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IL-17A, But Not IL-17F, Is Indispensable for Airway Vascular Remodeling Induced by Exaggerated Th17 Cell Responses in Prolonged Ovalbumin-Challenged Mice

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We previously demonstrated an essential role of Th17 cells in excessive mucous secretion and airway smooth muscle proliferation in a prolonged OVA-challenged C57BL/6 mouse model. However, the impact of Th17 cells in vascular remodeling, another characteristic feature of airway remodeling in asthma, remains elusive. This issue was further investigated in this study. The time-course experiments showed that progressively increasing levels of Th17 cells and IL-17A (not IL-17F) in the lungs of prolonged allergen-challenged mice were positively correlated with microvessel density in peribronchial tissues. In addition, exaggerated airway vascular remodeling in this mouse model was exacerbated by airway administration of IL-17A or adoptive transfer of Th17 cells. This effect was dramatically alleviated by the administration of anti-IL-17A Ab, but not anti-IL-17F Ab. Boyden chamber assays indicated that IL-17A accelerates endothelial progenitor cell (EPC) migration. Furthermore, EPC accumulation in the airways of allergen-exposed mice after adoptive transfer of Th17 cells was eliminated by blockade of IL-17A. We found that IL-17A promoted tubule-like formation rather than proliferation of pulmonary microvascular endothelial cells (PMVECs) *in vitro*. In addition, IL-17A induced PMVEC tube formation via the PI3K/AKT1 pathway, and suppression of the PI3K pathway markedly reduced the formation of tubule-like structures. Collectively, our results indicate that Th17 cells contribute to the airway vascular remodeling in asthma by mediating EPC chemotaxis, as well as PMVEC tube formation, via IL-17A rather than IL-17F. *The Journal of Immunology*, 2015, 194: 000–000.

Vascular remodeling is a characteristic feature of airway remodeling, which causes the symptoms associated with decreased pulmonary function in asthmatic individuals (1, 2). Specific changes to the vasculature in asthmatic airways are mainly caused by increased neovascularization, which encompasses both angiogenesis and vasculogenesis (3). Angiogenesis is a process in which mature pulmonary microvascular endothelial cells (PMVECs) break free from their basement membrane, then migrate and proliferate to form sprouts from parental vessels (4). Vasculogenesis refers to the process in which bone marrow-derived endothelial progenitor cells (EPCs) migrate, differentiate into mature endothelial cells, and then form new vessels (5).

Th17 cells, identified as a third subset of Th cells, are characterized by their production of IL-17A, IL-17F, IL-22, and other cytokines (6). Accumulating evidence has indicated that Th17 cells and the cytokines they produce play a key role in the development of asthma. Research on allergic asthmatics has shown a significant increase in the percentage of Th17 cells in PBMCs and the plasma concentrations of IL-17 and IL-22, in proportion to the disease severity (7). This is supported by several studies in which increased IL-17A and IL-17F expression and Th17 cells in the tissues of asthmatics were observed (8–10). However, the role of Th17 cells in vascular remodeling in asthma remains to be elucidated.

In the past decade, many studies have revealed a novel function for IL-17A and IL-17F, mainly expressed by Th17 cells, as angiogenic mediators. IL-17A induces endothelial migration and tube formation in rheumatoid arthritis through activation of the PI3K pathway and ligation of both IL-17Rs (11). IL-17F levels show a statistically significant correlation with the number of activated circulating endothelial cells and disease activity in systemic lupus erythematosus patients, suggesting a role in activating vascular endothelial cells and consequent blood vessel formation (12). These observations led us to speculate that Th17 cell and its associated cytokines are involved in asthmatic vascular remodeling.

In our previous study, increased Th17 cells and IL-17 production were observed along with increased collagen deposition, airway smooth muscle mass, and mucous hypertrophy in a mouse model of asthmatic airway remodeling (13). In this study, we further investigated the role of Th17 cell and its cytokines in airway vascular remodeling, mobilization, and recruitment of EPCs, as well as proliferation and tubule formation by PMVECs. Our results showed a critical requirement for IL-17A, but not IL-17F, produced by exaggerated Th17 responses in prolonged OVA-challenged mice

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Abbreviations used in this article: EBM, endothelial cell basal medium; EGM-2, endothelial cell growth medium-2; EPC, endothelial progenitor cell; MVD, microvessel density; PCNA, proliferating cell nuclear Ag; PMVEC, pulmonary microvascular endothelial cell; VEGF, vascular endothelial growth factor.

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is airway vascular remodeling by inducing EPCs chemotaxis as well as PMVEC tube formation.

Materials and Methods

Animals

Two groups of male C57BL/6 mice (aged 6–8 wk and 10 d) were purchased from the National Rodent Laboratory Animal Resources, Shanghai Branch (China) and housed under specific pathogen-free conditions. Experiments were performed according to protocols approved by the Animal Studies Committee of China.

Patient tissues

Lung tissues of asthmatic patients were acquired from The First Affiliated Hospital of Zhejiang University School of Medicine. The study was approved by the Ethics Committee of The First Affiliated Hospital, Zhejiang University, School of Medicine. All the patients provided written, informed consent and understood that their tissues would be used for research.

Cell culture

PMVECs were acquired from the lungs of 10-d-old C57BL/6 mice according to a previously reported protocol (14) with minor modifications. Mice were sacrificed through cervical dislocation for lung isolation. The edge of lung tissues were cut into pieces ($1 \times 1 \times 1$ mm), placed into 24-well plates coated with 1% gelatin, and cultured in endothelial cell basal medium (EBM)-2 (Lonza, Basel, Switzerland) supplemented with 20% FBS together with penicillin and streptomycin. After 60 h, the tissues were discarded and the medium was replaced with endothelial cell growth medium-2 (EGM-2) (Lonza) supplemented with 5% FBS. The plates contained only endothelial cells and blood cells, which were removed by changing the medium. EPCs were isolated from the bone marrow of C57BL/6 mice (aged 6–8 wk). The bone marrow of the long bones was washed out with PBS for preparation of a single-cell suspension. Cells were then layered onto Lymphoprep (TBD Science, Tianjin, China) for

isolation of the mononuclear cell layer by density gradient centrifugation. After washing twice with PBS, cells were cultured in EBM-2 supplemented with 5% FBS for 48 h. The nonadherent cells were collected and plated into fibronectin (Sigma-Aldrich, St. Louis, MO)-coated plates and cultured in EGM-2 supplemented with 15% FBS.

Tube formation assay

Matrigel matrix (BD Biosciences, San Jose, CA) was polymerized at 37°C in a 96-well plate before the assay was performed. PMVECs (2×10^4 /well) at passages 2–3 were added to polymerized Matrigel together with the increasing concentrations of IL-17A (1, 10, 100 ng/ml; eBioscience, San Diego, CA) after serum starvation for 12 h and then incubated at 37°C for 6–8 h. To investigate which signaling pathways contribute to IL-17A-mediated tube formation, we incubated cells with inhibitors to PI3K (LY294002; 2 and 5 μ M; Sigma-Aldrich) or DMSO for 45 min at 37°C before being added to polymerized Matrigel. IL-17 (10 ng/ml) was then added to the wells, and the plate was incubated for 6–8 h at 37°C. PBS and vascular endothelial growth factor (VEGF; 10 ng/ml; Peprotech, Rocky Hill, NJ) were used as negative and positive controls, respectively. Endothelial cell tubule formation was assessed using phase-contrast microscopy; findings were then photographed in five randomly selected microscopic fields ($\times 100$ magnification). The total perimeter of completely enclosed tubes in one field was calculated by Image-Pro Plus 5.1, and the average of the total perimeter measurements was used for quantification. In addition, node formation (defined as a nodular contact formation of at least three adherent endothelial cell tubes) in five randomly selected microscopic fields ($\times 100$ magnification) was evaluated blindly (11, 15).

