Endothelial Sox17 promotes allergic airway inflammation

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Background: IL-33, levels of which are known to be increased in patients with eosinophilic asthma and which is suggested as a therapeutic target for it, activates endothelial cells in which Sry-related high-mobility-group box (Sox) 17, an endothelium-specific transcription factor, was upregulated. Objective: We investigated the relationship between Sox17 and IL-33 and the possible role of Sox17 in the pathogenesis of asthma using a mouse model of airway inflammation. Methods: We used ovalbumin (OVA) to induce airway inflammation in endothelium-specific Sox17 null mutant mice and used IL-33 neutralizing antibody to evaluate the interplay between IL-33 and Sox17. We evaluated airway inflammation and measured levels of various cytokines, chemokines, and adhesion molecules. We also carried out loss- or gain-of-function experiments for Sox17 in human endothelial cells. Results: Levels of IL-33 and Sox17 were significantly increased in the lungs of OVA-challenged mice. Anti–IL-33 neutralizing antibody treatment attenuated not only OVA-induced airway inflammation but also Sox17 expression in pulmonary endothelial cells. Importantly, endothelium-specific deletion of Sox17 resulted in significant alleviation of various clinical features of asthma, including airway inflammation, immune cell infiltration, and airway hyperreactivity.

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infiltration, cytokine/chemokine production, and airway hyperresponsiveness. Sox17 deletion also resulted in decreased densities of Ly6chigh monocytes and inflammatory dendritic cells in the lungs. In IL-33–stimulated human endothelial cells, Sox17 showed positive correlation with CCL2 and intercellular adhesion molecule 1 levels. Lastly, Sox17 promoted monocyte adhesion to endothelial cells and upregulated the extracellular signal-regulated kinase–signal transducer and activator of transcription 3 pathway.

Conclusion: Sox17 was regulated by IL-33, and its genetic ablation in endothelial cells resulted in alleviation of asthma-related pathophysiologic features. Sox17 might be a potential target for asthma management. (J Allergy Clin Immunol 2019;144:561-73.)

Key words: Sox17, IL-33, asthma, allergic airway inflammation

Asthma is a chronic obstructive airway disease characterized by allergic inflammation and airway hyperresponsiveness (AHR).1 Despite recent improvements in the development of asthma treatment modalities, there are still unmet needs for novel therapeutic agents for severe and refractory asthma, which prompt in-depth investigation into the exact pathogenesis of asthma.2 A type 2–based immune reaction plays a central role in asthma development,3 and aside from immune cells, airway cells, such as bronchial epithelial cells and endothelial cells, have been shown to be actively involved in the pathogenesis of asthma.4,5 However, the precise functional mechanism of those cells underlying the development of allergic inflammation is yet to be clarified.

Airway endothelial cells constitute a protective physical barrier against invasion of environmental agents into inner tissues and orchestrate T2 immunity to allergens and pathogens by secreting various cytokines, such as IL-33, IL-25, and thymic stromal lymphopoietin (TSLP).6,7 Importantly, the genes of IL-33 and its receptor, ST2, have been suggested to be asthma susceptibility genes in a genome-wide association study.8 Accordingly, increased IL-33 expression was observed in the epithelial cells of patients with bronchial asthma,9 which was further increased in patients with severe and refractory asthma.10,11 By binding to ST2, IL-33 functions as a master regulator of T2-mediated inflammation.12 Activated vascular endothelial cells contribute to the development of asthmatic airway inflammation by recruiting immune cells,13 and IL-33 has been shown to stimulate endothelial cell inflammatory responses through upregulation of adhesion molecules.14,15 Indeed, increased expression of intercellular adhesion molecule 1 (ICAM-1) on endothelial cells and the interactions between ICAM-1 and its ligands have been suggested as the main mechanism by which various leukocyte populations are recruited and attached to the endothelium, which subsequently induces greater influx of inflammatory cells to the site of inflammation.16,17 However, the exact mechanism of IL-33–mediated endothelial cell activation is yet to be clearly defined.

Sry-related high-mobility-group box 17 (Sox17), an evolutionarily conserved transcription factor exclusively expressed in endothelial cells, regulates various signaling molecules, including Notch, TGF-β, Wnt, and vascular endothelial growth factor,18-23 which are known to play essential roles in asthmatic patients. Recently, a microarray analysis showed that Sox17 was upregulated in IL-33–stimulated endothelial cells, thereby suggesting a possible relationship between IL-33 and Sox17.24,25 Thus we aimed to examine the relationship between Sox17 and IL-33 during the development of asthmatic airway inflammation and the possible role of Sox17 in modulating asthma pathophysiology.

METHODS

Animals

All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee Facility at Korea Advanced Institute of Science and Technology. Six- to 8-week-old female wild-type (WT) mice were sensitized with 25 μg of OVA (grade V; Sigma-Aldrich, St Louis, Mo) mixed in 4 mg of Imject (Thermo Scientific, Rockford, Ill) on days 0 and 14, followed by intranasal challenges with 50 μg of OVA on days 21, 22, and 23, to generate an ovalbumin (OVA)–induced mouse model of asthma. Mice were killed on day 14 at 6 hours after the second sensitization, day 21 at 6 hours after a single OVA challenge, or day 24 at 24 hours after the last intranasal OVA application on day 23 (Fig 1, A).

WT mice were intraperitoneally injected with anti–IL-33 antibody (0.04, 0.4, and 4 μg per mouse; catalog no. AF3626; R&D Systems, Minneapolis, Minn) 30 minutes before each OVA sensitization and intranasal OVA challenge. As a control, the same amount of isotype control antibody (catalog no. AB-108-C; R&D Systems) was intraperitoneally injected into OVA-challenged mice. The lungs and bronchoalveolar lavage fluid (BALF) from the mice were collected in each experiment.

Cdh5(PAC)-CreER12 mice25 were crossed with Sox17fl/fl mice26 to generate endothelium-specific Sox17 knockout mice (Sox17PAC-ER12 mice); at 6 to 8 weeks of age, Sox17fl/flCdh5(PAC)-CreER12 mice were subcutaneously injected with tamoxifen (25 mg/kg; Sigma-Aldrich) dissolved in corn oil every 2 days for 6 days. C57BL/6 wild-type (WT) mice were purchased from Orient Bio (Seongnam-si, Korea). All animals were raised and bred in specific pathogen-free animal facilities.

