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Transcription Factors GATA-3 and ROR γ t Are Important for Determining the Phenotype of Allergic Airway Inflammation in a Murine Model of Asthma

Satoshi Ano,* Yuko Morishima,* Yukio Ishii,* Keigyou Yoh,[†] Yuichi Yageta,* Shigeo Ohtsuka,* Masashi Matsuyama,* Mio Kawaguchi,* Satoru Takahashi,[‡] and Nobuyuki Hizawa*

In refractory asthma, neutrophils, rather than eosinophils, often predominate in the airways. Neutrophilic airway inflammation appears to be resistant to steroids and may be related to the Th17, rather than the Th2, cytokine milieu. However, the role of GATA-3 and ROR γ t, transcription factors for Th2 and Th17 cell differentiation, respectively, in the pathogenesis of steroid-insensitive asthma remains unclear. To examine the effect of GATA-3- and ROR γ t-overexpression backgrounds on airway inflammation and steroid sensitivity, we generated two strains of transgenic mice overexpressing GATA-3 or ROR γ t. Mice were sensitized and challenged with OVA. Some OVA-sensitized/challenged mice were treated with dexamethasone, anti-IL-17 Ab, CXCR2 antagonist, or anti-IL-6R Ab to demonstrate their therapeutic effects on airway inflammation. Although Ag-specific airway inflammation and hyperresponsiveness were induced in each mouse, the phenotype of inflammation showed a distinct difference that was dependent upon the genotype. GATA-3-overexpressing mice exhibited steroid-sensitive eosinophilic inflammation with goblet cell hyperplasia and mucus hyperproduction under Th2-biased conditions, and ROR γ t-overexpressing mice developed steroid-insensitive neutrophilic inflammation under Th17-biased conditions. The levels of keratinocyte-derived chemokine, MIP-2, IL-6, and other neutrophil chemotaxis-related mediators were significantly elevated in OVA-exposed ROR γ t-overexpressing mice compared with wild-type mice. Interestingly, airway hyperresponsiveness accompanied by neutrophilic airway inflammation in ROR γ t-overexpressing mice was effectively suppressed by anti-IL-17 Ab, CXCR2 antagonist, or anti-IL-6R Ab administration. In conclusion, our results suggest that the expression levels of GATA-3 and ROR γ t may be important for determining the phenotype of asthmatic airway inflammation. Furthermore, blockade of the Th17-signaling pathway may be a treatment option for steroid-insensitive asthma. *The Journal of Immunology*, 2013, 190: 000–000.

Over the last two decades, the Th1/Th2 paradigm has offered important insights for investigating the pathogenesis of asthma. The current consensus on asthma is that the main underlying pathology is chronic airway inflammation that involves inflammatory cells, such as Th2 lymphocytes and eosinophils. Most asthma is mild or moderate and responds to conventional therapies, such as glucocorticosteroids, β_2 -agonists, leukotriene modifiers, and theophylline. However, 5–10% of asthma patients have severe symptoms that are unresponsive to these therapies (1). This subgroup of severe asthma is described as “refractory asthma,” requiring further research in the development

of novel therapeutic agents (2). Several studies suggest the involvement of eosinophils and neutrophils in refractory asthma (1–3). Recent studies also demonstrated that IL-17 is significantly increased in the airways in severe asthma compared with mild asthma (4). IL-17 is a proinflammatory cytokine mainly secreted from Th17 cells that is considered important for the induction of neutrophil influx into the airways by various mediators secreted from structural airway cells and inflammatory cells (5).

Th1, Th2, and Th17 are the major cell types derived from common T cell precursors in response to activation by a particular cytokine milieu. Transcription factors, namely T-bet, GATA-3, and ROR γ t, are crucial for naive CD4⁺ T cell differentiation into Th1, Th2, and Th17 cells, respectively. GATA-3, a member of the GATA family of zinc-finger transcription factors, promotes Th2 differentiation, suppresses Th1 differentiation, directly upregulates Th2 cytokine expression (6), and consequently enhances classical asthmatic responses. In contrast, T-bet, a member of the T-box family of transcription factors, promotes Th1 differentiation, suppresses Th2 differentiation, directly regulates IFN- γ induction, and attenuates asthmatic responses (7). A negative interaction has been reported between GATA-3 and T-bet. ROR γ t, a member of the nuclear receptor superfamily, was recently described as a master regulator for Th17 differentiation in the presence of TGF- β and IL-6 (8). Cytokines such as IL-1 β , IL-21, IL-23, and TNF- α also stimulate Th17 differentiation and activation (9). Th17 cells play critical roles in the induction of autoimmune diseases and host defense against pathogens by production of IL-17A, IL-17F, IL-21, and IL-22 (9). As described above, several recent studies indicated that

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; DEX, dexamethasone; GATA-3-tg, GATA-3-transgenic; KC, keratinocyte-derived chemokine; PAS, periodic acid-Schiff; ROR γ t-tg, ROR γ t-transgenic; WT, wild-type.

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refractory asthma may also be a Th17-type disease that cannot be explained by the classical Th1/Th2 theory. However, the precise role of ROR γ t in the pathogenesis of asthma remains unclear.

In the current study, we established an asthmatic model using transgenic mice overexpressing either GATA-3 or ROR γ t. Using these mice, we demonstrate that GATA-3 and ROR γ t are major factors that determine the phenotype of airway inflammation and steroid sensitivity in asthma. Moreover, we demonstrate a novel strategy for the treatment of severe and steroid-insensitive asthma by targeting ROR γ t/Th17-mediated inflammatory responses.

Materials and Methods

Mice

Wild-type (WT) C57BL/6 mice were purchased from Charles River (Yokohama, Japan). Transgenic mice overexpressing GATA-3 or ROR γ t under the control of the CD2 promoter were generated in our laboratory, as previously described (10, 11). All animal studies were approved by the Institutional Review Board.

Experimental protocols

To induce Ag-specific airway inflammation, 7–12-wk-old female mice were sensitized to 100 μ g OVA (Sigma-Aldrich, St. Louis, MO) s.c. on days 0 and 14 and then challenged with 10 μ g OVA intranasally on day 28. Nonimmunized mice received saline instead of OVA. In some mice, 2.5 mg/kg dexamethasone (DEX) was injected s.c. 4 h prior to challenge. Mice were sacrificed either 6 h after the last exposure to OVA or saline for the measurement of IL-4, IL-5, IL-6, IL-13, IL-17A, IL-17F, and IL-22 or 48 h after the last exposure for other measurements.