Transwell migration assay

EPC migration was evaluated using a modified Boyden chamber assay as described previously (15, 16). EPCs isolated from bone marrow were cultured for 14 d and then serum-starved for 12 h. Cells were plated (1.2×10^5 cells/cm²) into the upper compartments of transwell chambers separated by a membrane with 8- μ m pores (Corning, Corning, NY). EBM containing different concentrations of IL-17A (1, 10, 100 ng/ml) was

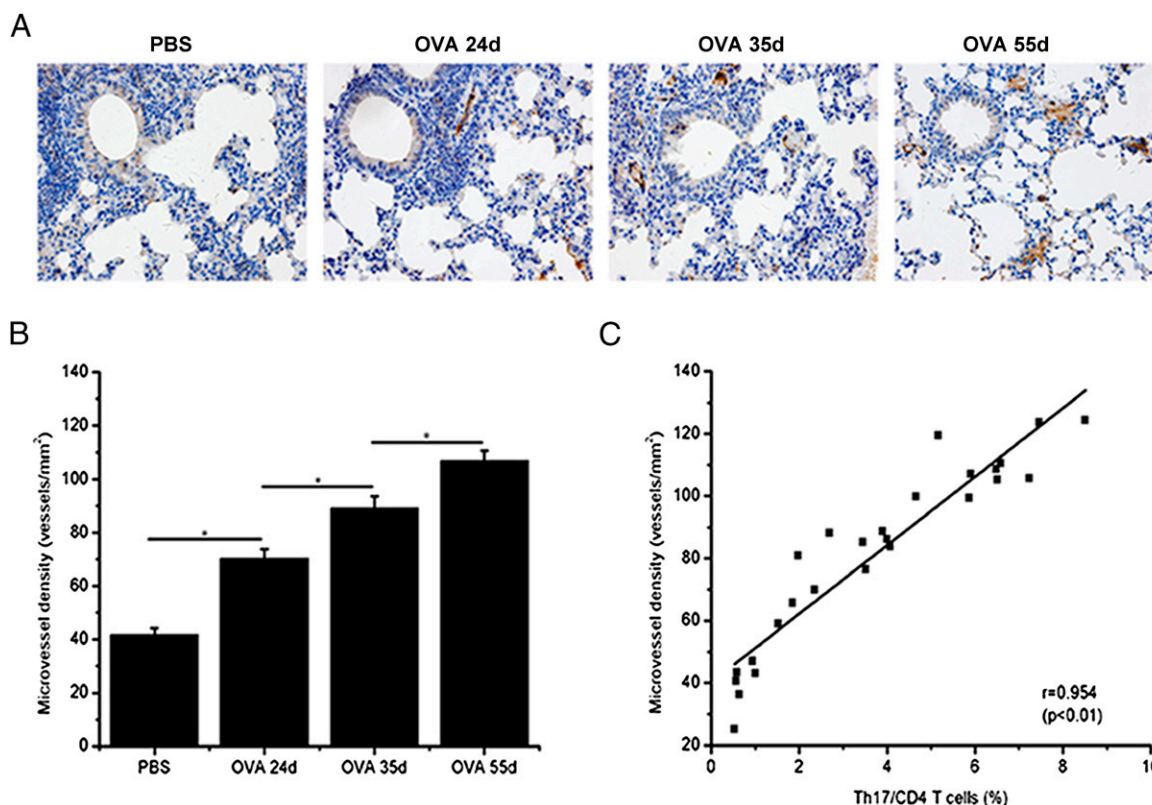


FIGURE 1. Significantly increased vascularity during OVA-challenge correlates positively with the number of Th17 cells in lungs. **(A)** After OVA sensitization and challenge, mice were sacrificed on days 24, 35, and 55 for lung tissue collection. Then the lung sections were stained with Factor VIII for identification of endothelial cells. Representative photomicrographs of lung tissue sections stained for Factor VIII (brown) from each group are shown (original magnification $\times 400$). **(B)** Airway vascularity was quantitated by assessment of MVD defined as the ratio of the number of stained microvasculature and the area of the lung tissue. Data represent the mean $\pm$ SEM of three different experiments ($n = 6$ /group). * $p < 0.05$ between groups. **(C)** Correlation between the levels of Th17 cells in the lung and vascular remodeling represented by MVD. Spearman regression analysis was used.

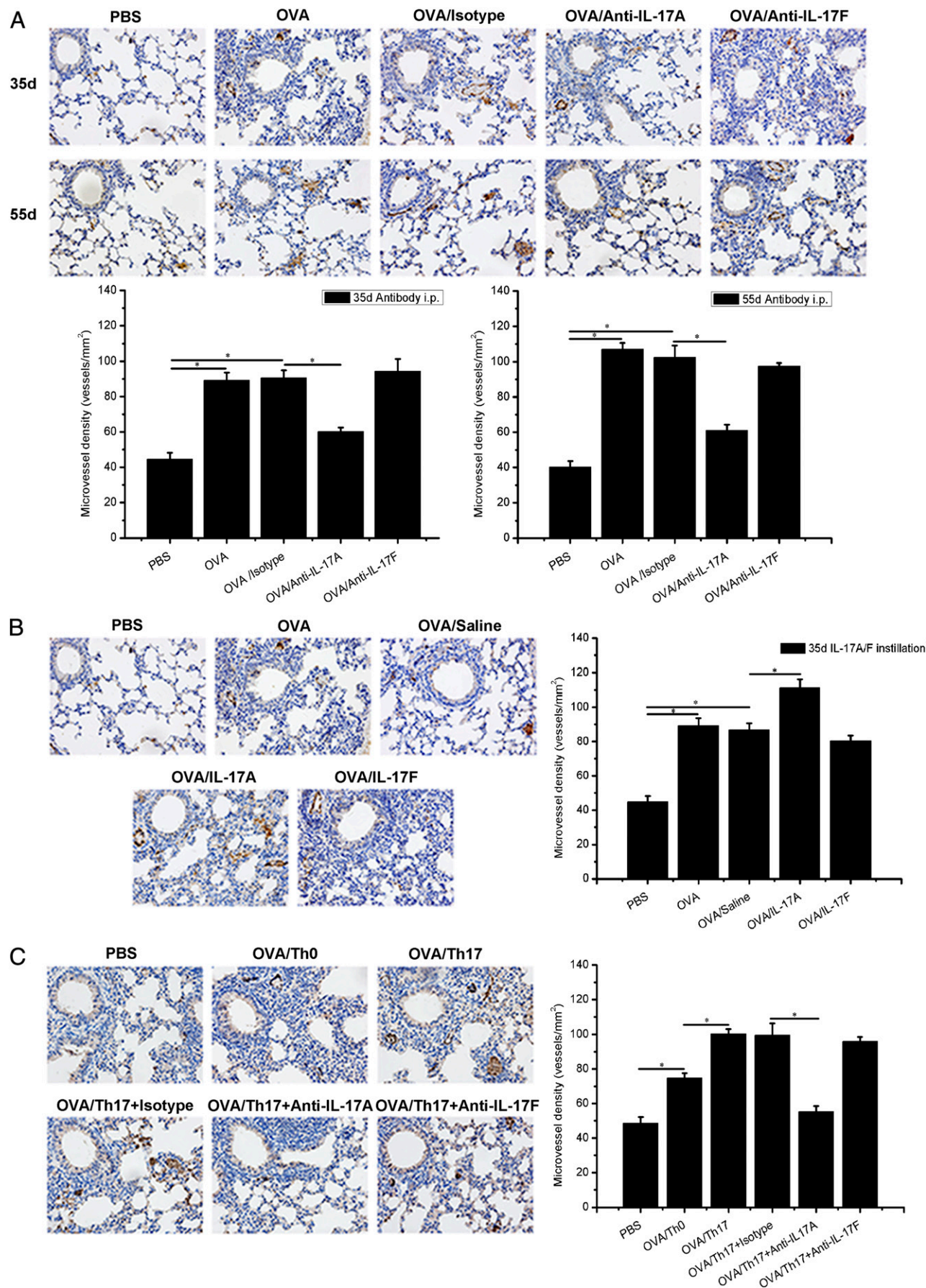


FIGURE 2. Th17/IL-17A mediates neovascularization of peribronchial tissues in prolonged OVA-challenge mice. **(A)** Anti-IL-17A mAb (50 μ g/mouse), anti-IL-17F mAb (50 μ g/mouse), or IgG isotype control (50 μ g/mouse) was administered i.p. twice each week from day 28 (*Figure legend continues*)

