



# The Overexpression of Heparin-Binding Epidermal Growth Factor Is Responsible for Th17-Induced Airway Remodeling in an Experimental Asthma Model

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Th17 cells that produce IL-17 have been found to participate in the development of allergy-triggered asthma. However, whether they play a causative role in the pathogenesis of airway remodeling in chronic asthma remains unclear. In this study, we investigated the role of Th17 cells in airway remodeling and the possible involvement of epidermal growth factor (EGF) receptor signals downstream of Th17. We established a C57BL/6 mouse model of prolonged allergen challenge that exhibits many characteristics of airway remodeling. Prolonged allergen challenge induced a progressive increase in the number of airway-infiltrating Th17 cells, and Th17 counts positively correlated with the severity of airway remodeling. Increases in mucus production, airway smooth muscle (ASM) mass, peribronchial collagen deposition, and airway heparin-binding EGF (HB-EGF) expression have been observed in sensitized mice following prolonged allergen exposure or adoptive Th17 transfer; remarkably, these effects can be abrogated by treatment with anti-IL-17 mAb. Both the EGFR inhibitor AG1478 and an anti-HB-EGF mAb ameliorated all of these effects, except for peribronchial collagen deposition in the presence of high levels of IL-17. In vitro, Th17 cells enhanced the airway epithelial expression of HB-EGF in a coculture of the two cells. The conditioned medium obtained from this coculture system effectively promoted ASM proliferation; this response was dramatically abolished by anti-HB-EGF mAb but not Abs against other EGF receptor ligands or IL-17. These observations demonstrated that overexpression of airway HB-EGF induced by IL-17 secreted from redundant expanding Th17 cells might contribute to excessive mucus expression and ASM proliferation in chronic asthma. *The Journal of Immunology*, 2010, 185: 834–841.

Helper 17 cells and their signature cytokine IL-17 have been found to play an important role in a number of autoimmune diseases, including rheumatoid arthritis, multiple sclerosis, psoriasis, and inflammatory bowel disease (1). Recent evidence indicates that Th17 cells and IL-17 might also participate in the airway remodeling that occurs in chronic asthma (2, 3). IL-17 mRNA and protein are increased in the sputum and lungs from asthmatic patients (4, 5), and the level of IL-17 correlates with the severity of airway hypersensitivity and mucus secretion (6–8). IL-17 may contribute to airway remodeling either through the accumulation of neutrophils or the release of matrix metalloproteinases (MMPs) as well as cytokines including IL-6, TNF- $\alpha$ , and IL-11 by resident cells (1–3). Moreover, it has been shown that the

overexpression of IL-17 can lead to significant airway inflammation, mucus production and subepithelial collagen deposition in genetically manipulated mice (9, 10). Although these studies provide evidence for the role of Th17 cells in the progression of airway remodeling, much less is known about the ability of Th17 cells to exacerbate airway remodeling and the mechanisms that may underlie this activity.

Current evidence indicates that the effects of IL-17 may be partly mediated by epidermal growth factor receptor (EGFR) signaling (11). The stimulation of EGFR by its ligands has been shown to cause excessive mucus secretion and smooth muscle cell proliferation in a mouse model of asthma (12, 13). Heparin-binding EGF (HB-EGF), a potent mitogen and chemotactic factor for smooth muscle cells in the EGF family (14), is expressed mainly in the airway epithelium and is significantly upregulated in asthmatic airways (15). In vitro, there is also evidence that IL-17 can stimulate HB-EGF secretion by synovial fibroblasts (16). These observations led us to investigate the role of the EGFR pathway in Th17-related airway remodeling.

In the current study, we used a prolonged allergen-challenged mouse model, which exhibits many features similar to those in human airway remodeling, to investigate the role of Th17 cells in the progression of airway remodeling. We found that the number of airway-infiltrated Th17 cells in sensitized mice increased gradually during prolonged OVA challenge and had a strong positive correlation with the severity of airway remodeling. Anti-IL-17 mAb effectively blocked the occurrence of airway remodeling induced by prolonged OVA challenge or adoptive Th17 transfer. These observations confirmed the causative role of Th17 cells in the pathogenesis of airway remodeling. Further investigation revealed that the overexpression of airway HB-EGF induced by IL-17 from redundant Th17 cells might contribute to excessive mucus production and airway smooth muscle (ASM) proliferation in chronic asthma.

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Abbreviations used in this paper: AHR, airway hyperresponsiveness; ASM, airway smooth muscle; BALF, bronchoalveolar lavage fluid; CM, conditioned medium; EGFR, epidermal growth factor receptor; HB-EGF, heparin-binding epidermal growth factor; KC, keratinocyte-derived chemokine; MMP, matrix metalloproteinase; PAS, periodic acid-Schiff; PCNA, proliferating cell nuclear Ag; PLN, paratracheal lymph node; SMA, smooth muscle actin.

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## Materials and Methods

### Animals

Male C57BL/6 mice (4–6 wk of age) were purchased from National Rodent Laboratory Animal Resources, Shanghai Branch (Shanghai, China), and housed under specific pathogen-free conditions. Experiments were performed according to protocols approved by the Animal Studies Committee of China.

### OVA sensitization and challenge

All mice were sensitized using 25  $\mu$ g OVA (grade V, Sigma-Aldrich, St. Louis, MO) in 0.1 ml alum i.p. on days 0 and 12. The experimental group was challenged with aerosolized 5% OVA for 30 min daily between days 18 and 23. Some mice were euthanized on day 24 to provide an acute asthmatic model. Prolonged inflammation was induced by subsequent exposure to aerosolized 5% OVA for 30 min three times per week from day 26 until the end of the study. Mice euthanized on days 35 or 55 represented chronic asthma (17). Control mice were subjected to the same protocol but received PBS instead of OVA in the challenge phase.

### Th17 cell generation and adoptive transfer

Naive CD4<sup>+</sup> T cells were purified from the spleens of C57BL/6 mice using anti-CD4 microbeads and a magnetic sorter (MACS; Miltenyi Biotec, Auburn, CA). The purity of the CD4<sup>+</sup> T cells was >90%. The cells were treated with Dynabeads Mouse CD3/CD28 T Cell Expander (Invitrogen, Carlsbad, CA) for 6 d in the presence of 5 ng/ml TGF- $\beta$  (Abcam, Cambridge, MA), 10 ng/ml recombinant murine IL-6 (Biolegend, San Diego, CA), 10  $\mu$ g/ml anti-IFN- $\gamma$ , and 10  $\mu$ g/ml anti-IL-4 mAbs (Biolegend) to stimulate their differentiation into Th17 cells (18). Before adoptive transfer, the concentrations of Th1/Th2/Th17 cells were determined by intracellular staining for IL-17, IL-4, and IFN- $\gamma$ . IL-17<sup>+</sup> cells accounted for 43.7–51.6% of the primary cultured cells, whereas IFN- $\gamma$ <sup>+</sup> and IL-4<sup>+</sup> cells were <0.9% and 0.5%, respectively.

