 10^6$ /bone marrow aspirate: PBS/PBS, 1.3 ± 0.14 ; OVA/PBS, 2.3 ± 0.15 ; OVA/OVA, 1.5 ± 0.1 ; ANOVA, *p* < 0.0001; Fig. 1C). The lower number of proangiogenic cells in the bone marrow of OVA allergen-challenged mice (OVA/OVA) compared with the PBS-challenged mice (OVA/PBS) was consistent with mobilization from the bone marrow and

FIGURE 1. Bone marrow proangiogenic progenitor cell and lung endothelial cell proliferation within 24 h of murine asthma model. OVA allergen or sham-sensitized mice were sensitized and then exposed to aerosolized OVA or sham inhalation. Twenty-four hours after the first allergen inhalation, samples were collected. **(A)** Bone marrow mononuclear cells were separated to isolate proangiogenic hematopoietic progenitor cells. Ag expression was analyzed by flow cytometry. Filled histograms show the level of cell surface expression; open histograms are control stainings. **(B)** Lung endothelial cells were isolated after whole organ digestion and dead cell removal, followed by CD45 depletion and sorting of CD31⁺ cells. CD31 purity of obtained endothelial cells, phase contrast image, and DiI-AcLDL uptake by endothelial cells are shown. Scale bar, 50 μ m. **(C)** Quantification of the number of isolated proangiogenic progenitor cells and lung endothelial cells. Mean and SEs of three mice per group are shown.



recruitment into the lungs, as suggested by greater numbers of proangiogenic progenitor cells in the lungs of only the OVA/OVA mice (proangiogenic progenitor cells $\times 10^6$ /lung: PBS/PBS, 0.1 ± 0.2 ; OVA/PBS, 0.1 ± 0.09 ; OVA/OVA, 0.37 ± 0.6 ; ANOVA, $p < 0.0001$). Parallel to the influx of proangiogenic progenitor cells in the lung, the number of CD45⁺CD31⁺ lung endothelial cells in the lung doubled (Fig. 1C; endothelial cells $\times 10^6$ /lung: PBS/PBS, 1.6 ± 0.02 ; OVA/PBS, 1.6 ± 0.1 ; OVA/OVA, 3.9 ± 1.6 ; ANOVA, $p = 0.04$). Overall, the data confirm that bone marrow-derived proangiogenic progenitor cell mobilization and recruitment to the

lung and lung endothelial cell proliferation are early events in the development of allergic airway disease.

Blockade of VE-cadherin inhibits angiogenesis in asthmatic lung

To investigate whether the neovascularization is actively involved in airway inflammation and remodeling, nascent endothelial cell formation of junctions was blocked by E4G10 rat anti-VE-cadherin. E4G10 (1.5 mg/mouse) or control Ab (cIgG) was administered i.p. three times per week as described previously (18, 19) (Fig. 2A). At