Six- to 8-week-old female Sox17PAC-ER12 mice and their littermates were sensitized to and challenged with OVA for 4 consecutive days from day 21 to day 24 to investigate the role of Sox17. The mice were killed for evaluation on day 25 at 24 hours after the last OVA intranasal challenge. Control mice were treated with saline according to the same protocol. Lungs, sera, and BALF from the mice were collected in each experiment. For experiments requiring sorted endothelial cells, lung hematopoietic cells, and other cells, the mice were sensitized and subjected to OVA administration at days 21 and 22 and were killed for evaluation on day 23. Tamoxifen was given at
days 2, 4, and 6 to the sensitized mice to induce Sox17 deletion after systemic sensitization with OVA. After the final tamoxifen treatment, mice were intranasally administered with 50 μg of OVA on days 16, 17, and 18 and killed on day 19. Lungs, sera, spleens, mediastinal lymph node (MLN) cells, and BALF from the mice were collected in each experiment.

Methods for other experiments, including BALF analysis, measurement of AHR, histopathologic examination, immunofluorescence staining, flow cytometric analysis, and cellular experiments with splenocytes and MLN cells are described in the Methods section in this article’s Online Repository at www.jacionline.org.

**Endothelial cell culture and experimental design**

Two types of human endothelial cells (human umbilical vein endothelial cells [HUVECs] and human pulmonary microvascular endothelial cells [HPMECs]) were used for the study. HUVECs (catalog no. CC-2519) and complete growth media (EGM2 BulletKit media, catalog no. CC-3162) were purchased from Lonza (Basel, Switzerland), as recommended by the manufacturer. HPMECs (catalog no. C-12281) and complete growth media (catalog no. C-20020) were obtained from PromoCell (Heidelberg, Germany). Both endothelial cells were plated in sterile culture dishes coated with 0.25% (wt/vol) gelatin (catalog no. 1393; Sigma-Aldrich) diluted in PBS and then incubated in a humidified atmosphere with 5% CO₂ at 37°C. We used 10 ng/mL IL-33 (catalog no. 200-33; PeproTech, Rocky Hills, NJ) to stimulate the HUVECs and HPMECs. Anti–ST2/IL-33 receptor antibody (1 μg/mL) was used to block IL-33/ST2 signaling (catalog no. MAB523; R&D Systems).

HUVECs were treated with conditioned medium (CM) obtained from cultured BEAS-2B cells in the presence or absence of anti-ST2 antibody for 6 hours to evaluate the effect of allergen-exposed bronchial epithelial cells on HUVECs. CM was prepared as follows: BEAS-2B cells (CRL-9609; ATCC, Manassas, Va) were cultured in RPMI 1640 (Welgene, Daegu, Korea) and then treated with 1 μg/mL house dust mite (HDM) extract or saline for 1 hour after overnight starvation. Cells were washed with PBS, and CM of the cells were collected for 4 hours. Next, levels of IL-33 in supernatants of HDM-treated BEAS-2B cells were measured by using DuoSet ELISA developmental kits (catalog no. DY3625B; R&D Systems), according to the manufacturer’s instructions.

Sox17 small interfering RNA (siRNA) and pCMV-Sox17 plasmids were transfected in HUVECs and HPMECs to knockdown or overexpress Sox17. Briefly, when HUVECs and HPMECs reached confluency of 40% to 60% and 80% to 90%, respectively, the cells were treated with 40 nmol/L Sox17 siRNA (5′-AGCGCCAGUCUCGGCGUAUA-3′; Bioneer, Daejeon, Korea) or scrambled siRNA in 500 μL of Opti-MEM mixed with 3.5 μL of Lipofectamine RNAiMAX (catalog no. 13778100; Invitrogen, Carlsbad, Calif) and added to each well (6-well plates). IL-33 (10 ng/mL) was added to the wells 2, 4, and 6 to the sensitized mice to induce Sox17 deletion after systemic sensitization with OVA. After the final tamoxifen treatment, mice were intranasally administered with 50 μg of OVA on days 16, 17, and 18 and killed on day 19. Lungs, sera, spleens, mediastinal lymph node (MLN) cells, and BALF from the mice were collected in each experiment.

Methods for other experiments, including BALF analysis, measurement of AHR, histopathologic examination, immunofluorescence staining, flow cytometric analysis, and cellular experiments with splenocytes and MLN cells are described in the Methods section in this article’s Online Repository at www.jacionline.org.

**FIG 1.** IL-33 secretion and pulmonary endothelial activation in an OVA-induced mouse model of allergic airway inflammation. A, Experimental design for generating a mouse model of allergic airway inflammation. Mice were sensitized by means of intraperitoneal (i.p.) injection of OVA/alum, followed by intranasal (i.n.) OVA challenges (n = 4-6 per group). B, Total cell counts and IL-33 levels in BALF from different groups on day 14 at 6 hours after the second sensitization or on day 21 at 6 hours after a single OVA challenge. C, Immunostaining for IL-33 (red) and nuclei (blue) in representative lung sections from different groups at 24 hours after the last intranasal OVA application on day 23. D, ICAM-1 (red) and CD31 (blue) immunostaining in the lungs. E, Sox17 and β-actin immunoblotting in the lungs. M.W., Molecular weight. F, Sox17 expression measured by means of immunoblotting from lung tissues (n = 5-14 per group). G, Sox17 (red), CD31 (green), and nuclei (blue) immunostaining in the lungs. Images are representative of independent lung sections from 3 different mice in 2 independent experiments. Ctrl, WT mice; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride. *Significance between the control and OVA groups: *P < .05, **P < .001, and ***P < .0001. Scale bars = 100 μm.
FIG 2. Sox17 upregulation in an IL-33–enriched environment of OVA-exposed mice. A and B, Immunoblotting of Sox17 and β-actin from HUVECs and HPMECs treated with increasing concentrations of IL-33 (0, 5, 10, and 50 ng/mL). C and D, HUVECs and HPMECs cotreated with IL-33 and anti-ST2 antibody and Sox17 expression normalized by β-actin expression. E, HUVECs treated with CM of BEAS-2B cells exposed to HDM and anti-ST2 antibody. F, Sox17 and β-actin expression measured by means of immunoblotting of the lungs from mice treated with anti-IL-33 antibody. G, Sox17 (red), CD31 (green), and nuclei (blue) immunostaining in respective lung sections. Sox17+ endothelial cells in lung tissues. DAPI, 4′,6-Diamidino-2-phenylindole dihydrochloride. H, mRNA levels of ST2 and Sox17 in lung tissues of OVA mice. I, Hematoxylin and eosin (H&E) staining of lungs from control mice and a mouse model of allergic airway inflammation with or without anti–IL-33 antibody. Original magnification ×40 (n = 5 per group). J, Total cell counts in BALF. K-M, IL-4, IL-13, and CCL2 levels in BALF. Similar results were obtained from 2 or 3 independent experiments. DAPI, 4′,6-Diamidino-2-phenylindole dihydrochloride; HDM, 1 µg/mL; IL-33 Ab, 4 µg of IL-33 neutralizing antibody; Iso, isotype; M.W, Molecular weight; N.D, not determined; ST2 Ab, 1 µg/mL ST2 neutralizing antibody. *Significance between groups: *P < .05, **P < .001, and ***P < .0001. Scale bars = 50 µm.
after 30 hours of transfection, and cells were collected 6 hours afterward for
immunoblotting or transcript analysis. For Sox17 overexpression, 600 ng of
pCMV-Sox17 plasmids containing the cytomegalovirus promoter for Sox17
were transfected by using Lipofectamine LTX with Plus reagent (catalog
no. 15338100; Invitrogen) in Opti-MEM reduced serum media (catalog no.
51985034; Thermo Scientific). After 24 hours, the cells were treated with
10 ng/mL IL-33 for 6 hours and harvested for Western blotting. For HPMECs,
retransfection of Sox17 was conducted by incubating the cells in complete
medium supplemented with siRNA or plasmid premixed 0.7 μL lipofectamine
after initial transfection.
Additional information on individual methods and tools are described in the Methods section in this article’s Online Repository.

