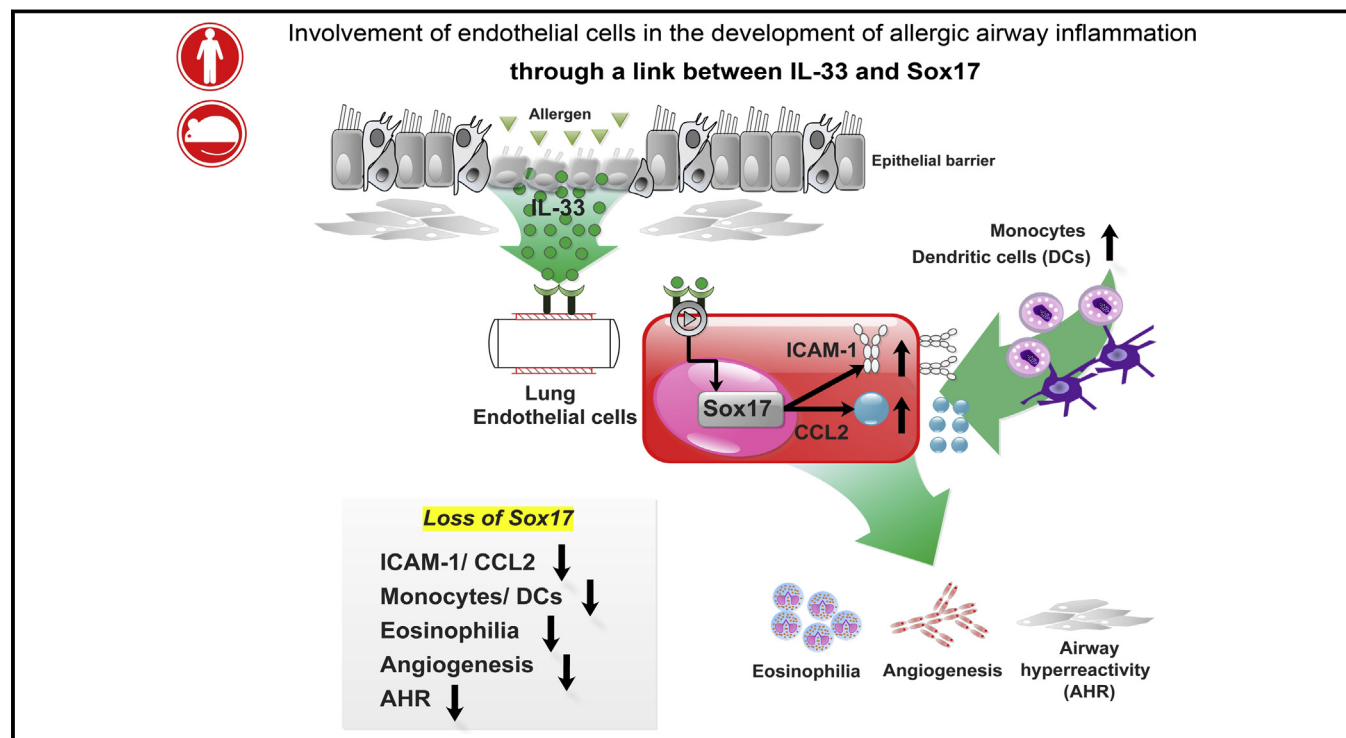


Endothelial Sox17 promotes allergic airway inflammation



Eun Hee Ha, BS,^a Jun-Pyo Choi, PhD,^c Hyouk-Soo Kwon, MD, PhD,^b Hyeung Ju Park, PhD,^a Sang Joon Lah, BS,^a Keun-Ai Moon, MS,^c Seung-Hyo Lee, PhD,^{a,d} Injune Kim, PhD,^{a,d} and You Sook Cho, MD, PhD^b *Daejeon and Seoul, Korea*

GRAPHICAL ABSTRACT



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

From ^athe Biomedical Science and Engineering Interdisciplinary Program, Korea Advanced Institute of Science and Technology (KAIST), Daejeon; ^bthe Division of Allergy and Clinical Immunology, Department of Internal Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul; ^cthe Asan Institute for Life Science, Seoul; and ^dGraduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology (KAIST), Daejeon.

This study was financially supported by a grant from the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), which was funded by the Ministry for Health and Welfare, South Korea (grant no. HI14C2628); National Research Foundation of Korea (NRF) MRC grant funded by the Korean government, South Korea (MSIT; grants NRF-2018R1A5A2020732 to Y.S.C.); and the National Research Foundation of Korea, South Korea (grants NRF-2018R1A2A1A05022672 to I.K.).

Disclosure of potential conflict of interest: The authors declare that they have no relevant conflicts of interest.

Received for publication August 14, 2018; revised January 24, 2019; accepted for publication February 22, 2019.

Available online March 28, 2019.

Corresponding author: You Sook Cho, MD, PhD, Division of Allergy and Clinical Immunology, Department of Internal Medicine, Asan Medical Center, University of Ulsan College of Medicine, 88, Olympic-ro 43-gil, Songpa-gu, Seoul 05505, Korea. E-mail: yscho@amc.seoul.kr.

The CrossMark symbol notifies online readers when updates have been made to the article such as errata or minor corrections

0091-6749/\$36.00

© 2019 Published by Elsevier Inc. on behalf of the American Academy of Allergy, Asthma & Immunology

<https://doi.org/10.1016/j.jaci.2019.02.034>

infiltration, cytokine/chemokine production, and airway hyperresponsiveness. *Sox17* deletion also resulted in decreased densities of $\text{Ly6c}^{\text{high}}$ 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 epithelial 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.¹⁴⁻¹⁶ 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.^{17,18} 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,¹⁹⁻²³ which are known to play essential roles in asthmatic patients. Recently, a microarray analysis showed that *Sox17* was upregulated in IL-33–stimulated

Abbreviations used

AHR:	Airway hyperresponsiveness
BALF:	Bronchoalveolar lavage fluid
CM:	Conditioned medium
DC:	Dendritic cell
ERK:	Extracellular signal-regulated kinase
HDM:	House dust mite
HPMEC:	Human pulmonary microvascular endothelial cell
HUVEC:	Human umbilical vein endothelial cell
ICAM-1:	Intercellular adhesion molecule 1
MLN:	Mediastinal lymph node
OVA:	Ovalbumin
PFA:	Paraformaldehyde
siRNA:	Small interfering RNA
Sox:	Sry-related high-mobility-group box
STAT3:	Signal transducer and activator of transcription 3
TSLP:	Thymic stromal lymphopoietin
WT:	Wild-type

endothelial cells, thereby suggesting a possible relationship between IL-33 and *Sox17*.²⁴ 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)-CreER^{T2} mice²⁵ were crossed with *Sox17^{fl/fl}* mice²⁶ to generate endothelium-specific *Sox17* knockout mice (*Sox17^{ΔEC}* mice); at 6 to 8 weeks of age, *Sox17^{fl/fl}Cdh5(PAC)-CreER^{T2}* 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 *Sox17^{ΔEC}* 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

