

IL-23 and Th17 Cells Enhance Th2-Cell-mediated Eosinophilic Airway Inflammation in Mice

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Rationale: The IL-23–IL-17A–producing CD4⁺ T-cell (Th17 cell) axis plays an important role in the development of chronic inflammatory diseases, including autoimmune diseases. However, the role of the IL-23–Th17 cell axis in the regulation of allergic airway inflammation is still largely unknown.

Objectives: To determine the role of IL-23 and Th17 cells in allergic airway inflammation.

Methods: We examined the effect of anti-IL-23 antibody on antigen-induced airway inflammation. We also investigated the effect of enforced expression of IL-23 on allergic airway inflammation by generating lung-specific IL-23 transgenic mice. Moreover, we examined the effect of adoptive transfer of antigen-specific Th17 cells on allergic airway inflammation.

Measurements and Main Results: IL-23 mRNA was expressed in the lung of sensitized mice upon antigen inhalation, and the neutralization of IL-23 decreased antigen-induced eosinophil recruitment and Th2 cytokine production in the airways. The enforced expression of IL-23 in the airways significantly enhanced antigen-induced eosinophil and neutrophil recruitment into the airways; Th2 cytokine, IL-17A, and tumor necrosis factor (TNF)- α production in the airways; goblet cell hyperplasia; and airway hyperresponsiveness. Moreover, IL-23-mediated enhancement of antigen-induced Th2 cytokine production and eosinophil recruitment in the airways was still observed in the mice lacking IL-17A. Furthermore, although adoptive transfer of antigen-specific Th17 cells alone induced neutrophil but not eosinophil recruitment into the airways upon antigen inhalation, cotransfer of Th17 cells with Th2 cells significantly enhanced antigen-induced Th2-cell-mediated eosinophil recruitment into the airways and airway hyperresponsiveness.

Conclusions: IL-23 and Th17 cells not only induce Th17-cell-mediated neutrophilic airway inflammation but also up-regulate Th2-cell-mediated eosinophilic airway inflammation.

Keywords: allergy; cytokines; eosinophils; transgenic/knockout mice

Asthma is a chronic airway inflammation that is characterized by intense eosinophil infiltrates, mucus hypersecretion, airway remodeling, and airway hyperresponsiveness (AHR) (1–4). These pathognomonic features are mediated mainly by anti-

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Th17 cells that produce proinflammatory cytokines have previously been linked to autoimmune inflammation, but their role in Th2-cell-dependent eosinophilic airway inflammation is uncertain.

What This Study Adds to the Field

The IL-23–Th17 cell axis enhances Th2-cell-mediated eosinophilic inflammation.

gen-specific Th2 cells and their cytokines such as IL-4, IL-5, and IL-13 (1–4). On the other hand, it has been reported that increased numbers of neutrophils in the airways are observed in severe asthma (5, 6). Furthermore, it has been shown that IL-17A is expressed in the airways of patients with asthma (7) and that its expression is correlated with the severity of asthma (8). In addition, in a mouse model of asthma, IL-17A and IL-17F, another IL-17 family cytokine, have been shown to induce neutrophilic airway inflammation (9, 10). These findings indicate the importance of IL-17A and IL-17F in the induction of neutrophilic airway inflammation.

Importantly, recent studies using murine asthma models have shown that IL-17 receptor-deficient (IL-17R^{−/−}) mice exhibit the reduced recruitment not only of neutrophils but also of eosinophils into the airways (11), resulting from the impaired priming of Th2 responses in IL-17R^{−/−} mice (11). It has also been demonstrated that IL-17A^{−/−} mice exhibit reduced Th2 responses to antigen sensitization (12). The reduced Th2 responses in IL-17A^{−/−} or IL-17R^{−/−} mice may be explained by the recently described role of IL-17A in germinal center reactions (13). On the other hand, it has been demonstrated that the neutralization of IL-17A at the effector phase of airway inflammation enhances eosinophilic inflammation (9, 11). Therefore, IL-17A seems to play a dual role in the regulation of eosinophilic airway inflammation; IL-17A promotes eosinophilic airway inflammation by mounting allergen-specific Th2 cell responses at the sensitization phase, but IL-17A inhibits eosinophilic airway inflammation by suppressing the local allergic response at the effector phase.

Over the past 5 years, much progress has been made in understanding IL-17A–producing CD4⁺ T cells (Th17 cells) (14–17). Th17 cells are a distinct population of CD4⁺ T cells that secrete IL-17A, IL-17F, IL-22, and tumor necrosis factor (TNF)- α (16, 17) and are implicated in the pathogenesis of a variety of inflammatory conditions including experimental

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autoimmune encephalomyelitis, collagen-induced arthritis, and psoriasis (18–20). IL-23, a heterodimeric cytokine that consists of a p19 subunit specific for IL-23 and the p40 subunit of IL-12 (21), has been shown to play a significant role in the maintenance of Th17 cells (15–17). Moreover, McGeachy and colleagues have recently shown that IL-23 is required for full acquisition of pathogenic function of effector Th17 cells, whereas the generation of Th17 cells with transforming growth factor (TGF)- β plus IL-6, in the absence of IL-23, completely abrogated their pathogenic function despite up-regulation of IL-17A production (22). These findings indicate the importance of IL-23–Th17 cell axis in inflammatory responses. However, it is still largely unknown whether IL-23–Th17 cell axis is involved in the regulation of allergic airway inflammation in which Th2 cells play critical roles.

In this study, we examined whether the IL-23–Th17 cell axis was involved in inducing antigen-induced allergic airway inflammation using a mouse model of asthma. We found that IL-23 mRNA was expressed in the lung of sensitized mice upon antigen inhalation and that neutralization of IL-23 decreased antigen-induced eosinophil recruitment into the airways. In accordance with these observations, the enforced expression of IL-23 in the airways significantly enhanced antigen-induced eosinophil recruitment and Th2 cytokine production in the airways. Moreover, adoptive transfer of antigen-specific Th17 cells significantly enhanced antigen-induced, Th2-cell-mediated eosinophil recruitment into the airways. Our results indicate that IL-23 and Th17 cells play an important role in enhancing antigen-induced, Th2-cell-mediated eosinophilic airway inflammation.

METHODS

Mice

The cDNA fragment of murine IL-23 in which IL-23/IL-12 p40 is flexibly linked to IL-23 p19 (23) was cloned into the NdeI/BglII site of transgenic construct pCC10-SV40 (a kind gift from Dr. R. Flavell, Yale University School of Medicine) (24) to generate pCC10 IL-23 SV40. Transgenic mice (CC10 IL-23 mice) were generated by standard procedures with pCC10 IL-23 SV40. All mice were housed in micro-isolator cages under pathogen-free conditions, and all experiments were performed according to the guidelines of Chiba University. See the online supplement for further details about animals and treatments.

Cytokine Assay

The amounts of IL-5, IL-13, IL-17A, IL-23, and IFN- γ in the bronchoalveolar lavage fluid (BALF) and in the culture supernatants were determined by enzyme immunoassay according to the manufacturer's instruction (see online supplement for details).