**Statistical analysis**

Results are presented as means ± SEMs. One-way ANOVA was performed to determine differences between multiple groups with post hoc comparisons followed by the Bonferroni or Newman-Keuls methods. For comparisons between 2 groups, Student t tests were carried out (GraphPad Prism Software, version 7.0; GraphPad Software, La Jolla, Calif). P values of less than .05 were considered statistically significant.

**RESULTS**

Antigen inhalation increases IL-33 expression and activates endothelial cells in a mouse model of asthma

To investigate whether IL-33 is associated with endothelial cells in the development of allergic airway inflammation, we first established a mouse model for asthma using OVA sensitization and intranasal OVA challenges (Fig 1, A). There were significant increases in total cell numbers and IL-33 levels in the BALF of WT mice on day 21 at 6 hours after a single OVA challenge (Fig 1, B).

We examined expression levels of endothelial adhesion molecules, Sox17, and IL-33 in endothelial cells on day 24, 24 hours after the 3 days of inhaled OVA challenges, the expression level of IL-33 in the lung tissues had significantly increased compared with that in control mice without OVA challenges (Fig 1, C). At the same time, ICAM-1 expression was upregulated in pulmonary endothelial cells of intranasal OVA-challenged mice (Fig 1, D); the density of Sox17-positive endothelial cells and the expression of Sox17 protein were also increased in the lungs of OVA-challenged mice (Fig 1, E-G).

IL-33 increases Sox17 expression in endothelial cells

To determine whether there is a direct effect of IL-33 on Sox17 expression in endothelial cells, we treated HUVECs and HPMECs with varying concentrations of IL-33 (Fig 2, A and B). Sox17 expression was significantly increased after IL-33 treatment, which was effectively blocked by treatment with antibody for ST2, a receptor for IL-33 (Fig 2, C and D).

To mimic the development of allergic airway inflammation, we cultured HUVECs in CM obtained from HDM-stimulated human bronchial epithelial cells (BEAS-2B cells) containing higher levels of IL-33 (see Fig E1 in this article’s Online Repository at www.jacionline.org). CM treatment enhanced Sox17 expression, whereas blockade of IL-33/ST2 signaling resulted in attenuated expression of Sox17 in HUVECs (Fig 2, E).

We next examined the effects of IL-33 blocking in vivo by administering IL-33 neutralizing antibody in OVA-exposed mice. Sox17 expression levels and numbers of Sox17-positive endothelial cells in the lung were significantly decreased on anti–IL-33 antibody treatment (Fig 2, F and G). Anti–IL-33–treated mice showed significantly decreased mRNA levels of ST2 and Sox17 (Fig 2, H), reduced asthmatic airway inflammation (Fig 2, I), and decreased numbers of immune cell infiltration into BALF (Fig 2, J). Levels of Th2 cytokines, such as IL-4 and IL-13, and the proinflammatory chemokine ligand CCL2 were attenuated in BALF of anti–IL-33–treated mice as well (Fig 2, K-M).

Endothelial Sox17 deletion attenuates various clinical features of asthma

Next, we investigated the role of endothelial Sox17 in OVA-challenged mice by using Sox17ΔEC mice with endothelium-specific deletion of Sox17. Sox17ΔEC mice showed no Sox17 expression in their pulmonary endothelial cells (Fig 3, A). We observed a significant reduction in infiltration of inflammatory cells, such as macrophages, eosinophils, neutrophils, and lymphocytes, in the BALF of OVA-challenged Sox17ΔEC mice (Fig 3, B). Serum IgE levels were not significantly increased in Sox17ΔEC asthmatic mice compared with those in WT asthmatic mice (Fig 3, C). Sox17ΔEC mice also did not show increased AHR to methacholine (Fig 3, D), and histopathologic examination revealed a decrease in the accumulation of inflammatory cells around the peribronchial and perivascular regions in OVA-challenged Sox17ΔEC mice (Fig 3, E and F). Amelioration of goblet cell hyperplasia was also observed in the lungs of OVA-challenged Sox17ΔEC mice (Fig 3, E and G). In terms of analysis of vessel morphology, pulmonary vessels in OVA-challenged WT mice exhibited greater numbers of sprouts after OVA challenge, which led to a larger vascular area; in contrast, vessels in Sox17-deficient mice showed no significant changes in the number of vessel sprouts (Fig 3, G and H).
branchings, resulting in little effect on vascular area (Fig 3, E, H, and I).