Primary cultured Th17 cells ( $5 \times 10^5$  cells/mouse) were adoptively transferred i.p. to sensitized mice 30 min before OVA or PBS challenge on every other day from day 18–23. Mice were euthanized on day 24 for tissue collection.

### Ab treatment

Anti-IL-17A mAb (100  $\mu$ g/mouse; clone 50104, R&D Systems, Minneapolis, MN), the EGFR inhibitor AG1478 (100  $\mu$ g/mouse dissolved in DMSO; Calbiochem, San Diego, CA), anti-HB-EGF mAb (50  $\mu$ g/mouse; Santa Cruz Biotechnology, Santa Cruz, CA), or control mAbs of the corresponding class (R&D Systems) were administered i.p. from day 35 onwards three times per week over the course of the prolonged OVA challenge. Mice were euthanized on day 55. In the mouse model of Th17 transfer, anti-IL-17A mAb, anti-keratinocyte-derived chemokine (KC) mAb (R&D Systems), anti-HB-EGF mAb, AG1478, or the corresponding control mAb was delivered to sensitized mice at a single dose of 100  $\mu$ g/mouse 30 min prior to the first Th17 cell transfer. Mice were euthanized on day 24.

### Determination of airway hyperresponsiveness

Airway hyperresponsiveness (AHR) was measured *in vivo* 24 h after the last aerosol exposure, as previously described (19). Briefly, mice were anesthetized by i.p. injection of phenobarbital (40 mg/kg), intubated, and placed in a whole-body plethysmography chamber. A small polyethylene catheter was placed in the jugular vein for i.v. administrations. Increasing methacholine doses (0.5, 1.0, 2.0, and 5.0  $\mu$ g/kg) were administered i.v. at 5-min intervals. Tidal volume, airway flow velocity, and transpulmonary pressure were measured for 2 min after each dose using a Transducer-PCLAB Medlab (Nanjing MedEase Science and Technology, Nanjing, China) to calculate airway resistance ( $R_L$ ) and dynamic compliance ( $C_{dyn}$ ). Results are expressed as the maximal resistance after each dose of methacholine minus the baseline value.

### Analysis of bronchoalveolar lavage fluid

After AHR measurements, the left lungs were lavaged three times with 0.5 ml D-HBSS. Bronchoalveolar lavage fluid (BALF) was centrifuged (2500 rpm, 10 min), and the supernatants were collected for cytokine analyses. Cell pellets were resuspended in PBS, and the total cell number was determined using a hemocytometer. Differential counts were performed on May-Grunwald/Giemsa-stained cytospun cells (Sigma-Aldrich).

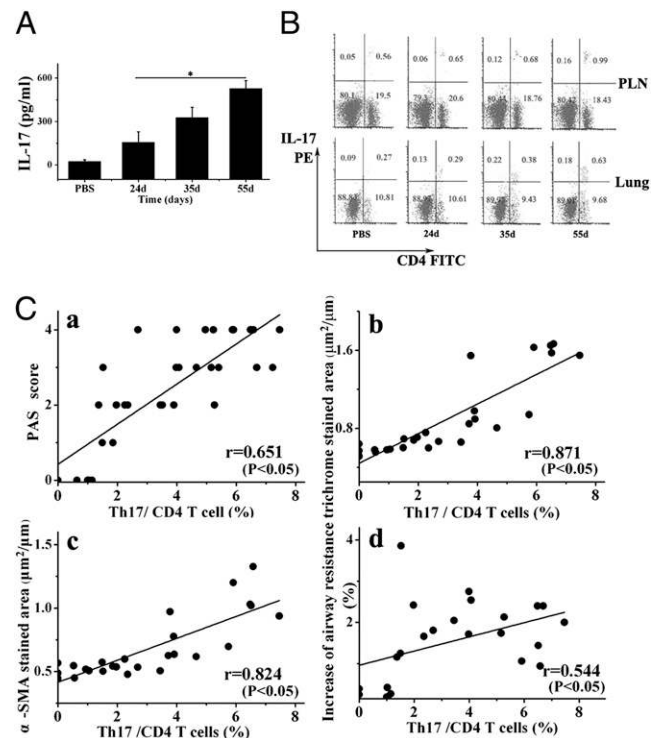
### Intracellular cytokine flow cytometry

FITC-labeled anti-mouse CD4 (isotype rat IgG<sub>2b</sub>; eBioscience, San Diego, CA) and PE-labeled anti-mouse IL-17 (isotype rat IgG<sub>1</sub>; BD Biosciences,

San Jose, CA) were used to detect Th17 cells. Rat IgG of the corresponding class (eBioscience) was used as an isotype control. Cells purified from mouse lungs and paratracheal lymph nodes (PLNs) were stimulated with 50 ng/ml PMA (BioVision, Mountain View, CA) and 500 ng/ml ionomycin (Fermentek, Jerusalem, Israel) in the presence of GolgiPlug (eBioscience) for 4 h, after which the cells were surface stained for CD4, permeabilized with Cytofix/Cytoperm (eBioscience), washed, and intracellularly stained with PE-labeled anti-IL-17. Flow cytometry acquisition was performed using an FACSCalibur (BD Biosciences), and results were analyzed with CellQuest software (BD Biosciences).

### Quantitative real-time PCR

Total RNA was extracted from right lungs and reverse transcribed with MMLV transcriptase (Invitrogen, Carlsbad, CA). The levels of mRNA expression were determined using an ABI7500 Detection System (Applied Biosystems, Foster City, CA) and FastStart DNA Master SYBR Green I (Roche, Basel, Switzerland). GAPDH was used as a reference control to normalize the loading of template cDNA. The sequences of each pair of primers were as follows: HB-EGF forward: 5'-TCCGCTGTCTTCTGTGCATCGT-3', HB-EGF reverse: 5'-TAGCCACGCCCAACTTCACT-3'; EGF forward: 5'-CCAAACGCGAAGACTTATCC-3', EGF reverse: 5'-TGATCCTCAAA-CACGGCTAGAGA-3'; TGF- $\alpha$  forward: 5'-GCCACAGATCCACACT CAGTA-3', TGF- $\alpha$  reverse: 5'-TCTGCATGCTCACAGCGAA-3'; EGFR forward: 5'-CATCTGTGCCAGCAATGTT-3', EGFR reverse: 5'-TTGC-ATGTGGCCTCATCTTG-3'; and GAPDH forward: 5'-TGCATCTGCA-CCACCAACTGCTTAG-3', GAPDH reverse: 5'-GGCATGGACTTGGT-CATGA-3' (20, 21).