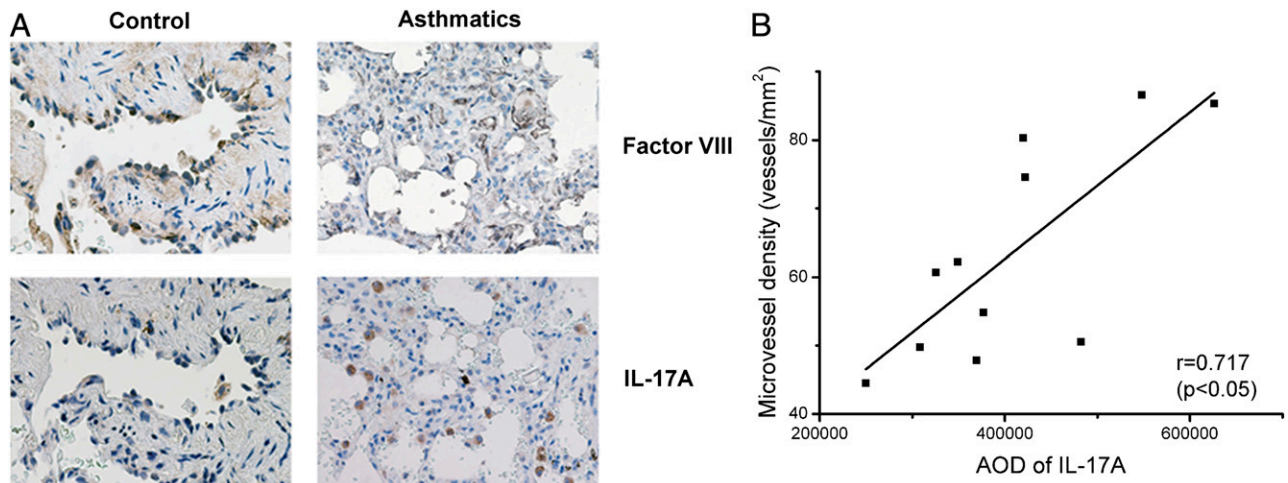


FIGURE 3. IL-17A in lung tissues of asthmatics was positive related to the pulmonary MVD. **(A)** Adjacent lung tissues sections from asthmatic patients were stained with IL-17A and Factor VIII, respectively. Representative photomicrographs were shown (original magnification $\times 400$). **(B)** Correlation between the levels of IL-17A and MVD in lung tissues. Spearman regression analysis was used.

added to the lower compartments of the chamber, using PBS as a negative control. After incubating at 37°C for 24 h, cells were fixed with 1% glutaraldehyde and stained with 0.1% crystal violet. Nonmigrated cells were removed from the upper surface of the transwell membrane. Images of representative fields were captured digitally, and cells in five randomly selected microscopic fields ($\times 200$ magnification) for each well were counted to assess the average number of migrated cells.

Proliferation assessment of endothelial cells

Proliferation of PMVECs was assessed by immunocytochemical staining of proliferating cell nuclear Ag (PCNA; Santa Cruz Biotechnology, Santa Cruz, CA). Poststarvation, PMVECs (5×10^3 cells/well) at passages 2–3 were cultured in the presence of different concentrations of IL-17A (1, 10, 100 ng/ml) for 24 h, using PBS and VEGF (10 ng/ml) as negative and positive controls, respectively. Then PCNA immunohistochemical staining was carried out to analyze the proliferation. The number of PCNA⁺ cells was divided by the total number of cells counted to yield the percentage of proliferating PMVECs (13).

OVA-challenge mouse model

The OVA-challenge mouse model was established as reported by Lloyd et al. (17) in their studies of OVA (Sigma-Aldrich) sensitization and challenge. Mice were sensitized using OVA (Sigma-Aldrich) at a concentration of 25 μg in 0.1 ml alum delivered i.p. on days 0 and 12. Control groups received the same volume of PBS in alum. Sensitized mice were exposed to 5% aerosolized OVA for 30 min daily between days 18 and 23 to provide an acute asthmatic model. Prolonged inflammation was induced subsequently by similar OVA exposure three times per week from day 26 until the end of the study (days 35 or 55). Control mice were subjected to the same protocol but received PBS instead of allergen.

Ab treatment

Anti-IL-17A mAb (50 μg /mouse; R&D Systems, Minneapolis, MN), anti-IL-17F mAb (eBioscience; 50 μg /mouse), or isotype IgG (50 μg /mouse; R&D Systems) was administered i.p. twice each week from day 28 onward during the course of the prolonged OVA-challenge. Mice were euthanized

through cervical dislocation on days 35 or 55. In the model of Th17 transfer and EPC transfer, anti-IL-17A mAb, anti-IL-17F mAb, or isotype IgG was administered i.p. from days 28 to 33 thirty minutes before the Th17 cell transfer. Mice were euthanized on day 35.

Airway administration

Mouse IL-17A (1 μg /mouse), IL-17F (1 μg /mouse), or saline was delivered into tracheas of mice three times from days 28 to 33. Mice were euthanized on day 35.

Th17 cell generation and adoptive transfer

Th17 cells were differentiated from naive CD4⁺ T cells in the presence of the required stimulating factors and associated Ig, according to a previously reported protocol (13). Naive CD4⁺ T cells were acquired from the spleens of C57BL/6 mice using anti-CD4 microbeads and a magnetic sorter (MACS; Miltenyi Biotec, Auburn, CA). Cells were then cultured in the presence of Dyna-beads Mouse CD3/CD28 T Cell Expander (Invitrogen, Carlsbad, CA), 5 ng/ml TGF- β (Abcam, Cambridge, MA), 10 ng/ml recombinant murine IL-6 (Biolegend), 10 mg/ml anti-IFN- γ , and 10 mg/ml anti-IL-4 mAb (Biolegend) for further differentiation into Th17 cells. Primary cultured Th17 cells (6×10^5 cells/mouse) were adoptively transferred i.v. twice to sensitized mice 30 min before OVA or PBS challenge from days 28 to 33. Mice were euthanized on day 35 for tissue collection.

Adoptive transfer of EPCs

EPCs were stained with the fluorescent imaging agent, Vivo Track 680 (Perkin Elmer) after culture for 14 d. Then the stained cells (1×10^6 cells) were injected i.v. into syngeneic OVA-challenged mice twice from days 28 to 33 after adoptive transfer of Th17 cells. Recipient mice were euthanized on day 35, and exogenous EPCs in lungs were detected by flow cytometry.

Flow cytometry

EPCs in the bone marrow or peripheral blood and Th17 cells in the lungs of OVA-challenged mice were detected by flow cytometry. To detect EPCs, we surface-stained cells purified from the bone marrow and blood of OVA-

onward during the course of the prolonged OVA-challenge. Mice were euthanized on days 35 and 55 for lung tissue collection. Representative photomicrographs of lung tissue sections from each group immunostained for Factor VIII (brown) are shown. Airway vascularity was quantitated by MVD. Data represent mean $\pm$ SEM of three different experiments ($n = 6$ /group). **(B)** IL-17A (1 μg /mouse), IL-17F (1 μg /mouse), or PBS was delivered into the airways three times from days 28 to 33 during prolonged OVA-challenge. Mice were euthanized on day 35 for lung tissue collection. Immunostaining for Factor VIII (brown) was carried out to identify the endothelial cells. Representative photomicrographs of lung tissue sections from each group are shown. Airway vascularity was quantitated by MVD. Data represent mean $\pm$ SEM of three different experiments ($n = 6$ /group). **(C)** Primary cultured Th17 cells were transferred i.v. into sensitized mice twice 30 min before OVA or PBS exposure from days 28 to 33. The control group received Th0 cells. Neutralizing anti-IL-17A or anti-IL-17F mAbs were administered before the Th17 transfer in the indicated mice. Mice were euthanized on day 35 for lung tissue collection. The lung sections were immunostained for Factor VIII (brown). Representative photomicrographs of lung tissues sections from each group were shown. Quantification of vascular remodeling is represented by MVD. Data represent mean $\pm$ SEM of three different experiments ($n = 4$ –5/group). (A–C) Original magnification $\times 400$. * $p < 0.05$ between groups.

challenged mice with FITC-labeled anti-Ly-6A/E (BD Biosciences), PE-labeled anti-CD117 (BD Biosciences), allophycocyanin-labeled anti-Flk-1 (BD Biosciences), and the respective isotype controls according to the manufacturer's instructions (18). For detecting Th17 cells, cells prepared from lungs were stimulated with 50 ng/ml PMA (BioVision, Mountain View, CA) and 500 ng/ml ionomycin (Fermentek, Jerusalem, Israel) in the presence of GolgiStop (eBioscience) for 4 h. Later, cells were surface-stained for CD4 (eBioscience), permeabilized with Cytotfix/Cytoperm (eBioscience), washed, and intracellularly stained with PE-labeled anti-IL-17 (BD Biosciences). Rat IgG of the corresponding class (eBioscience) was used as an isotype control (13). Flow-cytometry acquisition was performed using a FACSCalibur instrument (BD Biosciences), and the results were analyzed with Cell Quest software (BD Biosciences).