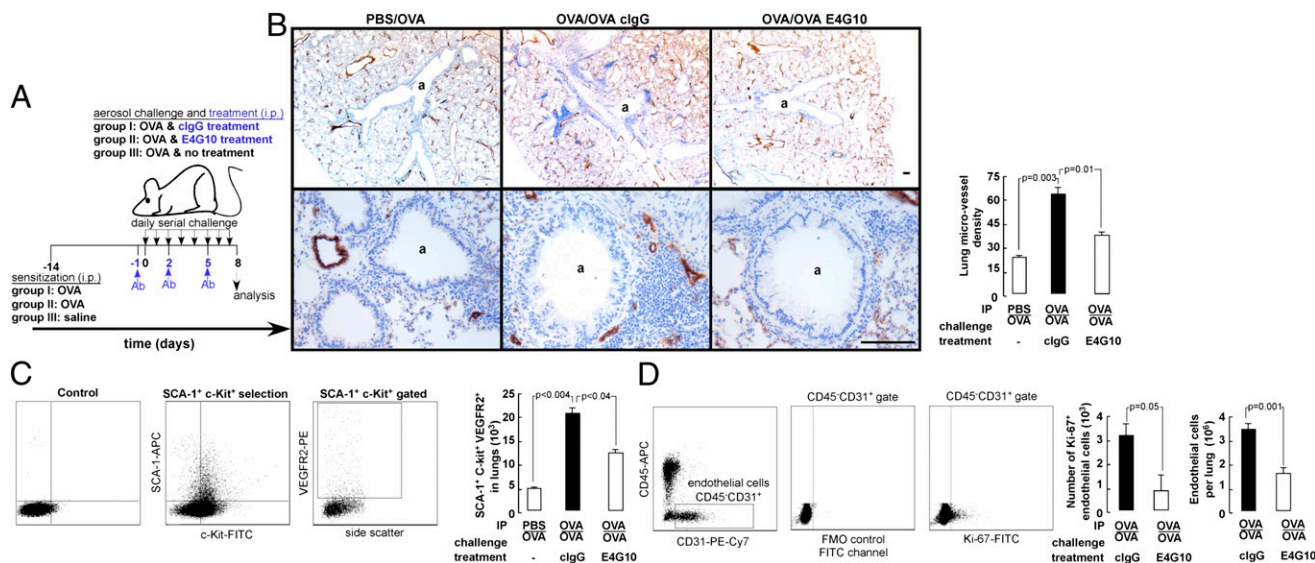


FIGURE 2. Inhibition of angiogenesis and progenitor cell recruitment to the lungs by blockade of VE-cadherin. **(A)** Model system for the investigation of the effects of VE-cadherin and angiogenesis in asthma. Female BALB/c mice (6–8 wk old) were sensitized with OVA allergen or vehicle. On indicated days (days -1, 2, and 5), mice received an i.p. injection of 1.5 mg E4G10 or control Abs. Two weeks after sensitization, mice were exposed to a series of aerosolized allergen or vehicle challenges for 1 wk, after which analyses were performed. Each group contained six mice. **(B)** Low- and high-power images of lung tissue sections stained for von Willebrand factor to visualize blood vessels and quantification of vessels density per lung tissue area. Scale bar, 150 μ m. **(C)** Flow cytometric enumeration of progenitor cells in the lungs. After tissue digestion, single-cell suspensions were stained for progenitor cell Ags. The numbers of progenitors per lung were quantified. **(D)** The effect of VE-cadherin on angiogenic endothelial cell proliferation, 24 h after first allergen challenge, was measured by flow cytometry. Endothelial cells were gated as pan-hematopoietic cell marker CD45⁺ and CD31⁺, followed by quantification of cell proliferation marker Ki-67 expression in the gated endothelial cells. The number of proliferating endothelial cells and the total number of endothelial cells were calculated based on total single-cell counts after whole lung digestion. Mean and SEs of six mice per group are shown.

indicated time points, samples were collected for analysis. In a pilot experiment, an OVA/OVA group without control Ab injection was included, and no differences were observed in airway inflammation and airway remodeling between cIgG-treated mice and control Ab-treated animals at day 8 of analysis. Animals sensitized and challenged with OVA (OVA/OVA) had greater microvessel density in the lungs compared with PBS/OVA mice, demonstrating increased angiogenesis during allergic airway inflammation (Fig. 2). Treatment of OVA/OVA mice with E4G10 inhibited angiogenesis (lung microvessel density: PBS/OVA, 25.9 ± 1.4 ; OVA/OVA with cIgG, 61.1 ± 5.8 ; OVA/OVA with E4G10, 37.1 ± 3.6 ; ANOVA, $p < 0.0001$; Fig. 2B). SCA-1⁺C-kit⁺VEGFR2⁺ proangiogenic progenitor cell recruitment into the lungs was also significantly reduced in E4G10-treated mice, indicating that the blockade of VE-cadherin also blocks bone marrow proangiogenic progenitor recruitment (Fig. 2C; SCA-1⁺C-kit⁺VEGFR2⁺ $\times 10^3$ /lung: PBS/OVA, 5.5 ± 0.7 ; OVA/OVA with cIgG, 20.8 ± 6.4 ; OVA/OVA with E4G10, 13.0 ± 2.0 ; ANOVA, $p < 0.0001$). In vivo blockade of VE-cadherin also decreased lung endothelial cell counts and endothelial cell proliferation, as measured by flow cytometric analyses for Ki-67 (Fig. 2D; % Ki-67⁺ lung endothelial cells: OVA/OVA with cIgG, 2.3 ± 0.2 ; OVA/OVA with E4G10, 1.5 ± 0.2 , $p = 0.049$; number of

endothelial cells/lung ($\times 10^6$): OVA/OVA with cIgG, 3.4 ± 0.2 ; OVA/OVA with E4G10, 1.7 ± 0.2 , $p = 0.001$). Thus, blockade of VE-cadherin reduced angiogenesis and inhibited mobilization or recruitment of proangiogenic progenitors into the lung from the circulation.