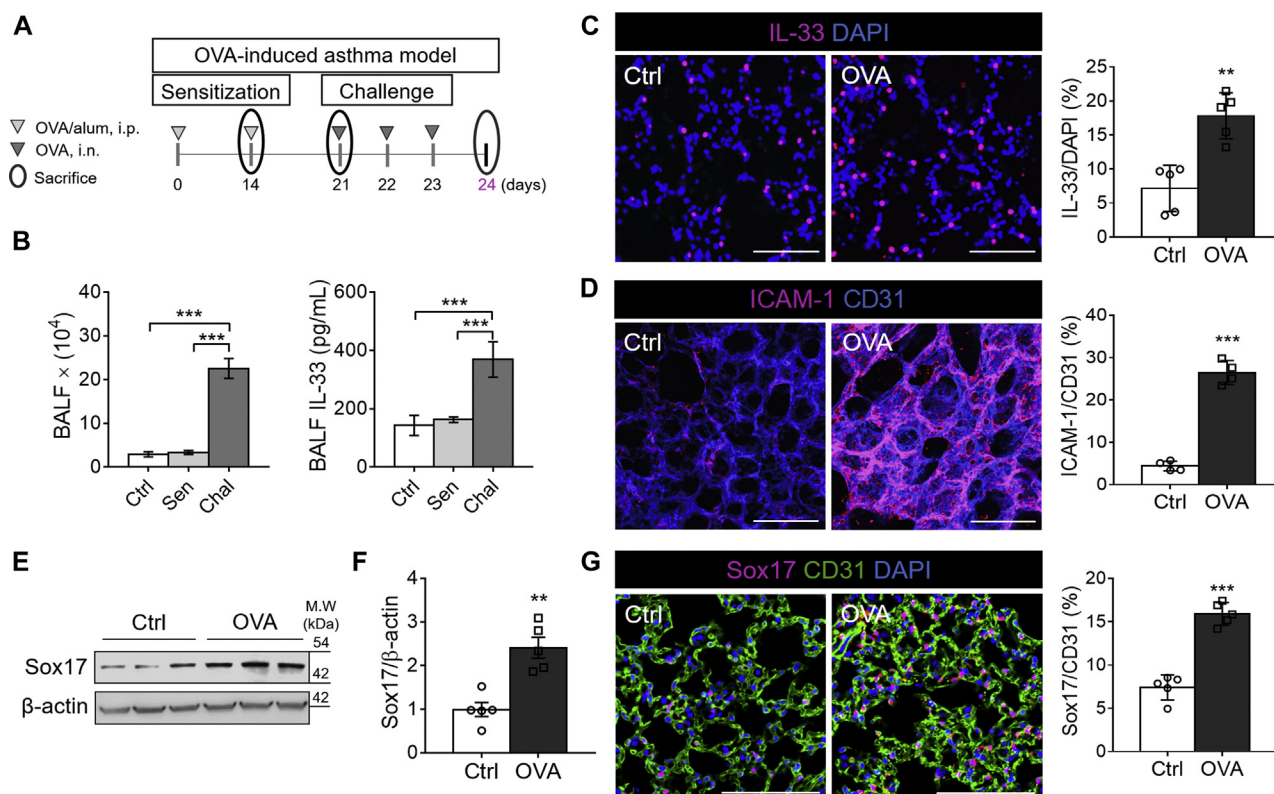


FIG 1. IL-33 secretion and pulmonary endothelial activation in an OVA-induced mouse model of allergic airway inflammation. **A**, Experimental design for generating a mouse model of allergic airway inflammation. Mice were sensitized by means of intraperitoneal (*i.p.*) injection of OVA/alum, followed 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.

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](http://www.jacionline.org) 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'-ACGCCAGCUCCGCGGUA UA-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

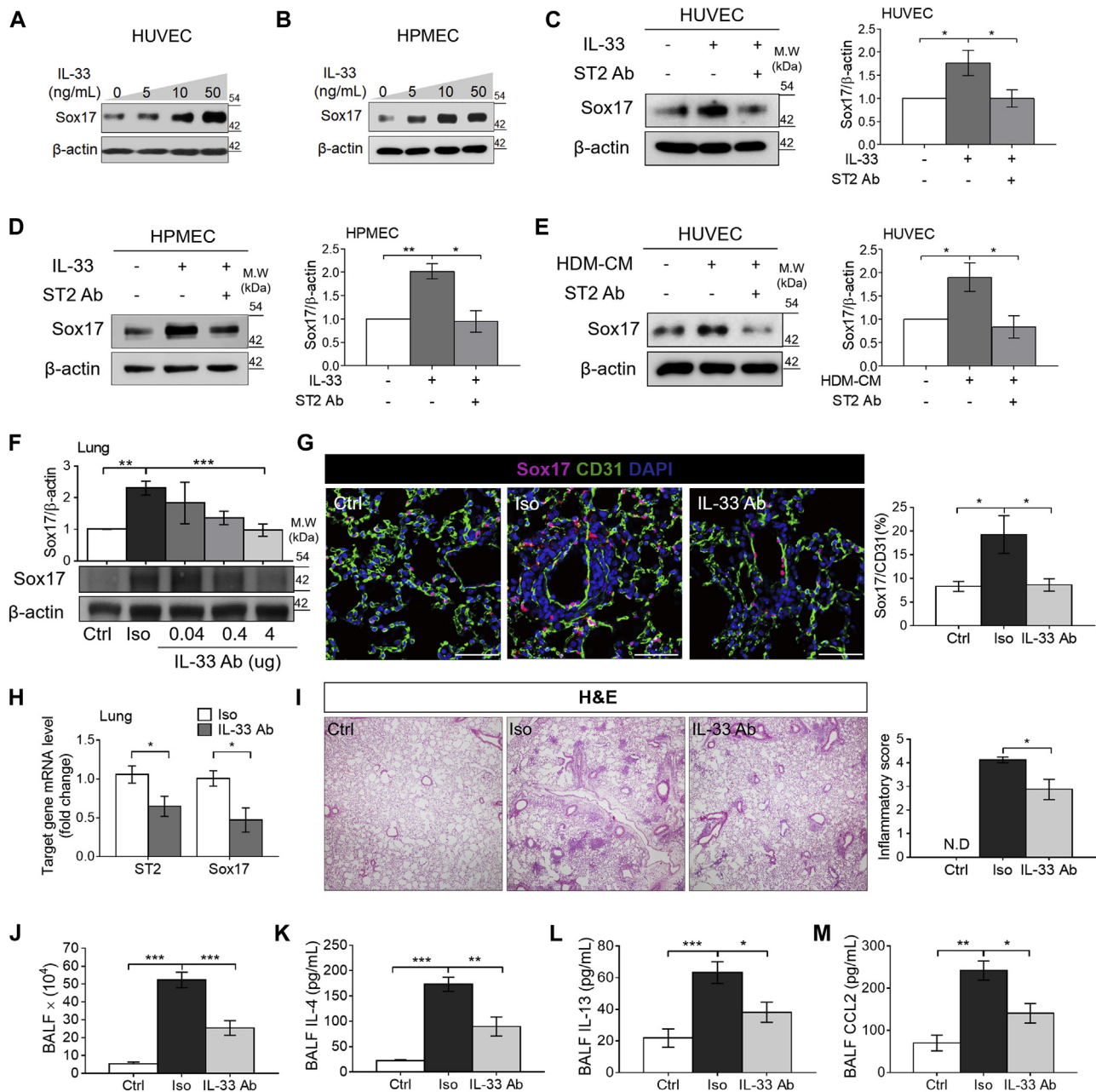


FIG 2. Sox17 upregulation in an IL-33-enriched environment of OVA-exposed mice. **A** and **B**, Immunoblotting of Sox17 and β -actin from HUVECs and HPMECs treated with increasing concentrations of IL-33 (0, 5, 10, and 50 ng/mL). **C** and **D**, HUVECs and HPMECs cotreated with IL-33 and anti-ST2 antibody 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 $\times 40$ ($n = 5$ 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 μ g/mL; IL-33 Ab, 4 μ g of IL-33 neutralizing antibody; Iso, isotype; M.W., Molecular weight; N.D., not determined; ST2 Ab, 1 μ g/mL ST2 neutralizing antibody. *Significance between groups: * $P < .05$, ** $P < .001$, and *** $P < .0001$. Scale bars = 50 μ m.