Histological analysis

The lung tissues from asthmatic model mice and patients with asthma were analyzed by immunohistochemistry. The lower lobe of the right lung of

asthmatic mice and lung tissues from asthmatic patients were fixed, embedded in paraffin, and cut into sections (4 μ m thickness). Factor VIII was detected with rabbit anti-human von Willebrand factor (DAKO, Carpinteria, CA) primary Ab for identifying endothelial cells. IL-17A was detected with rabbit anti-human IL-17A (Peprotech). Target Ag localization was visualized with HRP-conjugated goat anti-rabbit Ab (ZSGB-Bio, Beijing, China) coupled with 3, 3'-diaminobenzidine. Slides were then counterstained with Harris hematoxylin. The lung tissue area (mm^2) and the integrated OD of IL-17A⁺ staining were determined by digital image analysis of photographs using Image-Pro Plus5.1, and the number of stained microvessels was counted. Each slide was evaluated by a blinded observer. The average OD of the IL-17A⁺ staining area was represented by the ratio of integrated OD and the area of the lung tissue to indicate the expression level of IL-17A. The microvessel density (MVD) of the lung tissue was represented by the ratio of the number of stained microvessels and the area of lung tissue in each section to represent the level of neovascularization (19).

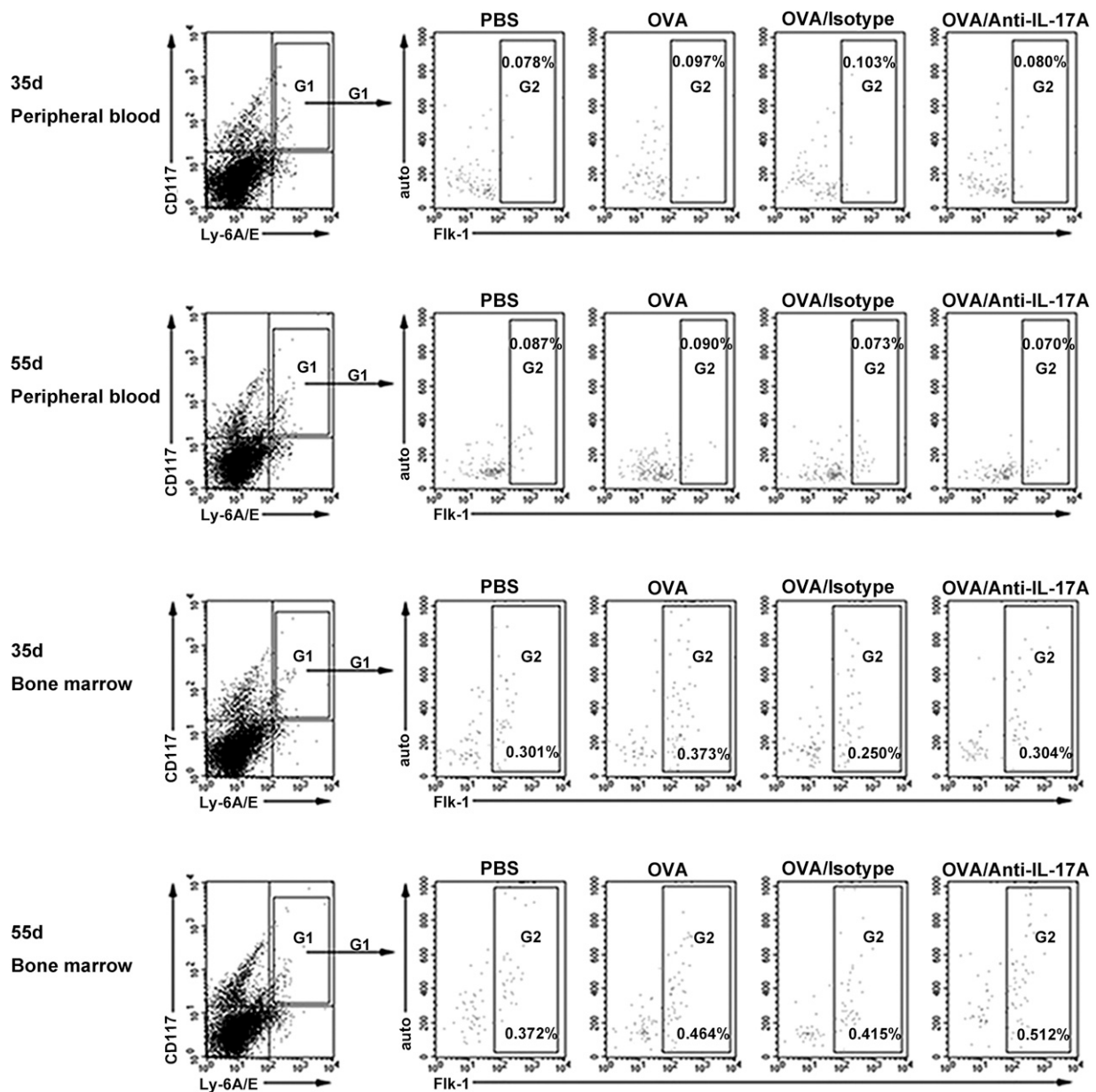


FIGURE 4. IL-17A does not promote the mobilization of EPCs from the bone marrow into the circulation. OVA-sensitized mice challenged with or without neutralization of IL-17A were euthanized on days 35 and 55. Cells isolated from the bone marrow and peripheral blood were surface stained with FITC-labeled anti-Ly-6A/E, PE-labeled anti-CD117, allophycocyanin-labeled anti-Flk-1, and the respective isotype controls and then analyzed by flow cytometry to detect EPCs. Representative flow plots and gating strategies are shown for Ly-6A/E⁺CD117⁺ cells (cells in G1), as well as for Ly-6A/E⁺CD117⁺Flk-1⁺ cells (cells in G2) within the Ly-6A/E⁺CD117⁺ gate. The ratio of Ly-6A/E⁺CD117⁺Flk-1⁺ cells to the total cells of each group is presented. Numbers indicated are the average values of three independent experiments ($n = 6/\text{group}$).

Western blot analysis

PMVECs (passages 2–3) were grown to 80% confluence in EGM-2 and then incubated in EBM supplemented with 0.1% FBS for 2 h before treatment with IL-17A (10 ng/ml). After treatment with IL-17A for 1–12 h, cell lysates were prepared and examined by Western blot analysis as described previously (13). Blots were probed with anti-phospho-AKT Ab (1:1000; Cell Signaling Technology, Beverly, MA) and anti-AKT Ab (1:1000; Cell Signaling Technology) overnight at 4°C. After incubation with HRP-conjugated secondary Ab (1:2000; Santa Cruz Biotechnology), antigenic bands were visualized by ECL (Thermo Scientific, Waltham, MA) according to the manufacturer's protocol.

Statistical analysis

Data are expressed as means $\pm$ SEM. Data were analyzed by ANOVA (Tukey honest significant difference), correlation analysis (Spearman), or independent *t* tests as appropriate with SPSS 17.0 (SPSS, Chicago, IL). Statistical significance was accepted at $p < 0.05$.