Decreased angiogenesis reduces airway inflammation, Th2 cytokines, and OVA-specific IgE

H&E staining of lung tissue sections revealed substantial reduction of airway inflammation in OVA/OVA mice treated with E4G10 (Fig. 3A). The numbers of eosinophils in lung tissue sections (Fig. 3B, 3C) and in the BALF (Fig. 3D) were reduced by VE-cadherin blockade (eosinophils 10^3 /ml BALF: PBS/OVA, 0.0 ± 0.0 ; OVA/OVA with cIgG, 57 ± 7 ; OVA/OVA with E4G10, 20 ± 6 ; ANOVA, $p < 0.0001$; 10^3 /mm² lung tissue: PBS/OVA, 0.11 ± 0.04 ; OVA/OVA with cIgG, 1.5 ± 0.48 ; OVA/OVA with E4G10, 0.61 ± 0.15 ; ANOVA, $p < 0.003$). Intranasal eotaxin-1 instillation induced eosinophilia in naive mice (number of eosinophils per 0.1 mm² lung tissue: naive mice, 21 ± 6 ; eotaxin-1-instilled naive mice, 44 ± 4 ; $p = 0.03$ [$n = 4$ mice/group]). Eotaxin-1-induced eosinophil influx was similar among E4G10 or control IgG-treated mice (number of eosinophils per 0.1 mm² lung tissue:

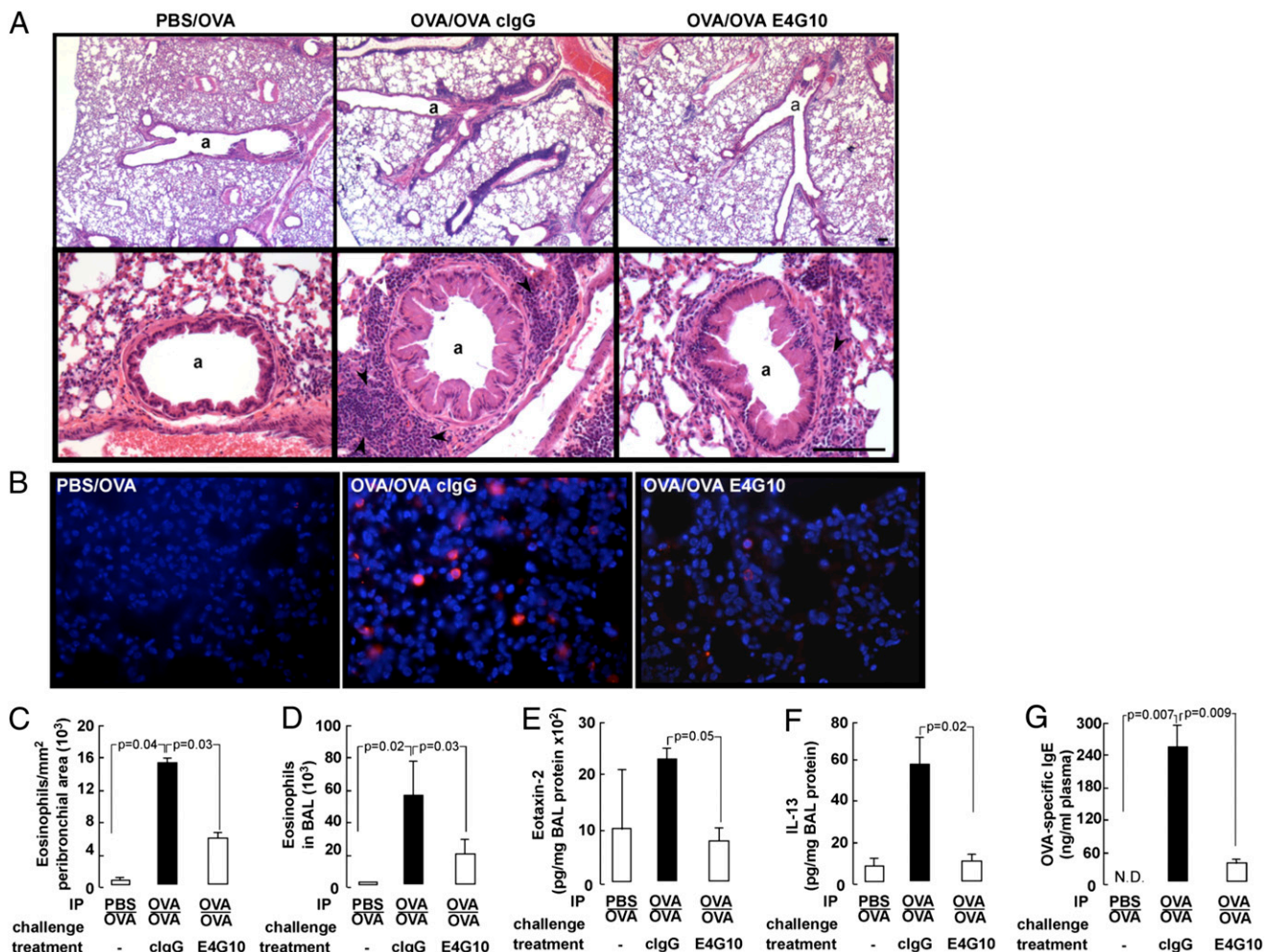


FIGURE 3. VE-cadherin blockade reduces inflammation in the mouse asthma model. (A) H&E staining of lung tissue sections at low-power magnification (original magnification $\times 5$; upper panel) and high-power magnification (original magnification $\times 20$; lower panel) show inhibition of inflammation by VE-cadherin blockade. The *a* indicates the airway, and black arrows indicate inflammatory infiltrates. Scale bar, 150 μ m. (B and C) Eosinophil-specific major basic protein staining shows a lower number of eosinophils per lung tissue area. Eosinophil specific major basic protein staining is shown in red, and nuclei is shown in blue (DAPI staining). (D) Eosinophils in BALF were reduced in mice receiving anti-VE-cadherin compared with control Abs and were similar to sham control mice. (E and F) Th2 cytokines eotaxin and IL-13 concentrations were lower in BALF of mice treated with anti-VE-cadherin. (G) Effect of VE-cadherin blockade on OVA-specific IgE levels. Mean and SEs of six mice per group are shown.

cIgG-treated and eotaxin-1-instilled naive mice, 40 ± 3 ; E4G10-treated and eotaxin-1-instilled naive mice, 44 ± 4 ; $p = 0.41$ [$n = 4$ mice/group]). In contrast, E4G10 treatment in the OVA model abrogated levels of Th2 cytokine eotaxin (Fig. 3E) and IL-13 (Fig. 3F) in BALF (eotaxin-2 in BALF [pg/mg protein]: OVA/OVA cIgG, 2434 ± 108 ; OVA/OVA E4G10, 566 ± 286 ; $p = 0.049$; IL-13 in BAL: OVA/OVA cIgG, 58 ± 18 ; OVA/OVA E4G10, 14 ± 4 pg/mg protein; $p = 0.02$). Statistical significance was not reached for levels of eotaxin-1 (eotaxin-1 in BALF [pg/mg protein]: OVA/OVA cIgG, 353 ± 197 ; OVA/OVA E4G10, 89 ± 21 ; $p = 0.19$). Immunohistochemistry