Real-Time Polymerase Chain Reaction Analysis

The expression of IL-23 p19, IL-12 p35, and IL-23/IL-12 p40 mRNA was determined by real-time quantitative Taqman polymerase chain reaction (PCR) with a standard protocol on an ABI PRISM 7300 instrument (Applied Biosystems, Foster City, CA). The expression of eotaxin-1, eotaxin-2, and RANTES (regulated upon activation, normal T-cell expressed and secreted) mRNA was determined by real-time quantitative PCR using a SYBR green reagent (Power SYBR Green PCR Master Mix; Applied Biosystems). See the online supplement for further details.

Measurement of Airway Responsiveness

Mice were challenged with inhaled ovalbumin (OVA) three times at a 48-hour interval. Twenty-four hours after the last inhaled OVA challenge, airway responsiveness to aerosolized acetylcholine was

assessed by a computer-controlled small-animal ventilator system (flexiVent; SCIREQ, Inc., Montreal, Canada) as described elsewhere (25).

Cytokine Expression of Lung-infiltrating CD4⁺ T Cells

Cytokine profiles of lung-infiltrating CD4⁺ T cells in CC10 IL-23 mice and littermate wild-type (WT) mice were evaluated by intracellular cytokine staining. See online supplement for further detail.

Generation of OVA-specific Th2 Cells and Th17 Cells

OVA-specific Th2 cells were generated from splenocytes of DO11.10 T-cell receptor (TCR) transgenic (DO11.10⁺) mice (26) as described previously with minor alterations (27). OVA-specific Th17 cells were generated from splenocytes of DO11.10 mice, as described previously but with minor alterations (28). See the online supplement for further details. Cytokine profiles and the expression of Foxp3 were evaluated by intracellular staining for IL-4, IFN- γ , IL-17A, and Foxp3, as described previously (29). We found that more than 50% of CD4⁺ T cells cultured in Th2-polarizing condition expressed IL-4, but few cells expressed IFN- γ , IL-17A, or Foxp3 (see Figure E1 in the online supplement). Similarly, more than 50% of CD4⁺ T cells cultured in Th17-polarizing condition expressed IL-17A, but few cells expressed IL-4, IFN- γ , or Foxp3 (see Figure E1).

Adoptive Transfer Experiments for Antigen-induced Airway Inflammation

Cultured DO11.10 Th2 cells (5 or 10 $\times$ 10⁶ cells/mouse), Th17 cells (5 or 10 $\times$ 10⁶ cells/mouse), or a mixture of Th2 cells and Th17 cells (5 $\times$ 10⁶ cells/mouse, each) were adoptively transferred intravenously into histocompatible BALB/c mice. Control mice received 0.9% saline intravenously. Twenty-four hours later, the mice were challenged with OVA inhalation. The numbers of eosinophils, neutrophils, and lymphocytes recovered in the BALF as well as the levels of IL-5 and IL-13 in the BALF were evaluated, and the histologic analysis of the lungs was performed at 48 hours after OVA inhalation. Where indicated, anti-eotaxin-1 antibody (100 μ g/body; clone 42285; R&D Systems, Inc., Minneapolis, MN) and anti-eotaxin-2 antibody (100 μ g/body; clone 106521; R&D Systems, Inc.) were administered intraperitoneally 24 hours before the inhaled OVA challenge. In some experiments, OVA-sensitized BALB/c mice were used as recipients of cultured DO11.10 Th17 cells and challenged with OVA inhalation.

Effect of IL-23 on Antigen-induced Th2 Cell Differentiation *In Vitro*

Effect of IL-23 on antigen-specific helper T-cell differentiation was evaluated, as described previously with minor alterations (29). See the online supplement for further details.

Data Analysis

Data are summarized as means $\pm$ SD. The statistical analysis of the results was performed by the unpaired Student's *t* test. *P* values less than 0.05 were considered significant.

RESULTS

IL-23 Is Involved in the Induction of Antigen-induced Allergic Airway Inflammation

To determine whether IL-23 is involved in the regulation of allergic airway inflammation, we first examined the expression of the IL-23–specific p19 subunit and the p40 subunit, which is shared with IL-12, in the lungs of sensitized mice upon antigen inhalation. We found that both p19 mRNA and p40 mRNA were induced in the lungs of OVA-sensitized mice at 2 hours after OVA inhalation, although p40 mRNA was detected in the lung before the inhaled OVA challenge (Figure 1A). On the other hand, the expression of IL-12–specific p35 subunit was not

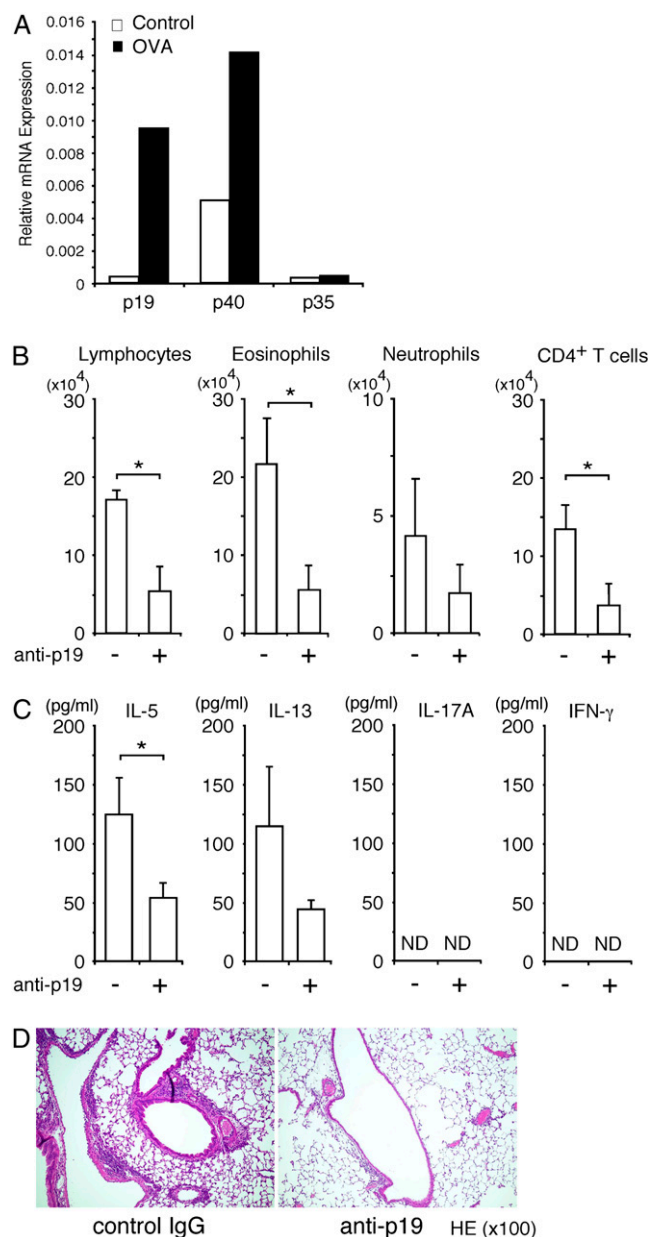


Figure 1. IL-23 is involved in the induction of antigen-induced eosinophil recruitment into the airways. (A) IL-23 mRNA is expressed in the lungs of sensitized mice upon antigen inhalation. Ovalbumin (OVA)-sensitized BALB/c mice were challenged with inhaled OVA or saline (control). Two hours after OVA or saline inhalation, total RNA was prepared from the lungs and real-time polymerase chain reaction (PCR) analysis for IL-23 p19, IL-12 p35, and IL-12/IL-23 p40 mRNA was performed. Shown are representative data from four independent experiments. (B–D) Anti-IL-23 antibody inhibits antigen-induced eosinophil recruitment into the airways. OVA-sensitized BALB/c mice were challenged with inhaled OVA. Antibody against IL-23 p19 or murine IgG1 was injected intraperitoneally at 24 hours before OVA inhalation. (B) The numbers of lymphocytes, eosinophils, neutrophils, and CD4⁺ T cells in the bronchoalveolar lavage fluid were evaluated at 48 hours after the OVA inhalation. Data are means $\pm$ SD; $n = 4$ mice in each group; $*P < 0.05$. (C) The amounts of IL-5, IL-13, IL-17A, and IFN- γ in the bronchoalveolar lavage fluid were determined by ELISA at 48 hours after OVA inhalation. Data are means $\pm$ SD; $n = 4$ mice in each group; $*P < 0.05$. ND = not detectable. (D) Histologic analysis of the lungs was performed with hematoxylin-and-eosin staining at 48 hours after OVA inhalation. Representative photomicrographs of lung sections are shown; $n = 4$ mice.