Results

Severity of peribronchial neovascularization resulting from prolonged OVA-challenge in sensitized mice is exacerbated progressively and is positively correlated with the proportion of Th17 cells

Prolonged OVA exposure has been demonstrated to lead to prominent changes similar to those previously reported in airway remodeling in sensitized mice (13, 17). In our experimental mouse model, the density of pulmonary microvasculature increased progressively with prolonged allergen challenge (Fig. 1A and 1B) together with the level of airway-infiltrating Th17 cells in the lung (Fig. 1C). These observations indicated a potent role of Th17 cells in airway vascular remodeling.

IL-17A, but not IL-17F, mediates Th17-induced airway vascular remodeling

To investigate the role of Th17 cells in vascular remodeling of OVA-challenged mice model, we focused on the effect of Th17 cell-associated cytokines, IL-17A and IL-17F. First, IL-17A mAb or IL-17F mAb was administered by i.p. injection over the course of the prolonged OVA exposure. The results suggest that neutralization of IL-17A significantly reduces the vascularity in the lung, whereas administration of the IL-17F mAb had no obvious

influence on microvascular formation (Fig. 2A). Airway administration of IL-17A during the prolonged OVA-challenge indeed increased the MVD, whereas application of IL-17F had no such effect (Fig. 2B). Furthermore, adoptive transfer of Th17 cells during the period of prolonged OVA exposure from days 28 to 34 promoted neovascularization in peribronchial tissues, which was abrogated by the administration of anti-IL-17A mAb (Fig. 2C). From these results we inferred that Th17 cells are involved in promoting airway vascular remodeling via IL-17A, rather than IL-17F.

Pulmonary MVD in asthmatic patients correlates positively with the level of IL-17A in the lung tissues

We also analyzed the relation between MVD and the level of IL-17A in the lung tissues of asthmatic patients by immunohistochemistry. For this purpose, we stained adjacent sections of lung tissues for IL-17A and Factor VIII, respectively. The results indicated that the MVD of lung tissues correlated positively with the expression level of IL-17A in the airways of asthma patients (Fig. 3A and 3B).

IL-17A is not involved in the mobilization of EPCs from the bone marrow into the circulation

The results of our *in vivo* experiments indicated that Th17 cells enhance peribronchial neovascularization via its associated cytokine, IL-17A. To investigate the underlying mechanisms, we first examined the effect of IL-17A on EPCs. We detected the endogenous EPCs in the peripheral blood and bone marrow of OVA-challenged mice with or without neutralization of IL-17A and found that there were no significant differences in the proportion of cells between the groups (Fig. 4). These results suggest that IL-17A has no biological function in the mobilization of EPCs from the bone marrow into the circulation.

IL-17A contributes to the migration of EPCs *in vitro*

For the purpose of determining whether IL-17A is chemotactic for EPCs, we performed an EPCs migration assay in a Boyden chamber with varying concentrations of IL-17A (1–100 ng/ml), as well as a negative control. The results showed that IL-17A possesses the chemotactic activity for EPCs and induces significantly enhanced migration *in vitro* at concentrations of 10 and 100 ng/ml, compared with that observed in the negative control (Fig. 5A and 5B).

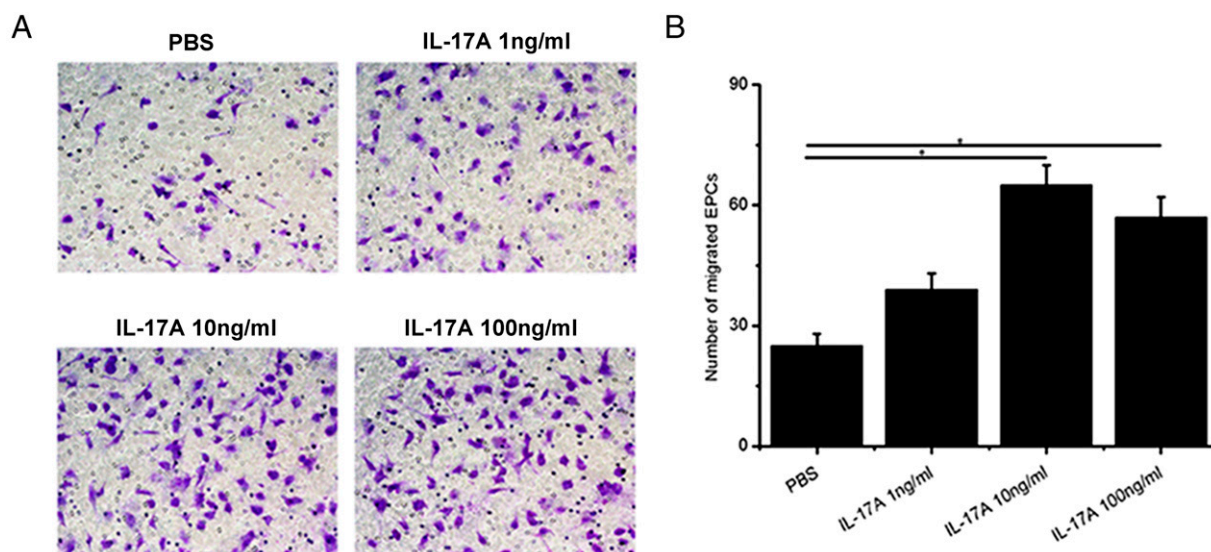


FIGURE 5. IL-17A exerts a chemotactic on EPCs *in vitro*. (A) Representative photomicrographs showing EPC migration after stimulation by different concentrations of IL-17A (1, 10, and 100 ng/ml), as well as a negative control. At 24 h, migrating cells attached to the lower membrane were fixed with 1% glutaraldehyde and stained with 0.1% crystal violet (original magnification $\times 200$). (B) The average number of migrated EPCs from five randomly selected microscopic fields in each well was calculated. Data represent the mean $\pm$ SEM of four different experiments ($n = 3$ wells/group). * $p < 0.05$ compared with the control group.

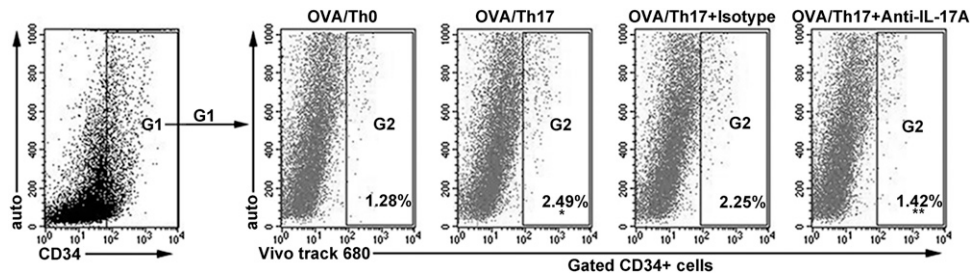


FIGURE 6. IL-17A enhances EPC recruitment to the lung in OVA-challenged mice. The Vivo Track 680–stained exogenous EPCs were infused into the tail vein two times during prolonged OVA-challenge from days 28 to 33 after adoptive transfer of Th17 cells. Recipient mice were euthanized on day 35 and exogenous EPCs in the lungs were detected by flow cytometry ($n = 6/\text{group}$). Representative flow plots and gating strategies are shown for CD34⁺ cells (cells in G₁), as well as for Vivo Track 680⁺ cells (cells in G₂) within the CD34⁺ gate. The proportion of Vivo Track 680⁺ cells to CD34⁺ cells of each group is presented. Numbers indicated are the averaged values of three independent experiments ($n = 3/\text{group}$). * $p < 0.05$ compared with the OVA/Th0 group, ** $p < 0.05$ compared with the OVA/Th17+Isotype group.

