

Exogenous Interleukin-17A Inhibits Eosinophil Differentiation and Alleviates Allergic Airway Inflammation

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Abstract

IL-17 is known to play important roles in immune and inflammatory disease, such as in asthma, but its functions in allergic airway inflammation are still controversial, and the molecular mechanisms mediating these functions remain unclear. Increased production of eosinophils in bone marrow and their emergence in the airway have been linked to the onset and progression of allergic asthma. In this study, we investigated the effects of exogenous IL-17 on allergic airway inflammation and explored the underlying molecular mechanisms through eosinophil generation. Exogenous IL-17 significantly attenuated the features of allergic inflammation induced by ovalbumin in mice. It inhibited eosinophil differentiation both *in vivo* and *in vitro*, accompanied by down-regulated expression of CC chemokine receptor 3, GATA binding protein 1 (GATA-1), and GATA binding protein 2 (GATA-2), as well as reduced formation of common myeloid progenitors and eosinophil progenitors, but without influencing eosinophil apoptosis. IL-17 also significantly decreased the number of eosinophils in IL-5–transgenic mice, although it notably increased the levels of IL-3, IL-5, and granulocyte/macrophage colony-stimulating factor. In addition, IL-17 had little effect on secretion of the inflammatory cytokines by eosinophils. Neutralization

of endogenous IL-17 significantly augmented eosinophil recruitment in the airways. Together, these findings suggest that exogenous IL-17 protects against allergic airway inflammation, most likely through inhibition of the eosinophil differentiation in bone marrow.

Keywords: IL-17A; eosinophil; differentiation; allergic airway inflammation

Clinical Relevance

The present study represents an initial effort to demonstrate that exogenous IL-17 attenuates airway hyperresponsiveness and airway inflammation in allergic asthma, most likely through inhibition of eosinophil differentiation followed by decreased eosinophil recruitment to inflamed tissue, rather than down-regulation of the survival and cytokine secretion of eosinophils. The study also re-emphasizes the importance of targeting the inhibition of eosinophil differentiation in allergic inflammation, which may lead to new therapeutic targets for asthma.

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Eosinophils are multifunctional leukocytes implicated in the pathogenesis of various inflammatory diseases, including infection by parasitic helminths, bacteria, and viruses, allergic diseases, and even cancer (1). Eosinophils in the bloodstream and inflamed tissues differentiate from hematopoietic stem cells under the control of several critical factors, such as IL-5, which induces the differentiation of eosinophil progenitors (EoPs) from granulocyte/macrophage progenitors (GMPs) and stimulates the release of eosinophils from the bone marrow into the peripheral circulation (2). GATA binding protein (GATA) transcription factors, such as GATA-1 and GATA-2, have been detected in mature eosinophils as well as in early stage EoPs (3). The appropriate accumulation of GATA factors is not only essential for myeloid progenitor cells to commit to mature eosinophils, but also impacts the function of eosinophils (4, 5). The combination of CC chemokine receptor 3 (*Ccr3*) with its ligand, eotaxin, contributes greatly to the mobilization and trafficking of mature eosinophils from the bone marrow to sites of inflammation (6–8). We have recently reported that treatment with anti-CCR3 inhibits asthmatic eosinophilia and airway hyperresponsiveness (AHR), partly by modulating the *Ccr3* expression, migration, and differentiation of CD34⁺ progenitors in asthmatic C57BL/6 mice (9). Thus, blockade of eosinophil production could be an efficacious approach for the treatment or prevention of asthma at the initial stage. However, to the best of our knowledge, few efforts have targeted the inhibition of eosinophil differentiation to prevent allergic airway diseases.

IL-17, a proinflammatory cytokine, has been closely associated with a range of inflammatory diseases. Functional research studies on subjects with asthma as well as animal models of allergic airway inflammation indicate that IL-17 is a proinflammatory regulator implicated in mediating the migration and activation of inflammatory cells and perpetuating the inflammatory response in chronic airway inflammatory disease by inducing the expression of various inflammatory mediators, such as cytokines, chemokines, adhesion molecules, and growth factors (10, 11). Upon sensitization and stimulation by an allergen, IL-17-producing T helper (Th) type 17 cells home to the lung and enhance not only neutrophilic, but also eosinophilic, airway inflammation in mouse models (12, 13). It is reported that

neutrophils are recruited to the inflamed airway by the attraction of IL-8/C-X-C motif ligand 8 and/or other chemokines upon stimulation of IL-17 (14, 15), and increasing evidence also shows that elevated IL-17 levels are correlated with increased neutrophilic inflammation, a characteristic of severe and steroid-resistant asthma (16, 17). However, the role of IL-17 in allergen-induced eosinophilic inflammation is controversial. It has been suggested that IL-17 enhances the production of cytokines from eosinophils (18, 19), and amplifies eosinophil activation in the Th2-mediated eosinophilic inflammatory response, possibly by modulating eosinophil function (19, 20). Conversely, treatment of ovalbumin (OVA)-sensitized asthmatic mice with exogenous IL-17 inhibits airway inflammation, and reduces peribronchial mucus hypersecretion, as well as decreasing AHR, thus having an inhibitory effect during the course of asthma (21). IL-17 also exerts a dose-dependent effect in the modulation of IL-13-induced airway inflammation (22).