staining showed expression of VEGF by airway epithelial cells, but no obvious differences were observed between cIgG and E4G10 groups (not shown). TSLP plays a critical role in IgE production via activation of dendritic cells, which primes Th2 T cells to induce isotype switching in B cells (9). OVA-specific IgE levels were strikingly reduced in the E4G10-treated group (Fig. 3G): (OVA-specific IgE [ng/ml plasma]: PBS/OVA, undetectable levels; OVA/OVA cIgG, 250.4 ± 48.8 ; OVA/OVA E4G10, 37.9 ± 3.4 ; ANOVA, $p = 0.0002$). Overall, blockade of new vessel formation by VE-cadherin Ab reduced Th2 cytokines and IgE production, indicating that blockade of angiogenesis inhibits the polarization of T cells and differentiation of B cells to IgE production, all of which resulted in the lesser airway inflammation.

Blockade of VE-cadherin inhibits airway remodeling

Airway fibrosis and goblet cell metaplasia are important remodeling outcomes of Th2-reactions in asthma. Airway fibrosis analyzed by trichrome staining for collagen deposition was lower in animals treated with E4G10 compared with cIgG (trichrome area mm^2 per 100mm^2 lung: PBS/OVA, 1.7 ± 0.2 ; OVA/OVA with

cIgG, 2.7 ± 0.3 ; OVA/OVA with E4G10, 1.3 ± 0.5 ; ANOVA, $p = 0.037$; Fig. 4A). Mucus-producing goblet cells in the airways detected with PAS histochemistry were $\sim 50\%$ lower in mice treated with E4G10 compared with control IgG (% PAS⁺ cell/bronchiole: PBS/OVA, 4 ± 2 ; OVA/OVA with cIgG, 84 ± 3 ; OVA/OVA with E4G10, 36 ± 3 ; ANOVA, $p < 0.0001$; Fig. 4B). These results show that asthmatic airway remodeling, including airway fibrosis and goblet cell hypertrophy, requires nascent endothelium with available VE-cadherin.