Adoptive transfer of Th17 leads to EPC accumulation in the airways of allergen-exposed mice

In the *in vitro* migration assay, IL-17A induced significant EPC migration. Furthermore, we also discovered that Th17 promoted the recruitment of EPCs to the lung in allergen-exposed mice. Primary cultured EPCs labeled with Vivo Track 680 were injected *i.v.* into the OVA-challenged mice after adoptive transfer of Th17 cells. The stained exogenous EPCs were detected in the lungs of recipient mice by flow cytometry, and the level of cells in the Th17 adoptive transfer group was higher than that in the Th0 adoptive transfer group. Furthermore, administration of IL-17A mAb led to a reduction in the amount of labeled cells that migrated to the lung (Fig. 6).

IL-17A does not induce proliferation of PMVECs

Because proliferation of endothelial cells is one of the most important aspects of angiogenesis, we investigated the role of IL-17A in PMVEC proliferation. For this purpose, PMVECs at passages 2–3 were incubated with different concentrations of IL-17A for 24 h before immunocytochemical analysis of PCNA expression. IL-17A had no significant effect on the proliferation of microvascular endothelial cells *in vitro* (Fig. 7).

IL-17A enhances tubule formation of PMVECs

Because IL-17A did not affect the growth of PMVECs, we focused on its effect on tubule formation. Endothelial tubule formation by PMVECs was assayed in the presence of increasing concentrations of IL-17A (1–100 ng/ml). Analysis of the results indicated that at concentrations of 10 and 100 ng/ml, IL-17A enhanced the formation of tubelike structures and significantly increased the number of nodular tube contacts in Matrigel compared with the negative control (Fig. 8A and 8B).

IL-17A activates the PI3K pathway in PMVECs and inhibition of PI3K reduces IL-17A–induced PMVEC tube formation

To determine whether the PI3K/AKT pathway is activated by IL-17A in PMVECs, we examined AKT1 and phosphorylated AKT1 by Western blots analysis. We found that AKT1 was still phosphorylated after IL-17A stimulation for 12 h, although the level of p-AKT1 was slightly lower than that detected after stimulation for 1 and 6 h (Fig. 9A). An inhibitor of PI3K (2 and 5 μM) was used to determine whether tube formation by PMVECs is mediated via the PI3K/AKT pathway. PI3K inhibition effectively suppressed PMVEC tube formation induced by IL-17A (Fig. 9B and 9C). These results suggested that IL-17A induced tube formation *in vitro* through activation of the PI3K/AKT pathway.

Discussion

In this study, we demonstrated a progressive enhancement of new microvascular formation during prolonged allergen exposure, together with an increase in the proportion of Th17 cells in the lung. These results indicate that Th17 cells are involved in the process of peribronchial neovascularization. Th17 cells are characterized by the predominant production of IL-17A, IL-17F, IL-6, IL-21, and IL-22 (6). IL-17A and IL-17F, which are the main members of the IL-17 family, have the greatest homology (20) and share the same receptor and regulatory elements (21). IL-17A and IL-17F have been reported to be involved in vascular regeneration in some diseases (11, 12, 16, 22–24). In our study, we found that neutralization of IL-17A led to reduced vascularity in the lung of OVA-challenged mice, whereas IL-17F antagonism did not markedly influence the microvascular regeneration. Furthermore, airway administration of IL-17A, but not IL-17F, promoted peribronchial neovascularization. On the basis of these results, we conclude that Th17 cells influence the process of peribronchial neovascularization in OVA-challenged mice through the specific production of IL-17A. This conclusion was further validated by the observations that adoptive transfer of Th17 in mice obviously increased the MVD of bronchia and that this effect was inhibited by IL-17A neutralization. In addition, analysis of the relation between IL-17A and MVD in

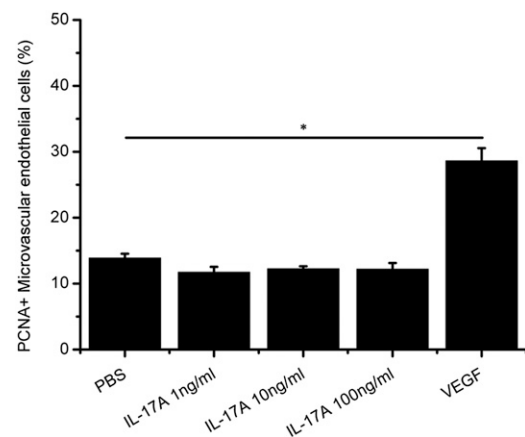


FIGURE 7. IL-17A does not induce proliferation of PMVECs *in vitro*. PMVECs at passages 2–3 were cultured in the presence of different concentrations of IL-17A (1, 10, 100 ng/ml) for 24 h poststarvation. PBS and VEGF (10 ng/ml) were used as negative and positive controls, respectively. Then PCNA immunohistochemical staining was carried out to analyze the proliferation. Endothelial cell proliferation was assessed by the percentage of PCNA-immunoreactive cells. Data represent the mean $\pm$ SEM of three different experiments. * $p < 0.05$ compared with the control group.