Levels of various cytokines and chemokines (IL-4, IL-13, IL-33, CCL2, and CCL11) in BALF were significantly reduced in Sox17 mice on day 25 at 24 hours after the last allergen exposure in OVA-induced airway inflammation (Fig 3, J-N). Meanwhile, we found no significant difference in IL-25 and TSLP expression between lung tissues of OVA-induced control and Sox17 mice (see Fig E2, A and B, in this article’s Online Repository at www.jacionline.org). IL-25 and TSLP were not detected by means of ELISA in the BALF. As for adhesion molecules, ICAM-1 levels were decreased at both the mRNA and protein levels in the lungs of OVA-challenged Sox17 mice compared with OVA-challenged control mice (Fig 3, O and P).

In addition, we evaluated whether global Sox17 deletion in systemic endothelial cells resulted in suppressed immunization responses. First, we measured TGF cytokine profiles in the spleens of WT and Sox17 mice sensitized/challenged by OVA. As a result, we found that OVA and subsequent costimulation of splenocytes with anti-CD3/CD28 resulted in significant upregulation of IL-4 and IL-13 concentrations in culture supernatants of both WT and Sox17 mice; importantly, there were no significant differences between WT and Sox17 mice (Fig E3 in this article’s Online Repository at www.jacionline.org).

Next, we tested the effects of late Sox17 deletion, which was carried out after systemic sensitization with OVA and before OVA challenge (see Fig E4, A, in this article’s Online Repository at www.jacionline.org). Interestingly, late Sox17 deletion also alleviated allergic airway inflammation and reduced IL-4, IL-13, and CCL2 levels in BALF (see Fig E4, B-E). Serum IgE levels were also significantly decreased (see Fig E4, F). Total cell counts and IL-4 and IL-13 levels from MLN cell culture supernatants were lower in OVA-challenged Sox17 mice than in WT mice (see Fig E4, G and H).

**Endothelium-specific Sox17 deletion affects mounting type 2 immune response in patients with allergic airway inflammation**

We evaluated whether Sox17 affects the mounting immune response in OVA exposed-mice. OVA-challenged Sox17 mice showed reduced infiltration of CD11b+ and CD11c+ cells in their

![FIG 4. Endothelium-specific Sox17 deletion suppresses inflammatory DC and monocyte homing into the lung. A, CD11b (green) and CD11c (red) immunostaining of lung tissues from control and OVA groups in endothelium-specific Sox17 knockout mice. Quantification of CD11b+ and CD11c+ immune cells in random microscopic fields (n = 4-7 per group). B and C, Fluorescence-activated cell sorting plots and frequencies (means ± SEMs) of inflammatory DCs (CD3+CD19+CD45+MHCIILy6g+CD11b+CD11c+) and Ly6chigh monocytes (CD45+CD19+Ly6g+Ly6c+CD11b+) in the respective lungs. Graphs show absolute counts of DCs or monocytes in the respective lungs (n = 4-6 per group). Similar results were obtained from 2 independent experiments. Ctrl, WT mice; iD, endothelium-specific Sox17 knockout mice. *Significance within groups: *P < .05, **P < .001, and ***P < .0001. Scale bars = 100 μm.](image-url)
lung tissues (Fig 4, A). Moreover, numbers of CD11b\(^+\)CD11c\(^+\) inflammatory dendritic cells (DCs) and Ly6c\(^{\text{high}}\) monocytes were decreased in the lungs of OVA-challenged Sox17\(^{\text{iD}}\) EC mice (Fig 4, B and C).

We sorted endothelial cells, lung hematopoietic cells, and other cells from the lungs of each study group after OVA challenge to study the effect of Sox17 on lung endothelial cell responses during airway inflammation (Fig 5, A). In these cells we measured expression levels of various chemical mediators associated with the allergic immune response: Sox17 deletion reduced expression of CCL2 and ICAM-1 in endothelial cells but not in other cell types, and there were no remarkable changes in endothelium-specific expression of various mediators related to endothelial activation, such as vascular cell adhesion molecule 1, IL-33, and ST2 (Fig 5, B).

### Sox17 is involved in IL-33–mediated endothelial activation

We investigated whether Sox17 modulates the expression of chemokines and adhesion molecules in human endothelial cells exposed to IL-33. Knockdown of Sox17 in HUVECs and HPMECs was carried out by using Sox17 siRNA (Fig 6, A and B). IL-33 stimulation resulted in dramatic increase of CCL2 and ICAM1 transcripts in both endothelial cells. Notably, CCL2 and ICAM1 transcripts were significantly reduced when Sox17 was silenced (Fig 6, C and D). ELISA analysis of supernatants from cultured HUVECs and HPMECs of each study group showed that CCL2 secretion in cells with Sox17 knockout was significantly decreased. In addition, we found that ICAM-1 expression was significantly reduced in Sox17 knockout endothelial cells after IL-33 stimulation (Fig 6, E and
Meanwhile, expression levels of CCL2 and ICAM-1 were greatly increased in HUVECs and HPMECs, which stably expressed platelet endothelial cell adhesion molecule 1 with Sox17 overexpression, which were further enhanced on IL-33 stimulation (Fig 6, G and H).

We also tested whether Sox17 regulates monocyte adhesion to endothelial cells under IL-33 stimulation. For the cell adhesion assay, 2 different human monocytic cell lines (U937 and THP-1) were used. Adhesion of U937 cells to monolayers of HUVECs or HPMECs was significantly decreased on Sox17 knockdown. THP-1 cell adhesion to monolayers of HPMECs showed similar results (Fig 6, I). Finally, we evaluated IL-33 signaling pathway in endothelial cells in relation to Sox17. HUVECs with Sox17 knockdown showed reductions in IL-33–induced phosphorylation of extracellular signal-regulated kinase (ERK) and signal transducer and activator of transcription 3 (STAT3), as well as ST2 expression (Fig 6, J).

**DISCUSSION**

Here we demonstrated that Sox17 expression in endothelial cells was regulated by IL-33 and involved in development of
allergic airway inflammation by promoting inflammatory DC and monocyte homing to the lungs in a mouse model of asthma.

Endothelial cells carry important roles in the development of allergic airway inflammation,\(^2\) although the precise molecular mechanism remains undefined. Recently, the critical role of IL-33, a major player in the development of allergic airway inflammation in asthmatic patients, has been highlighted. Aside from its effects on immune cells, IL-33 acts directly on endothelial cells to produce various cytokines and chemokines in TH2-inflamed lungs.\(^1\) On the contrary, a recent report showed that IL-33 regulates retinal angiogenesis through direct inhibition of fibroblasts and endothelial cells.\(^3\) The precise roles and mechanisms of IL-33 in endothelial cells during the pathogenesis of bronchial asthma are yet to be clarified; nevertheless, the pivotal roles of endothelial cells in asthmatic airways seem to be closely linked to IL-33.