Real-time PCR analysis

The lungs were removed from each mouse genotype under unstimulated conditions, cut into small pieces, incubated in RPMI 1640 containing 10% FCS and 75 U/ml Type I Collagenase (Sigma-Aldrich) for 2 h at 37°C, and then filtered through a nylon mesh to remove large tissue fragments. After collection of lung interstitial cells, T cells were stained with allophycocyanin-anti-TCR- β Ab (Pharmingen, San Diego, CA) and isolated by FACS. Total RNA was then extracted, and the mRNA levels of T-bet, GATA-3, ROR γ t, and Foxp3 were quantified using real-time PCR. Primers sequences were as follows: T-bet, 5'-TTCCATTCCTGTCCTTCAC-3' and 5'-CCACATCCAAACATCCTG-3'; GATA-3, 5'-GGAACTCCGTCAGGGCTA-3' and 5'-AGAGATCCGTCAGCAGAG-3'; ROR γ t, 5'-TGAGGCCATTGAGTGTGG-3' and 5'-CTTCCATTGCTCCTGCTTTC-3'; Foxp3, 5'-CCTGCC-TTGGTACATTCGTG-3' and 5'-TGTTGTGGGTGAGTGTCTTG-3'; and GAPDH, 5'-TGTGTCCGTCGTGGATCTGA-3' and 5'-CCTGCTTACC-ACCTTCTTGAT-3'.

Histopathological assessment

Lung paraffin sections were stained with H&E to determine general morphology. Sections were also stained with periodic acid-Schiff (PAS) to demonstrate the presence of mucin within goblet cells. The number of goblet cells was determined as the percentage of PAS-positive cells of the total airway epithelial cells. To quantify subepithelial fibrosis, the sections were stained with Masson's trichrome technique. The thickness of the subepithelial layer was defined by measuring the distance between the basement membrane and the luminal border of the tracheal cartilage and smooth muscle layer using a computer-assisted imaging system (FLOVEL Filing System, Tokyo, Japan). The analyses were performed at four sites tangential to each airway cross-section by an investigator blinded to the genotypes examined.

Bronchoalveolar lavage

The lungs were lavaged with five repeat instillations of 1 ml saline via the trachea. Cells were counted using a hemocytometer, and a differential cell count was performed using standard light microscopic techniques based on staining with Diff-Quik (American Scientific Products, McGaw Park, IL).

Measurement of OVA-specific IgE

Serum was obtained from each group, and OVA-specific IgE levels were determined by ELISA, according to the manufacturer's instructions (DS Pharma Biomedical, Osaka, Japan).

Measurement of airway responsiveness

Mice were anesthetized and connected to a mechanical ventilator (Fine-Pointe; Buxco, Wilmington, NC). Aerosolized methacholine (Sigma-Aldrich) was administered at increasing doses (0–25 mg/ml), and the peak value of respiratory resistance for each dose was recorded.

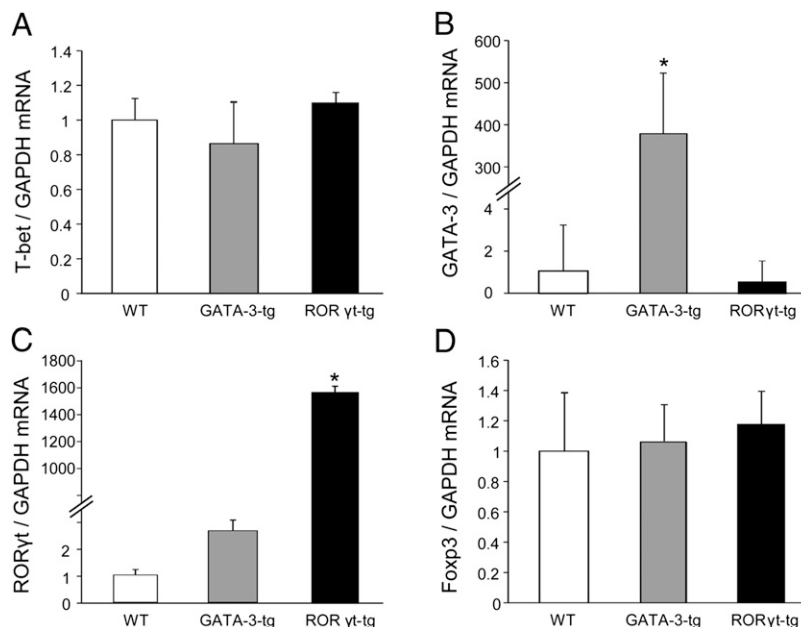
Analysis of inflammatory molecules in the lung

The lungs were removed and homogenized in PBS containing a mixture of protease inhibitors (Roche Diagnostics, Indianapolis, IN). Homogenates were then centrifuged at 90,000 $\times$ g for 30 min at 4°C. The supernatants were collected, and their protein concentrations were determined. The concentrations of GM-CSF, IL-1 α , IL-1 β , IL-4, IL-5, IL-6, IL-13, IL-17A, IL-17F, IL-22, keratinocyte-derived chemokine (KC), MIP-2, TGF- β , and TNF- α were determined by ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). The results were normalized to total protein content.

Neutralization studies

For neutralization studies, 7.5 mg/kg anti-IL-17 Ab (clone 50104; R&D Systems), 10 mg/kg CXCR2 antagonist SB225002 (Merck, Darmstadt, Germany), or 400 mg/kg anti-IL-6R Ab (Chugai Pharmaceutical, Shizuoka, Japan) was injected i.p. into mice 4 h prior to challenge. In some

FIGURE 1. GATA-3 and ROR γ t gene overexpression in the lungs of the corresponding transgenic mice. The mRNA expression levels of T-bet (A), GATA-3 (B), ROR γ t (C), and Foxp3 (D) in lung T cells from un-sensitized WT, GATA-3-tg, and ROR γ t-tg mice were quantified using real-time PCR. Relative quantitation of the gene transcripts was calculated by the $2^{-\Delta\Delta CT}$ method. Data are expressed as the mean $\pm$ SEM of five to six mice in each group. * p < 0.05 versus the other genotypes.