In this study, we investigated the effect and possible mechanisms of exogenous IL-17 on eosinophil generation in a mouse model of allergic asthma *in vivo* and *in vitro*. In addition, the effects of IL-17 on the survival and function of eosinophils were explored.

Materials and Methods

Mice

Female C57BL/6 mice (wild-type, 6–8 wk old) were purchased from the Animal Center of Zhejiang University (Hangzhou, Zhejiang, China) and housed in a conventional animal facility. An IL-5-transgenic line (NJ.1638; females, 6–8 wk old), which constitutively expresses IL-5 exclusively from mature T cells using an enhancer/promoter construct derived from the mouse CD3 δ gene, was generated as described previously (23). All protocols were approved by the Ethics Committee for Animal Studies at Zhejiang University.

OVA-Induced Airway Inflammation and Recombinant Mouse IL-17 Treatment

See SECTION 1 of the online supplement.

NJ.1638 Mice Given Recombinant Mouse IL-17

See SECTION 2 of the online supplement.

Quantitative RT-PCR

Cells were lysed with TRIzol reagent (Invitrogen, Grand Island, NY) and total RNA extracted according to the manufacturer's protocol. See SECTION 3 of the online supplement.

Preparation of Mouse Bone Marrow Cells

See SECTION 4 of the online supplement.

Liquid Culture of Mouse Bone Marrow-Derived Eosinophils *In Vitro*

See SECTION 5 of the online supplement.

Generation of Mature Mouse Bone Marrow-Derived Eosinophils *In Vitro*

Bone marrow cells were collected and cultured in RPMI 1,640 (Invitrogen) with 20% FBS (Gibco, Carlsbad, CA), 100 IU/ml penicillin, 10 μ g/ml streptomycin, 2 mM glutamine, 25 mM HEPES, 1 $\times$ nonessential amino acids, 1 mM sodium pyruvate, and 50 μ M 2-ME supplemented with 100 ng/ml stem cell factor (SCF; PeproTech, Rocky Hill, NJ) and 100 ng/ml FLT3 ligand (PeproTech) from Days 0 to 4 at a concentration of 1×10^6 /ml in six-well plates. For eosinophil differentiation, the SCF and FLT3 ligand were washed out and replaced by 10 ng/ml recombinant mouse IL-5 (R&D Systems, Minneapolis, MN) on Day 4, and the cells were cultured for an additional 8 days in the presence of IL-5 only (24). One-half of the culture medium was replaced with fresh medium every other day. Eosinophil maturity was assessed by fluorescence-activated cell sorting (FACS) staining for sialic acid-binding immunoglobulin-like lectin-F (Siglec-F; phycoerythrin-conjugated anti-Siglec-F) and F4/80 (allophycocyanin [APC]-conjugated anti-F4/80) and/or Wright-Giemsa staining of Cytospin preparations.

Assay for Eosinophil Colony-Forming Units

Eosinophil colony-forming units (CFUs) were generated as previously described (9). Bone marrow cells were isolated and cultured (1×10^5 cells/ml) in semisolid medium (RPMI 1,640 supplemented with 0.9% methylcellulose, 20% FBS, 1% penicillin-streptomycin, 2 mM L-glutamine, and 0.006% β -mercaptoethanol) with 10 ng/ml SCF (R&D Systems) and 10 ng/ml IL-5 (R&D Systems) with or without 10 ng/ml IL-17 (R&D Systems) in 3.5-cm dishes at 37°C