enhanced in the lungs of OVA-sensitized mice at 2 hours after OVA inhalation (Figure 1A).

We therefore examined the effect of anti-p19 monoclonal antibody (mAb), which neutralizes the activity of IL-23 but not of IL-12, on antigen-induced airway inflammation in sensitized mice. The inhalation of aerosolized OVA induced lymphocyte, eosinophil, and neutrophil recruitment into the airways of OVA-sensitized mice at 48 hours after OVA inhalation (Figure 1B). Fluorescence-activated cell sorter analysis of lymphocytes in BALF revealed that the majority of lymphocytes were CD4⁺ T cells in the mice (Figure 1B). The administration of anti-p19 mAb at 24 hours before the inhaled antigen challenge significantly inhibited antigen-induced lymphocyte and eosinophil recruitment into the airways of sensitized mice ($n = 4$ mice/group; $P < 0.05$) (Figure 1B). Anti-p19 mAb also significantly inhibited antigen-induced IL-5 production in the airways ($n = 4$; $P < 0.05$) (Figure 1C). In addition, anti-p19 mAb tended to inhibit antigen-induced IL-13 production in the airways, although the difference did not reach statistical significance ($n = 4$; $P = 0.06$) (Figure 1C). IL-17A and IFN- γ were undetectable in the airways in both antigen-inhaled control IgG1-treated mice and anti-p19 mAb-treated mice (Figure 1C). Histologic analysis showed that peribronchial and perivascular inflammatory cell infiltration in the lung, which mainly consisted of lymphocytes and eosinophils, at 48 hours after OVA inhalation was also significantly inhibited by anti-p19 mAb (Figure 1D). These results suggest that endogenously produced IL-23 enhances antigen-induced, Th2-cell-mediated eosinophil recruitment into the airways.

Generation of CC10 IL-23 Mice That Express IL-23 in the Lung

To determine the mechanisms by which IL-23 enhances antigen-induced allergic airway inflammation, we generated CC10 IL-23 transgenic mice (CC10 IL-23 mice) that express murine IL-23 in which IL-23/IL-12 p40 is flexibly linked to IL-23 p19 (23), specifically in the lung, under the control of CC10 promoter (24). We obtained two lines of CC10 IL-23 mice and confirmed that both lines of the offspring secreted IL-23 protein in the airways (Figure 2A). On the other hand, IL-23 was undetectable in the sera of CC10 IL-23 mice. CC10 IL-23 mice did not show gross abnormalities in organs, including the lung (data not shown). In addition, no abnormality was found in the numbers of T cells and B cells and their activation status in the spleen of CC10 IL-23 mice (see Figure E2). Because the expression levels of IL-23 in the airways were similar between two lines of CC10 IL-23 mice, we used transgenic line 1 in the following experiments.

Enforced Expression of IL-23 in the Airways Enhances Antigen-induced Airway Inflammation and AHR

We then examined the effect of enforced expression of IL-23 in the airways on antigen-induced airway inflammation. CC10 IL-23 mice and littermate WT mice were immunized with OVA and then challenged with inhaled OVA or saline (as a control). With inhaled saline challenge, no significant inflammatory cell infiltration was observed in the airways in WT mice and in CC10 IL-23 mice (Figure 2B), indicating that the expression of IL-23 in the airways itself does not cause airway inflammation. With the inhaled OVA challenge, antigen-induced recruitment of lymphocytes, eosinophils, and neutrophils into the airways was significantly increased in CC10 IL-23 mice compared with WT mice ($n = 5$; $P < 0.05$) (Figure 2B). Histologic analysis of lung sections showed that peribronchial and perivascular inflammatory cell infiltration, which consisted of lymphocytes, eosinophils, and some neutrophils, was enhanced in antigen-inhaled CC10 IL-23 mice compared with antigen-inhaled WT mice