The transcription factor Sox17 plays a crucial role in vascular development through regulation of complex signaling networks for modulating angiogenesis\(^2\) and pulmonary vascular morphogenesis.\(^3\) Current evidence supports the role of Sox17 during arterial endothelial cell differentiation and vascular remodeling\(^3\) as an upstream regulator of Notch signaling, which induces IL-33 expression in quiescent vessels.\(^9\) A recent study showed that another member of the Sox family, Sox7, modulates IL-33 to trigger signaling pathways related to inflammation-associated tumor invasion.\(^3\) Although Sox17 is considered an important regulator of the biological processes of endothelial cells, its significance in immune vascular interaction in allergic airway inflammation is only beginning to be recognized. It remains unknown whether Sox17 exerts IL-33-dependent biological effects on activated endothelial cells to maintain chronic inflammation. Thus we aimed to identify the role of Sox17, an endothelium-specific transcription factor that contributes to endothelial cell activation associated with TH2 inflammatory responses.

We first tried to test whether IL-33 and endothelial cells are involved in allergic airway inflammation. We observed significantly increased levels of IL-33 and immune cell recruitment into the airways of OVA-challenged mice. At the same time, expression levels of endothelial adhesion molecules, such as ICAM-1 and enhanced Sox17, were also increased. Recent studies demonstrated that pulmonary microvascular endothelial cells had greater expression of IL-33 receptors compared with lung fibroblasts or smooth muscle cells and thus were preferentially responsive to IL-33 as well. Thus IL-33 might be closely associated with activated endothelial cells during development of allergic airway inflammation.

We also observed that IL-33 directly increased the expression of Sox17 in endothelial cells. By using a neutralizing antibody for ST2, a receptor for IL-33, we observed that IL-33 acts as an inducer of Sox17 in HUVECs and HPMECs in an ST2-dependent manner. Previous studies have demonstrated that anti–IL-33 antibody and anti-ST2 antibody behave as negative regulators in OVA-mediated allergic airway inflammation.\(^3\) Accordingly, our results also showed that Sox17 expression and TH2-mediated inflammatory responses were reduced by anti–IL-33 antibody treatment. These data show that IL-33 directly influences the increase in Sox17 expression in endothelial cells during allergic airway inflammation.

To date, a couple of reports have demonstrated the role of Sox17 in other airway diseases. Sox17 expression was increased in the tissues of patients with eosinophilic chronic rhinosinusitis with nasal polyps, suggesting that upregulation of Sox17 is involved in establishment of type 2 responses.\(^3\) Sox17 was also suggested as a candidate transcription factor associated with thromboxane A\(_2\) receptor polymorphisms, which is implicated in childhood-onset asthma.\(^3\) Therefore we further investigated the specific role of Sox17 in lung endothelial cells in the course of allergic airway inflammation by using Sox17 endothelium-specific knockout mice. We observed that deletion of Sox17 in endothelial cells resulted in suppression of key clinical features of allergic asthma, such as eosinophilic inflammation, AHR, increased angiogenesis, and goblet cell hyperplasia. Furthermore, absence of Sox17 in endothelial cells was linked to reduction in levels if various cytokines, chemokines, and adhesion molecules that are critical in the development of allergic airway inflammation.
involved in airway remodeling in asthmatic patients through increased angiogenesis and resulting higher vascularity, as well as mucus gland and goblet cell hyperplasia. Previous reports showed that IL-33 is a potent endothelial activator and carries proangiogenic role by promoting endothelial cell growth from pre-existing vessels.\textsuperscript{54,29,30,39} Our results indicate that the proangiogenic role of IL-33 might be closely related to Sox17. Conversely, endothelial Sox17 deletion did not seem to suppress systemic immune responses, suggesting that Sox17 carries a specific role in the IL-33–mediated inflammatory response that is confined to lungs exposed to culprit allergens. Taken together, for the first time, our results show that Sox17 deletion in pulmonary endothelial cells ameliorates the critical features of allergic asthma.

These findings prompted us to evaluate the role of Sox17 in mounting T2 immune responses. Inflammatory DCs and monocytes have the ability to polarize CD4\textsuperscript{+} T cells, which is leading to activation of pulmonary adaptive immunity.\textsuperscript{40} Specifically, the pool of CD11b\textsuperscript{+}CD11c\textsuperscript{+} DCs and Ly6C\textsuperscript{high} monocytes has been reported to be recruited to the lungs with ongoing inflammation after exposure to HDM or OVA allergen.\textsuperscript{41,42} Among lung DC subsets, resident CD11b\textsuperscript{+}Ly6C\textsuperscript{DCs} are responsible for T\textsubscript{H}2 cell differentiation and subsequent allergic airway sensitization to HDM or OVA allergen.\textsuperscript{43} Interestingly, we observed that infiltration of CD11b\textsuperscript{+}CD11c\textsuperscript{+} DCs and Ly6C\textsuperscript{high} monocytes to the lungs was greatly reduced in OVA-challenged Sox17\textsuperscript{-/} mice. Our results indicate that endothelial Sox17 is closely linked to development of the T\textsubscript{H}2 immune response by modulating DC and monocyte recruitment to the lungs in patients with allergic airway inflammation.

We also examined whether Sox17 in endothelial cells modulates the expression of various chemokines and adhesion molecules in the lungs during allergic airway inflammation. We sorted each cell type (endothelial cells, lung hematopoietic cells, and other cells) from the lungs of OVA-challenged mice and observed that endothelial cells from endothelium-specific Sox17 null mutant mice had significantly reduced expression of CCL2 and ICAM-1.