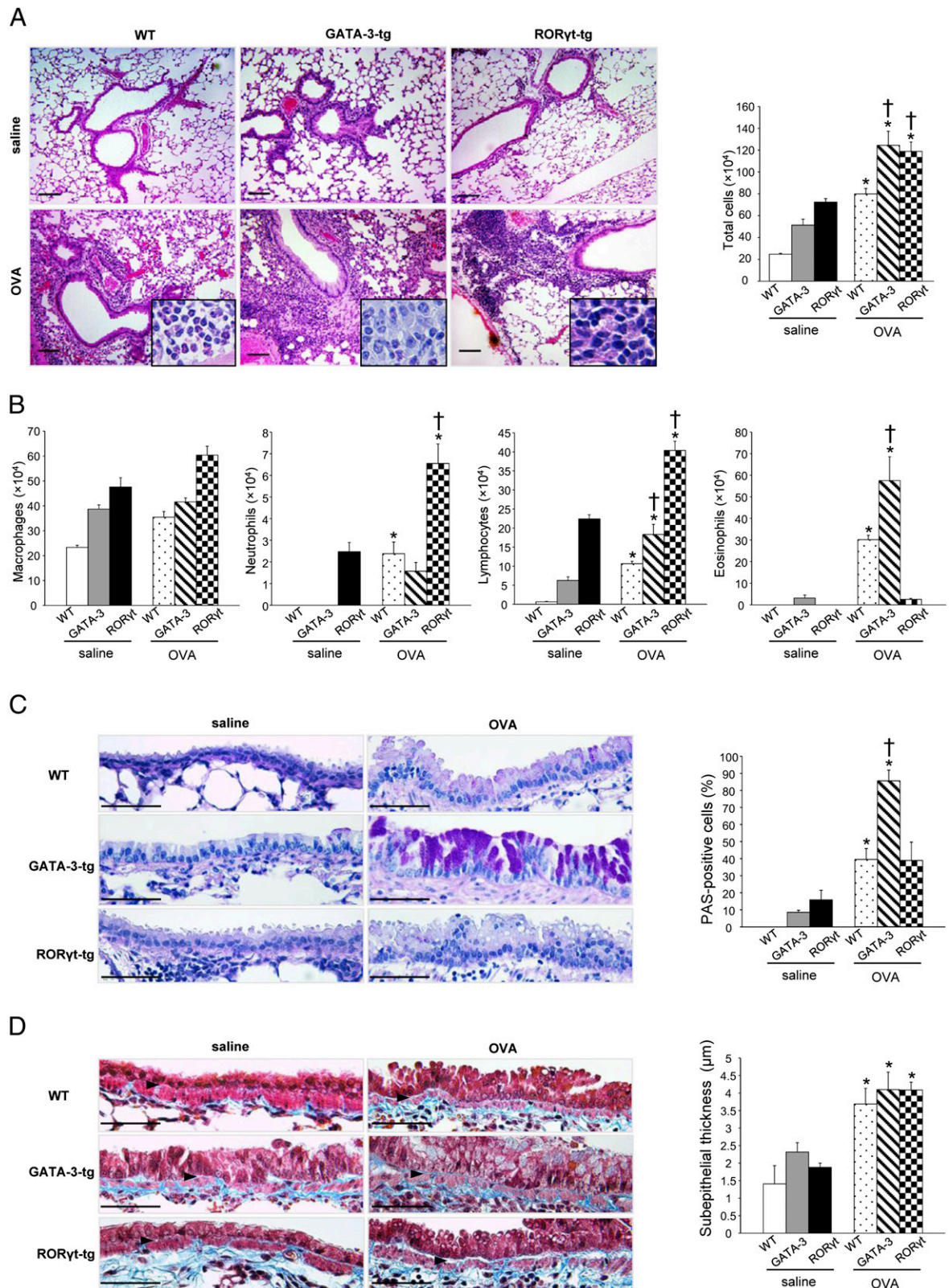


FIGURE 2. Development of eosinophilic airway inflammation and goblet cell hyperplasia is significantly enhanced in OVA-exposed *GATA-3*-tg mice, whereas neutrophilic airway inflammation is enhanced in OVA-exposed *RORγt*-tg mice. **(A)** H&E-stained lungs of WT, *GATA-3*-tg, and *RORγt*-tg mice 48 h after challenge with saline or OVA. Scale bars, 100 μm . *Insets* show infiltration of inflammatory cells at higher magnification. **(B)** Numbers of total cells, macrophages, neutrophils, lymphocytes, and eosinophils in the BALF of WT, *GATA-3*-tg, and *RORγt*-tg mice 48 h after challenge with saline or OVA. **(C)** Representative images of PAS-stained lungs of WT, *GATA-3*-tg, and *RORγt*-tg mice 48 h after challenge with saline or OVA. Scale bars, 50 μm . Corresponding quantitative results are expressed as the percentage of PAS-positive cells of the total airway epithelial cells (*right panel*). **(D)** Representative images of Masson's trichrome-stained lungs of WT, *GATA-3*-tg, and *RORγt*-tg mice 48 h after challenge with saline or OVA. Scale bars, 50 μm . Corresponding quantitative results are expressed as the thickness of the subepithelial layer (arrowheads). Data are the mean $\pm$ SEM of 5–13 mice in each group. * $p < 0.05$ versus corresponding saline-exposed mice, $^{\dagger}p < 0.05$ versus OVA-exposed WT mice.

mice, 100 mg/kg anti-IL-6R Ab was administered every week during OVA sensitization. The dosage of each compound was determined as previously described (12–14).

Statistical analysis

Data are presented as the mean $\pm$ SEM. Statistical analysis was conducted using StatMate III (ATMS, Tokyo, Japan). Analysis was performed by one-way ANOVA with post hoc Tukey analysis for multiple-group comparisons and an unpaired Student *t* test to compare between two groups. Airway responsiveness to methacholine was analyzed by repeated-measures ANOVA. Differences were considered statistically significant when *p* < 0.05.

Results

GATA-3 and ROR γ t genes are overexpressed in lung T cells of corresponding transgenic mice

We first confirmed the effect of genetic modulation of GATA-3 and ROR γ t on the expression levels of T-bet, GATA-3, ROR γ t, and Foxp3, master transcription factors for the differentiation of Th1, Th2, Th17, and regulatory T cells, respectively, in lung T cells under unstimulated conditions (Fig. 1). GATA-3 expression was

379-fold higher in the lung T cells of *GATA-3*-transgenic (*GATA-3*-tg) mice than in WT mice, whereas ROR γ t expression did not differ from that in WT mice. In addition, ROR γ t expression was 1557-fold higher in the lung T cells of *ROR γ t*-transgenic (*ROR γ t*-tg) mice than in WT mice, whereas GATA-3 expression did not differ between the two genotypes. Genetic modulation of GATA-3 and ROR γ t did not affect the expression of either T-bet or Foxp3 in either genotype.

Eosinophilic airway inflammation and goblet cell hyperplasia are enhanced in GATA-3-tg mice, and neutrophilic airway inflammation is enhanced in ROR γ t-tg mice

To clarify the effects of GATA-3 and ROR γ t overexpression on the development of OVA-specific airway inflammation, lung tissues were evaluated by light microscopy 48 h after the last exposure to OVA or saline (Fig. 2A). Inflammatory cell infiltration in the peribronchial and perivascular regions was remarkable in all genotypes of OVA-exposed mice compared with that in saline-exposed controls, and the degree of inflammatory cell infiltration was higher in *GATA-3*-tg and *ROR γ t*-tg mice than in WT