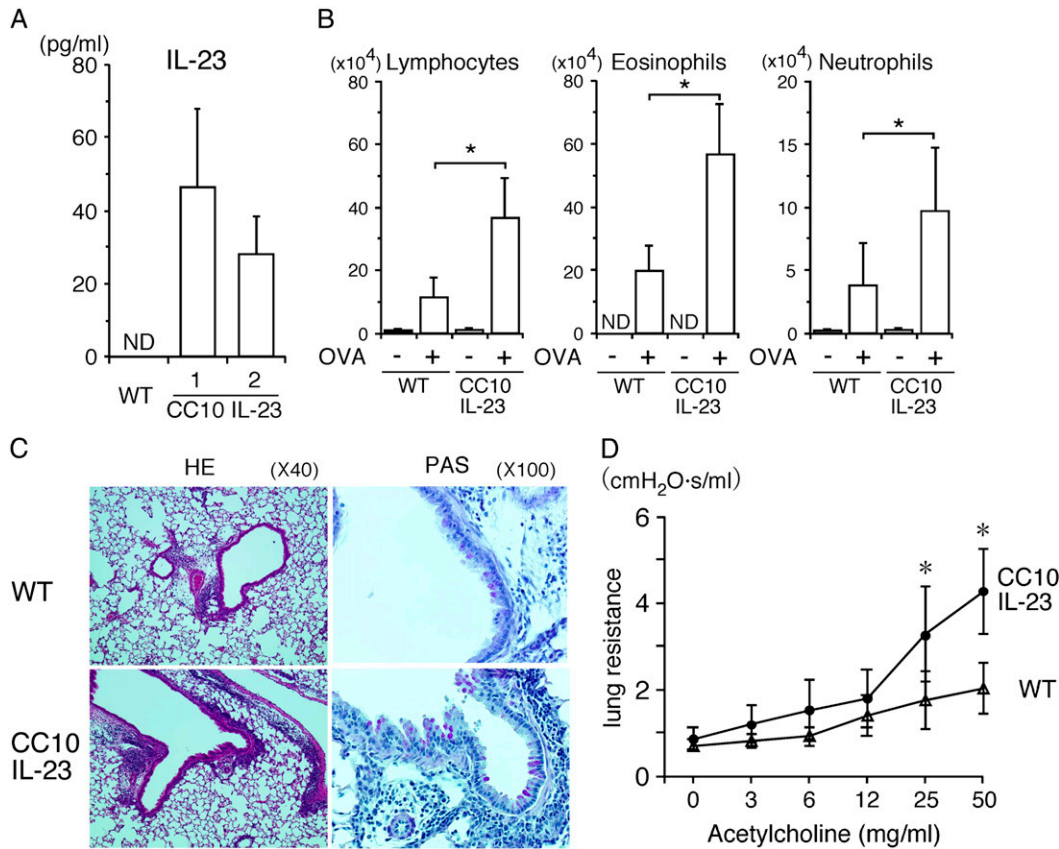


Figure 2. Enforced expression of IL-23 in the airways enhances antigen-induced airway inflammation and airway hyperresponsiveness. (A) IL-23 expression in the airways of CC10 IL-23 mice (line 1 and line 2) and littermate wild-type (WT) mice were subjected to bronchoalveolar lavage fluid (BALF) and the amounts of IL-23 in the BALF were determined by ELISA. Data are means $\pm$ SD; $n = 4$ mice in each group. ND = not detectable. (B–C) Enforced expression of IL-23 in the airways enhances antigen-induced airway inflammation. Ovalbumin (OVA)-sensitized CC10 IL-23 mice and littermate WT mice were challenged with inhaled OVA or saline. (B) The numbers of lymphocytes, eosinophils, and neutrophils in the BALF were evaluated at 48 hours after OVA or saline inhalation. Data are means $\pm$ SD; $n = 5$ mice in each group; $*P < 0.05$. (C) Histologic analysis of the lungs was performed with

hematoxylin-and-eosin and periodic acid Schiff staining at 48 hours after OVA inhalation. Representative photomicrographs of lung sections are shown; $n = 5$ mice. (D) Enforced expression of IL-23 in the airways enhances antigen-induced airway hyperresponsiveness. OVA-sensitized CC10 IL-23 mice and littermate WT mice were challenged with inhaled OVA three times at a 48-hour interval. Airway resistance to acetylcholine was measured at 24 hours after the final OVA inhalation by flexiVent systems (SCIREQ, Inc., Montreal, Canada) as described in METHODS. Data are means $\pm$ SD; $n = 5$ mice in each group; $*P < 0.05$.

(Figure 2C, left panels). Moreover, periodic acid Schiff staining of lung sections revealed that goblet cell hyperplasia was also enhanced in antigen-inhaled CC10 IL-23 mice (Figure 2C, right panels). Consistent with the enhanced airway inflammation and goblet cell hyperplasia in CC10 IL-23 mice, AHR to acetylcholine was significantly increased in CC10 IL-23 mice compared with WT mice ($n = 5$; $P < 0.05$) (Figure 2D). These results indicate that the expression of IL-23 in the airways enhances antigen-induced lymphocyte, eosinophil, and neutrophil recruitment into the airways; goblet cell hyperplasia; and AHR.

IL-23 Enhances Antigen-induced IL-13 and IL-17A Production in the Airways

To determine the basis for IL-23-mediated enhancement of antigen-induced airway inflammation, we examined cytokine levels in the BALF of OVA-sensitized CC10 IL-23 mice and WT mice upon OVA inhalation. Figure 3A shows that IL-13 levels in the BALF were significantly enhanced in antigen-inhaled CC10 IL-23 mice compared with those in antigen-inhaled WT mice ($n = 7$; $P < 0.05$), which is consistent with the enhanced goblet cell hyperplasia and AHR (Figures 2C and 2D). IL-17A levels in the BALF were also significantly enhanced in antigen-inhaled CC10 IL-23 mice compared with those in antigen-inhaled WT mice ($n = 7$; $P < 0.05$) (Figure 3A). Furthermore, IL-5, IL-13, and IL-17A production from anti-CD3-stimulated draining lymph node cells was also significantly enhanced in antigen-inhaled CC10 IL-23 mice compared antigen-inhaled WT mice ($n = 5$; $P < 0.05$) (Figure 3B). On the

other hand, in the absence of OVA inhalation, IL-17A was not detectable in the airways even in CC10 IL-23 mice (Figure 3A). Intracellular cytokine analysis of lung-infiltrating CD4⁺ T cells revealed that both IL-4-producing CD4⁺ T cells and IL-17A-producing CD4⁺ T cells were increased in antigen-inhaled CC10 IL-23 mice compared with antigen-inhaled WT mice (Figure 3C). In addition, the number of TNF- α -producing CD4⁺ T cells was increased in antigen-inhaled CC10 IL-23 mice (Figure 3C). Moreover, the induction of eotaxin-1 and eotaxin-2 mRNA was enhanced in antigen-inhaled CC10 IL-23 mice compared with that in antigen-inhaled WT mice ($n = 4$) (Figure 3D). These results suggest that IL-23 enhances antigen-induced activation of both Th2 cells and Th17 cells as well as the induction of eotaxin(s) during the effector phase of allergic airway inflammation.

IL-23 Enhances Antigen-induced Th2 Cell Differentiation *In Vitro*

We then examined the effect of IL-23 on IL-4 production from antigen-stimulated CD4⁺ T cells *in vitro*. Isolated CD4⁺ T cells from DO11.10 mice were stimulated with OVA peptide presented on CD11c⁺ dendritic cells (DCs) in the presence or absence of IL-23, and the number of IL-4-producing CD4⁺ T cells was evaluated by intracellular cytokine staining. Figure 4 shows that IL-23 enhanced IL-4 production from antigen-stimulated CD4⁺ T cells ($n = 4$; $P < 0.05$). In contrast, IL-23 did not enhance IL-4 production when isolated CD4⁺ T cells were stimulated with anti-CD3 mAb plus anti-CD28 mAb in