Meanwhile, although we observed a positive association between the degree of airway inflammation and protein levels of IL-33 in BALF, no difference was found in IL33 mRNA levels from other lung constitutional cells (CD45\textsuperscript{+}CD11c\textsuperscript{+}CD31\textsuperscript{+} cells), such as epithelial cells, smooth muscle cells, and fibroblasts. Such discrepancy might have stemmed from different experimental settings or the fact that protein expression levels are not necessarily consistent with mRNA levels. However, a more feasible explanation might be that there are no fundamental differences in the amount of IL-33 production from lung constitutional cells under various degrees of lung inflammatory situations. Thus differences in IL-33 levels in BALF, which represent the total sum of IL-33 secreted from all cellular sources, could mainly result from differences in the situations of inflammatory cells between study groups. Specifically, an abundance of IL-33 increases levels of the transcription factor Sox17 in endothelial cells and expression of ICAM-1 and CCL2, thereby leading to increased recruitment of inflammatory cells. These results further support the notion that endothelial cells are involved in the development of allergic airway inflammation by facilitating the recruitment of IL-33–producing inflammatory cells into the lungs through Sox17 regulation, which might be an important pathogenic mechanism in asthma.

We performed Sox17 loss- and gain-of-function studies using human endothelial cells and observed results similar to those of in vivo experiments. Endothelial cells are a major source of CCL2 and are responsible for recruitment of CCR2-expressing cells, such as monocytes and DCs,\textsuperscript{44} therefore we further examined whether IL-33–induced CCL2 secretion is modulated by Sox17 and found that Sox17 silencing resulted in downregulation of CCL2 in IL-33–stimulated HUVECs and HPMECs. In line with recent microarray data,\textsuperscript{23,45} we found a positive correlation between CCL2 levels and Sox17 expression.

We observed that endothelial Sox17 deletion resulted in reduced recruitment of DCs to the lungs in OVA-induced asthma models. Reduced ICAM-1 expression was also observed in Sox17-silenced endothelial cells. Other groups have previously reported that DCs are able to adhere to ICAM-1–presenting endothelial cells through constitutively expressed ligands, such as the cell-surface integrins lymphocyte function–associated antigen 1 (CD11a/CD18), Mac-1 (CD11b/CD18), and gp150,95 (CD11c/CD18), and that such interaction is responsible for promoting the trafficking of DCs through endothelium-driven pulmonary inflammation.\textsuperscript{46,47} Therefore it might be reasonable to suggest that decreased migration of DCs into the airways in our study is associated with reduced ICAM-1 expression on endothelial cells. Moreover, we observed that adherence of monocytes to HUVECs and HPMECs was enhanced by IL-33 treatment, which was effectively inhibited by Sox17 silencing. Taken together, these results reveal the relationship between IL-33 and Sox17 in endothelial cells in the course of persistent allergic airway inflammation in asthmatic patients. In addition, similar effects of Sox17 were observed in both HUVECs and HPMECs, indicating that the role of Sox17 is conserved in various endothelial cells.

Indeed, we confirmed that IL-33 influenced endothelial activation through activation of the ERK and STAT3 pathways and that Sox17 promotes IL-33–mediated intracellular signaling in endothelial cells. In line with our results, several reports have suggested that IL-33 induces production of adhesion molecules and chemokines after ST2 ligation.\textsuperscript{4,24} In addition, IL-33 exerts its action in signaling cascades composed of myeloid differentiation response gene 88, IL-1 receptor–associated kinases 1 and 4, and TNF receptor–associated factor 6, which lead to phosphorylation of I\textkappaB-\alpha, mitogen-activated protein kinase, ERK, p38, c-Jun N-terminal kinase, and STAT3.\textsuperscript{39,50}

In conclusion, we show that the endothelial transcription factor Sox17 contributes to IL-33–mediated endothelial activation in the development of allergic airway inflammation. Endothelial Sox17 mediated the recruitment of inflammatory DCs and monocytes, which is suspected to occur through increased expression of CCL2 and ICAM-1 in endothelial cells exposed to IL-33. This study expands the current understanding on IL-33 in terms of its disease-associated roles and suggests that Sox17 might be a target for managing allergic inflammation.

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Key messages

- IL-33 upregulated Sox17, an endothelium-specific transcription factor, in a receptor- and dose-dependent manner in a mouse model of asthmatic airway inflammation.

- Endothelium-specific deletion of Sox17 resulted in significant alleviation of various clinical features of asthma, including airway inflammation, immune cell infiltration, cytokine/chemokine production, AHR, and homing of monocytes and inflammatory DCs to the lungs.

REFERENCES


METHODS

BALF analysis

After tracheostomy, a total of 2 mL of BALF was obtained by means of lavage with PBS. Cells were prepared by means of centrifugation at 14,000 rpm for 1 minute at 4°C, and the pellets were resuspended in PBS after removal of RBCs. For total and differential cell counts, BALF cells were cytospun onto slides (StatSpin CytoFuge 12; Iris, Norwood, Mass) at 400g for 5 minutes at room temperature, followed by a Diff-Quik stain (Sysmex, Kobe, Japan) and common fixation for light microscopy with synthetic mounting medium (Histomount; Ted Pella, Redding, Calif). Differential counts of 300 cells were used in each preparation to examine numbers of eosinophils, macrophages, neutrophils, and lymphocytes in the BALF.

Histopathologic evaluation

Mouse lungs were fixed with 4% neutral-buffered formalin for 24 hours and processed by means of paraffin embedding. Tissue sections (4 μm) were obtained and stained with hematoxylin and eosin and periodic acid–Schiff. Degrees of lung tissue inflammation and mucus secretion were graded on a scale of 0 to 5 (0, none; 1, minimal; 2, mild; 3, moderate; 4, marked; and 5, severe).

Tissue immunofluorescence staining and characterization of lung morphometry

For vibratome sectioning, tissues were embedded in 1% agarose, and 150-μm sections were produced with a Leica VT1200S vibrating blade microtome (Leica). Vibratome sections were then fixed in 2% paraformaldehyde (PFA) in PBS before staining with a standard immunofluorescence protocol. For cryosectioning, mouse lungs were fixed in 4% PFA on ice overnight and dehydrated in PBS with 30% sucrose. After tissue sectioning was performed with a cryomicrotome (Leica), and immunofluorescence staining was carried out. In brief, tissues were blocked in PBS with 0.1% Triton X-100 containing 5% donkey or goat serum and then incubated with primary antibodies, including anti-CD31 (catalog no. 550274; BD Biosciences), anti-Sox17 (1:100; catalog no. AF1924; R&D Systems), anti–IL-33 (1:100; catalog no. AF5626; R&D Systems), anti–ICAM-1 (1:100; catalog no. MA5-407; Invitrogen), anti–CD11b (1:100; catalog no. ab88878; Abcam), and anti–CD11c (1:100; catalog no. ab33483; Abcam), followed by fluorescence-labeled secondary antibodies. Nuclei were stained with 4′,6-diamidino-2-phenylindole dihydrochloride and mounted in Vectashield (Vector laboratories, Peterborough, United Kingdom). We used a Zeiss LSM780 confocal fluorescence microscope (Carl Zeiss, Jena, Germany) to obtain immunofluorescence images. ImageJ software (http://rsb.info.nih.gov/ij/) was used to measure the fluorescence intensities of IL-33, Sox17, ICAM-1, and CD31 in lung tissues. To further characterize the morphometry of lung tissues, computer-assisted color image segmentation was carried out on CD31+ vessels for vessel area (random 0.26-mm² regions), and the number of vascular branchings was counted on at least 3 representative fields of 1 mm² in area in randomly selected regions.