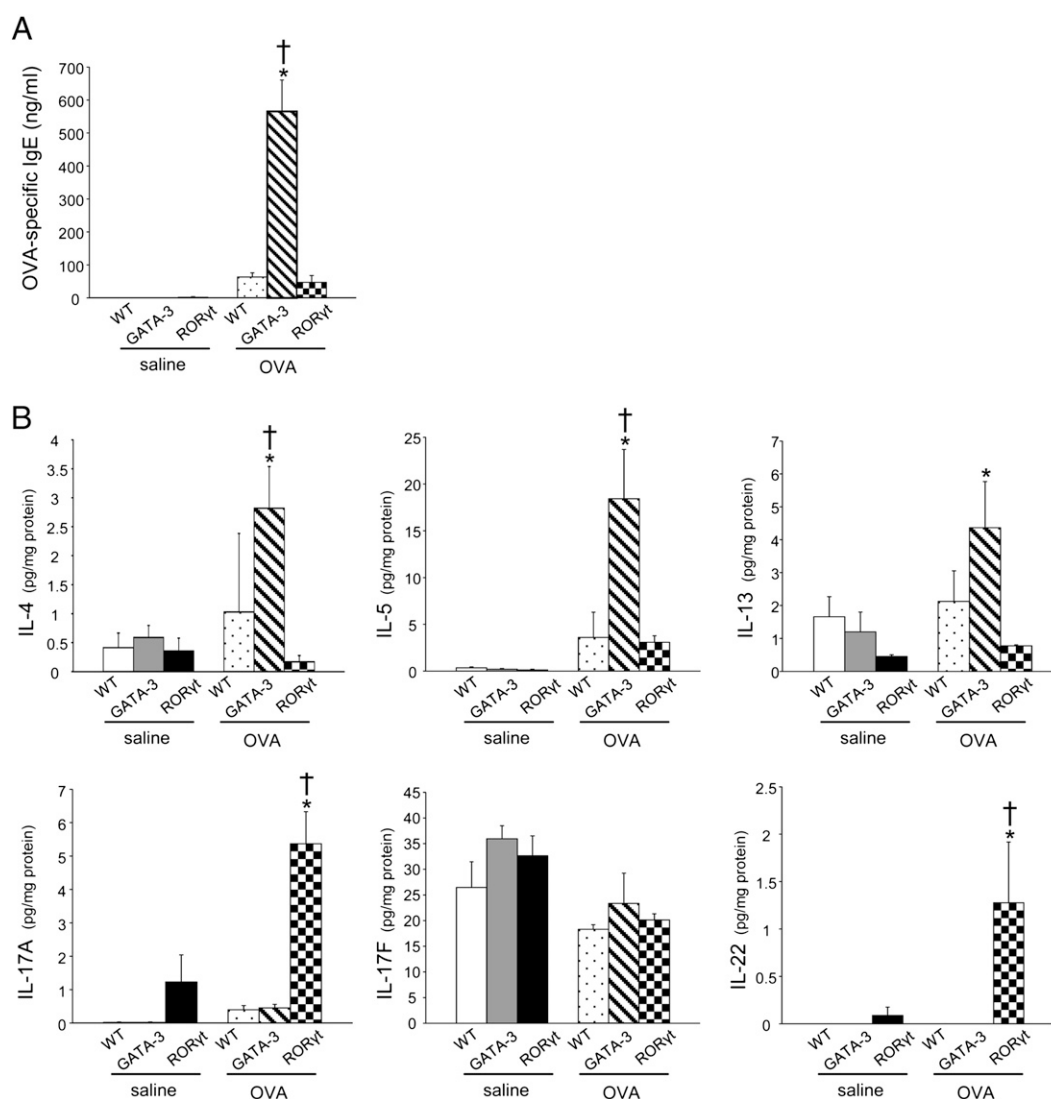


FIGURE 3. OVA sensitization/challenge enhances allergen-induced IgE and Th2 cytokine production in *GATA-3*-tg mice and allergen-induced Th17 cytokine production in *ROR γ t*-tg mice. **(A)** OVA-specific IgE concentrations in the serum of WT, *GATA-3*-tg, and *ROR γ t*-tg mice 48 h after challenge with saline or OVA. **(B)** Concentrations of IL-4, IL-5, IL-13, IL-17A, IL-17F, and IL-22 in lung homogenates obtained from WT, *GATA-3*-tg, and *ROR γ t*-tg mice after challenge with saline or OVA. Results are the mean $\pm$ SEM of five to eight mice in each group. **p* < 0.05 versus corresponding saline-exposed mice, $\dagger$ *p* < 0.05 versus OVA-exposed WT mice.

mice. The numbers of total cells, macrophages, eosinophils, neutrophils, and lymphocytes in the bronchoalveolar lavage fluid (BALF) were also determined at the same time. As shown in Fig. 2B, following OVA exposure the number of total cells and lymphocytes was increased in all genotypes of mice. The number of total cells and lymphocytes in OVA-exposed *RORγt*-tg and *GATA-3*-tg mice was significantly higher than in OVA-exposed WT mice. OVA exposure significantly increased the numbers of neutrophils in WT and *RORγt*-tg mice but not in *GATA-3*-tg mice. Neutrophil counts in OVA-exposed *RORγt*-tg mice were significantly higher than in OVA-exposed WT mice. In contrast, the numbers of eosinophils were significantly elevated after OVA exposure in WT and *GATA-3*-tg mice but not in *RORγt*-tg mice. Eosinophil counts in OVA-exposed *GATA-3*-tg mice were significantly higher than in OVA-exposed WT mice.

We next evaluated the presence of mucin within goblet cells and the degree of airway fibrosis beneath the epithelial basement membrane. OVA exposure resulted in goblet cell hyperplasia and mucus hyperproduction in the airways of WT and *GATA-3*-tg mice but not in *RORγt*-tg mice (Fig. 2C). Epithelial cells that were positive for PAS staining in OVA-exposed *GATA-3*-tg mice were significantly increased compared with OVA-exposed WT mice. Although the subepithelial deposition of extracellular matrix in-

creased after OVA exposure in all genotypes of mice, there was no significant difference among the mouse genotypes (Fig. 2D).

These results suggest that the development of eosinophilic airway inflammation is significantly enhanced in mice overexpressing *GATA-3*, whereas neutrophilic airway inflammation is enhanced in mice overexpressing *RORγt* after exposure to the same Ag. In addition, overexpression of *GATA-3* promoted the development of goblet cell hyperplasia and mucus hyperproduction, whereas overexpression of *RORγt* did not affect these processes.

OVA-specific IgE and Th2/Th17 cytokine levels are regulated by overexpression of GATA-3 and RORγt

Because the asthmatic response is traditionally considered to be mediated by IgE and Th2 cytokines, we evaluated the serum levels of OVA-specific IgE in each mouse genotype (Fig. 3A). OVA-specific IgE was not detected in serum samples from saline-exposed control mice. However, the level of OVA-specific IgE became significantly high in OVA-exposed *GATA-3*-tg mice compared with other genotypes.

We then examined the expression levels of IL-4, IL-5, IL-13, IL-17A, IL-17F, and IL-22 to analyze the Th2 and Th17 cell balance in the lungs of each mouse genotype (Fig. 3B). As expected, fol-