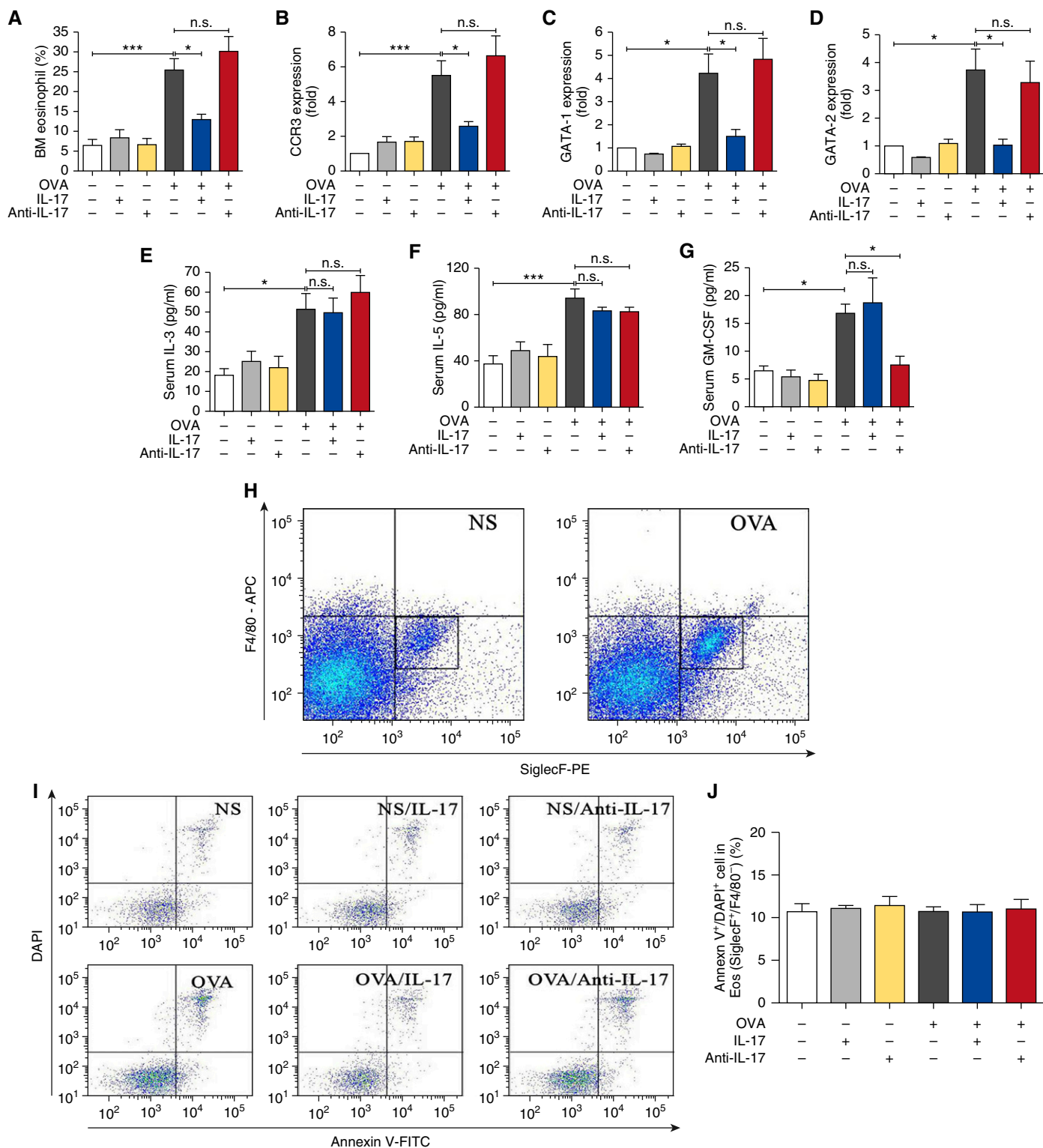


Figure 1. IL-17 inhibits the differentiation rather than the survival of bone marrow eosinophils *in vivo*. Allergic mice were sensitized and challenged with ovalbumin (OVA) and treated intratracheally with 40 μ l saline containing 100 ng IL-17 (OVA/IL-17) or 100 ng neutralizing IL-17 antibody (OVA/anti-IL-17) 1 hour before each OVA challenge. Control mice were challenged with saline alone and received IL-17 (NS/IL-17) or anti-IL-17 (NS/anti-IL-17) in an equal dosage and volume at each treatment time point. NS, normal saline. At 24 hours after the final OVA challenge, bone marrow cells and serum were harvested for the further steps. (A) Percentages of eosinophil in bone marrow (BM) cells. (B–D) Expression of mRNA encoding CC chemokine receptor 3 (*Ccr3*) (B), GATA binding protein 1 (GATA-1) (C), and GATA binding protein 2 (GATA-2) (D) determined by quantitative RT-PCR (qRT-PCR), the relative level

under 5% CO₂ for 7 days. On Day 7 in culture, individual colonies with more than 40 cells were counted under an inverted light microscope. Colonies were randomly selected and identified by Wright-Giemsa staining for eosinophil differentiation.

Flow Cytometric Analysis of Bone Marrow EoPs *In Vivo*

EoPs were detected by flow cytometry. Specifically, total bone marrow cells were flushed from the femurs and tibiae of asthmatic mice and controls with PBS containing 0.1% BSA. Erythrocytes were lysed (RBC lysis buffer; BD Biosciences, San Jose, CA) and the cells were washed once in PBS containing 0.1% BSA. The cells were stained for expression of the markers Lin (Lineage)[−] Sca-1 (stem cell antigen-1)[−] C-kit (v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog)^{low} CD34⁺ IL-5Rα⁺ (Lin, PE-cy7-conjugated anti-Sca-1; ebioscience, San Diego, CA), APC-conjugated anti-C-kit (ebioscience), AF700-conjugated anti-CD34 (ebioscience), and AF488-conjugated anti-IL-5Rα (BD Biosciences), and analyzed by FlowJo software (Cologne, Germany).