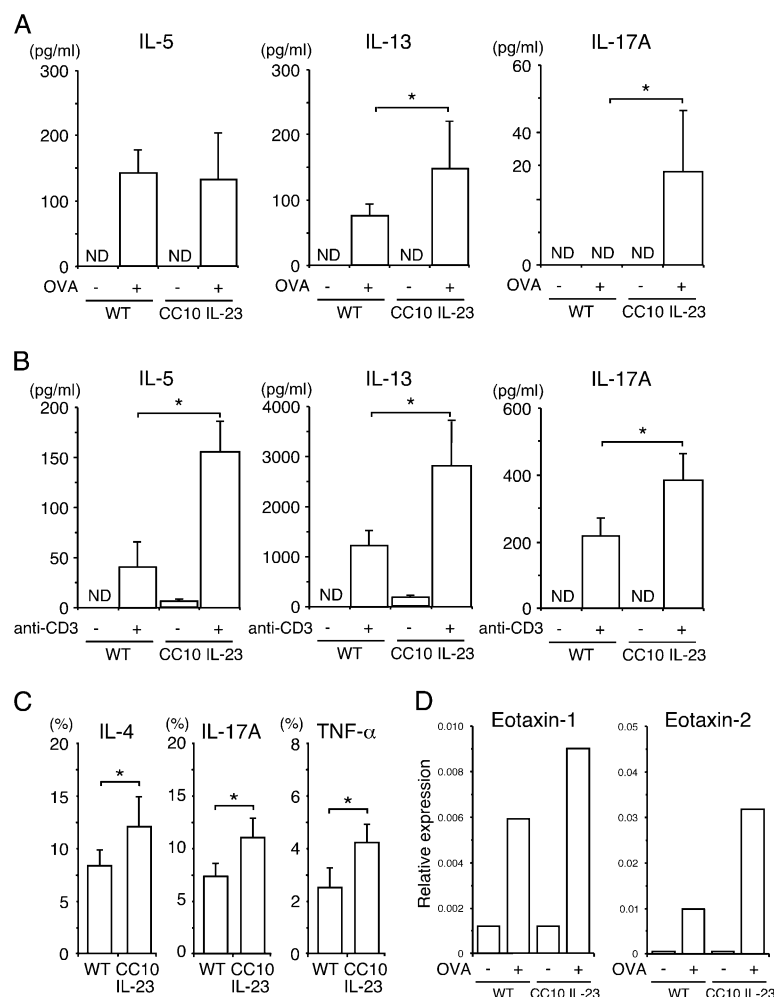


Figure 3. Enforced expression of IL-23 enhances antigen-induced IL-13 and IL-17 production in the airways. (A–D) Ovalbumin (OVA)-sensitized CC10 IL-23 mice and littermate wild-type (WT) mice were challenged with inhaled OVA or saline. (A) The amounts of IL-5, IL-13, and IL-17A in the bronchoalveolar lavage fluid were determined by ELISA at 48 hours after OVA or saline inhalation. Data are means $\pm$ SD; $n = 7$ mice in each group; $*P < 0.05$. (B) Cytokine production from draining lymph node T cells of CC10 IL-23 mice. Mediastinal lymph nodes were harvested from OVA-sensitized CC10 IL-23 mice and littermate WT mice at 48 hours after OVA inhalation, and lymphocytes were stimulated with anti-CD3 antibody for 24 hours. The amounts of IL-5, IL-13, and IL-17A in the supernatants were determined by ELISA. Data are means $\pm$ SD; $n = 5$ mice in each group; $*P < 0.05$. ND = not detectable. (C) Cytokine profiles of lung-infiltrating CD4⁺ T cells in CC10 IL-23 mice. Forty-eight hours after the inhaled OVA challenge, lung-infiltrating lymphocytes were harvested from OVA-sensitized CC10 IL-23 mice and littermate WT mice and intracellular cytokine staining for IL-4, IL-17A, or tumor necrosis factor (TNF)- α was performed on CD4⁺ T cells as described in METHODS. Data are means $\pm$ SD of the percentage of IL-4-, IL-17A-, or TNF- α -producing cells in CD4⁺ T cells; $n = 3$ mice; $*P < 0.05$. (D) Total RNA was prepared from the lung tissues of each mouse at 48 hours after OVA inhalation, and real-time polymerase chain reaction for eotaxin-1 and eotaxin-2 mRNA was performed. Representative data from four independent experiments are shown.

the absence of DCs (Figure 4). These results suggest that IL-23 functions on DCs to modulate their activity on Th2 cell differentiation and that this pathway may be involved in the enhanced Th2 responses in the airways of CC10 IL-23 mice.

IL-17A Is Involved in the Enhanced Neutrophil but not Eosinophil Recruitment into the Airways in CC10 IL-23 Mice

Recent studies have shown that IL-23 enhances the maintenance of Th17 cells (16, 17); indeed, the levels of IL-17A were increased in the airways of CC10 IL-23 mice upon antigen challenge (Figure 3). Therefore, we next examined whether IL-17A was involved in the IL-23-mediated enhancement of antigen-induced eosinophil and neutrophil recruitment into the airways by generating CC10 IL-23 IL-17A-deficient (CC10 IL-23 IL-17A^{-/-}) mice. CC10 IL-23 IL-17A^{-/-} mice, CC10 IL-23 mice, IL-17A^{-/-} mice, and littermate WT mice were immunized with OVA and then challenged with OVA inhalation. Forty-eight hours after OVA inhalation, the numbers of lymphocytes, eosinophils, and neutrophils in the BALF were evaluated. As shown in Figure 5A, the numbers of lymphocytes and eosinophils in the BALF were comparable between CC10 IL-23 IL-17A^{-/-} mice and CC10 IL-23 mice. The numbers of lymphocytes and eosinophils in the BALF were also comparable between IL-17A^{-/-} mice and WT mice (Figure 5A). In addition, the levels of IL-5 and IL-13 in the BALF were not significantly affected by the absence of IL-17A (Figure 5B). On the other hand, antigen-induced neutrophil recruitment tended to be decreased in CC10 IL-23 IL-17A^{-/-} mice compared with that in CC10 IL-23 mice (Figure 5A). These

results suggest that IL-17A is involved in the enhanced antigen-induced neutrophil recruitment but not eosinophil recruitment into the airways in CC10 IL-23 mice.

Antigen-specific Th17 Cells Enhance Th2 Cell-mediated Allergic Airway Inflammation

To investigate further the mechanisms for IL-23-mediated enhancement of antigen-induced eosinophil recruitment into the airways, we next evaluated the roles of Th17 cells in the regulation of allergic airway inflammation by the adoptive transfer experiments of antigen-specific Th17 cells. OVA-specific Th17 cells were generated by stimulating CD4⁺ T cells from DO11.10 mice (26) with OVA323–339 peptide in Th17-polarizing condition and were expanded in the presence of IL-23. OVA-specific Th2 cells were concurrently generated by stimulating CD4⁺ T cells from DO11.10 mice with OVA323–339 peptide in Th2-polarizing condition (27). BALB/c mice were injected intravenously with Th2 cells, Th17 cells, or a mixture of Th2 cells and Th17 cells and then challenged with OVA inhalation. As shown in Figure 6A, antigen-induced eosinophil recruitment into the airways was induced at 48 h after inhaled antigen challenge in the mice injected with antigen-specific Th2 cells but not with antigen-specific Th17 cells. Moreover, importantly, the cotransfer of antigen-specific Th17 cells with antigen-specific Th2 cells significantly enhanced antigen-induced Th2-cell-mediated eosinophil recruitment into the airways ($n = 4$; $P < 0.05$) to levels similar to the eosinophil recruitment induced by double numbers of antigen-specific Th2

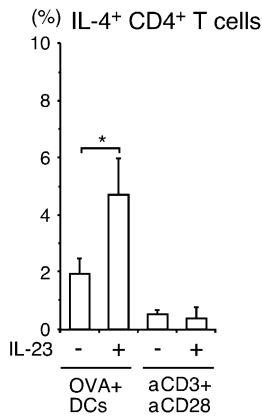


Figure 4. IL-23 enhances IL-4 production from antigen-stimulated CD4⁺ T cells. Isolated CD4⁺ T cells from DO11.10 mice were stimulated with ovalbumin peptide presented on CD11c⁺ dendritic cells or with plate-coated anti-CD3 monoclonal antibody (mAb) plus anti-CD28 mAb in the presence or absence of IL-23 at 37°C for 4 days. Cells were harvested and intracellular cytokine staining against IL-4 on CD4⁺ T cells was performed. Data are means $\pm$ SD of the percentage of IL-4-producing cells in CD4⁺ T cells; $n = 4$ mice; * $P < 0.05$.

cells (Figure 6A). On the other hand, antigen-induced neutrophil recruitment into the airways was induced in the mice injected with antigen-specific Th17 cells but not with antigen-specific Th2 cells (Figure 6A). Histologic analysis of the lung also showed the markedly enhanced peribronchial inflammation with eosinophil and neutrophil infiltrates in the mice injected with both Th2 cells and Th17 cells compared with mice injected with Th2 cells alone (Figure 6B).