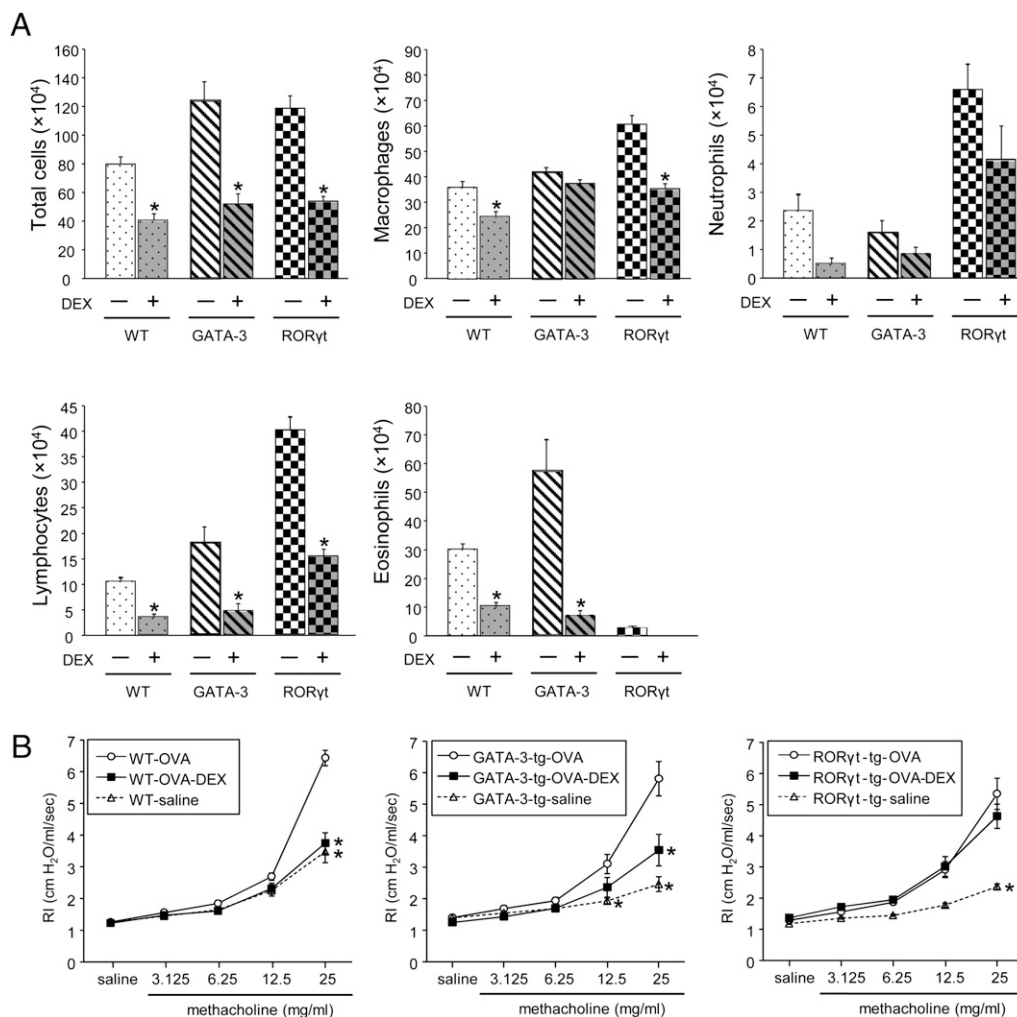


FIGURE 4. DEX treatment significantly reduces airway inflammation and AHR in OVA-exposed WT and *GATA-3*-tg mice but not in *RORγt*-tg mice. (A) Numbers of total cells, macrophages, neutrophils, lymphocytes, and eosinophils in the BALF of WT, *GATA-3*-tg, and *RORγt*-tg mice 48 h after the final exposure to OVA, with or without DEX treatment. (B) AHR to methacholine in WT, *GATA-3*-tg, and *RORγt*-tg mice 48 h after the final exposure to saline or OVA, with or without DEX treatment. Data are the mean $\pm$ SEM of six mice. * $p < 0.05$ versus corresponding OVA-exposed mice without DEX treatment.

lowing OVA exposure the expression levels of IL-4, IL-5, and IL-13 in *GATA-3*-tg mice, as well as IL-17A and IL-22 in *ROR γ t*-tg mice, were enhanced in lung homogenates. The concentrations of IL-4 and IL-5 were significantly higher in OVA-exposed *GATA-3*-tg mice compared with OVA-exposed WT mice, whereas IL-17A and IL-22 were significantly higher in OVA-exposed *ROR γ t*-tg mice compared with OVA-exposed WT mice. Interestingly, the expression level of IL-17F was not different among saline- and OVA-exposed mice of all genotypes.

These findings indicate that the immune responses and cytokine production deviated to Th2 in *GATA-3*-tg mice, whereas those in *ROR γ t*-tg mice deviated to Th17.

Effects of steroids on airway inflammation are regulated by overexpression of GATA-3 and ROR γ t

Steroids are considered one the most effective treatments for asthma. Therefore, we assessed the effects of DEX on airway inflammation in each OVA-exposed mouse genotype. As shown in Fig. 4A, our findings clearly demonstrated that the numbers of total cells and lymphocytes were significantly decreased in all mouse genotypes after DEX treatment. The numbers of eosinophils, which were increased in WT and *GATA-3*-tg mice after OVA exposure, were significantly decreased after DEX treatment. However, the number of neutrophils, which was elevated in WT and *ROR γ t*-tg mice after OVA exposure, was not suppressed by DEX treatment.

We then assessed the effects of DEX on airway hyperresponsiveness (AHR) in each OVA-exposed mouse genotype. To evaluate AHR, airway resistance in response to increasing doses of aerosolized methacholine was measured 48 h after OVA exposure (Fig. 4B). In each mouse genotype, airway resistance to methacholine was significantly increased after OVA exposure compared with that in saline-exposed controls. DEX treatment significantly reduced airway resistance to methacholine in OVA-exposed WT and *GATA-3*-tg mice but not in *ROR γ t*-tg mice. These results indicated that eosinophilic airway inflammation and AHR, which occurred in WT and *GATA-3*-tg mice under Th2-biased conditions, could be suppressed by steroid administration. In contrast, neutrophilic airway inflammation and AHR, which occurred in *ROR γ t*-tg mice under Th17-biased conditions, was resistant to steroids.

Th17-related cytokines and chemokines are overexpressed in OVA-exposed ROR γ t-tg mice

IL-17 stimulates the production of various inflammatory mediators, such as GM-CSF, IL-1, IL-6, KC, MIP-2, TGF- β , and TNF- α , from various types of inflammatory cells and airway structure cells, which can directly or indirectly lead to the recruitment and activation of neutrophils (15). Therefore, we examined the expression levels of these mediators in the lungs of each mouse genotype to determine the involvement of Th17-related cytokines and chemokines in neutrophilic inflammation. As shown in Fig. 5, OVA exposure significantly increased the concentrations of GM-CSF, IL-1 α , IL-1 β , IL-6, KC, MIP-2, and TGF- β in the lung homogenates of *ROR γ t*-tg mice. Furthermore, after OVA exposure, the concentrations of IL-1 β , IL-6, KC, MIP-2, TGF- β , and TNF- α were significantly higher in *ROR γ t*-tg mice than in WT mice.