**FIGURE 1.** The number of airway-infiltrated Th17 cells increased progressively during prolonged OVA challenge and is positively correlated to the severity of airway remodeling. *A*, IL-17 levels in BALF from sensitized mice challenged with OVA or PBS on day 24 (acute phase) and on days 35 and 55 (chronic phase) were measured by ELISA. *B*, CD4<sup>+</sup>IL-17<sup>+</sup> cell numbers in lungs and PLNs from acute and prolonged OVA-challenged mice and PBS controls were analyzed by flow cytometry. The frequency of CD4<sup>+</sup>IL-17<sup>+</sup> cells in the lymphocyte region of the forward/side scatter plot is shown. The numbers indicated are the averaged values of three independent experiments. *C*, Correlation between the levels of Th17 cells in the lung and the degree of excessive mucus secretion represented by PAS score (*a*), collagen deposition represented by Masson trichrome-stained area (*b*), ASM mass thickness represented by  $\alpha$ -SMA-stained area (*c*), and AHR represented by increase of airway resistance (*d*). Spearman regression analysis was used. Data represent the means  $\pm$  SD of three independent experiments.  $n = 6$ /group/time point. \* $p < 0.05$  compared with the previous time point.

### Histology and immunohistochemistry

Paraffin-embedded lung sections (4  $\mu\text{m}$ ) were stained with periodic acid-Schiff (PAS) to evaluate the degree of mucus secretion. Mucus secretion was scored according to the percentage of PAS-positive cells in the total number of airway epithelial cells (0, <5% goblet cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75%). Masson trichrome staining and  $\alpha$ -smooth muscle actin (SMA) immunostaining were used to assess peribronchial collagen deposition and the thickness of the ASM layer, respectively. The staining area was outlined and quantified using a light microscope (DP20, Olympus, Melville, NY) and the attached image analysis system (Image-Pro Plus 5.1). Results were expressed as the area of staining per micrometer length of the basement membrane of bronchioles with a 150–200- $\mu\text{m}$  internal diameter at comparable sites of each slide (22, 23). At least 10 bronchioles were evaluated per slide.

### Measurement of collagen

Lung tissue (100 mg) was homogenized in 1 ml PBS containing protease inhibitor mixture (Roche) and then centrifuged at  $800 \times g$  for 30 min. The supernatants were collected and analyzed for total lung collagen content using the Sircol Collagen Assay Kit (Biocolor, County Antrim, U.K.) according to the manufacturer's instructions (17).

### Western blot analysis

Lung homogenates were boiled and separated by SDS-PAGE (12% SDS tricine gel) pretransfer to polyvinylidene difluoride membranes. Membranes were blocked and then incubated with anti-HB-EGF mAb (1:1000; Santa Cruz Biotechnology) overnight at 4°C. Postincubation with HRP-conjugated secondary Ab (1:3000; Santa Cruz Biotechnology), membranes were washed extensively, and antigenic bands were visualized by ECL (Thermo Scientific, Waltham, MA) according to the manufacturer's protocol.

### Cytokine analysis

The concentrations of IL-17 in BALF supernatants and the levels of HB-EGF in lung homogenates were measured by standardized sandwich ELISA according to the manufacturer's protocol. All paired Abs and kits were purchased from R&D Systems.

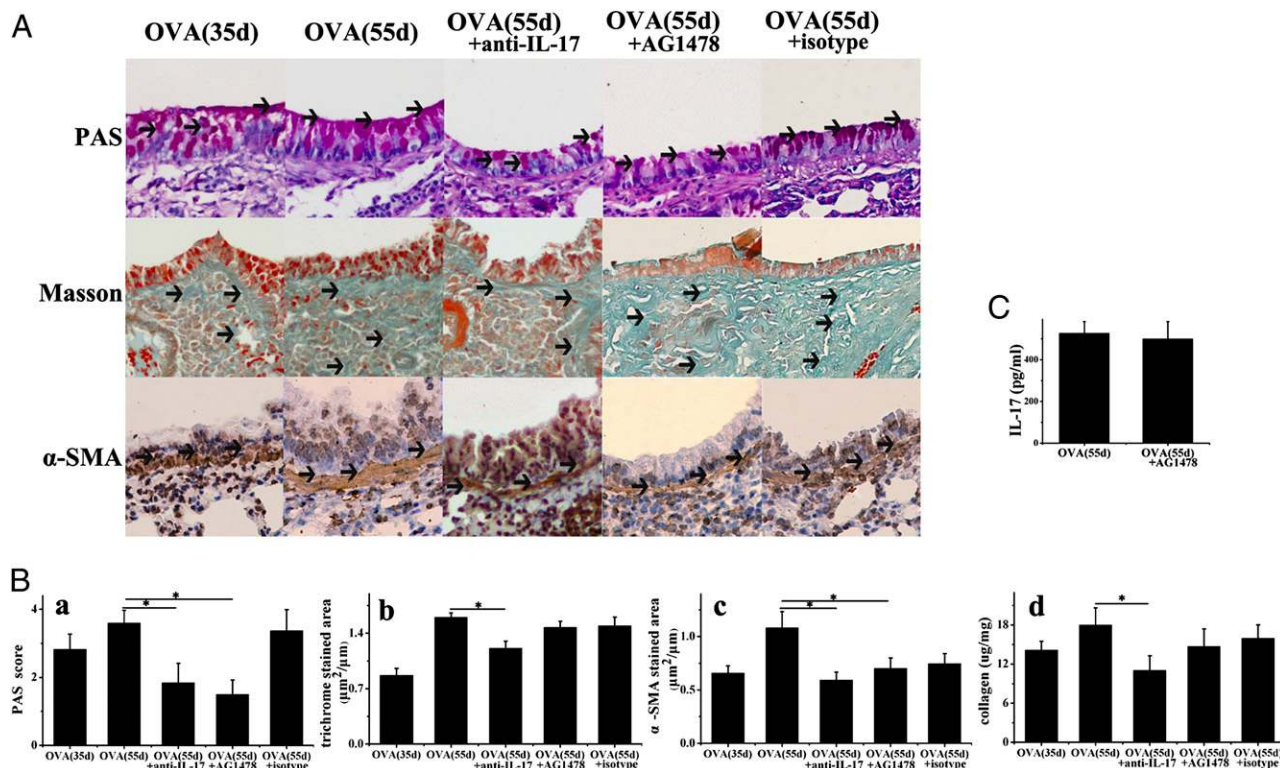
### Airway epithelial cell preparation and coculture with Th17 cells