To address the mechanisms underlying a synergistic increase in antigen-induced eosinophil recruitment into the airways in the mice injected with both Th2 cells and Th17 cells, we examined the levels of cytokines and chemokines shown to promote eosinophil recruitment into the airways. Analysis of Th2 cytokines in the BALF revealed that the levels of IL-5 and IL-13 were increased in the mice injected with Th2 cells, but they were not significantly enhanced by the concomitant transfer of Th17 cells with Th2 cells (Figure 6C). On the other hand, as shown in Figure 6D, the expression levels of eotaxin-1 mRNA in the lung were increased in mice injected with both Th2 cells and Th17 cells compared with mice injected with Th2 cells alone. The expression levels of eotaxin-2 but not of RANTES in the lung were also increased in the mice injected with both Th2 cells and Th17 cells (Figure 6D). In addition, neutralization of eotaxin-1 and eotaxin-2 before the inhaled antigen challenge decreased eosinophil recruitment into the airways of the mice injected with Th2 cells or a combination of Th2 cells and Th17 cells (Figure 6E), suggesting that eotaxin-1 and/or eotaxin-2 is involved in the enhanced antigen-induced eosinophil recruitment into the airways in these mice. Consistent with the enhanced eosinophilic airway inflammation, AHR to acetylcholine was also increased in the mice injected with both Th2 cells and Th17 cells compared with mice injected with Th2 cells or Th17 cells ($n = 4$) (Figure 6F).

We further examined the effect of Th17 cells on antigen-induced allergic airway inflammation by another adoptive transfer experiment using sensitized mice as recipients. OVA-sensitized mice were injected with OVA-specific Th17 cells and then challenged with OVA inhalation. As shown in Figure 7, the transfer of antigen-specific Th17 cells to sensitized mice significantly enhanced not only antigen-induced neutrophil recruitment into the airways but also antigen-induced eosinophil recruitment into the airways ($n = 4$; $P < 0.05$). In the absence of sensitization of recipient mice with OVA, OVA inhalation induced no significant eosinophil recruitment into the airways even in the mice injected with OVA-specific Th17 cells (Figure 7). Taken together, these results indicate that Th17 cells enhance antigen-induced Th2 cell-mediated eosinophil recruitment into the airways.

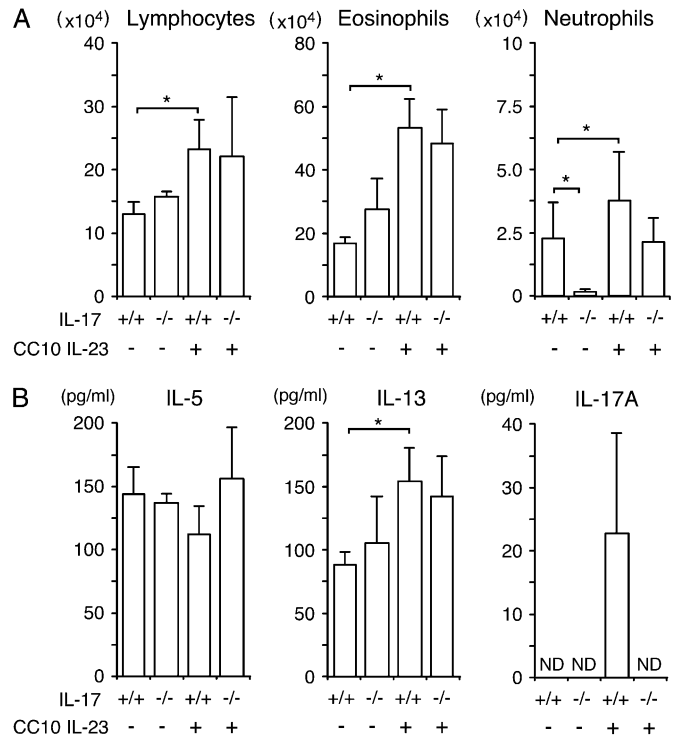


Figure 5. IL-17A is not essential for the enhancement of antigen-induced eosinophil recruitment into the airways of CC10 IL-23 mice. (A–B) Ovalbumin (OVA)-sensitized CC10 IL-23 IL-17A^{-/-} mice, CC10 IL-23 mice, IL-17A^{-/-} mice, and wild-type (WT) mice were challenged with OVA inhalation. (A) The numbers of lymphocytes, eosinophils, and neutrophils in the bronchoalveolar lavage fluid were evaluated at 48 hours after the OVA inhalation. Data are means $\pm$ SD; $n = 4$ mice in each group; * $P < 0.05$. (B) The amounts of IL-5, IL-13, and IL-17A in the bronchoalveolar lavage fluid were determined by ELISA at 48 hours after OVA inhalation. Data are means $\pm$ SD; $n = 4$ mice in each group; * $P < 0.05$. ND = not detectable.

DISCUSSION

We show that IL-23 and Th17 cells enhance Th2 cell-mediated eosinophilic airway inflammation. Our findings indicated that IL-23 mRNA was expressed in the lung of sensitized mice upon antigen inhalation (Figure 1) and that neutralization of IL-23 attenuated antigen-induced eosinophil recruitment and Th2 cytokine production in the airways (Figure 1). We also found that the enforced expression of IL-23 in the airways enhanced antigen-induced eosinophil recruitment into the airways, goblet cell hyperplasia, and AHR (Figure 2). The enforced expression of IL-23 in the airways also enhanced Th2 cytokine production in the airways (Figure 3). Moreover, adoptive transfer of antigen-specific Th17 cells significantly enhanced antigen-induced, Th2-cell-mediated eosinophil recruitment into the airways and AHR (Figures 6 and 7). These results suggest that IL-23 and Th17 cells up-regulate Th2-cell-mediated eosinophilic airway inflammation in two phases: IL-23 enhances antigen-induced activation of Th2 cells in the airways and antigen-specific Th17 cells cooperate to enhance Th2-cell-mediated eosinophil recruitment into the airways.

We show that IL-23 enhances antigen-induced activation of both Th2 cells and Th17 cells during the effector phase of allergic airway inflammation and thereby increases Th2-cell-mediated eosinophil recruitment and Th17-cell-mediated neutrophil recruitment into the airways. The expression of IL-23 p19 mRNA was induced in the lung of sensitized mice at 2 hours