Angiogenesis affects airway mechanics

Airway reactivity to methacholine was measured to analyze the effect of angiogenesis inhibition on AHR. As anticipated, airway resistance was greater in the cIgG-treated OVA/OVA group compared with the OVA/PBS group (Fig. 5A). E4G10-treated OVA/OVA mice had methacholine reactivity significantly lower than cIgG-treated OVA/OVA mice. Airway elastance was significantly higher in the cIgG OVA/OVA mice compared with E4G10 OVA/OVA mice (Fig. 5B). The data are consistent with the decreased collagen deposition and mucus secretion in E4G10 OVA/OVA mice, supporting the notion that angiogenesis participates in the cellular mechanisms that lead to airway inflammation and hyperresponsiveness in asthma.

Mechanisms by which VE-cadherin blockade inhibits asthma phenotype development: IL-25 and TSLP expression and STAT6 activation

To investigate mechanisms underlying the inhibitory effect of VE-cadherin blockade on the genesis of atopic inflammation, IL-25 and TSLP production by endothelial cells and proangiogenic progenitor cells isolated from mice after challenge or Ab exposure,

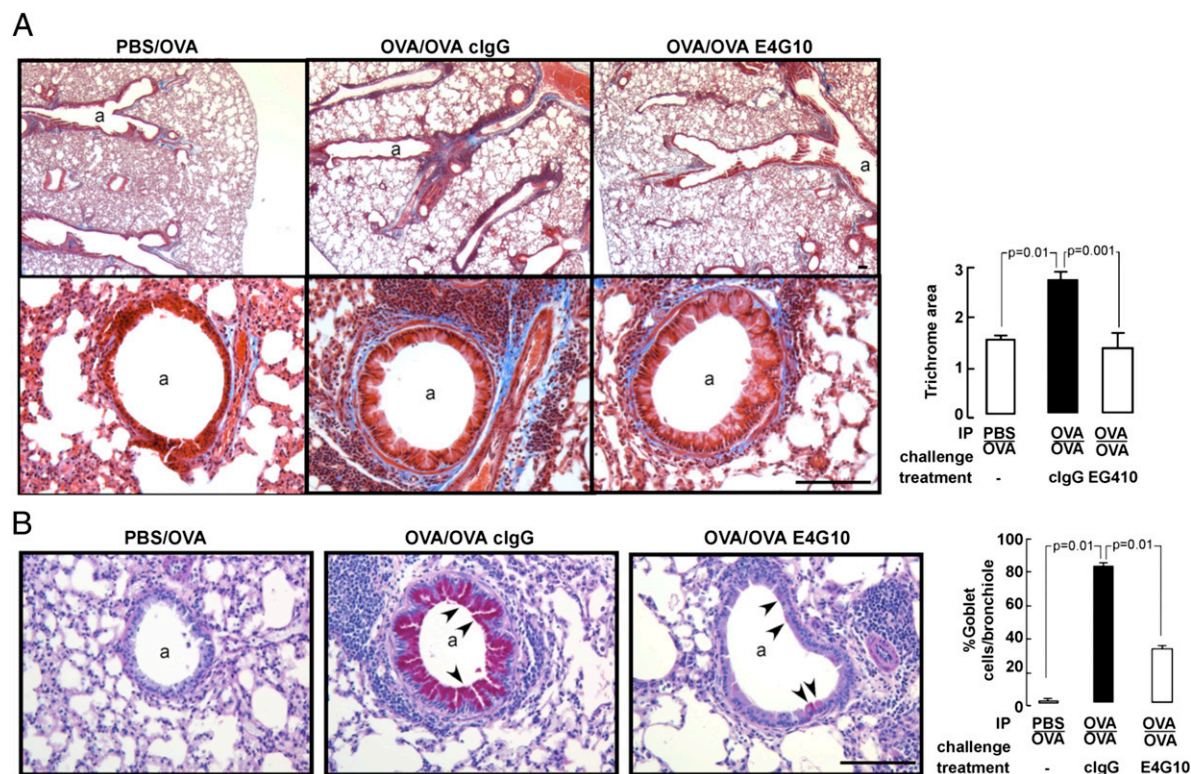


FIGURE 4. Airway fibrosis and goblet cell numbers reduced by VE-cadherin blockade. **(A)** Masson trichrome staining of lung tissue sections shows collagen deposition (blue) in low- and high-power field pictures. Collagen deposition was less in mice treated with anti-VE-cadherin Abs. Scale bar, $150 \mu\text{m}$. For quantification, μm^2 collagen tissue/ $100 \mu\text{m}^2$ lung tissue is shown. **(B)** Mucus-producing goblet cells stained with PAS to detect carbohydrate macromolecules, such as mucus, were stained purple-magenta (black arrows). Asthmatic mice showed increased numbers of goblet cells that were inhibited by VE-cadherin blockade. Representative bronchiole from each group is shown. Scale bar, $100 \mu\text{m}$. Mean and SEs of six mice per group are shown.

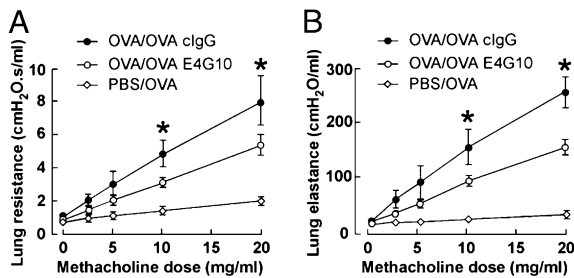


FIGURE 5. VE-cadherin blockade abrogates airway reactivity. (A) Dose-response curves of AHR show significantly lower airway resistance in mice treated with anti-VE-cadherin Abs. (B) Dose-response curves of lung elastance, which is inversely related to lung elasticity, were also reduced by VE-cadherin blockade. Mean and SEs of six mice per group are shown. *Significant difference between groups ($p < 0.05$).

or both, were analyzed. Lung endothelial cells from OVA/OVA mice secreted substantial levels of IL-25, but proangiogenic progenitor cells did not (Fig. 6A, 6B). IL-25 expression by lung endothelial cells was reduced by VE-cadherin blockade in the OVA/OVA group. TSLP production by isolated endothelial cells was low (Fig. 6C). Conversely, there was significant increase of TSLP production by proangiogenic progenitor cells isolated from OVA/OVA mice, which was prevented by VE-cadherin blockade (Fig. 6D). Expression of IL-33, another main Th2 cytokine, was

not observed. Recruitment of CD3⁺ T cells was not affected by E4G10 (Number of CD3⁺ cells per 0.1 mm² lung tissue: OVA/OVA with cIgG, 125 ± 14 ; OVA/OVA with E4G10, 116 ± 11 ; $p = 0.51$), which, taken together with the low levels of Th2-cytokines, indicated that the T cells present in the lung were not polarized to the Th2 type.

EMSA for STAT6 DNA binding activity revealed STAT6 activation in OVA/OVA lungs, which was reduced by the VE-cadherin blockade (Fig. 6E, 6F). Immunostaining for phospho-STAT6 confirmed greater STAT6 activation in airway epithelial cells and endothelial cells of OVA/OVA mice (Fig. 6G). The VE-cadherin blockade resulted in less STAT6 activation in the endothelium and epithelium of OVA/OVA mice. Interestingly, cIgG treatment appeared to potentiate STAT6 activation. We cannot exclude that cIgG potentially provided an adjuvant-like effect, but the fact that E4G10, which is of the same isotype as the control IgG, inhibited STAT6 activation further supports the concept that VE-cadherin blockade regulates the origins of atopic asthma.

Discussion

More than a century has passed since the initial observation by Ellis that blood vessel density is increased in the airways of asthma patients (11). Since then, the dogma has been that angiogenesis in asthma is a result of inflammation, supported by the notion that inflammation induces angiogenesis (34) and by lack of evidence