Treatment with anti-IL-17 Ab, CXCR2 antagonist, or anti-IL-6R Ab attenuates neutrophilic airway inflammation and AHR in ROR γ t-tg mice

To clarify the role of IL-17 in neutrophilic airway inflammation in *ROR γ t*-overexpressing mice, we evaluated the effects of anti-IL-17 Ab on OVA-induced airway responses in *ROR γ t*-tg mice. Treat-

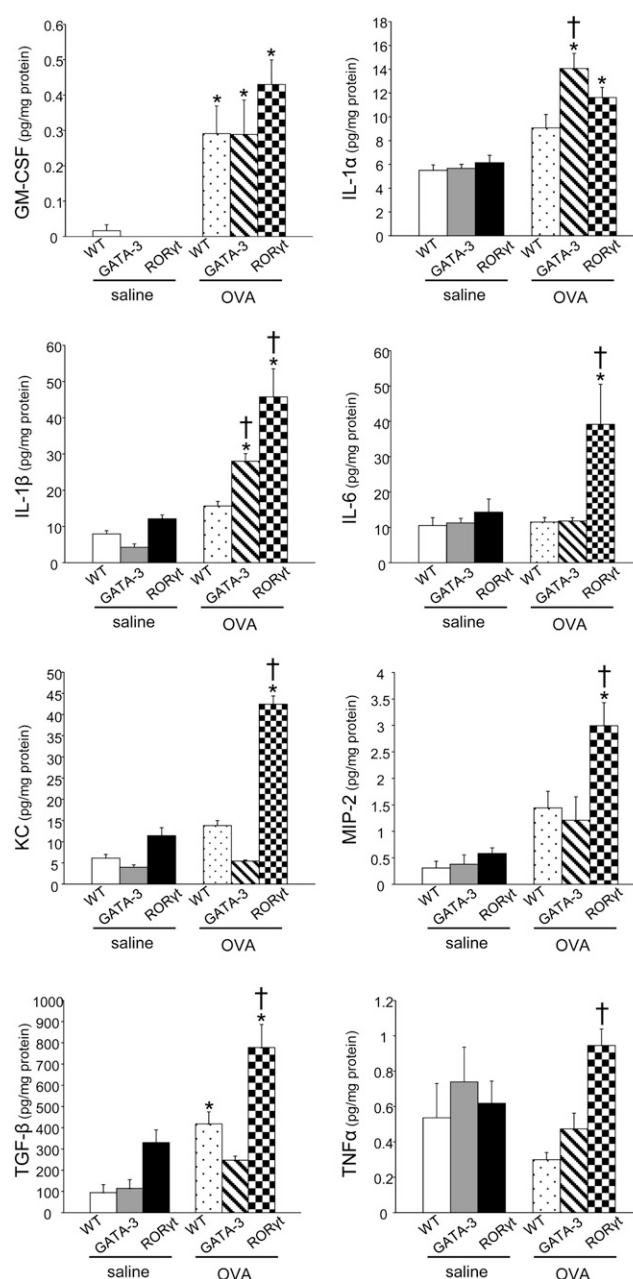


FIGURE 5. The levels of KC, MIP-2, IL-6, and other mediators related to neutrophil chemotaxis were elevated in the lungs of OVA-exposed *ROR γ t*-overexpressing mice. Concentrations of GM-CSF, IL-1 α , IL-1 β , IL-6, KC, MIP-2, TGF- β , and TNF- α in lung homogenates obtained from WT, *GATA-3*-tg, and *ROR γ t*-tg mice after challenge with saline or OVA. Results are the mean $\pm$ SEM of five to eight mice in each group. * p < 0.05 versus corresponding saline-exposed mice, $\dagger p$ < 0.05 versus OVA-exposed WT mice.

ment with anti-IL-17 Ab significantly decreased peribronchial inflammatory cell infiltration, neutrophil counts in the BALF, and airway resistance to methacholine in OVA-exposed *ROR γ t*-tg mice (Fig. 6A–C). Moreover, the expressions of KC and MIP-2, which had not decreased as a result of DEX treatment, were also suppressed by treatment with anti-IL-17 Ab in OVA-exposed *ROR γ t*-tg mice (Fig. 6D). Because CXCR2 is a chemokine receptor expressed by neutrophils that binds with high affinity to CXC chemokines, such as KC and MIP-2, which are potent mediators of neutrophil chemotaxis, we evaluated the effects of a CXCR2 antagonist on OVA-induced airway responses in *ROR γ t*-tg mice.