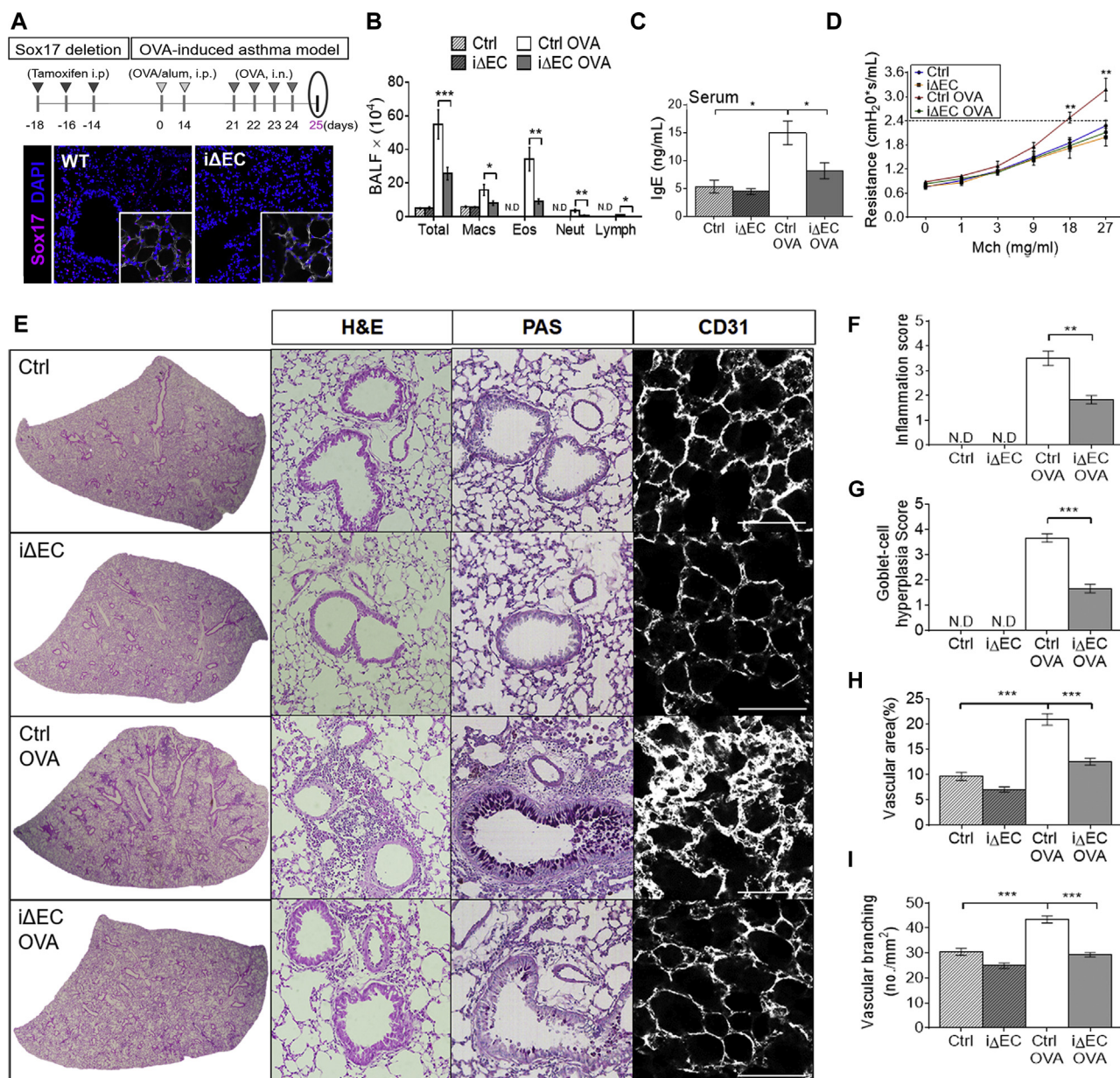


FIG 3. Endothelium-specific *Sox17* deletion suppresses OVA-induced allergic airway inflammation, AHR, and goblet cell hyperplasia. **A**, Experimental design for generating an OVA-induced asthma model in WT and *Sox17*^{ΔEC} mice (iΔEC; upper portion) and *Sox17* expression in the lung tissues of each mouse model (lower portion; n = 4–15 per group). i.n., Intranasal, i.p., intraperitoneal. **B**, Total and differential cell analysis of BALF. **C**, Levels of serum IgE in each study group. **D**, Changes in airway resistance. **E** and **F**, Hematoxylin and eosin (H&E) staining in the lungs. Original magnification, ×200 and ×10. **E** and **G**, Periodic acid-Schiff (PAS) staining in the lungs. Original magnification ×200. **E**, **H**, and **I**, CD31 immunostaining for assessment of CD31⁺ vessels in each study group. Quantification of vessel area and vessel branching. **J–N**, Levels of IL-4, IL-13, IL-33, CCL2, and CCL11 in BALF of WT and *Sox17*^{ΔEC} mice on day 25, 24 hours after the last OVA challenge on day 24. Similar results were obtained from at least 3 independent experiments. **O**, Total lung mRNA levels of ICAM-1 (n = 5–7 per group). **P**, ICAM-1 (red) and CD31 (blue) immunostaining in respective lung sections. Images are representative of independent lung sections from 3 different mice from 2 independent experiments. Ctrl, WT mice; DAPI, 4'-6-diamidino-2-phenylindole dihydrochloride; iΔEC, endothelium-specific *Sox17* knockout mice (*Sox17*^{ΔEC} mice); M.W., molecular weight; N.D., not determined. *Significance within groups: *P < .05, **P < .001, and ***P < .0001. Scale bars = 100 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.

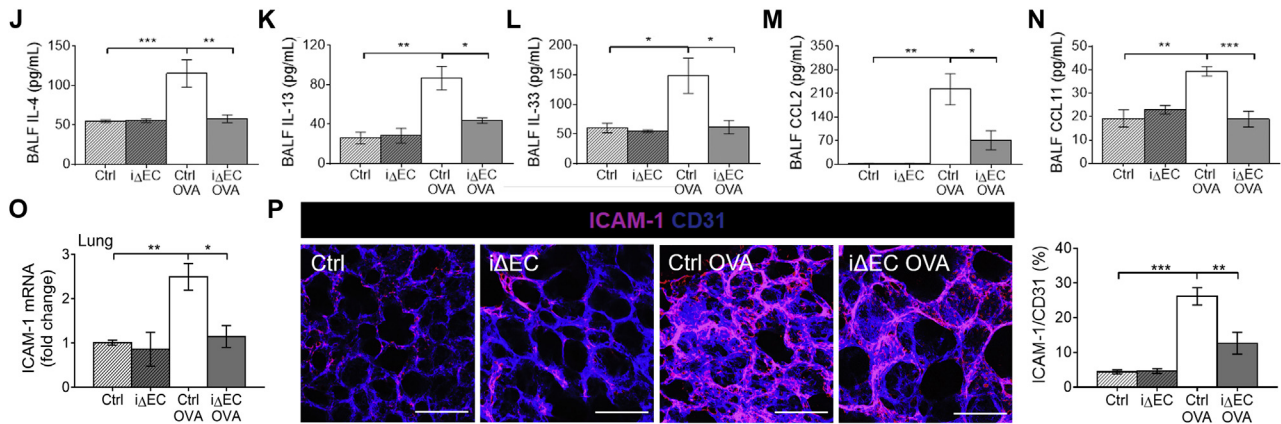


FIG 3. (Continued).

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 $\pm$ 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 *Sox17*^{iΔEC} mice with endothelium-specific deletion of *Sox17*. *Sox17*^{iΔEC} 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 *Sox17*^{iΔEC} mice (Fig 3, B). Serum IgE levels were not significantly increased in *Sox17*^{iΔEC} asthmatic mice compared with those in WT asthmatic mice (Fig 3, C). *Sox17*^{iΔEC} 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 *Sox17*^{iΔEC} mice (Fig 3, E and F). Amelioration of goblet cell hyperplasia was also observed in the lungs of OVA-challenged *Sox17*^{iΔEC} 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