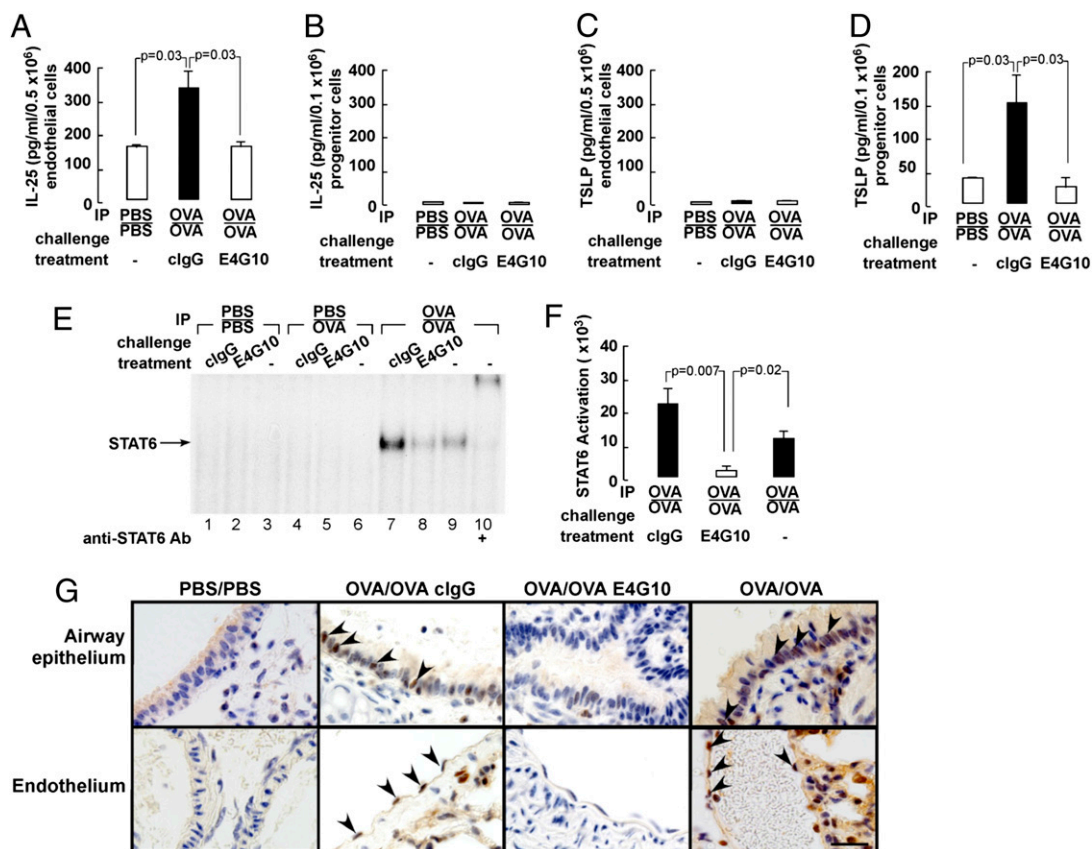


FIGURE 6. Inhibition of Th2 cytokine production and signal transduction by VE-cadherin blockade. (A–D) Animals were treated with VE-cadherin Abs, sensitized, and challenged as shown in Fig. 2A. In these experiments, analyses were performed 24 h after the first allergen exposure. Lung endothelial cells and bone marrow progenitor cells were incubated ex vivo, and levels of IL-25 and TSLP in overlaying media were analyzed by ELISA, showing reduced secretion by in vivo VE-cadherin inhibition. (E–G) Analyses were performed 30 min to 1 h after the first allergen exposure. Animals were treated with VE-cadherin Abs, sensitized, and challenged as depicted in Fig. 2A, whole lung tissue was used for analysis. (E) EMSA for STAT6 DNA binding was performed. E4G10 treatment inhibits OVA allergen induced STAT6 activation in the lung (lane 8), whereas control Ab did not (lane 7). (F) Densitometric quantification of STAT6 activation. (G) Phospho-STAT6 (P-STAT6) in lung tissue was analyzed by immunohistochemistry. STAT6 activation in airway epithelial cells and endothelial cells, indicated by arrows in OVA/OVA groups, were attenuated by E4G10 treatment. Scale bar, 200 μ m. Mean and SEs of three mice per group are shown.

that pathologic angiogenesis itself can induce inflammation. In parallel to tumors, an angiogenic switch occurs early in the genesis of asthma (12), supporting the possibility that angiogenesis can regulate airway inflammation and remodeling. In this study, we provide evidence by blocking nascent endothelial cells that angiogenesis is a formative first step in the development of Th2 inflammation via IL-25 and TSLP production by the resident endothelium and recruited bone marrow–derived proangiogenic progenitors. To our knowledge, this study is the first to report that nascent endothelium is an essential early step in the origins of the Th2 response.