AHRR measurement

As previously described, AHR in response to increasing doses of methacholine (0, normal saline only, 1, 3, 9, 18, and 27 mg/mL; Sigma-Aldrich) was measured 16 hours after the last intranasal OVA challenge by using the forced oscillation technique (flexiVent; SCIREQ, Montreal, Quebec, Canada). After anesthesia with 60 mg/kg pentobarbital sodium (Hanlim Pharma, Seoul, Korea) and a direct tracheal intubation cannula (20-gauge cannula), mice were connected to the flexiVent system through the endotracheal cannula. After neuromuscular blockade was induced by means of intraperitoneal injection of 1 mg/kg pancuronium bromide (Sigma-Aldrich), the mice were ventilated at a respiratory rate of 150 breaths/min and tidal volume of 10 mL/kg against a positive end-expiratory pressure of 3 cm H₂O. Then a snapshot perturbation maneuver was imposed to measure the resistance of the whole respiratory system.

Preparation of murine lung cells

Mice were killed, and their lungs were harvested under sterile conditions. Lungs were digested into single-cell suspension by incubating in collagen type IV (catalog no. C5138; Sigma-Aldrich) and DNase I for 30 minutes at 37°C to obtain total lung cells for flow cytometry. Lung cells were filtered through a 40-μm strainer to remove cell clumps. Total lung cell suspensions were prepared, and RBCs were removed by means of incubation in ammonium chloride solution (catalog no. 07800; STEMCELL technologies, Vancouver, British Columbia, Canada).

Flow cytometry–based analysis and sorting of lung cells

Isolated cells from lung tissues from each study group were stained with antibodies against anti-mouse CD3, CD19, CD45, MHC class II, Ly6c, Ly6G, CD11c, and CD11b that conjugated to fluorescein isothiocyanate, phycoerythrin, peridinin-chlorophyll-protein complex–Cy5.5, phycoerythrin-Cy7, allophycocyanin (APC), APC-Cy7, or Pacific Blue (all from eBioscience, San Diego, Calif; BD; Tonbo Bioscienes, San Diego, Calif; and BioLegend, San Diego, Calif) after live cell gating. The stained cells were analyzed by using a FACSCanto (BD Biosciences). For experiments requiring sorted endothelial cells, lung hematopoietic cells, and other cells, lung cells were resuspended with CD31, CD45, and Ter119 antibody on ice as follows: fluorescein isothiocyanate–conjugated anti-CD31 (clone MEC13.3, 1:500, BioLegend), APC–conjugated anti-CD45 (clone 30-F11; 1:500; BioLegend), and APC-conjugated anti-Ter119 (clone TER119; 1:500; BioLegend). CD31+ CD45+ Ter119+ endothelial cells, CD45+ TER119+ CD31+ lung hematopoietic cells, and other cells were fractionated by using a cell sorter (FACSAria II; BD Biosciences). Data were analyzed with FlowJo software (TreeStar, Ashland, Ore).

Monocyte–endothelial cell adhesion assays

A monocyte–endothelial cell adhesion assay was performed, as previously reported. In brief, primary human endothelial cell lines of HUVECs and HPMECs were grown on gelatin-coated 60-μm dishes (catalog no. 81156, ibidi, Cambridge, Mass) with complete culture media. After transfection with scrambled siRNA or Sox17 siRNA, the cells were treated with IL-33 (10 ng/mL) for an additional 6 hours. U937 (catalog no. 21593; Korea Cell Line Bank) and THP-1 (catalog no. TIB-202; ATCC) are widely used monocytic cell lines that originated from tissue and blood leukemia, respectively. Nonadherent U937 and THP-1 cells were suspended overnight in RPMI 1640 with 2% FBS and then freshly harvested before coculture with endothelial cells. A density of 1 × 10⁶ U937 and 1 × 10⁶ THP-1 cells per well was added to plates containing endothelial cells pretreated with IL-33. Prepared endothelial cells were fixed with 4% PFA for 10 minutes, and all treatments were removed from the endothelial and mononcyte cells. After incubation at 37°C for 1 hour, nonadherent monocytic cells were removed by means of gentle washing with PBS 3 times, and monolayers were fixed with 4% PFA in PBS. The images were captured with an optical microscope (Olympus, Center Valley, Pa).