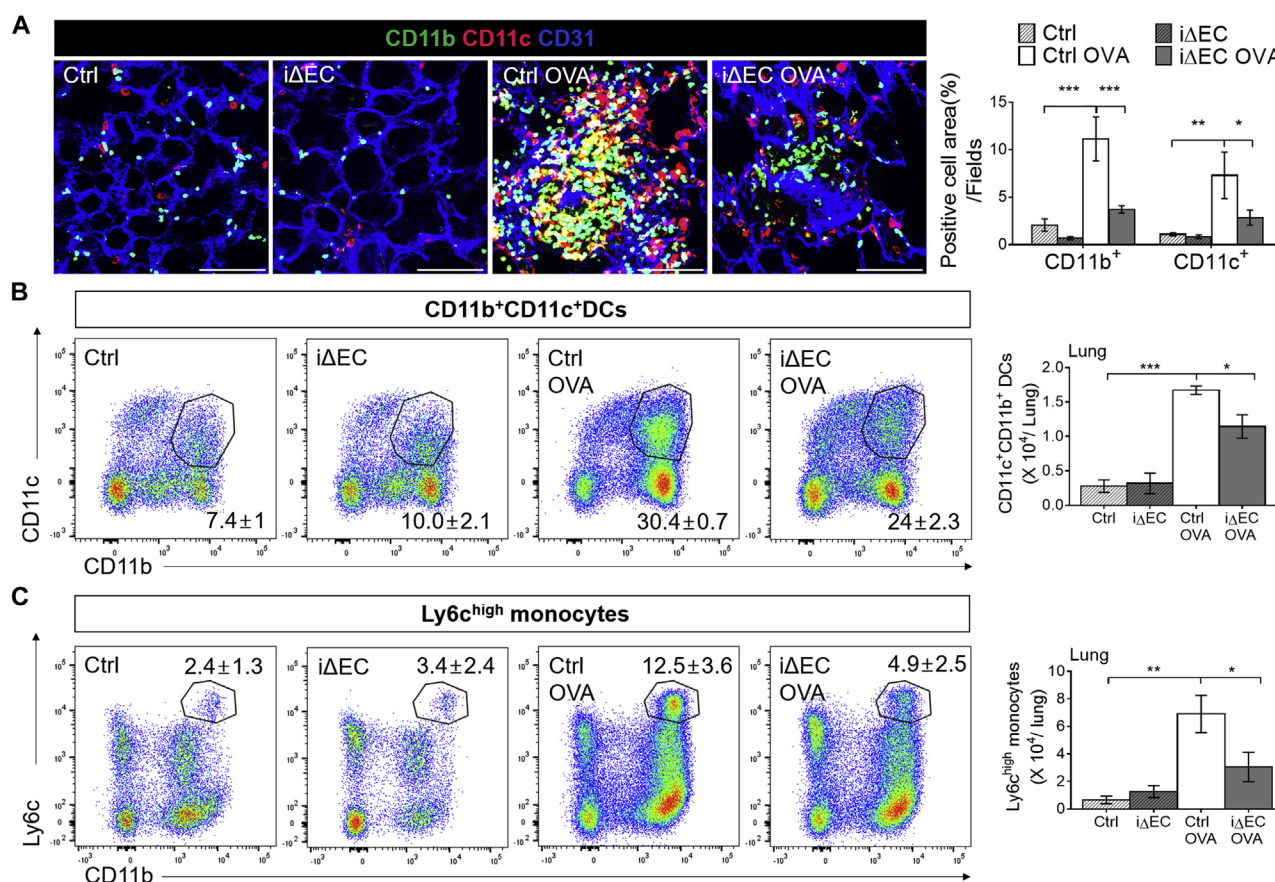


FIG 4. Endothelium-specific *Sox17* deletion suppresses inflammatory DC and monocyte homing into the lung. **A**, CD11b (green) and CD11c (red) immunostaining of lung tissues from control and OVA groups in endothelium-specific *Sox17* knockout mice. Quantification of CD11b⁺ and CD11c⁺ immune cells in 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⁺MHCII⁺Ly6g⁺CD11b⁺CD11c⁺; Fig 4, **B**) and Ly6c^{high} monocytes (CD45⁺CD3⁺CD19⁺Ly6g⁺Ly6c⁺CD11b⁺; Fig 4, **C**) 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; iΔEC, endothelium-specific *Sox17* knockout mice. *Significance within groups: *P < .05, **P < .001, and ***P < .0001. Scale bars = 100 μm.

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*^{iΔEC} 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*^{iΔEC} 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*^{iΔEC} 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 T_H2 cytokine profiles in the spleens of WT and *Sox17*^{iΔEC} 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*^{iΔEC} mice; importantly, there were no significant differences between WT and *Sox17*^{iΔEC} mice (see 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*^{iΔEC} 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*^{iΔEC} mice showed reduced infiltration of CD11b⁺ and CD11c⁺ cells in their

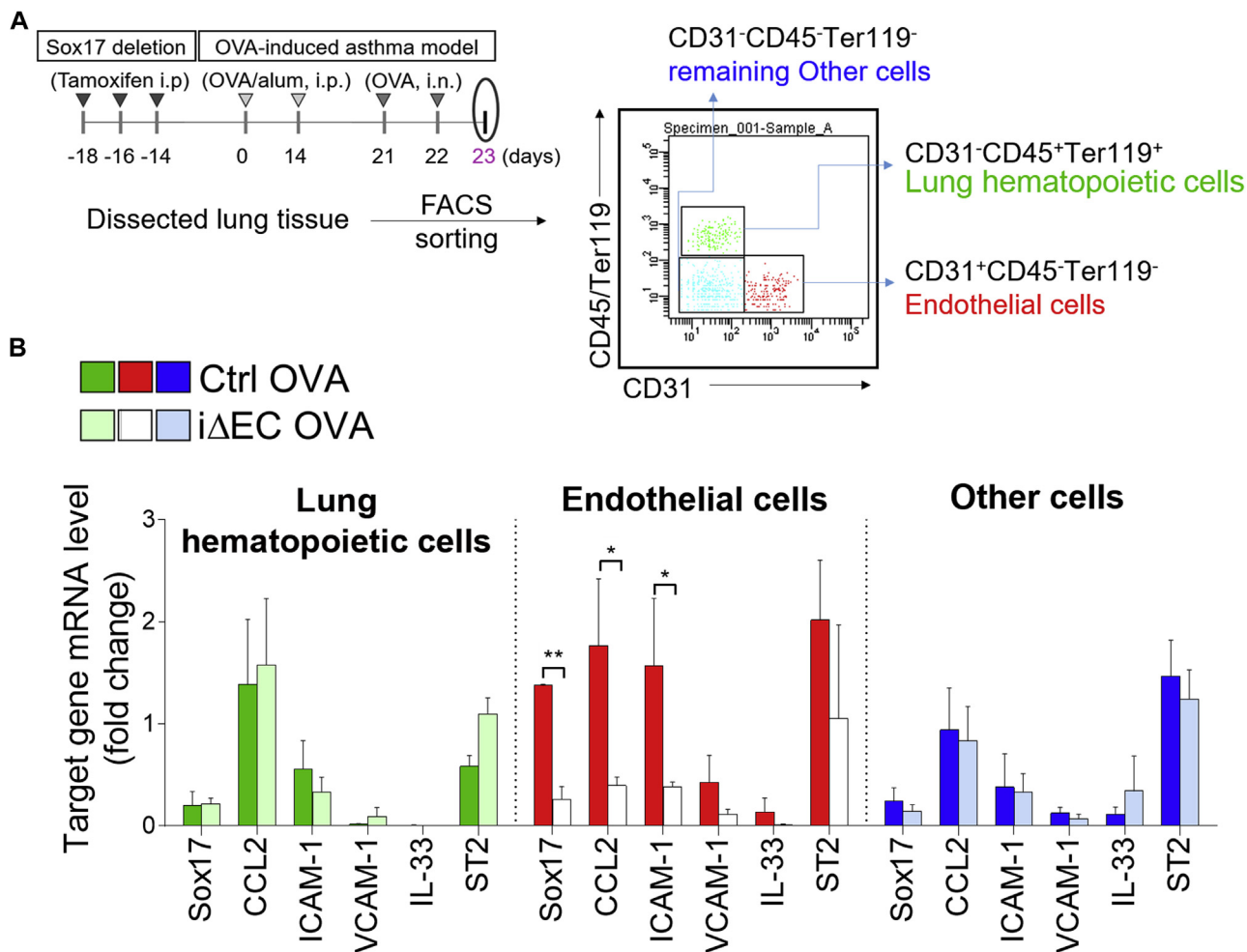


FIG 5. Endothelial Sox17 contributes to endothelial activation in an OVA-induced allergic airway inflammation mouse model. **A**, Flow cytometric gating strategy for isolating lung endothelial cells and lung hematopoietic cells from other types of cells from OVA-exposed mice. Flow cytometric plot shows endothelial cells (CD31⁺CD45⁻Ter119⁻, red), lung hematopoietic cells (CD31⁻CD45⁺Ter119⁺, green), and other cells (CD31⁻CD45⁻Ter119⁻, blue), including smooth muscle cells and epithelial cells. *i.n.*, Intranasal; *i.p.*, intraperitoneal. **B**, End point quantitative real-time PCR in sorted endothelial cells, lung hematopoietic cells, and other cells from lung tissues. Similar results were obtained from 3 independent experiments. Ctrl, WT mice; Δ EC, endothelium-specific Sox17 knockout mice. *Significance between the Ctrl OVA group and the Δ EC OVA group: * $P < .05$ and ** $P < .001$.

lung tissues (Fig 4, A). Moreover, numbers of CD11b⁺CD11c⁺ inflammatory dendritic cells (DCs) and Ly6c^{high} monocytes were decreased in the lungs of OVA-challenged Sox17 ^{Δ 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 knockdown was significantly decreased. In addition, we found that ICAM-1 expression was significantly reduced in Sox17 knockdown endothelial cells after IL-33 stimulation (Fig 6, E and