Airway epithelial cells were obtained by scraping the lumen of the trachea with a cell scraper (15) and then cocultured with Th17 cells in RPMI 1640 medium at 37°C. Transwell Permeable Support (Corning, Corning, NY) or anti-IL-17 mAb was added to the coculture system to separate the cell types or neutralize the IL-17 activity, respectively. Th17 cells were depleted by anti-CD4 microbeads and a magnetic sorter, and purified airway epithelium was recovered 48 h later. Total RNA was extracted, and real-time PCR was performed to evaluate the expression of HB-EGF, EGF, and TGF- $\alpha$  mRNAs.

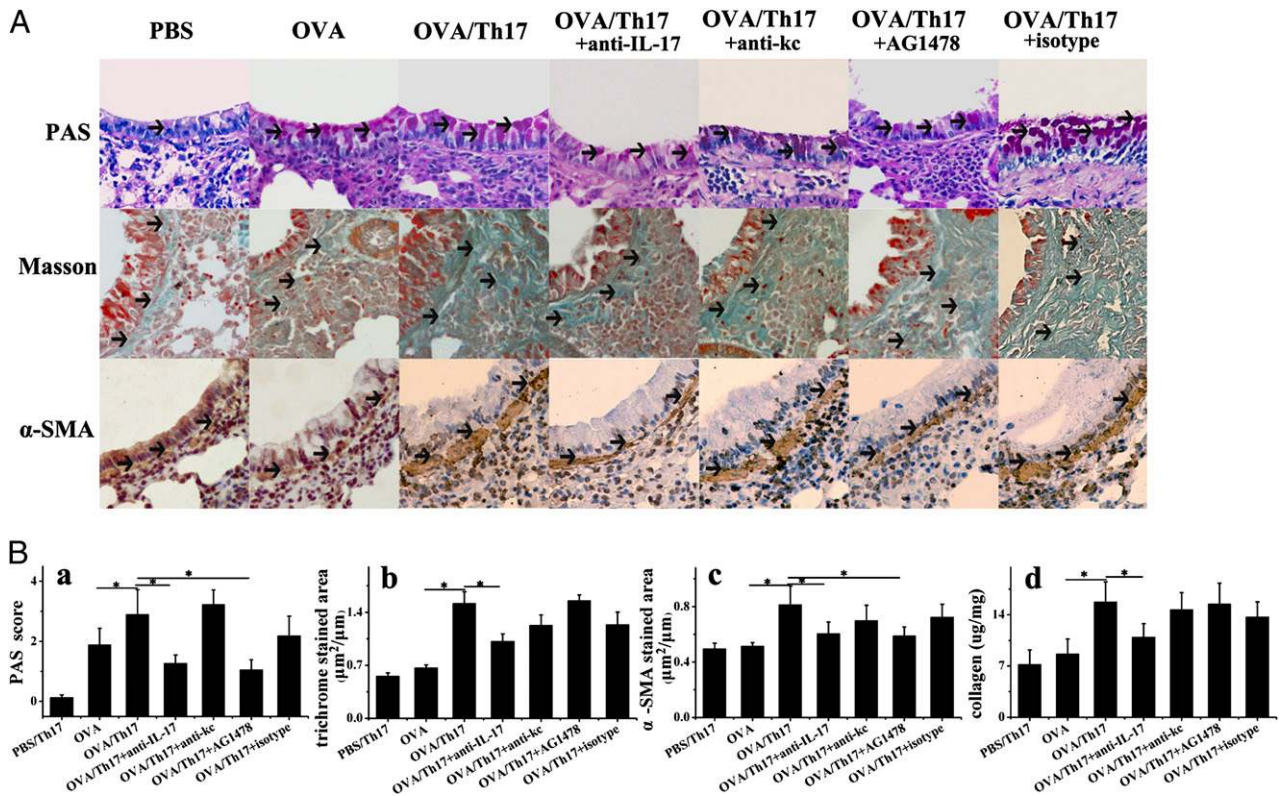
### ASM cell proliferation assay

ASM cells were obtained as described previously (24). Briefly, tracheae of sensitized mice were excised, washed, and digested with 0.2% collagenase IV (Sigma-Aldrich) in PBS for 30 min at 37°C. The tissue was allowed to stand, and supernatant was collected and centrifuged ( $500 \times g$ , 6 min). The pellet containing ASM cells was resuspended in 1:1 DMEM/Ham's F12 (PAA Laboratories, Pasching, Austria), then plated in six-well plates.

Cells at passages 2–4 were plated in six-well plates ( $8 \times 10^4$  cells/well in 2 ml medium). The cells were starved in DMEM with 0.5% BSA when they had reached 70% confluence for 48 h. Poststarvation, the cells were stimulated with HB-EGF (1.25–10 ng/ml, R&D) for 48 h and