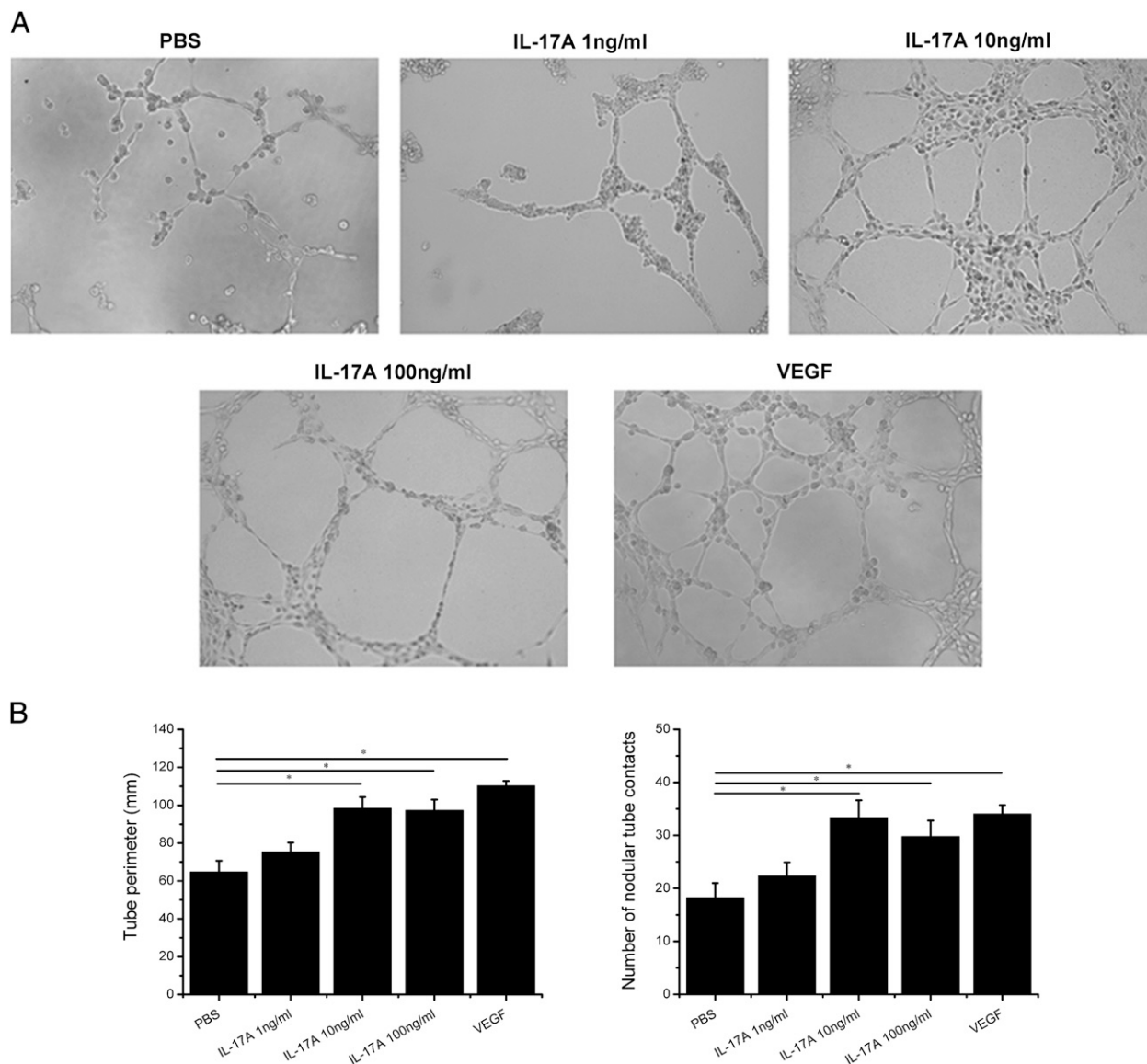


FIGURE 8. IL-17A promotes tube formation of PMVECs in vitro. **(A)** PMVECs at passages 2–3 were serum starved for 12 h and then added to a polymerized Matrigel-coated well and incubated for 6–8 h at 37°C in the presence of increasing concentrations of IL-17A (1, 10, 100 ng/ml). PBS and VEGF (10 ng/ml) were used as negative and positive controls, respectively. Photomicrographs of representative wells of each group are shown (original magnification $\times 100$). **(B)** The average of the total perimeter of full tubes in each group was calculated by Image-Pro Plus5.1. Node formation (defined as a nodular contact formation of at least three adherent endothelial cell tubes) was evaluated blindly under a phase-contrast microscope. Data represent the mean $\pm$ SEM of five different experiments. * $p < 0.05$ compared with the negative control.

the lungs of asthmatic patients suggested that level of IL-17A correlated positively with the severity of airway vascular remodeling.

Accumulating evidence has revealed that bone marrow-derived EPCs are involved in the process of vasculogenesis in physiological or pathological neovascularization. Dome et al. (25) found that the levels of bone marrow-derived EPCs significantly increased in patients with non-small-cell lung cancer and were arrested in small intratumoral capillaries or located in the capillary walls. Asosingh et al. (18) demonstrated that circulating EPCs were increased in asthma patients and were related to the increased numbers of submucosal vessels. In our experimental model, we found that there was no significant difference between the levels of EPCs in the peripheral blood or bone marrow from mice treated with or without anti-IL-17A mAb. This indicates that Th17 and IL-17A have no effect on EPC mobilization from the bone marrow to the circulation. Th17 cells and IL-17A did not affect EPC mobilization. This raised the question of their role in

inducing EPCs migration. In our in vitro studies, we observed that IL-17A mediated chemotactic effects on the migration of EPCs from the upper chamber to lower compartment in a Boyden chamber. Furthermore, adoptive transfer of Th17 cells to OVA-challenged mice and subsequent transfer of fluorescently labeled EPCs via the tail vein resulted in increased migration of exogenous EPCs to the lungs compared with the group that received Th0 cells. In addition, this effect was attenuated by neutralization of IL-17A. Thus, we suggest that IL-17A produced mainly by Th17 cells exerts a chemotactic on EPCs and induces the recruitment of EPCs to the lung in allergen-challenged mice. IL-17A potentiates allergic inflammation by inducing the release of proinflammatory cytokines, as well as chemokines, including CXCR2-associated ligands (26). CXCR2 is expressed by EPCs in both humans and rodents (27, 28). Recently, it has been shown that the ligation of CXCR2 with its ligands, CXCL1 and CXCL2, is critical for EPC recruitment into the lung of allergen-sensitized mice (29). In our

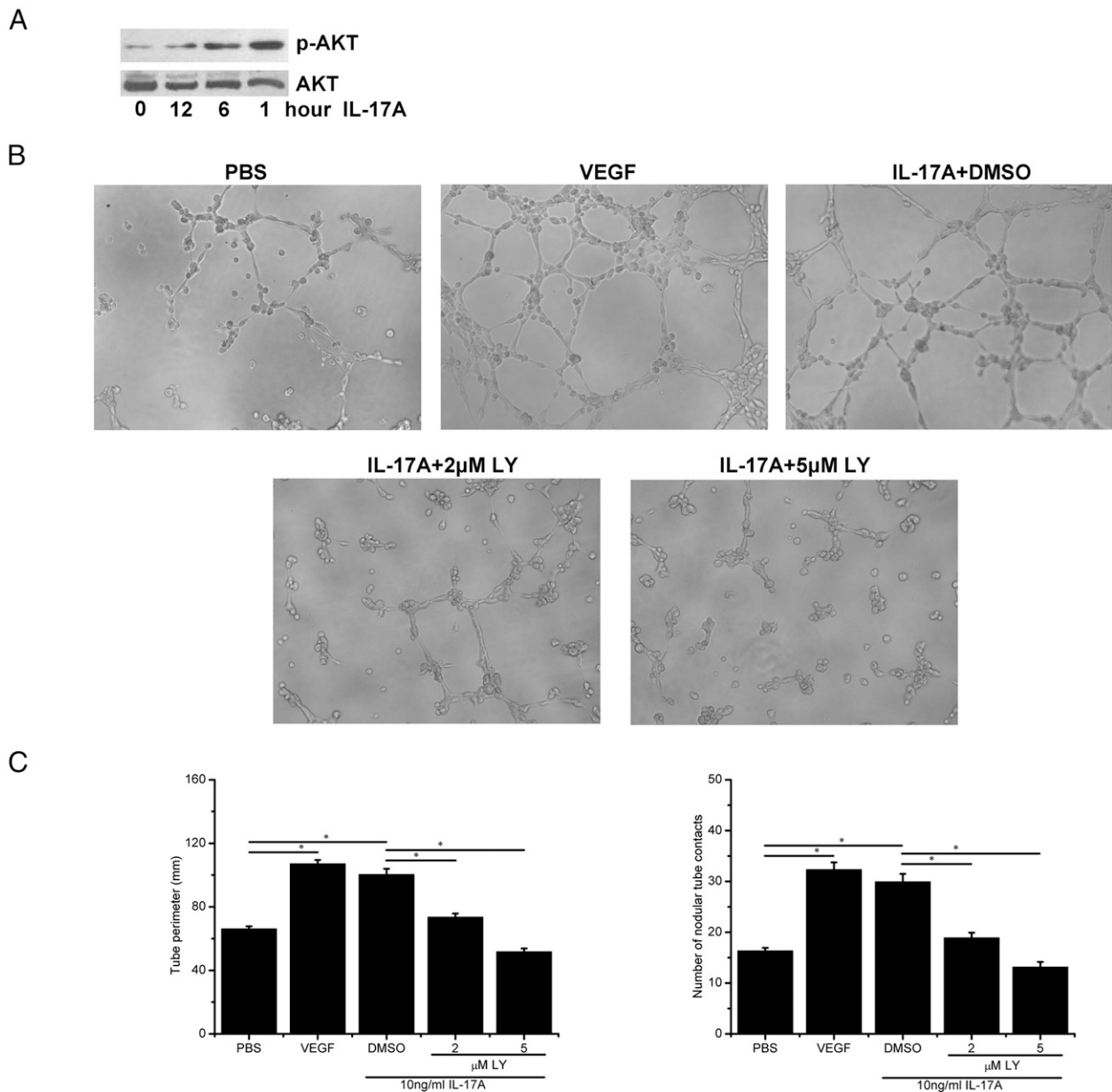


FIGURE 9. IL-17A activates the PI3K pathway in PMVECs, and inhibition of PI3K reduces IL-17A-induced PMVEC tube formation. **(A)** To explore the effects of IL-17A in PMVECs, we stimulated cells with IL-17 (10 ng/ml) for 0–12 h and the cell lysates were probed for p-AKT. **(B)** To determine signaling pathways associated with PMVEC tube formation, we incubated cells with inhibitors to PI3K (LY294002; 2 and 5 μM) or DMSO for 45 min at 37°C before being added to polymerized Matrigel. IL-17 (10 ng/ml) was then added to the wells, and the plate was incubated for 6–8 h at 37°C. Photomicrographs of representative wells of each group are shown (original magnification $\times 100$). **(C)** The average of the total perimeter of full tubes in each group was calculated by Image-Pro Plus 5.1. Node formation was evaluated blindly under a phase-contrast microscope. Data represent the mean $\pm$ SEM of five different experiments. * $p < 0.05$ compared with the negative control.

study, analysis of the cytokines present in the bronchoalveolar lavage fluid of asthmatic mice indicated that acute and prolonged allergen exposure led to a significant increase in CXCL1 and CXCL2 at 24 and 35 d when compared with the PBS controls. However, levels of these factors decreased by day 55 (Supplemental Fig. 1). Thus, we hypothesize that, in addition to the direct chemotactic effect of IL-17A, EPC recruitment induced by IL-17A in vivo is also related to the activation of CXCR2. In addition, Miyake et al. (30) reported that CXCL1 was related to the viability of endothelial cells and induced migration and tube formation via the ERK1/2 signaling pathway. Thus, we propose that CXCL1 is also involved in angiogenesis of endothelial cells.