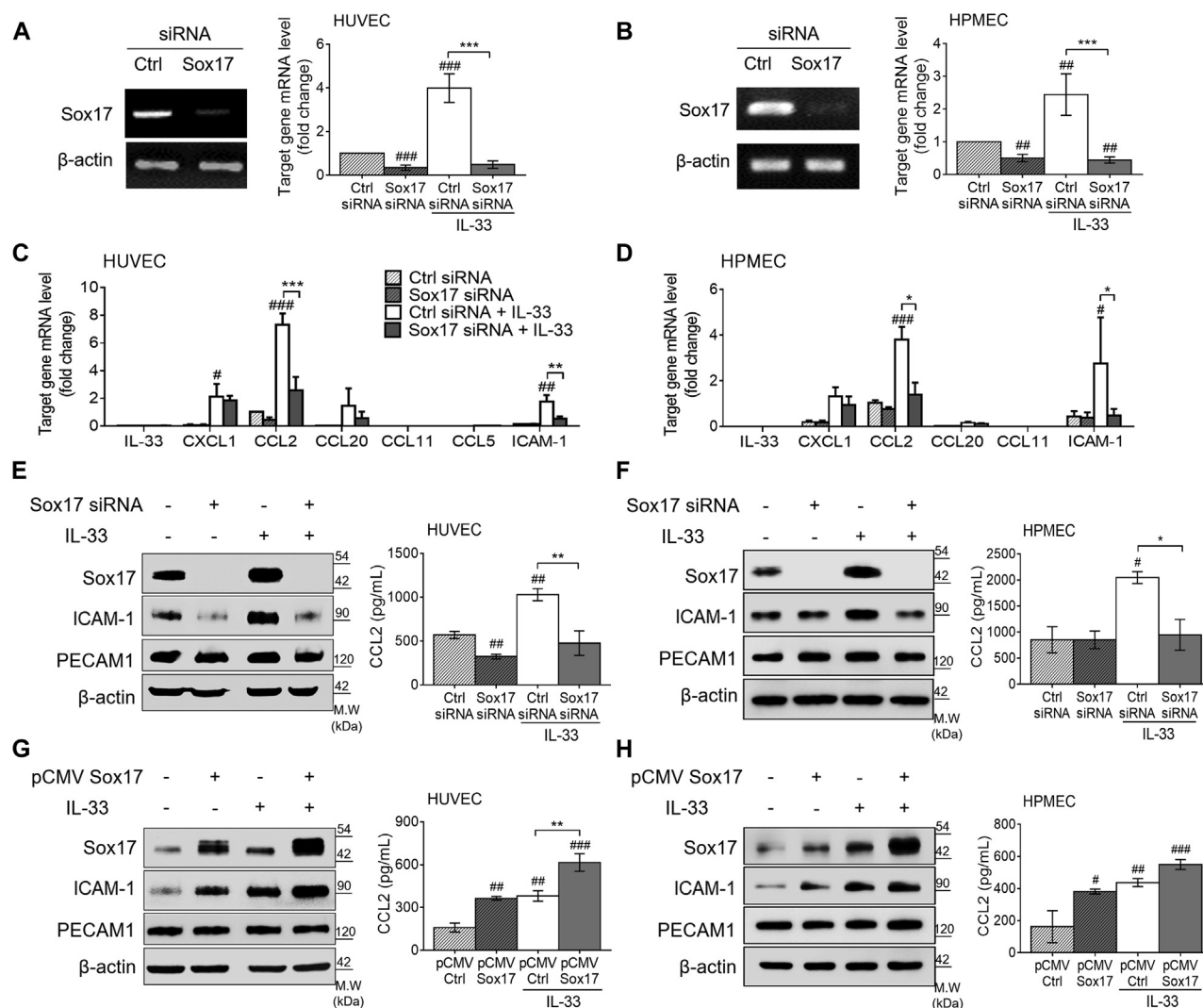


FIG 6. Sox17 promotes IL-33-mediated endothelial activation. **A** and **B**, Sox17 mRNA in IL-33-treated HUVECs and HPMECs transfected with Sox17 siRNA or control (Ctrl) siRNA. **C** and **D**, mRNA levels of IL-33, CXCL1, CCL2, CCL5, CCL11, CCL20, and ICAM-1 in HUVECs and HPMECs Sox17 knockdown with IL-33 treatment. **E** and **F**, Sox17, platelet endothelial cell adhesion molecule 1 (PECAM1), and ICAM-1 protein expression levels in lysates of Sox17-silenced HUVECs and HPMECs with IL-33 treatment and CCL2 levels in supernatants. **G** and **H**, Sox17, PECAM1, and ICAM-1 expression levels in lysates of Sox17-overexpressed HUVECs and HPMECs with IL-33 treatment and CCL2 levels in supernatants. **I**, Photomicrographs showing the attachment abilities of monocytic cell lines (U937 and THP-1) to Sox17-silenced HUVECs (or HPMECs) with IL-33 treatment. Original magnification $\times 40$. **J**, Immunoblotting for phosphorylated ERK, phosphorylated STAT3, and ST2 in HUVECs with Sox17 knockdown after IL-33 treatment. Similar results were obtained from 3 independent experiments. Ctrl siRNA, scrambled siRNA; pCMV-Ctrl, negative control plasmid vector; Sox17 siRNA, Sox17-targeting siRNA; pCMV-Sox17, Sox17 expression vector. #Significance compared with the control group: $\#P < .05$, $\#\#P < .001$, and $\#\#\#P < .0001$. *Significance between IL-33-treated mouse groups: $*P < .05$, $**P < .001$, and $***P < .0001$.

F). 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

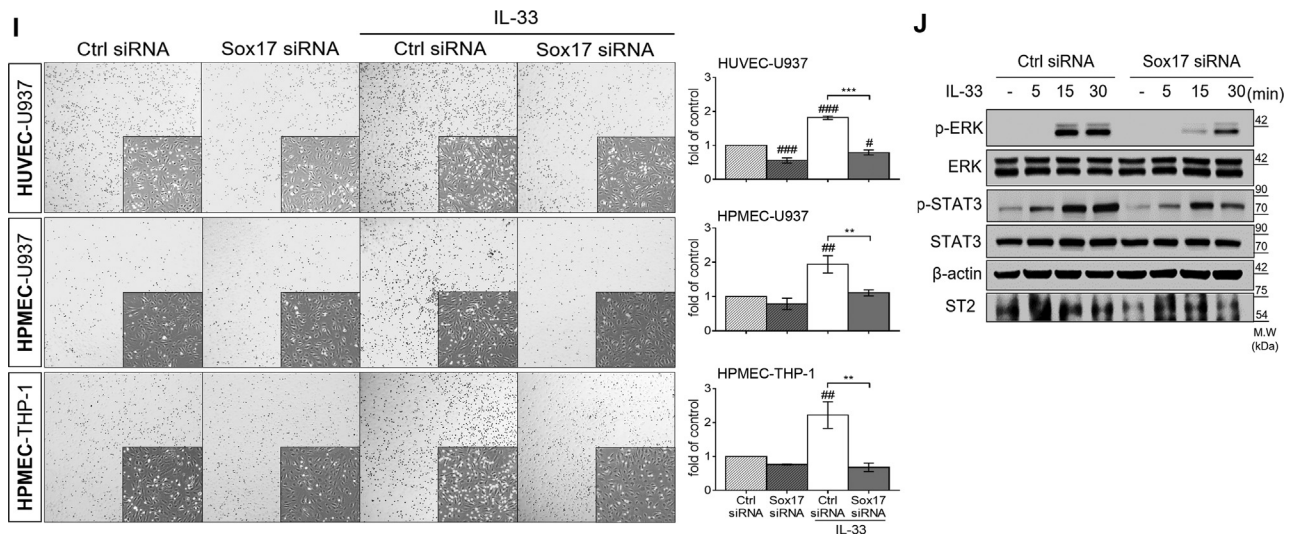


FIG 6. (Continued).

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,²⁷ 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 T_H2 -inflamed lungs.^{14,24,28} IL-33 also carries vascular functions by increasing angiogenesis and vascular permeability.^{29,30} On the contrary, a recent report showed that IL-33 regulates retinal angiogenesis through direct inhibition of fibroblasts and endothelial cells.³¹ 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^{22,32} and pulmonary vascular morphogenesis.³³ Current evidence supports the role of Sox17 during arterial endothelial cell differentiation and vascular remodeling¹⁹ as an upstream regulator of Notch signaling, which induces IL-33 expression in quiescent vessels.²⁸ 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.³⁴ 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 T_H2 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.¹⁴ 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.^{35,36} Accordingly, our results also showed that Sox17 expression and T_H2 -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.³⁷

Sox17 was also suggested as a candidate transcription factor associated with thromboxane A_2 receptor polymorphisms, which is implicated in childhood-onset asthma.³⁸ 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 of various cytokines, chemokines, and adhesion molecules that are critical in the development of allergic airway inflammation. Sox17 was

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.^{24,29,30,39} 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⁺ T cells, which is leading to activation of pulmonary adaptive immunity.⁴⁰ Specifically, the pool of CD11b⁺CD11c⁺ DCs and Ly6C^{high} monocytes has been reported to be recruited to the lungs with ongoing inflammation after exposure to HDM or OVA allergen.^{41,42} Among lung DC subsets, resident CD11b⁺Ly6C[−] DCs are responsible for T_H2 cell differentiation and subsequent allergic airway sensitization to HDM.⁴³ Also, Ly6C^{high} monocytes have potential to differentiate into lung DCs during persistent inflammation.⁴¹ Interestingly, we observed that infiltration of CD11b⁺CD11c⁺ DCs and Ly6C^{high} monocytes to the lungs was greatly reduced in OVA-challenged *Sox17*^{ΔEC} mice. Our results indicate that endothelial Sox17 is closely linked to development of the T_H2 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[−]Ter119[−]CD31[−] 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 DCs⁴⁴; 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,^{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.^{46–48} 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.^{14,24} 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 IκB-α, mitogen-activated protein kinase, ERK, p38, c-Jun N-terminal kinase, and STAT3.^{49,50}

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.