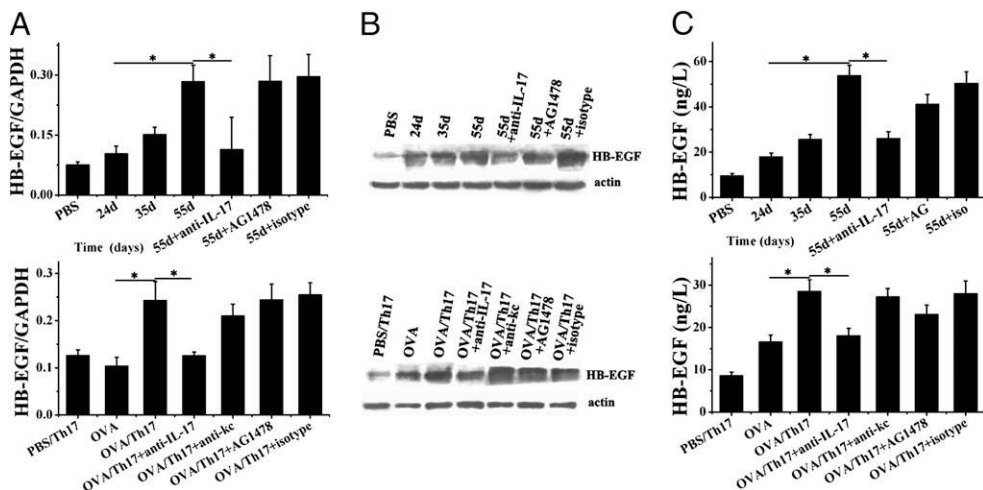
**FIGURE 2.** Anti-IL-17 mAb or AG1478 administration attenuated prolonged OVA-induced airway remodeling. Repeated OVA sensitization and challenges were performed, and anti-IL-17 mAb, AG1478, or isotype IgG was administered (100  $\mu\text{g}/\text{mouse}$  i.p.) from day 35 onwards three times per week 30 min prenebulization. Mice were euthanized on day 55 for tissue collection. **A**, Representative photomicrographs of PAS-, Masson trichrome-, and  $\alpha$ -SMA-stained lung sections from each group. Original magnification  $\times 200$ . **B**, Mucus hypersecretion, subepithelial collagen deposition, and ASM mass thickness were respectively quantified by PAS score (PAS-positive cells in the airway epithelium of random bronchioles were counted and divided by the total number of airway epithelial cells; 0, <5% goblet cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75%) (a), the area of Masson trichrome staining (b),  $\alpha$ -SMA staining per micrometer length of the bronchiolar basement membrane ( $\mu\text{m}^2/\mu\text{m}$ ) (c), and levels of total collagen measured in the lung homogenate samples (d). **C**, IL-17 expression. The results represent the means  $\pm$  SD of four different experiments.  $n = 6/\text{group}$ .  $*p < 0.05$  between groups.



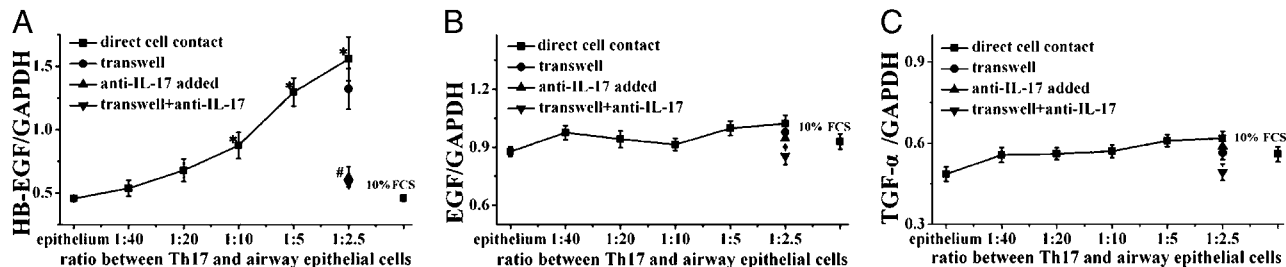
**FIGURE 3.** Adoptive Th17 transfer accelerated the development of airway remodeling, which could be attenuated by AG1478 and anti-IL-17 mAb but not anti-KC mAb. Primary cultured Th17 cells ( $5 \times 10^5$  cells/mouse) were transferred i.p. to sensitized mice 30 min before OVA or PBS aerosolization on every other day from days 18–23. Meanwhile, anti-IL-17 mAb, anti-KC mAb, AG1478, or isotype IgG was administered i.p. at a single dose of 100 μg prior to the first Th17 transfer in some of the mice. Mice were euthanized 24 h after the final challenge. *A*, Representative photomicrographs of paraffin-embedded PAS-, Masson trichrome-, and α-SMA-stained lung sections from each group were shown. Original magnification  $\times 200$ . *B*, Mucus hypersecretion, subepithelial collagen deposition, and ASM mass thickness were respectively quantitated by PAS score (PAS-positive cells in the airway epithelium of random bronchioles were counted and divided by the total number of airway epithelial cells; 0, <5% goblet cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4, >75%) (*a*), the area of Masson trichrome staining (*b*), α-SMA staining per micrometer length of the bronchiolar basement membrane ( $\mu\text{m}^2/\mu\text{m}$ ) (*c*), and levels of total collagen measured in the lung homogenate samples (*d*). The results represent the means  $\pm$  SD of four different experiments.  $n = 6/\text{group}$ .  $*p < 0.05$  between groups.

then counted and immunostained for proliferating cell nuclear Ag (PCNA; Santa Cruz Biotechnology) to evaluate the proportion of proliferating ASM cells (15). ASM cells were counted in four randomly selected fields ( $\times 40$

magnification), and the number of PCNA-positive cells was divided by the total number of cells counted to yield the percentage of proliferating ASM cells.



**FIGURE 4.** Elevated lung levels of HB-EGF were observed after prolonged OVA challenge or adoptive Th17 transfer. The prolonged OVA challenge model was established, and anti-IL-17 mAb, AG1478, or isotype IgG was given as previously detailed. In another series of experiments, Th17 cells were transferred i.p. to an acute OVA-challenged model with concomitant anti-IL-17 mAb, anti-KC mAb, or AG1478 administration as described. *A*, mRNA expression of HB-EGF were detected by real-time PCR and normalized to the expression of the housekeeping gene GAPDH. *B*, Western blot using 100 μg lung protein extract per lane and probed with a HB-EGF Ab. *C*, HB-EGF levels in lung homogenates from each group were analyzed by ELISA. The upper panel represents the prolonged OVA challenge model, and the lower panel represents the adoptive Th17 transfer model. Data represent the means  $\pm$  SD of four different experiments.  $n = 6/\text{group}$ .  $*p < 0.05$  between groups.



**FIGURE 5.** Airway epithelial expression of HB-EGF was upregulated upon coculture with Th17 cells in vitro. Increasing concentrations of Th17 cells were cocultured with airway epithelium under various conditions, including transwell separation and anti-IL-17 addition, for 48 h. Th17 cells were then depleted to recover the purified airway epithelium. HB-EGF, EGF, and TGF- $\alpha$  mRNA expressions were measured by real-time quantitative PCR normalized to the expression of the housekeeping gene GAPDH. Dose-response curves were drawn to show the effects of Th17 on HB-EGF (A), EGF (B), and TGF- $\alpha$  (C) expressions in airway epithelial cells. Data represent the means  $\pm$  SD of four independent experiments per data point. \* $p$  < 0.05 versus baseline; # $p$  < 0.05 versus transwell or anti-IL-17 mAb addition.