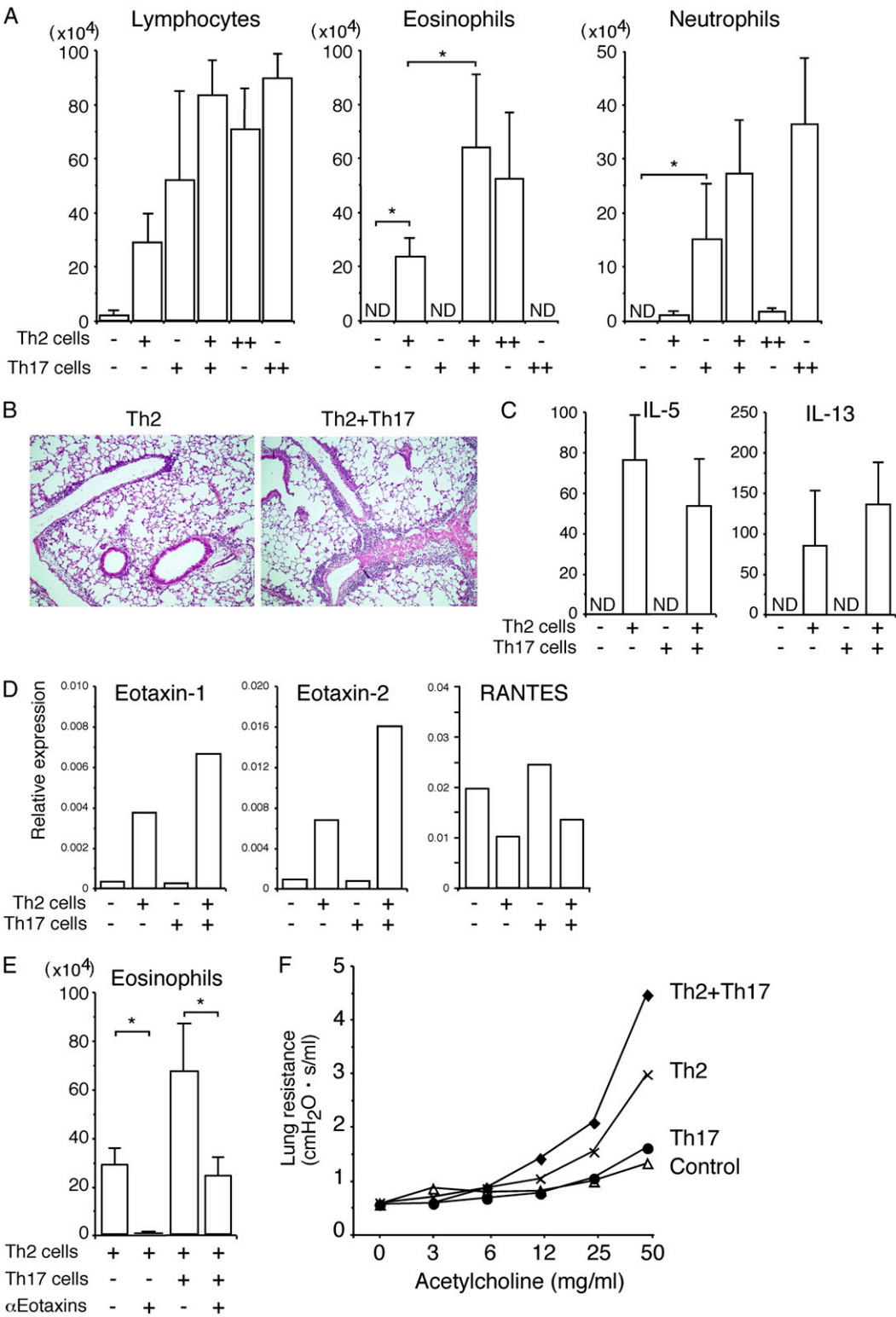


Figure 6. Antigen-specific Th17 cells enhance Th2-cell-mediated airway inflammation. (A–E) Ovalbumin (OVA)-specific Th2 cells and Th17 cells were prepared from splenocytes of DO11.10 mice, as described in METHODS. BALB/c mice were injected intravenously with OVA-specific Th2 cells (5×10^6 [+] or 10×10^6 [++]), OVA-specific Th17 cells (5×10^6 [+] or 10×10^6 [++]), or a combination of OVA-specific Th2 cells (5×10^6) and Th17 cells (5×10^6). Twenty-four hours later, the recipient mice were challenged with inhaled OVA. (A) The numbers of lymphocytes, eosinophils, and neutrophils in the bronchoalveolar lavage fluid (BALF) were evaluated at 48 hours after OVA inhalation. Data are means $\pm$ SD; $n = 4$ mice in each group; * $P < 0.05$; ND = not detectable. (B) Histologic analysis of the lungs was performed with hematoxylin-and-eosin staining at 48 hours after OVA inhalation. Shown are representative microphotographs of lung sections from four mice injected with OVA-specific Th2 cells or a combination of OVA-specific Th2 cells and Th17 cells. (C) The amounts of IL-5 and IL-13 in the BALF were determined by ELISA at 48 hours after OVA inhalation. Data are means $\pm$ SD; $n = 4$ mice in each group. (D) Total RNA was prepared from the lung tissues of each recipient mouse at 48 hours after OVA inhalation, and real-time polymerase chain reaction for eotaxin-1, eotaxin-2, and RANTES mRNA was performed. Shown are representative data from four independent experiments. (E) Anti-eotaxin-1 antibody and anti-eotaxin-2 antibody (α -eotaxins) or control antibody was administered intraperitoneally 24 hours before the inhaled OVA challenge. The numbers of eosinophils in the BALF were evaluated at 48

hours after OVA inhalation. Data are means $\pm$ SD; $n = 3$ mice in each group; * $P < 0.05$. (F) As in (A), BALB/c mice were injected intravenously with OVA-specific Th2 cells (5×10^6), Th17 cells (5×10^6), or a combination of Th2 cells and Th17 cells (5×10^6 , each). Twenty-four hours later, the recipient mice were challenged with inhaled OVA three times at a 48-hour interval. Twenty-four hours after the last inhaled OVA challenge, airway responsiveness to aerosolized acetylcholine was assessed as described in METHODS. Shown is a representative experiment from four independent experiments.

after antigen inhalation (Figure 1A), consistent with a recent report showing that IL-23 levels are increased in the lung homogenates of sensitized mice by antigen inhalation (11). We found that a neutralizing anti-p19 antibody attenuated antigen-

induced eosinophil recruitment and Th2 cytokine production in the airways (Figures 1B and 1C). Furthermore, we found that the enforced expression of IL-23 in the airways enhanced antigen-induced eosinophil recruitment and IL-5 and IL-13 production in

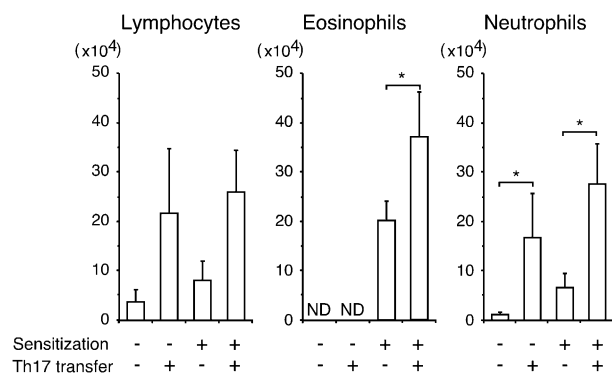


Figure 7. Antigen-specific Th17 cells enhance antigen-induced eosinophil recruitment into the airways of sensitized mice. Ovalbumin (OVA)-sensitized BALB/c mice or nonsensitized BALB/c mice were injected with OVA-specific Th17 cells or received saline (control). Twenty-four hours after the cell transfer, mice were challenged with inhaled OVA. Forty-eight hours after the inhalation, the numbers of lymphocytes, eosinophils, and neutrophils in the BALF were evaluated. Data are means $\pm$ SD; $n = 4$ mice in each group; * $P < 0.05$.

the airways, goblet cell hyperplasia, and AHR as well as antigen-induced neutrophil recruitment and IL-17A production in the airways (Figures 2 and 3). Therefore, our findings suggest that IL-23 is expressed at the site of allergic airway inflammation, enhances both Th2 cell and Th17 cell activation upon antigen stimulation, and thus increases eosinophilic and neutrophilic airway inflammation. Because IL-23 is produced by dendritic cells and resident macrophages upon a variety of stimulation such as TNF- α , CD40, LPS, or oligodeoxynucleotides containing a CpG motif (CpG ODN) (17, 21, 30), IL-23-mediated enhancement of allergic airway inflammation could be induced by a variety of stimuli including not only the antigen exposure but also bacterial or viral infection.