We thank Dr Joon Seo Lim from the Scientific Publications Team at Asan Medical Center for his editorial assistance in preparing this manuscript. The Korea Advanced Institute of Science and Technology Venture Research Program for Graduate and PhD students was instrumental in the development of this article.

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

- Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 2012;18:716-25.
- Pelaia G, Vatrella A, Maselli R. The potential of biologics for the treatment of asthma. *Nat Rev Drug Discov* 2012;11:958-72.
- Paul WE, Zhu J. How are T(H)2-type immune responses initiated and amplified? *Nat Rev Immunol* 2010;10:225-35.
- Hammad H, Lambrecht BN. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat Rev Immunol* 2008;8:193-204.
- Shoda T, Futamura K, Orihara K, Emi-Sugie M, Saito H, Matsumoto K, et al. Recent advances in understanding the roles of vascular endothelial cells in allergic inflammation. *Allergol Int* 2016;65:21-9.
- Lambrecht BN, Hammad H. Asthma: the importance of dysregulated barrier immunity. *Eur J Immunol* 2013;43:3125-37.
- Yi L, Cheng D, Zhang K, Huo X, Mo Y, Shi H, et al. Intelectin contributes to allergen-induced IL-25, IL-33, and TSLP expression and type 2 response in asthma and atopic dermatitis. *Mucosal Immunol* 2017;10:1491-503.
- Portelli M, Sayers I. Genetic basis for personalized medicine in asthma. *Expert Rev Respir Med* 2012;6:223-36.
- Prefontaine D, Nadigel J, Chouiali F, Audusseau S, Semlali A, Chakir J, et al. Increased IL-33 expression by epithelial cells in bronchial asthma. *J Allergy Clin Immunol* 2010;125:752-4.
- Prefontaine D, Lajoie-Kadach S, Foley S, Audusseau S, Olivenstein R, Halayko AJ, et al. Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. *J Immunol* 2009;183:5094-103.
- Janeva E, Kaeva B, Breskovska G, Goseva Z, Angelko G, Zdravetska M, et al. IL-33 in patients with severe uncontrolled allergic asthma. *Eur Respir J* 2017;50: PA572.
- Milovanovic M, Volarevic V, Radosavljevic G, Jovanovic I, Pejnovic N, Arsenijevic N, et al. IL-33/ST2 axis in inflammation and immunopathology. *Immunol Res* 2012;52:89-99.
- Soveg F, Abdala-Valencia H, Campbell J, Morales-Nebreda L, Mutlu GM, Cook-Mills JM. Regulation of allergic lung inflammation by endothelial cell transglutaminase 2. *Am J Physiol Lung Cell Mol Physiol* 2015;309:L573-83.
- Yagami A, Orihara K, Morita H, Futamura K, Hashimoto N, Matsumoto K, et al. IL-33 mediates inflammatory responses in human lung tissue cells. *J Immunol* 2010;185:5743-50.
- Demyanets S, Konya V, Kastl SP, Kaun C, Rauscher S, Niessner A, et al. Interleukin-33 induces expression of adhesion molecules and inflammatory activation in human endothelial cells and in human atherosclerotic plaques. *Arterioscler Thromb Vasc Biol* 2011;31:2080-9.
- Gautier V, Cayrol C, Farache D, Roga S, Monsarrat B, Burlet-Schiltz O, et al. Extracellular IL-33 cytokine, but not endogenous nuclear IL-33, regulates protein expression in endothelial cells. *Sci Rep* 2016;6:34255.
- Liu T, Laidlaw TM, Feng C, Xing W, Shen S, Milne GL, et al. Prostaglandin E2 deficiency uncovers a dominant role for thromboxane A2 in house dust mite-induced allergic pulmonary inflammation. *Proc Natl Acad Sci U S A* 2012;109:12692-7.
- Liu T, Garofalo D, Feng C, Lai J, Katz H, Laidlaw TM, et al. Platelet-driven leukotriene C4-mediated airway inflammation in mice is aspirin-sensitive and depends on T prostanoid receptors. *J Immunol* 2015;194:5061-8.
- Corada M, Orsenigo F, Morini MF, Pitulescu ME, Bhat G, Nyqvist D, et al. Sox17 is indispensable for acquisition and maintenance of arterial identity. *Nat Commun* 2013;4:2609.
- Lange AW, Keiser AR, Wells JM, Zorn AM, Whitsett JA. Sox17 promotes cell cycle progression and inhibits TGF-beta/Smad3 signaling to initiate progenitor cell behavior in the respiratory epithelium. *PLoS One* 2009;4:e5711.
- Sinner D, Rankin S, Lee M, Zorn AM. Sox17 and beta-catenin cooperate to regulate the transcription of endodermal genes. *Development* 2004;131:3069-80.
- Kim K, Kim IK, Yang JM, Lee E, Koh BI, Song S, et al. SoxF transcription factors are positive feedback regulators of VEGF signaling. *Circ Res* 2016;119:839-52.
- Yang H, Lee S, Kim K, Yang Y, Kim JH, Adams RH, et al. Sox17 promotes tumor angiogenesis and destabilizes tumor vessels in mice. *J Clin Invest* 2013;123:418-31.
- Pollheimer J, Bodin J, Sundnes O, Edelmann RJ, Skanland SS, Sponheim J, et al. Interleukin-33 drives a proinflammatory endothelial activation that selectively targets nonquiescent cells. *Arterioscler Thromb Vasc Biol* 2013;33:e47-55.
- Okabe K, Kobayashi S, Yamada T, Kurihara T, Tai-Nagara I, Miyamoto T, et al. Neurons limit angiogenesis by titrating VEGF in retina. *Cell* 2014;159:584-96.
- Kim I, Saunders TL, Morrison SJ. Sox17 dependence distinguishes the transcriptional regulation of fetal from adult hematopoietic stem cells. *Cell* 2007;130:470-83.
- Tonnel AB, Lassalle P, Delneste Y. Role of endothelial cells in the pathogenesis of bronchial asthma. *Ann Allergy* 1993;71:306-11.
- Sundlisaeter E, Edelmann RJ, Hol J, Sponheim J, Kuchler AM, Weiss M, et al. The alarmin IL-33 is a notch target in quiescent endothelial cells. *Am J Pathol* 2012;181:1099-111.
- Stojkovic S, Kaun C, Heinz M, Krychtiuk KA, Rauscher S, Lemberger CE, et al. Interleukin-33 induces urokinase in human endothelial cells—possible impact on angiogenesis. *J Thromb Haemost* 2014;12:948-57.
- Choi YS, Choi HJ, Min JK, Pyun BJ, Maeng YS, Park H, et al. Interleukin-33 induces angiogenesis and vascular permeability through ST2/TRAF6-mediated endothelial nitric oxide production. *Blood* 2009;114:3117-26.
- Theodoropoulou S, Copland DA, Liu J, Wu J, Gardner PJ, Ozaki E, et al. Interleukin-33 regulates tissue remodelling and inhibits angiogenesis in the eye. *J Pathol* 2017;241:45-56.
- Lee SH, Lee S, Yang H, Song S, Kim K, Saunders TL, et al. Notch pathway targets proangiogenic regulator Sox17 to restrict angiogenesis. *Circ Res* 2014;115:215-26.
- Lange AW, Haitchi HM, LeCras TD, Sridharan A, Xu Y, Wert SE, et al. Sox17 is required for normal pulmonary vascular morphogenesis. *Dev Biol* 2014;387:109-20.
- Yang Y, Andersson P, Hosaka K, Zhang Y, Cao R, Iwamoto H, et al. The PDGF-BB-SOX7 axis-modulated IL-33 in pericytes and stromal cells promotes metastasis through tumour-associated macrophages. *Nat Commun* 2016;7:11385.
- Lee HY, Rhee CK, Kang JY, Byun JH, Choi JY, Kim SJ, et al. Blockade of IL-33/ST2 ameliorates airway inflammation in a murine model of allergic asthma. *Exp Lung Res* 2014;40:66-76.
- Hayakawa H, Hayakawa M, Kume A, Tominaga S. Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation. *J Biol Chem* 2007;282:26369-80.
- Wang W, Gao Z, Wang H, Li T, He W, Lv W, et al. Transcriptome analysis reveals distinct gene expression profiles in eosinophilic and noneosinophilic chronic rhinosinusitis with nasal polyps. *Sci Rep* 2016;6:26604.
- Buroker NE. TBXA2R rNPs, transcriptional factor binding sites and asthma in Asians. *Open J Pediatr* 2014;4:148-61.
- Cao K, Liao X, Lu J, Yao S, Wu F, Zhu X, et al. IL-33/ST2 plays a critical role in endothelial cell activation and microglia-mediated neuroinflammation modulation. *J Neuroinflammation* 2018;15:136.
- Lambrecht BN, Hammad H. Biology of lung dendritic cells at the origin of asthma. *Immunity* 2009;31:412-24.
- Tashiro H, Takahashi K, Hayashi S, Kato G, Kurata K, Kimura S, et al. Interleukin-33 from monocytes recruited to the lung contributes to house dust mite-induced airway inflammation in a mouse model. *PLoS One* 2016;11:e0157571.
- Lambrecht BN, Hammad H. Lung dendritic cells in respiratory viral infection and asthma: from protection to immunopathology. *Annu Rev Immunol* 2012;30:243-70.
- Mesnil C, Sabatel CM, Marichal T, Toussaint M, Cataldo D, Drion PV, et al. Resident CD11b(+)Ly6C(-) lung dendritic cells are responsible for allergic airway sensitization to house dust mite in mice. *PLoS One* 2012;7:e3242.
- Braunersreuther V, Mach F, Steffens S. The specific role of chemokines in atherosclerosis. *Thromb Haemost* 2007;97:714-21.