The supernatant from the Th17 and epithelium coculture system was also collected and mixed with DMEM/Ham's F12 at a ratio of 2:1; this mixture was used as conditioned medium (CM). ASM cells were cultured in CM with or without anti-IL-17A, anti-EGF (Merck, Whitehouse Station, NJ), anti-TGF- $\alpha$  (Merck), anti-HB-EGF, or AG1478 at 10  $\mu$ g/ml each for 48 h, and the proliferative response was determined as described above.

#### Statistical analysis

Results are expressed as means  $\pm$  SD with a group size of six from four different experiments. Data were analyzed by ANOVA (Tukey honestly significant difference), correlation analysis (Spearman) or independent  $t$  tests as appropriate with SPSS 13.0 (SPSS, Chicago, IL). Statistical significance was accepted at  $p$  < 0.05.

## Results

### Airway-infiltrated Th17 cells increased progressively during prolonged OVA challenge, positively correlated with airway remodeling

It has been demonstrated that prolonged OVA challenge leads to prominent airway remodeling in sensitized mice, characterized by excessive mucus secretion, peribronchial collagen deposition, and ASM cell proliferation (17). These traits were reproduced in our experiments. Concomitantly, the levels of IL-17 in BALF (Fig. 1A) and the numbers of Th17 cells in the lungs or PLNs (Fig. 1B) increased progressively during prolonged allergen exposure. Further regression analysis showed that the percentage of Th17 cells among all lung CD4<sup>+</sup> T cells was positively correlated with the extent of mucus production, peribronchial collagen deposition, and ASM mass thickness. A weak correlation between Th17 and AHR was also discovered (Fig. 1C). These observations indicated a potential role for Th17 cells in airway remodeling.

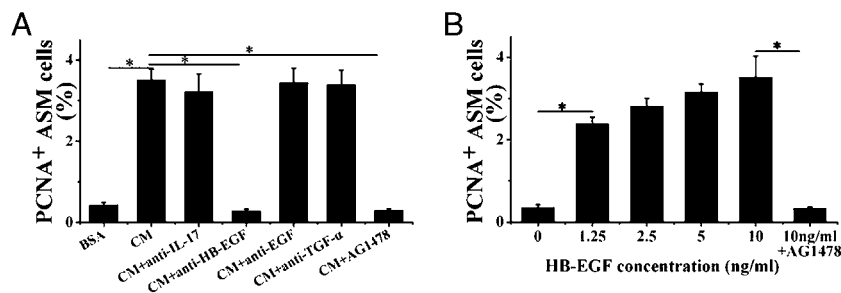
### IL-17 is essential for Th17-mediated airway remodeling

In order to further investigate the effect of Th17 cells on OVA-induced airway remodeling, anti-IL-17 mAb was given during prolonged allergen challenges. As expected, anti-IL-17 mAb administration obviously attenuated OVA-induced mucus secretion, ASM layer thickening, and lung collagen deposition (Fig. 2A, 2B). Furthermore, adoptive Th17 transfer during the course of acute allergen exposure significantly accelerated mucus secretion, lung collagen deposition, and ASM layer thickening, and these effects could be abrogated by the addition of anti-IL-17 mAb (Fig. 3).

Th17 cells and IL-17 have been reported to be the key regulators of the recruitment and activation of neutrophils. In our experiments, a time-dependent increase in the number of BALF neutrophils was detected during prolonged OVA challenge (Supplemental Fig. 1A), in parallel with the increase in Th17 cell number. In addition, the adoptive transfer of Th17 cells stimulated massive neutrophil recruitment to the airways, an effect that could be reversed by anti-IL-17 mAb (Supplemental Fig. 1B). Therefore, we tested whether Th17-triggered neutrophil infiltration was involved in Th17-induced airway remodeling by using a neutralizing Ab to KC, a murine neutrophil chemoattractant. The administration of anti-KC mAb during Th17 transfer significantly reduced Th17-triggered neutrophil influx (Supplemental Fig. 1B), but the extent of airway remodeling was hardly affected (Fig. 3).

### EGFR blockade suppresses Th17-induced airway remodeling

To determine whether EGFR mediates Th17-induced airway remodeling, the EGFR inhibitor AG1478 was given to sensitized mice, which were then subjected to prolonged OVA challenge or adoptive Th17 transfer. In the prolonged OVA challenge model,



**FIGURE 6.** HB-EGF can promote ASM cell proliferation in vitro. A, The supernatant from the Th17/epithelial cell coculture was collected and mixed with DMEM/Ham's F12 at the ratio of 2:1 to make CM. ASM cells were cultured in CM with or without anti-IL-17, anti-EGF, anti-TGF- $\alpha$ , anti-HB-EGF, or AG1478 at 10  $\mu$ g/ml for 48 h, and the proliferative response was assayed. B, ASM cells were treated with increasing concentrations of HB-EGF, and their proliferative response was evaluated. The extent of ASM cell proliferation was assessed by the percentage of PCNA-immunoreactive cells. Data represent the means  $\pm$  SD of four independent experiments. \* $p$  < 0.05 between groups.

AG1478 did not affect the concentration of BALF IL-17 (Fig. 2C), but mucus secretion and ASM layer thickness were dramatically decreased. However, no marked reduction in lung collagen deposition was induced by AG1478 (Fig. 2A, 2B). Similar results were observed in Th17-transferred mice treated with AG1478 (Fig. 3).

*Elevated expression of HB-EGF is observed in the lung after prolonged OVA challenge or adoptive Th17 transfer*

The effects of prolonged OVA challenge and adoptive Th17 transfer on the mRNA levels of three EGFR ligands (TGF- $\alpha$ , EGF, and HB-EGF), as well as EGFR itself, were examined in the lung. We found that HB-EGF mRNA expression was significantly upregulated following prolonged OVA challenge and that this effect could be reversed by anti-IL-17 mAb administration. Accordingly, HB-EGF expression was increased by the adoptive transfer of Th17 cells and decreased following treatment with anti-IL-17 (Fig. 4A). A similar change in HB-EGF protein level was also observed in both the prolonged OVA challenged model and the adoptive Th17-transferred model, as confirmed by Western blot and ELISA (Fig. 4B, 4C).

In contrast, the expression of EGF, TGF- $\alpha$ , and EGFR mRNAs was almost unaffected by both treatments (Supplemental Fig. 2).