The process of angiogenesis requires bone marrow–derived proangiogenic hematopoietic progenitors and the proliferation of endothelial cells. Proangiogenic progenitor cell mobilization or recruitment and angiogenesis during asthmatic airway inflammation are reduced moderately by blockade of adherens junction formation of nascent endothelial cells. However, inhibition of angiogenesis resulted in marked attenuation of airway inflammation and remodeling. Our findings are in line with reports that overexpression of VEGF, a key angiogenic factor (35, 36), in airway epithelial cells results in angiogenesis and airway inflammation in mouse models via development of a spontaneous Th2 response (37). Interestingly, VE-cadherin association with VEGFR2 is critical for VEGF signaling during angiogenesis (16), indicating crucial cross-talk between these two pathways in new vessel formation and inflammation. The primary role of VE-cadherin in vascular stability (35, 38, 39) makes it challenging to use molecular approaches targeting the whole protein, which results in lethal vascular leakage (18–20, 40). However, fine distinctions in the different epitopes allowed development of a new class of inhibitors, such as humanized E4G10 Abs, which only affect angiogenic endothelial cells, and not quiescent endothelial cells, which form the majority of the endothelium (18–20).

Although proangiogenic progenitors are known to be essential to the process of new vessel formation in the murine OVA-model of lung inflammation, we show for the first time, to our knowledge, that TSLP is provided delivered to the vascular bed *in trans* by the circulating bone marrow–derived progenitors, complementing the IL-25 produced by the resident endothelium. Prior studies indicate that TSLP is not expressed by most hematopoietic cells, with the exception of myeloid cells including neutrophils, macrophages, and mast cells (41–43). In this context, proangiogenic and mast cell progenitors share the same identifying surface markers (12, 15), suggesting that the hematopoietic myeloid progenitors that promote angiogenesis required in the early stages of atopic inflammation perhaps, in part, by delivery of TSLP to the tissue bed. In support of this concept, TSLP is central to dendritic cell–driven T and B cell expansion and differentiation of B cells that results in allergen-specific IgE production (2). In this study, the inhibition of new vessel formation partially reduced recruitment of proangiogenic progenitors; however, more importantly the recruited proangiogenic progenitors did not produce TSLP, and this was associated with the expected loss of IgE production. The findings reveal a vital link between circulating proangiogenic progenitors required for new vessel formation and initiation of Th2 inflammation and IgE production in atopic lung inflammation.

Epithelial-derived IL-25, TSLP, and IL-33 are currently defined as the three most proximal activators of the Th2 cascade (9). Epithelium lining body cavities is the first cellular contact with the external environment. The epithelial IL-33– and IL-25–induced Th2 response is the host response to pathogens, including allergens, fungi, and helminth infections (9). In this study, we show that endothelium lining vessels is also capable of activating Th2 responses in synergy with bone marrow–derived proangiogenic

progenitor cells via IL-25 and TSLP. Although they do not differentiate into endothelial cells, proangiogenic progenitors are potent paracrine-acting angiogenic cells (44) and are shown to be important paracrine-acting cells for development of atopy. A recent study reported that in organ transplantation, human endothelial cells promoted proliferation of proinflammatory Th17 and regulatory CD4⁺ T lymphocytes (45), which is more evidence that the endothelium directly modulates the immune system. It is also possible that unengaged VE-cadherin monomers on nascent endothelial cells are essential in autoactivation of vascular immune response (46). In this study, the results of eotaxin administration in mice show that E4G10 blockade does not act by affecting recruitment of eosinophils or T cells. Similarly, expression of VE-cadherin by murine proangiogenic progenitors (20) was not observed in our hands (data not shown), in line with recent findings by others (44), reducing the possibility of off-target effects of the Ab.

Traditionally, asthma and atopy have been viewed as mucosal diseases. The data in this study suggest that the origin of mucosal atopic inflammation requires new vessel formation, a process in which endothelial cells and bone marrow–derived progenitors provide the combined signals to initiate Th2 inflammation. In this context, subsets of bone marrow–derived progenitor cells, such as IL-25 responsive “type-2 multipotent progenitors” (47), “nuocytes” (48) and “natural helper cells” (49) have been reported to have a critical role in the initiation of gut Th2-response and “type 2 myeloid cells” in asthma (50). Bone marrow–derived proangiogenic progenitors and these other innate type-2 cells most probably originate from a common ancestor as indicated by their coexpression of SCA-1 and C-kit (47, 49). Overall, our findings expand the emerging concept of vascular immunology by adding Th2 response to the list of immune responses initiated by the endothelium.

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Disclosures

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