The canonical Th17 cytokine, IL-17A, was identified by Pickens and coworkers (11) as a proangiogenic factor in rheumatoid ar-

thritis. In addition, Numasaki et al. (19) revealed a novel role for IL-17A in the induction of vascular endothelial cell migration and cord formation and regulation of the production of a variety of proangiogenic factors in tumors. In our study, we found that IL-17A had no direct effect on the proliferation of PMVECs, although it promoted the development of microvessel-like structures in vitro. From these results we conclude that Th17 exhibits an IL-17A-dependent enhancement of angiogenesis via promoting tube formation rather than the proliferation of endothelial cells. Receptors of IL-17 make up a distinct family of cytokine receptors (31). IL-17A binds to its receptors, IL-17RA and IL-17RC, to activate downstream signal transduction (32, 33). Our RT-PCR results indicated that both EPCs and PMVECs expressed higher mRNA levels of IL-17RA and IL-17RC compared with human

embryonic kidney 293 cells (Supplemental Fig. 2). To determine which signaling pathway was involved in the process of PMVEC tube formation, we first used IL-17A to stimulate PMVECs for 1, 6, and 12 h. We discovered that AKT1 was phosphorylated at 1 h and the state of phosphorylation was maintained to 12 h. PMVECs were then incubated with a chemical inhibitor of PI3K before the tube formation assay, and formation of tubule-like structure was suppressed in the presence of IL-17A. Therefore, we conclude that IL-17A induced tube formation in vitro through activation of the PI3K/AKT pathway. VEGF plays a fundamental role in physiological and pathophysiological forms of angiogenesis (34). Early studies showed that VEGF promotes endothelial cell survival, migration, and permeability via the PI3K/AKT pathway (35, 36). In our study, analysis of VEGF levels in the supernatants of cultured PMVECs stimulated with IL-17A indicated that IL-17A increased the expression of VEGF in PMVECs (Supplemental Fig. 3). Thus, we hypothesize that the increased VEGF expression induced by IL-17A also contributes to the enhancement of PMVEC tube formation in the presence of IL-17A.

In conclusion, our findings indicate that Th17 cells mediate airway vascular remodeling in asthma by inducing the recruitment of EPCs and the PMVEC tubule formation in the lungs via IL-17A rather than IL-17F.

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Disclosures

The authors have no financial conflicts of interest.

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Supplemental Materials

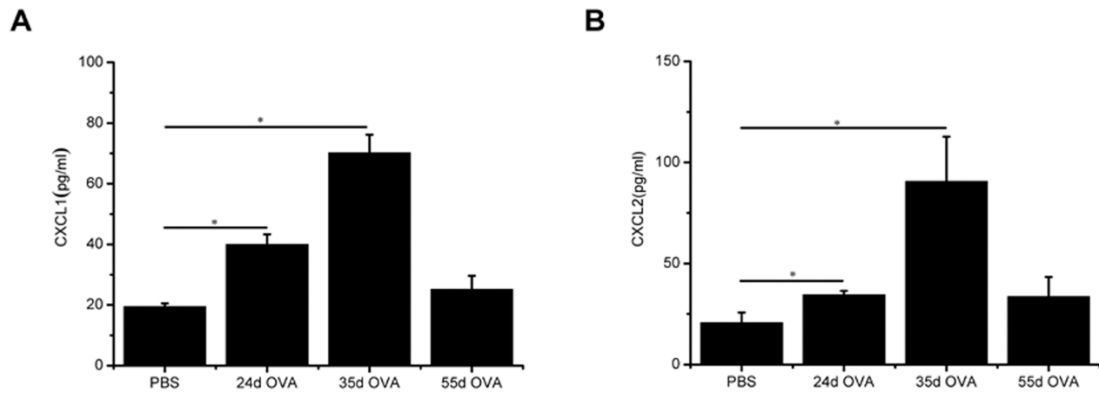


Fig. S1 Concentrations of CXCL1 (A) and CXCL2 (B) in the BALF of asthma model mice were examined by standardized sandwich ELISA. Data represent the mean $\pm$ SEM of four different experiments (n = 6/group). *Indicates $P < 0.05$ compared to the PBS group.

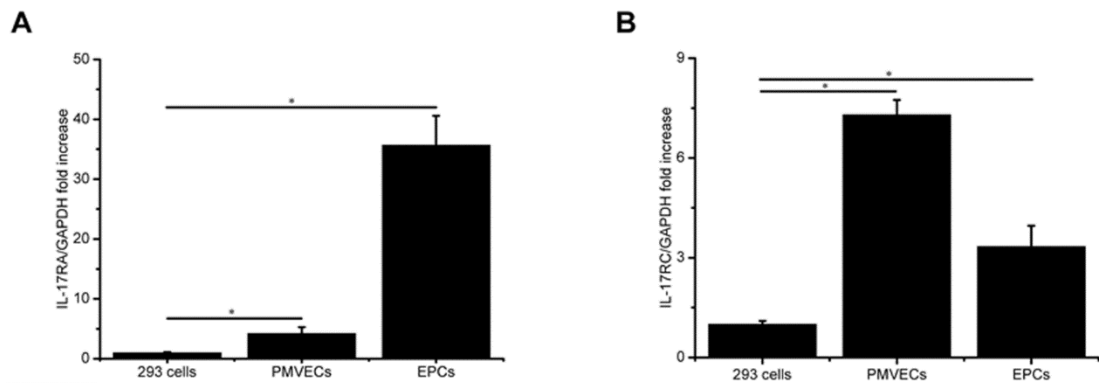


Fig. S2 EPCs and PMVECs express IL-17RA (A) and RC (B). Real-time RT-PCR analysis was performed to determine IL-17RA and IL-17RC expression level in EPCs, PMVECs and HEK293 cells. The relative gene expression levels were normalized by GAPDH and determined by the $\Delta\Delta C_t$ method, and results were expressed as fold increase above the levels detected in HEK293 cells. Data represent the mean $\pm$ SEM (n = 3). *Indicates $P < 0.05$ compared to the the control.

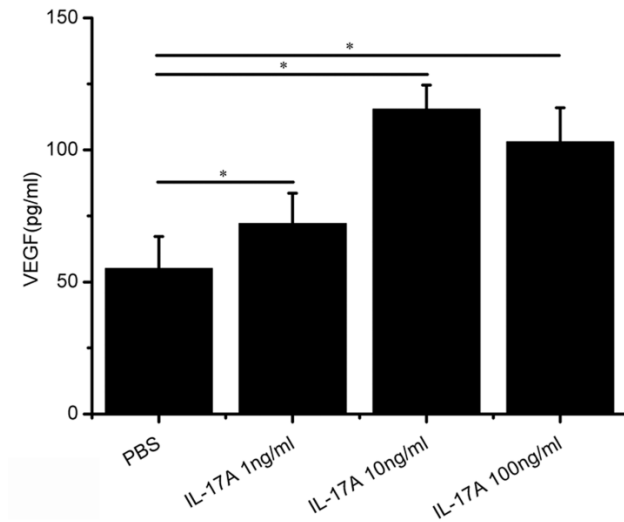


Fig. S3 Concentrations of VEGF in culture supernatants of PMVECs after treatment with different concentrations of IL-17A (1, 10 and 100 ng/ml) were examined by standardized sandwich ELISA. PBS was used as a negative control. Data represent the mean $\pm$ SEM of five different experiments. * Indicates $P < 0.05$ compared to the negative control.