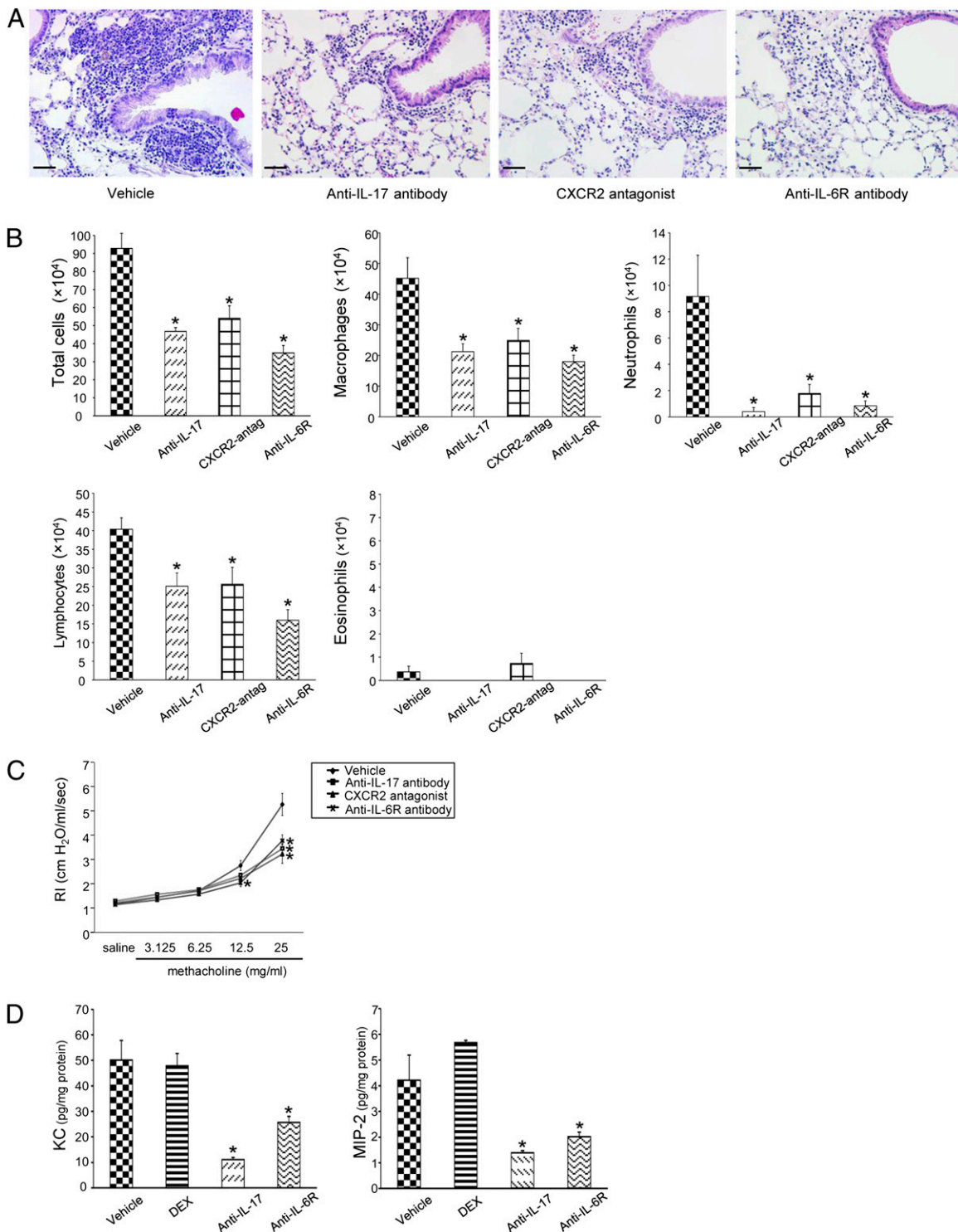


FIGURE 6. Treatment with anti-IL-17 Ab, CXCR2 antagonist, or anti-IL-6R Ab at the Ag-challenge phase reduces neutrophilic airway inflammation and AHR in *RORγt*-tg mice. **(A)** H&E-stained lungs of OVA-exposed *RORγt*-tg mice treated with anti-IL-17 Ab, CXCR2 antagonist, or anti-IL-6R Ab 4 h before OVA challenge. Scale bars, 50 μm. **(B)** Numbers of total cells, macrophages, neutrophils, lymphocytes, and eosinophils in the BALF of OVA-exposed *RORγt*-tg mice treated with anti-IL-17 Ab (Anti-IL-17), CXCR2 antagonist (CXCR2-antag), or anti-IL-6R Ab (Anti-IL-6R) 4 h before OVA challenge. **(C)** Airway responsiveness to methacholine in OVA-exposed *RORγt*-tg mice treated with anti-IL-17 Ab, CXCR2 antagonist, or anti-IL-6R Ab 4 h before OVA challenge. **(D)** Concentrations of KC and MIP-2 in the lung homogenates of OVA-exposed *RORγt*-tg mice treated with DEX, anti-IL-17 Ab, or anti-IL-6R Ab 4 h before OVA challenge. Results are the mean $\pm$ SEM from five to seven mice in each group. * $p < 0.05$ versus OVA-exposed *RORγt*-tg mice treated with vehicle.

Treatment with a CXCR2 antagonist significantly decreased peribronchial inflammatory cell infiltration, neutrophil counts in the BALF, and airway resistance to methacholine in OVA-exposed *RORγt*-tg mice (Fig. 6A–C). We next examined the inhibitory

effects of IL-6R Ab on OVA-induced airway responses in *RORγt*-tg mice, because IL-6 is an important cytokine for the induction of Th17 differentiation, as well as a downstream target of IL-17. Treatment with 400 mg/kg anti-IL-6R Ab 4 h before OVA chal-

challenge significantly decreased peribronchial inflammatory cell infiltration, neutrophil counts in the BALF, and airway resistance to methacholine in OVA-exposed ROR γ t-g mice (Fig. 6A–C). The expression of KC and MIP-2 was also suppressed by treatment with anti-IL-6R Ab during the challenge phase in OVA-exposed ROR γ t-g mice (Fig. 6D). Treatment with the smaller amount (100 mg/kg) of anti-IL-6R Ab before OVA challenge did not suppress either neutrophilic inflammation or airway resistance in OVA-exposed ROR γ t-g mice (data not shown).

To examine the effects of IL-6 during the Ag-sensitization phase, we further administered 100 mg/kg of anti-IL-6R Ab every week during OVA sensitization. Treatment with anti-IL-6R Ab during the sensitization significantly decreased peribronchial inflammatory cell infiltration, neutrophil counts in the BALF, and airway resistance to methacholine in OVA-exposed ROR γ t-g mice (Fig. 7A–C). The expressions of KC and MIP-2 were also suppressed by treatment with anti-IL-6R Ab in these mice (Fig. 7D).

Discussion

Asthma has been widely accepted as an eosinophilic airway disease that can be explained by imbalances in Th1/Th2 cytokine expression. According to this concept, at the transcription factor level, the upregulation of GATA-3 (16–18) and downregulation of T-bet (18, 19) were clinically demonstrated in T cells from the airways and peripheral blood of asthmatics. These transcription factors also have a crucial role in the development of airway remodeling by

mediating Th1/Th2 cell differentiation (20). Consistent with previous findings, our observations revealed that OVA sensitization/challenge induced enhanced Th2-type responses, such as elevation of serum OVA-specific IgE levels; increased lung expression levels of IL-4, IL-5, and IL-13; eosinophilic airway inflammation; and the development of goblet cell hyperplasia and mucus hyperproduction under GATA-3-overexpressed conditions.

Interestingly, neutrophils predominantly infiltrated the airways of ROR γ t-overexpressing mice, with enhanced lung expression of IL-17A and IL-22 after exposure to the same Ag. IL-17 is increased in the airways of persistent asthma sufferers (4, 21) and in asthma animal models (22–24), and the increased levels of IL-17 in the airways were accompanied by neutrophilic infiltration. Although IL-17 can be produced by numerous cell types, we propose that Th17 cells are the main source of IL-17A in our ROR γ t-overexpressing asthmatic mouse model. In contrast, the expression levels of IL-17F were not different among mice of all genotypes. A possible explanation may be that Th17 cells are not the main source of IL-17F and, therefore, the expression of IL-17F was not enhanced in the ROR γ t-g mice, in which only ROR γ t is overexpressed under the control of the CD2 promoter. The precise role of ROR γ t in asthma has not been fully clarified, but it was reported that expression of ROR γ t or RORC, which encodes ROR γ t, is increased in the PBMCs of asthmatics (18, 25). Therefore, it appears that an increased level of ROR γ t may be implicated in the mechanism underlying severe asthma.