45. Lee S, Kim IK, Ahn JS, Woo DC, Kim ST, Song S, et al. Deficiency of endothelium-specific transcription factor Sox17 induces intracranial aneurysm. *Circulation* 2015;131:995-1005.
46. Alun Brown K. Adhesion of dendritic cells to endothelia. *Methods Mol Med* 2001; 64:313-23.
47. Karta MR, Rosenthal PS, Beppu A, Vuong CY, Miller M, Das S, et al. β_2 integrins rather than β_1 integrins mediate *Alternaria*-induced group 2 innate lymphoid cell trafficking to the lung. *J Allergy Clin Immunol* 2018;141: 329-38.e12.
48. D'Amico G, Bianchi G, Bernasconi S, Bersani L, Piemonti L, Sozzani S, et al. Adhesion, transendothelial migration, and reverse transmigration of in vitro cultured dendritic cells. *Blood* 1998;92:207-14.
49. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. *Immunity* 2005;23:479-90.
50. Ryu WI, Lee H, Bae HC, Jeon J, Ryu HJ, Kim J, et al. IL-33 down-regulates CLDN1 expression through the ERK/STAT3 pathway in keratinocytes. *J Dermatol Sci* 2018;90:313-22.

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 lung tissues 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. AF3626; R&D Systems), anti-ICAM-1 (1:100; catalog no. MA5407; Invitrogen), anti-CD11b (1:100; catalog no. ab8878; 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.

AHR measurement

As previously described,^{E1} 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 4 (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 Biosciences, 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.^{E2} 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 monocytic 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. 3531317001; 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 instructions. Primer sequences for quantitative real-time PCR are as follows: anti-mouse primers for ICAM-1 (forward, GTGGC GGGAAAGTTCCTG; reverse, CGTCTGCAGGTCGTCATCT TAGGAG), CCL2 (forward, TTAAAAACCTGGATCGGAACCAA; reverse, GCATTA GCTTCAGATTACGGGT), ST2 (forward, ATTCAGGGGACCATCA AGTG; reverse, CGTCTTGGAGGCTCTTTCTG), CD31 (forward, AGAGA

CGGTCTTGTCGAGT; reverse, TACTGGGCTTCGAGAGCATT), IL-33 (forward, ATGGGAAGAAGCTGATG GTG; reverse, CCGAGGACTTTT TGTGAAGG), Sox17 (forward, GAGGGCCAGAAGC AGTGTTACAC; reverse, CTGGCTAAACTGGACAGTGATTGTG), and the housekeeping gene GAPDH (forward, GAGCCAAAAGGGTCATCA; reverse, CATAT TTCTCGTGGTTTACACAC). Human primers for ICAM-1 (forward, CCTT CCTCACCG TGTACTGG; reverse, AGCGTAGGGTAAGGTTCTTGC), CCL2 (forward, AGTCTC TGCCGCCCTTCT; reverse, GTGACTGGGGCA TTGATTG), CCL5 (forward, CCT CATTGCTACTGCCCTCT; reverse, GGT GTGGTGTCCGAGGAATA), CCL11 (forward, AAACCACCACCTCTCA CGCCA; reverse, GCAGCAGGTGGTTGGGACAGAAG), CCL20 (forward, ACTCCACCTCTGCGGCGAATCA; reverse, ATTGGCCAGCTGCCGTGT GAA), CXCL1 (forward, TCCTGCATCCCCCATAGTTA; reverse, CTTCAG GAACAGCCACC AGT), IL-33 (forward, CAAAGAAGTTTGCCCCATGT; reverse, AAGGCAAAGCACT CCACAGT), Sox17 (forward, CAGACTC CTGGGTTTTGTGTGTGCTG; reverse, GAAATGGAGGAAGCTGTTTT GGGACAC), and the housekeeping gene β -actin (forward, CCAACCGCGA GAAGATGA; reverse, CCAGAGGCGTACAGGGATAG) were included. Data were analyzed by using LightCycler Software (version 3.5; Roche). Relative quantitation was calculated by using the comparative cycle threshold ($2^{-\Delta\Delta C_t}$) 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 (density of 1×10^6 cell/s per well) was cultured in 48-well round-bottom plates in complete culture medium (RPMI 1640; Welgene) containing 1 μ g/mL soluble or plate-bound anti-CD3 (catalog no. 16-0037; eBioscience) and 1 μ g/mL soluble anti-CD28 (catalog no. 16-0289; eBioscience). After incubation at 37°C for 3 days, a 300- μ L culture supernatant was collected from the cells and preserved in -80°C until used for ELISA to determine the levels of IL-4 and IL-13.

Analysis of cytokine levels from mouse lung tissue homogenates

For protein extraction, mouse lung tissues were dissolved in tissue lysis buffer (Cell Signaling, Danvers, Mass) containing protease inhibitors and then sonicated with ultrasonic homogenizer for 2 minutes on ice. Each extraction was prepared by using the following steps: vortex mixing (15 seconds, 3 times), centrifugation (20 minutes at 14,000g), and transfer of supernatants

(200 μ L) to fresh microcentrifuge tubes. Supernatants were preserved at -80°C and used for analysis of cytokine levels in the lungs by using ELISA developmental kits (R&D Systems), according to the manufacturer's instructions.

ELISA

Concentrations of IL-4, IL-13, IL-33, CCL2, and CCL11 in BALF; IL-25 and TSLP in lung homogenates of mice with OVA-induced asthma; and CCL2 in HUVEC and HPMEC culture supernatants were measured in *in vivo* and *in vitro* experiments by using the DuoSet ELISA Development Kit (R&D Systems), according to the manufacturer's instructions. Serum levels of total IgE were detected by using the mouse IgE ELISA Quantitation Set (Bethyl Laboratories, Victoria, Australia), 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 $\times$ 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. 9145S; 1:1000; Cell Signaling), anti-total STAT3 (catalog no. 9139P; 1:1000; Cell Signaling), and anti-ST2 (catalog no. MAB523; 1:1000; R&D Systems).

REFERENCES

- E1. Jeon JO, Kim S, Choi E, Shin K, Cha K, So IS, et al. Designed nanocage displaying ligand-specific Peptide bunches for high affinity and biological activity. *ACS Nano* 2013;7:7462-71.
- E2. Choi S, Park MS, Lee YR, Lee YC, Kim TW, Do SG, et al. A standardized bamboo leaf extract inhibits monocyte adhesion to endothelial cells by modulating vascular cell adhesion protein-1. *Nutr Res Pract* 2013;7:9-14.

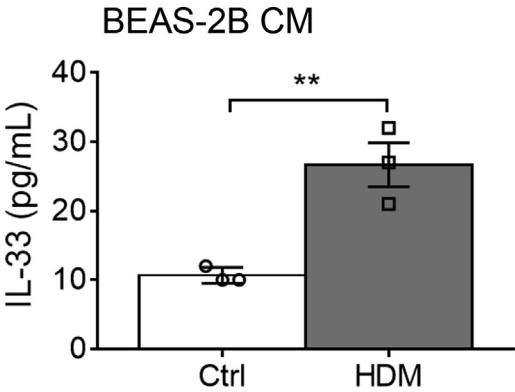


FIG E1. ELISA analysis of IL-33 in CM of BEAS-2B cells after exposure to HDM (1 μ g/mL) for 4 hours. IL-33 levels were detected in CM obtained from HDM-treated BEAS-2B cells and vehicle control (n = 3 per group). *Significance compared with the control group: ** $P < .001$.