Regarding the mechanism underlying IL-23-mediated Th2-cell differentiation, we found that IL-23 increased the number of IL-4-producing CD4⁺ T cells when CD4⁺ T cells were stimulated with antigenic peptide presented on DCs (Figure 4). In contrast, IL-23 did not enhance IL-4 production when CD4⁺ T cells were stimulated with anti-CD3 mAb plus anti-CD28 mAb (Figure 4), which confirms a previous finding that naive CD4⁺ T cells do not express IL-23 receptor (31). Thus, it is suggested that IL-23 may function on DCs to modulate their activity on Th2-cell differentiation and this machinery could be involved in part in the IL-23-mediated enhancement of allergic airway inflammation.

We also demonstrate that Th17 cells enhance Th2-cell-mediated eosinophil recruitment into the airways (Figures 6 and 7). We found that adoptive transfer of antigen-specific Th17 cells to nonsensitized mice did not induce eosinophil recruitment into the airways upon antigen inhalation, but the cotransfer of antigen-specific Th17 cells with antigen-specific Th2 cells significantly enhanced antigen-induced Th2-cell-mediated eosinophil recruitment into the airways (Figures 6A and 6B). In addition, when antigen-specific Th17 cells were transferred to sensitized mice, Th17 cells also significantly enhanced eosinophil recruitment into the airways of sensitized mice upon antigen inhalation (Figure 7). Therefore, it is indicated that Th17 cells themselves do not induce eosinophil recruitment into the airways but cooperate to enhance Th2-cell-mediated eosinophil recruitment into the airways.

At present, the mechanism underlying Th17-cell-mediated enhancement of Th2-cell-mediated eosinophil recruitment into the airway is unknown. Because antigen-induced eosinophil

recruitment into the airways was not reduced by the absence of IL-17A, regardless of the enforced expression of IL-23 (Figure 5), it is unlikely that IL-17A plays a critical role in the Th17-cell-mediated enhancement of eosinophilic airway inflammation. Moreover, because Yang and colleagues have shown that IL-17F-deficient mice exhibit enhanced Th2 cytokine production and eosinophilic airway inflammation in asthma models (32), IL-17F seems not to be responsible for the Th17-cell-mediated enhancement of eosinophilic airway inflammation. Future experiments using antigen-specific Th17 cells generated from DO11.10 IL-17A^{-/-} mice or DO11.10 IL-17F^{-/-} mice will reveal the precise role of IL-17A or IL-17F in Th17-cell-mediated enhancement of allergic airway inflammation.

On the other hand, we found that the enhancement of antigen-induced eosinophil recruitment into the airways by the cotransfer of Th17 cells and Th2 cells was associated with the enhanced expression of eotaxin-1 and eotaxin-2 (Figure 6D), both of which are potent chemokines for inducing the recruitment of eosinophils (33, 34). We also found that the neutralization of eotaxin-1 and eotaxin-2 inhibited the eosinophil recruitment into the airways in the mice co-injected with Th2 cells and Th17 cells (Figure 6E), suggesting that the induction of eotaxin-1 and/or eotaxin-2 is responsible in part for the enhanced eosinophil recruitment into the airways at the effector phase.

In this regard, it has been shown that IL-17A, a major product of Th17 cells, inhibits the expression of eotaxin-1 in murine asthma models (11). On the other hand, TNF- α , which is also produced by Th17 cells (15), has been shown to induce eotaxin production from epithelial cells (35). Because eotaxin-1 is secreted from epithelial cells and fibroblasts by the activation of Stat6 and nuclear factor (NF)- κ B in a synergistic fashion (36) and because IL-13 activates Stat6 pathways and TNF- α activates NF- κ B pathways, it is possible that a synergistic induction of eotaxin-1 and eotaxin-2 by the coactivation of Th2 cells and Th17 cells is mediated by IL-13 and TNF- α . Because DCs have also been shown to produce TNF- α , depending on the status of DCs (37), it is possible that IL-23 may stimulate DCs to produce TNF- α and thus DC-derived TNF- α may be involved in the induction of eotaxins in CC10 IL-23 mice. It is also interesting that the cotransfer of Th1 cells and Th2 cells similarly enhances Th2-cell-mediated eosinophilic airway inflammation (27, 38) and that this eosinophilic airway inflammation is inhibited by the administration of anti-TNF- α antibody (27). The importance of TNF- α in the induction of airway inflammation is supported by our previous study showing that the neutralization of TNF- α attenuates antigen-induced eosinophil recruitment into the airways even in WT mice (39) as well as by another report showing the efficacy of TNF- α neutralization on patients with asthma (40).

We showed that the adoptive transfer of antigen-specific Th17 cells significantly induced neutrophil recruitment into the airways upon antigen inhalation (Figures 6 and 7). This is in agreement with previous studies showing that IL-17A is involved in the neutrophil recruitment into the airways (9). In addition, our findings, which showed that the enforced expression of IL-23 in the airways enhanced antigen-induced IL-17A production and neutrophil recruitment in the airways (Figures 2 and 3) and that IL-23-mediated enhancement of antigen-induced neutrophil recruitment in the airways was reduced by the absence of IL-17A (Figure 5), suggest that IL-23 contributes to neutrophilic airway inflammation through the activation of Th17 cells in the effector phase. The recent findings that IL-23 preferentially expands Th17 cells (15) also suggest that IL-23 can induce neutrophilic airway inflammation possibly through the induction of Th17 cells. Because studies have demonstrated that both IL-17A and IL-17F induce the expression of a variety of cytokines and chemokines,

such as IL-6, IL-8, granulocyte-macrophage colony-stimulating factor, and CXCL10 from epithelial cells, vascular endothelial cells, and fibroblasts (41, 42), it is possible that these cytokines and chemokines are involved in the Th17-cell-mediated neutrophil recruitment into the airways.

The ability of Th17 cells to evoke migration of neutrophils suggests that Th17 cells are involved in the pathogenesis of severe asthma or acute exacerbations, in which accumulation of neutrophils in the airways is a hallmark of disease (5, 6). Indeed, it has been shown that IL-17A is expressed in the airways of patients with asthma (7) and that its expression is increased in patients with moderate-to-severe asthma compared with patients with mild asthma and normal control subjects (8). In addition, our study demonstrates that Th17 cells significantly enhance Th2-cell-mediated eosinophilic airway inflammation (Figures 6 and 7). Thus, we suggest that the preferential induction of IL-23 and the activation of Th17 cells together with Th2 cells may be involved in the pathogenesis of severe asthma.

In conclusion, we have shown that IL-23 and Th17 cells are involved not only in causing antigen-induced neutrophil recruitment into the airways but also in the enhancement of Th2-cell-mediated eosinophil recruitment into the airways. Although additional studies are required to address the molecular mechanisms for IL-23- and Th17-cell-mediated enhancement of allergic airway inflammation, our results raise the possibility that IL-23 and/or Th17 cells could be a novel therapeutic target for asthma.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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