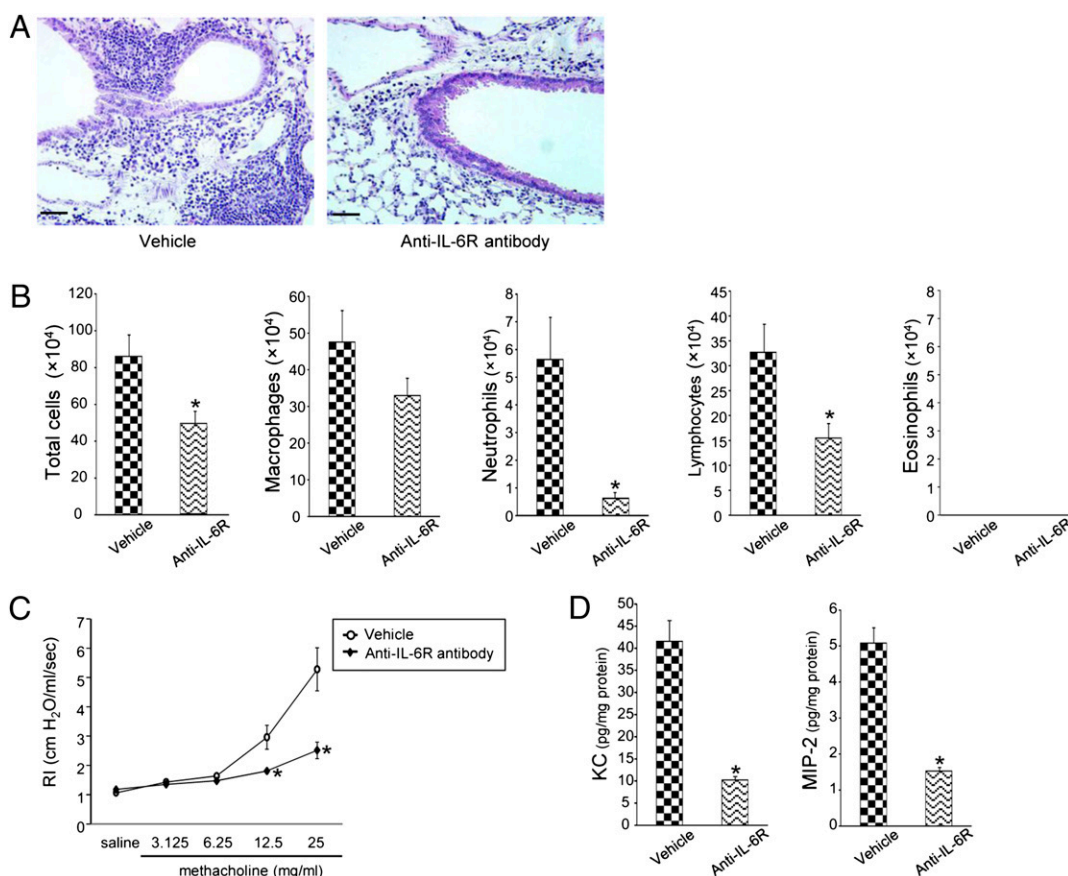


FIGURE 7. Treatment with anti-IL-6R Ab at the Ag-sensitization phase reduces neutrophilic airway inflammation and AHR in ROR γ t-g mice. **(A)** H&E-stained lungs of OVA-exposed ROR γ t-g mice treated or not with anti-IL-6R Ab every week during OVA sensitization. Scale bars, 50 μ m. **(B)** Numbers of total cells, macrophages, neutrophils, lymphocytes, and eosinophils in the BALF of OVA-exposed ROR γ t-g mice treated or not with anti-IL-6R Ab (Anti-IL-6R) every week during OVA sensitization. **(C)** Airway responsiveness to methacholine in OVA-exposed ROR γ t-g mice treated or not with anti-IL-6R Ab every week during OVA sensitization. **(D)** Concentrations of KC and MIP-2 in the lung homogenates of OVA-exposed ROR γ t-g mice treated or not with anti-IL-6R Ab every week during OVA sensitization. Results are mean $\pm$ SEM from 5–10 mice in each group. * p < 0.05 versus OVA-exposed ROR γ t-g mice treated with vehicle.

The present study also revealed that the lung expression levels of KC, MIP-2, and other mediators related to neutrophil chemotaxis were elevated in *RORγt*-tg mice after Ag exposure. MIP-2 is a mouse homolog of human IL-8 (CXCL8), and CXCL8 expression was shown to be elevated and correlated with IL-17A levels and neutrophil counts in the sputum of asthmatic patients (21), indicating the involvement of CXC chemokines in the pathogenesis of neutrophilic airway inflammation in asthma. Previous studies suggested that IL-17 induces CXCL8 secretion from synoviocytes (26), as well as enhances IL-1β-induced CXCL8 secretion in lung microvascular endothelial cells (27). Therefore, the upregulation of CXC chemokines in *RORγt*-tg mice may occur in a Th17 cell-dependent manner. Taken together, *RORγt*- and IL-17-mediated CXC chemokine secretion may be a major molecular mechanism of enhanced neutrophilic airway inflammation induced by Ag under Th17-biased conditions.

We found that the neutrophilic airway inflammation observed in *RORγt*-tg mice was steroid insensitive. Clinically, asthmatics with neutrophilic airway inflammation respond poorly to steroid therapy (28, 29). Steroids have the capacity to induce apoptosis in eosinophils, but they increase neutrophil release from the bone marrow, reduce egress of neutrophils from the circulating pool into the marginating pool, and prevent apoptosis in neutrophils (30, 31). In the current study, it is interesting to note that AHR observed in *GATA-3*-tg mice was attenuated by DEX treatment, whereas AHR in *RORγt*-tg mice was steroid insensitive. Similarly, McKinley et al. (32) demonstrated that Ag-induced neutrophilic inflammation and AHR observed in Th17 cell-reconstituted SCID mice were also steroid resistant. Neutrophil elastase was shown to induce AHR in guinea pigs (33), and depletion of neutrophils inhibited ozone-induced AHR (34). These findings suggest that the difference in steroid sensitivity of AHR between *GATA-3*-tg and *RORγt*-tg mice is due to differences in inflammatory cell profiles between these two genotypes. Parallel with the resistance of airway neutrophilia to steroids, lung expression of neutrophil chemoattractant KC and MIP-2 was not attenuated by DEX treatment in *RORγt*-tg mice.

Finally, we demonstrated that neutrophilic airway inflammation, AHR, and enhanced expression of CXC chemokines in OVA-exposed *RORγt*-tg mice were clearly suppressed by treatment with anti-IL-17 Ab, CXCR2 antagonist, or anti-IL-6R Ab. It is interesting to note that *RORγt* overexpression-triggered airway responses were attenuated by inhibiting not only IL-17 or CXC chemokines but also IL-6. Recent studies showed that IL-6 is important for promoting Th17 differentiation and orchestrating downstream Th17 immune responses to cause inflammatory and autoimmune disorders, such as rheumatoid arthritis and multiple sclerosis (35–37). The role of Th17 in the pathogenesis of autoimmune diseases is currently being studied, and inhibition of the Th17 pathway by IL-6 blockade was recently proposed as a treatment option (38). In the current study, the expression level of IL-6 was significantly upregulated after OVA sensitization/challenge in *RORγt*-tg mice, suggesting that IL-6, together with IL-17, may affect the development of neutrophilic airway inflammation by augmenting the production of neutrophil chemokines. Moreover, it is worth mentioning that OVA-induced airway responses in *RORγt*-tg mice were attenuated by blocking IL-6 signaling at the challenge phase, as well as at the sensitization phase. Because IL-17 promotes autoimmunity by triggering a positive-feedback loop via IL-6 induction (37), we propose that *RORγt*-regulated IL-17-biased immune conditions may accelerate IL-6 production; in turn, an excessive amount of IL-6 may amplify upstream of Th17 immune responses to promote Th17-driven neutrophilic airway inflammation. Indeed, the expression of IL-6 and soluble IL-6R is increased in the serum and airways of

asthmatic patients (39–41). Therefore, in addition to IL-17 and CXC chemokines, IL-6 may be a target for the control of Th17-driven steroid-insensitive inflammation.

In conclusion, the current study demonstrates that the phenotype of asthmatic airway inflammation is determined by T cell-specific transcription factors. GATA-3 induces steroid-sensitive eosinophilic airway inflammation by enhancing Th2 cell differentiation and Th2 cytokine production, whereas *RORγt* induces steroid-insensitive neutrophilic airway inflammation by enhancing Th17 cell differentiation and Th17 cytokine production. Clinically, the former condition may correspond to mild to moderate asthma, whereas the latter corresponds to treatment-resistant severe asthma. Because human asthma is a heterogeneous disease, we believe that our animal model may provide an important first step to clarify its intricate pathogenesis. Furthermore, our results demonstrate that treatment with anti-IL-17 Ab, CXCR2 antagonist, and anti-IL-6R Ab inhibits *RORγt*-induced neutrophilic airway inflammation and AHR. Thus, blockade of the Th17-signaling pathway may be a novel strategy for the treatment of asthmatic patients, particularly for those who are resistant to steroid treatment.

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Disclosures

The authors have no financial conflicts of interest.

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