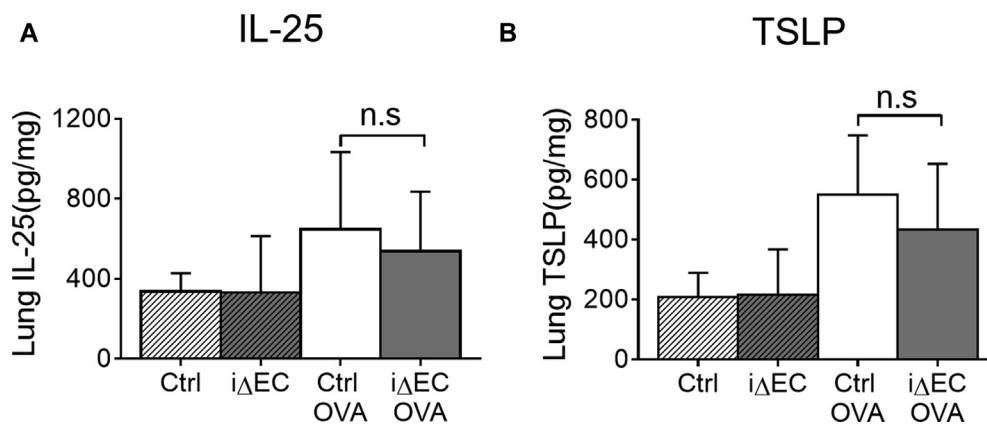


FIG E2. IL-25 and TSLP in lung homogenates of OVA-induced asthma models. **A** and **B**, Protein levels of IL-25 and TSLP in lung tissues of OVA-induced asthma models from *Sox17*^{ΔEC} and WT mice, as measured by means of ELISA, are shown (n = 4-8 mice per group). *Ctrl*, WT mice; *iΔEC*, endothelium-specific *Sox17* knockout mice (*Sox17*^{ΔEC} mice); *n.s.*, not significant.

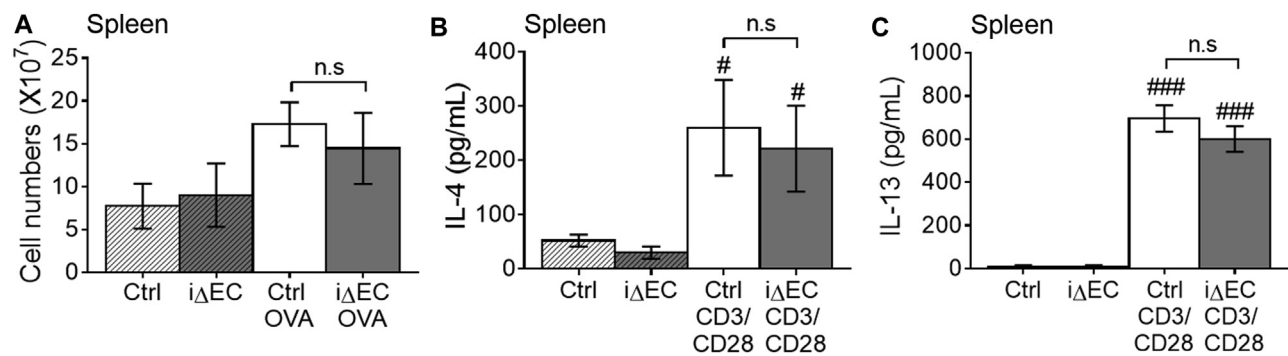


FIG E3. Immune responses in the spleens of WT and *Sox17*-deleted mice. **A**, Total count of splenocytes in each study group. **B** and **C**, T_H2 cytokines (IL-4, IL-13) production by splenocytes from WT C57BL/6 and *Sox17*^{ΔEC} mice 72 hours after anti-CD3/anti-CD28 stimulation is shown (n = 5-11 mice per group). *Ctrl*, WT mice; *iΔEC*, endothelium-specific *Sox17* knockout mice (*Sox17*^{ΔEC} mice); *n.s.*, not significant. #Significance compared with the control group: #*P* < .05 and ###*P* < .0001.

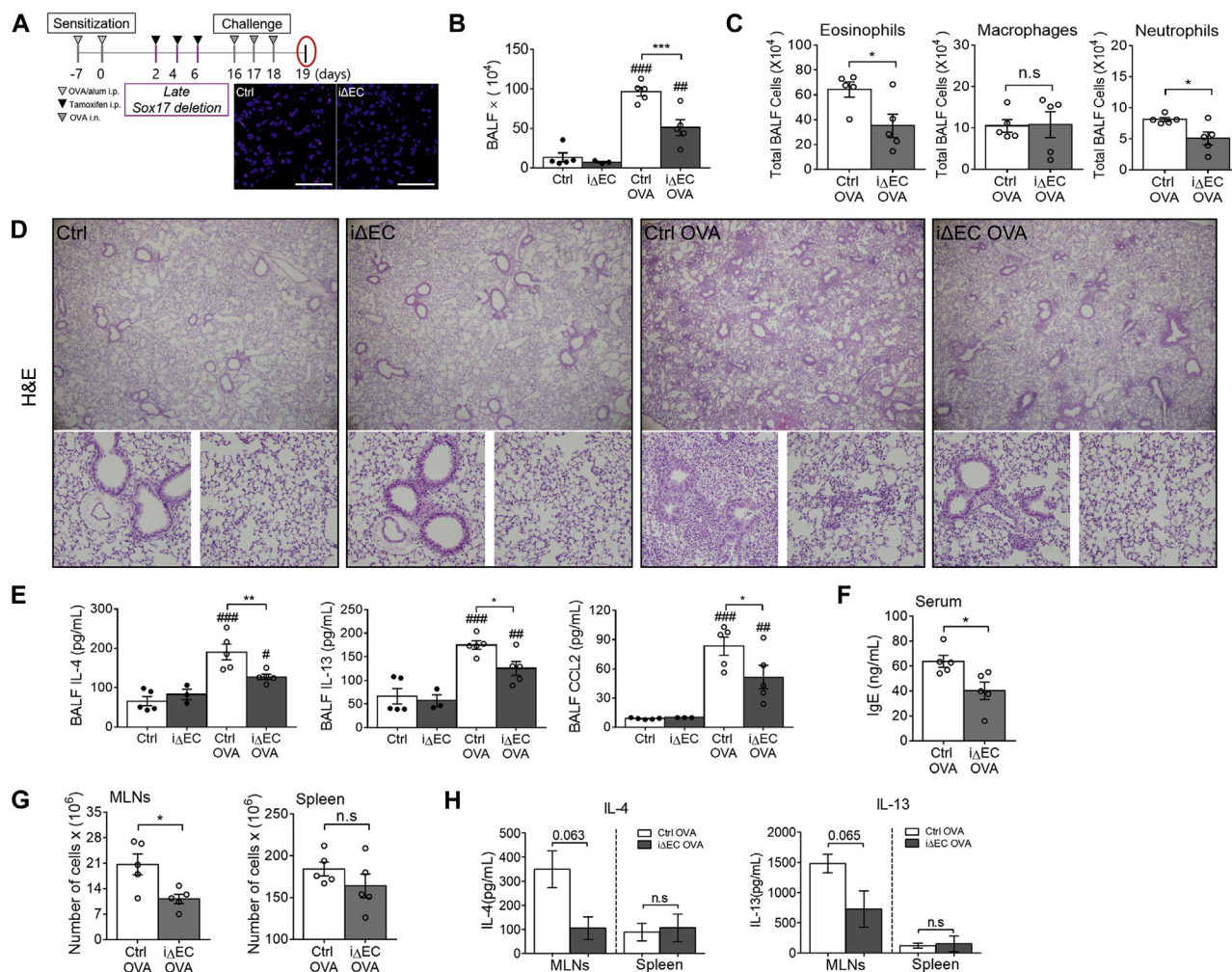


FIG E4. Modulation of OVA-induced airway inflammation and T_H2 immune responses by deletion of endothelial *Sox17* between sensitization and OVA challenge. **A**, Experimental design for late *Sox17* deletion after sensitization and OVA challenge in WT and *Sox17* Δ EC mice (Δ EC; upper portion) and *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 $\times 40$ and $\times 200$. **E**, Levels of IL-4, IL-13, and CCL2 in BALF of WT and *Sox17* Δ EC 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 μ g/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; Δ EC, endothelium-specific *Sox17* knockout mice (*Sox17* Δ EC 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 μ m.