*Airway epithelial expression of HB-EGF is upregulated by coculture with Th17 cells in vitro*

Next, we tested the hypothesis that Th17 could promote epithelial HB-EGF expression in vitro by developing a coculture system of these two cells. After 48 h of coculture, the expression of HB-EGF in the airway epithelia was significantly elevated commensurate with the increased Th17 cell concentration. This effect could be

almost completely inhibited by anti-IL-17 mAb. Moreover, separating these two cells with a transwell membrane did not affect the expression of HB-EGF, suggesting that some soluble molecule (possibly IL-17), but not direct cell contact, is required for this effect (Fig. 5A). However, the expression of EGF and TGF- $\alpha$  in airway epithelial cells did not fluctuate significantly in the presence of Th17 cells (Fig. 5B, 5C).

*HB-EGF can promote ASM cell proliferation in vitro*

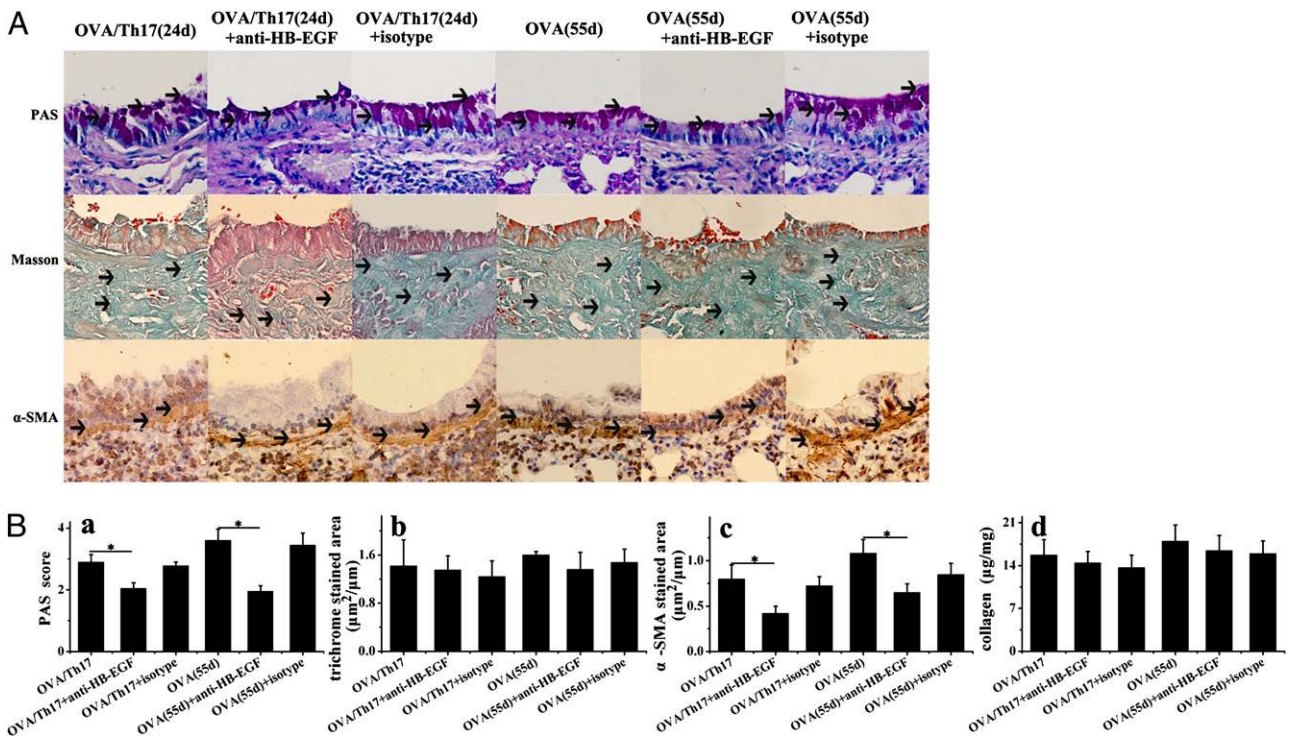
CM from the Th17 and airway epithelium coculture system effectively promoted ASM proliferation, and this effect was dramatically abolished by the addition of anti-HB-EGF or AG1478 but not Abs specific for other EGFR ligands or IL-17 (Fig. 6A). Meanwhile, HB-EGF stimulated ASM cell proliferation in a dose-dependent manner, which could be fully inhibited by AG1478 (Fig. 6B).

*Anti-HB-EGF can reverse Th17-mediated mucus secretion and ASM mass thickening in vivo*

We also performed analogous in vivo experiments by administering anti-HB-EGF mAb in a mouse model of prolonged allergen exposure or during adoptive Th17 transfer. The administration of anti-HB-EGF mAb clearly attenuated the excessive mucus secretion and ASM mass thickening induced by prolonged OVA but had little effect on lung collagen deposition. Similar results were observed in Th17-transferred mice (Fig. 7).

**Discussion**

Prolonged OVA challenge induces many of the same features as airway remodeling, including excessive mucus secretion, peribronchial collagen deposition, and ASM proliferation (17, 25, 26).



**FIGURE 7.** Anti-HB-EGF mAb attenuated Th17-mediated mucus secretion and ASM mass thickening. The prolonged OVA challenge and adoptive Th17 transfer were repeated, and 50  $\mu\text{g}$  anti-HB-EGF was given to some of the mice at the indicated times. *A*, Representative photomicrographs of PAS-, Masson trichrome-, and  $\alpha$ -SMA-stained lung sections from each group. Original magnification  $\times 200$ . *B*, Mucus hypersecretion, subepithelial collagen deposition, and ASM mass thickness were quantified by PAS score (PAS-positive cells in the airway epithelium of random bronchioles were counted and divided by the total number of airway epithelial cells; 0,  $<5\%$  goblet cells; 1, 5–25%; 2, 25–50%; 3, 50–75%; and 4,  $>75\%$ ) (*a*), the area of Masson trichrome staining (*b*),  $\alpha$ -SMA staining per micrometer length of the bronchiolar basement membrane ( $\mu\text{m}^2/\mu\text{m}$ ) (*c*), and levels of total collagen measured in the lung homogenate samples (*d*). The results represent the means  $\pm$  SD of four independent experiments.  $n = 6/\text{group}$ .  $*p < 0.05$  between groups.