Quantitative real-time PCR

Total RNA was derived from lung tissues and sorted cells of mice by using TRizol (Thermo Scientific). Purified RNA (1 μg) was reverse transcribed to cDNA by using oligo (dT) primer and reverse transcriptase (catalog no. 353137001; Roche, Basel, Switzerland). The resulting cDNA was amplified by means of PCR with a LightCycler 480 SYBR green I step kit and the LightCycler 480 instrument II real-time PCR system (Roche), according to the manufacturer’s instruments. Primer sequences for quantitative real-time PCR are as follows: anti-mouse primers for ICAM-1 (forward, GTGCC GGAAAAATTCCTCG; reverse, CGTCTGCGATGGCTGATCT TAGGAG), CCL2 (forward, TTTAAAACCTGGATCGAACCA; reverse, GCATTA GCTTCAGATTACCGGT), ST2 (forward, ATTCAGGGGACCACATCA AGTG; reverse, CGTCTTGGAGGCTCTTCTG), CD31 (forward, AGAGA
CGTCTTGTGCAG; reverse, TACTGGGCTCTCGAGAGCAT), IL-33 (forward, ATGGGAGGCTGATG; reverse, CCGAGGACTTTTGTGAAAG), Sox17 (forward, GAGGGCCAGAAGCAGTGTTACAC; reverse, CATATTTCTCAGTTGTCAC), and the housekeeping gene GAPDH (forward, GAGCCAAAAGGGTCTCATCA; reverse, CATATTTCTCAGTTGTCAC). Human primers for ICAM-1 (forward, CCTCTCAGTTGTCAGTGC; reverse, AGGCTGAAAGGTTCTGTC), CCL2 (forward, GTGGTGTCCAGCTGAGTCTG; reverse, GGTGTGTGCTCCAGAGGATA), CCL11 (forward, AAACCAACCCCTCTCAGGA; reverse, GCAGGAAGGTGGACGACAGAAG), CCL20 (forward, ACTCCACCTGGCGCAATCA; reverse, ATGGGCAGCTGCGGTGGA), CXCL1 (forward, TCTTGCACTCCCCCATATGTA; reverse, CCTTCAGGAACAGCCACCAGT), IL-33 (forward, CAGACTCCACCTCTGCGGCGAATCA; reverse, ATTGGCCAGCTGCCGTGT), and the housekeeping gene β-actin (forward, CCAACCGCGACTCACA; reverse, CAGACCGCACTCAGGATG). Data were analyzed by using LightCycler Software (version 3.5; Roche). Relative quantitation was calculated by using the comparative cycle threshold (2^ΔΔCT) method. Expression levels of each gene were then normalized to those of β-actin.

**Cytokine analysis in culture supernatants obtained from splenocyte and MLN cell cultures**

Splenocytes and MLN cells were prepared after RBC removal by means of incubation in ammonium chloride solution (catalog no. 07800; STEMCELL Technologies). For T-cell activation experiments, total splenocytes and MLNs were counted with Trypan blue, and the same amount of freshly obtained cells (3 × 10^6 cell/well) was cultured in 48-well round-bottom plates in complete culture medium (RPMI 1640; Welgene) containing protease inhibitor cocktail (Roche). After incubation at 37°C for 3 days, a 300-µL culture supernatant was collected and preserved at −80°C for storage. Supernatants were preserved at −80°C and used for analysis of cytokine levels in the lungs by using ELISA development kits (R&D Systems), according to the manufacturer’s instructions.

**Western blotting**

Mouse lung tissues were lysed on ice in lysis buffer (Cell Signaling) containing protease inhibitors. Primary human endothelial cell lines were harvested and dissolved in RIPA buffer (50 mmol/L Tris-HCl [pH 7.4], 150 mmol/L sodium chloride, 1 mmol/L EDTA, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate) with 1 mmol/L sodium orthovanadate and 1× protease inhibitor cocktail (Roche). Protein samples were incubated on ice for 20 minutes and then centrifuged at 14,000 rpm for 10 minutes at 4°C. Proteins were separated by using SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat skim milk (or 5% BSA; Sigma) for 1 hour and incubated with primary antibody overnight at 4°C and horseradish peroxidase-conjugated secondary antibody for 2 hours. The following primary antibodies were used: anti-Sox17 (catalog no. AF1924; 1:2500; R&D Systems), anti-β-actin (catalog no. BS6007; 1:5000; GeneTex, Irvine, Calif), anti-phosphorylated ERK (catalog no. 9106S; 1:1000; Cell Signaling), anti-total ERK (catalog no. 9102; 1:1000; Cell Signaling), anti-phosphorylated STAT3 (catalog no. 9155S; 1:1000; Cell Signaling), and anti-ST2 (catalog no. MAB523; 1:1000; R&D Systems).

**REFERENCES**


FIG E1. ELISA analysis of IL-33 in CM of BEAS-2B cells after exposure to HDM (1 µg/mL) for 4 hours. IL-33 levels were detected in CM obtained from HDM-treated BEAS-2B cells and vehicle control (n = 3 per group). *Significance compared with the control group: **P < .001.
FIG E2. IL-25 and TSLP in lung homogenates of OVA-induced asthma models. A and B, Protein levels of IL-25 and TSLP in lung tissues of OVA-induced asthma models from Sox17\textsuperscript{iD}EC and WT mice, as measured by means of ELISA, are shown (n = 4-8 mice per group). Ctrl, WT mice; \textit{iD}EC, endothelium-specific Sox17 knockout mice (Sox17\textsuperscript{iD}EC mice); n.s., not significant.
FIG E3. Immune responses in the spleens of WT and Sox17-deleted mice. A, Total count of splenocytes in each study group. B and C, Th2 cytokines (IL-4, IL-13) production by splenocytes from WT C57BL/6 and Sox17EC mice 72 hours after anti-CD3/anti-CD28 stimulation is shown (n = 5–11 mice per group). Ctrl, WT mice; iΔEC, endothelium-specific Sox17 knockout mice (Sox17iΔEC mice); n.s., not significant. #Significance compared with the control group: #P < .05 and ###P < .0001.
FIG E4. Modulation of OVA-induced airway inflammation and Th2 immune responses by deletion of endothelial Sox17 between sensitization and OVA challenge. **A**, Experimental design for late Sox17 deletion after sensitization and OVA challenge in WT and Sox17KOEC mice (KOEC, upper portion) and Sox17 expression in the lung tissues of each mouse model (lower portion; n = 3-5 mice per group). i.p., Intraperitoneal. **B**, Total cell count in BALF measured with a hemocytometer. **C**, Differential BALF cell analysis. **D**, Hematoxylin and eosin (H&E) staining in the lungs of peribronchial (lower left panel) and alveolar (lower right panel) spaces. Original magnification ×40 and ×200. **E**, Levels of IL-4, IL-13, and CCL2 in BALF of WT and Sox17KOEC mice on day 19 at 24 hours after the last OVA challenge on day 18. **F**, Levels of serum IgE in each study group. **G**, Total cell counts in splenocytes and MLNs at day 19. **H**, Splenocytes and MLNs were cultured in the presence or absence of 1 μg/mL anti-CD3 or anti-CD28 for 3 days. Concentrations of IL-4 and IL-13 were determined in culture supernatants by using ELISA. Images are representative of 3 independent lung sections, each from 3 different mice. Ctrl, WT mice; KOEC, endothelium-specific Sox17 knockout mice (Sox17KOEC mice); n.s., not significant. #Significance compared with the control group: #P < .05, ##P < .001, and ###P < .0001. *Significance within groups: *P < .05, **P < .001, and ***P < .0001. Scale bars = 50 μm.