These features were also observed in our mouse model. Meanwhile, a progressively elevated Th17 immunological response was detected during prolonged OVA challenge and correlated with the severity of airway remodeling. Th17 cells have been demonstrated to induce an intense airway inflammatory response (1) that is generally believed to cause subsequent airway remodeling (27), and Th17 cells have been linked with several remodeling-associated cytokines, such as IL-6, IL-11, vascular EGF, and MMPs (2, 3); however, the current study is the first to demonstrate that Th17 cells can mediate OVA-induced airway remodeling and that this effect is mainly dependent on IL-17 secretion. Various studies have shown that Th17 cells and IL-17 can potentially activate and recruit a huge number of neutrophils through KC and MIP-2 upregulation in an asthmatic mouse model (28–31). In this study, both prolonged OVA challenge and adoptive Th17 transfer induced significant neutrophil influx, in agreement with the previous studies. However, we also found that the administration of a mAb that neutralized KC almost blocked Th17-triggered neutrophil influx entirely but had very little inhibitory effect on airway remodeling. This led us to propose that the neutrophils might not contribute significantly to the pathogenesis of Th17-mediated airway remodeling. Instead, other downstream factors should be investigated.

The airway epithelium, although playing an important role as a physical barrier, is believed to be central to asthma pathogenesis (32, 33). Airway epithelium may participate in airway remodeling through the production of cytokines and growth factors that induce the remodeling of other airway tissues. One group of growth factors that are central to epithelial repair and its altered phenotype is the EGF family cytokines, of which EGF, HB-EGF, and TGF- $\alpha$  have been studied most extensively (15, 34, 35). EGFR is expressed in the airway epithelium as well as in ASM cells, and its expression is substantially upregulated in asthmatic tissues (34–36). EGFR stimulation of the damaged epithelium may generate a mucus-secretory phenotype by promoting MUC5AC expression through the EGFR/Ras/Raf/ERK signaling pathway (12, 34, 37). In addition, EGFR and its ligands are capable of promoting proliferation in various cell types including epithelial cells, smooth muscle cells, and fibroblasts (13, 34, 35, 38). Our study revealed that Th17 cells could enhance the expression of HB-EGF in airway epithelia at both the mRNA and protein levels. HB-EGF was first discovered as a 22-kDa heparin-binding growth factor secreted by macrophage-like U-937 cells (39). It is synthesized as a transmembrane precursor with a signal peptide, propeptide, mature protein, transmembrane, and cytoplasmic domains and functions in a juxtacrine manner to stimulate cell growth (40). Mature secreted HB-EGF containing an N-terminal heparin-binding region and a C-terminal EGF-like region is cleaved from the membrane-bound precursor (41). This cleavage process requires the participation of MMPs (42), the production of which can also be regulated by Th17 cells (2, 43). HB-EGF acts as a mitogen in various cell types, including mouse fibroblasts, human epithelial cells, keratinocytes, and rat hepatocytes; it is the only mitogen in the EGF family that has been confirmed to stimulate smooth muscle cells *in vivo*, and it appears to be more potent than EGF in this cell type (13–15). In this study, we observed that the specific EGFR inhibitor AG1478 and a neutralizing anti-HB-EGF mAb were both able to reverse Th17-induced excessive mucus secretion and ASM mass thickening without affecting IL-17 levels. Thus, we suggest that Th17-mediated excessive mucus secretion and ASM mass thickening are at least partly regulated through the HB-EGF/EGFR signaling pathway. Studies by Zhang and coworkers (44) showed that the p38 MAPK and ERK pathways are both important intermediates in the elevation of HB-EGF expression in airway epithelial cells, and both were confirmed to be

involved in IL-17-induced proinflammatory cytokine secretion from these cells (45, 46). However, the specific pathway that is responsible for the Th17-mediated expression of mature HB-EGF is yet to be identified, and the precise role of HB-EGF in Th17-induced airway remodeling must be explored further.

Meanwhile, we examined the direct effect of Th17 cells on ASM cell proliferation by coculturing the two cell types *in vitro*, which revealed a relatively very weak proliferative response (data not shown). Therefore, we speculated that Th17/IL-17 stimulation of ASM cell proliferation occurs mainly through indirect mechanisms. In addition to the HB-EGF/EGFR pathway studied in this paper, cytokines, such as IL-6 and vascular EGF have also been implicated in ASM cell proliferation (38), and their production can be regulated by IL-17 (1–3). Therefore, several pathways might be involved in Th17-mediated ASM cell proliferation and need further research.

Chen et al. (7) observed that IL-17 could stimulate MUC5B and MUC5AC gene expression and mucus overproduction through the IL-6 paracrine/autocrine loop. Studies on IL-17-transgenic mice and OVA-exposure mice indicate a parallel relationship between the extent of mucus secretion and the lung level of IL-17 (6, 9). These results are consistent with our findings that IL-17 plays a promotive role in mucus hyper-secretion. However, Schnyder-Candrian et al. (30) recently reported that IL-17 reduced OVA-induced mucus hypersecretion in a mouse model. We guess this contrary result may be due to the difference in sensitization and challenge protocol and different IL-17 administration routes.

Peribronchial collagen deposition is another characteristic feature of airway remodeling that contributes significantly to airway wall thickness and the associated decreases in lung function. We found no evidence of HB-EGF/EGFR involvement in the pathogenesis of collagen deposition, although IL-17 did participate in this process. Heon Park's study (9) in IL-17-transgenic mice yielded parallel results, showing obvious alveolar wall thickening and aggregations of subepithelial collagen deposition in mice overexpressing IL-17 compared with control mice. The expression of TGF- $\beta$  and IL-6, which were both confirmed to be potential profibrotic cytokines and in combination can drive Th17 differentiation, are both elevated upon OVA-induced airway remodeling (5, 17, 47, 48). In turn, IL-17 can promote IL-6 expression in structural lung cells, ASM cells, and fibroblasts, which triggers a positive feedback loop (1–4, 45). In addition, IL-17 may contribute to lung collagen deposition through the stimulation of and synergy with various other profibrotic cytokines, such as IL-11 and TNF- $\alpha$  (1–5), to promote the local accumulation of fibroblasts, myofibroblasts, and smooth muscle cells (5, 47). The production of MMP-9 and tissue inhibitor of metalloproteinase-1, which may have roles in collagen deposition (22), can also be regulated by IL-17 (2, 9, 43). Moreover, IL-17 itself is probably involved in the proliferation of fibroblasts, with IL-17RA/IL-17RC, NF- $\kappa$ B, and Act-1 potentially acting downstream (1, 2, 49). However, we also observed that anti-IL-17 mAb could only partly block the development of prolonged OVA-induced subepithelial collagen deposition, suggesting that a complex network of cytokines and molecules might be involved in the course of deposition.

In conclusion, we have shown that Th17 cells promoted prolonged OVA induced-airway remodeling through the secretion of IL-17. Th17/IL-17 increased the expression of HB-EGF and could therefore act through EGFR to promote mucus secretion and ASM mass proliferation.

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## Disclosures

The authors have no financial conflicts of interest.

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