Flow Cytometric Analysis of Bone Marrow-Derived Eosinophil *In Vitro* and *In Vivo*

To evaluate the differentiation level of eosinophils (SiglecF⁺ and F4/80[−]) from bone marrow, eosinophils from culture were stained with PE-conjugated anti-Siglec-F (ebioscience), an early eosinophil differentiation marker (25), and APC-conjugated anti-F4/80 (ebioscience). Staining was performed for 30 minutes at 4°C in buffer (PBS with 0.1% BSA), then the cells were washed with buffer. Data were acquired with a FACS Calibur flow cytometer (BD FACSCalibur, San Jose, CA) and analyzed using FlowJo software.

Apoptosis Evaluation of Bone Marrow-Derived Eosinophils *In Vitro* and *In Vivo*

Apoptosis of bone marrow eosinophil (BmEos) was analyzed by double staining with AnnexinV-FITC and propidium iodide (PI; ebioscience) and assessed by

flow cytometry. For induced mature BmEos in culture (or *in vivo*), an aliquot of 100-μl suspension containing 1×10^5 cells was collected and stained with PE-conjugated anti-Siglec-F and APC-conjugated anti-F4/80 for 30 minutes in the dark at 4°C, followed by washing with flow cytometry buffer. The cells were suspended in 500 μl binding buffer with 5 μl Annexin V and 10 μl PI, and kept in the dark for 15 minutes at room temperature. The cells were then analyzed by flow cytometry within 1 hour. Apoptosis levels of eosinophil were gated on SiglecF⁺ and F4/80[−] staining.

ELISA of Cytokine Levels

Cytokine levels in serum and cell culture supernatant were analyzed by ELISA using paired antibodies (R&D Systems) following the manufacturer's instructions.

Statistical Analysis

Results are presented as mean ($\pm$ SEM). Data were analyzed with Student's *t* test (two-tailed) or one-way ANOVA. Differences were considered statistically significant at a *P* value of less than 0.05. Values are from three separate experiments.

Results

IL-17 Inhibits the Differentiation Rather Than the Survival of Bone Marrow Eosinophils *In Vivo*

First, we explored the possible mechanisms mediating the effect of IL-17 in suppressing allergic inflammation through eosinophil generation. As regulation of eosinophil differentiation in bone marrow is known to play an important role in asthma pathogenesis, we therefore determined whether exogenous IL-17 affects eosinophil differentiation in a mouse model of asthma. Bone marrow from OVA-challenged mice treated with IL-17 showed a significantly reduced percentage of eosinophil, but not of neutrophil, compared with those receiving OVA alone (Figure 1A, bar 5 versus 0.4). IL-17 alone slightly, but not significantly, increased the neutrophil ratio in both control and OVA-treated mice (see Figure E1 in the online supplement). Notably, IL-17 antibody administration slightly

augmented the levels of eosinophils induced by OVA, but it did not reach statistical significance (Figure 1A, bar 6 versus 0.4).

Factors associated with eosinophil differentiation, such as *Ccr3*, *Gata-1*, and *Gata-2* in total bone marrow were further investigated using quantitative real-time PCR analysis (26). Not surprisingly, OVA markedly induced the expression of transcripts of *Ccr3*, *Gata-1*, and *Gata-2*, all of which were significantly attenuated by IL-17 treatment (Figures 1B–1D, bar 5 versus 0.4). Although IL-17 was administered intratracheally, it was elevated in serum, which indicated that airway administration can have systemic effects (Figure E2). To determine whether the inhibitory effect of IL-17 on eosinophil differentiation is dependent on IL-3, IL-5, and granulocyte/macrophage colony-stimulating factor (GM-CSF), the levels of these cytokines were analyzed (Figures 1E–1G). Interestingly, all of them were significantly elevated in the OVA-sensitized and -challenged mice, whereas IL-17 administration had no effect on them when compared with the OVA-challenged group (Figures 1E–1G, bar 5 versus 0.4). Notably, IL-17 antibody significantly inhibited the allergen-induced GM-CSF (Figure 1G, bar 6 versus 0.4).

Next, we sought to determine whether the decreased number of eosinophils is due to eosinophil death caused by IL-17. Mouse eosinophils were labeled as SiglecF⁺, but were F4/80[−] (30–32), as shown in Figure 1H. Bone marrow cells from OVA-sensitized and -challenged mice showed a higher proportion of eosinophils than those in control mice, and IL-17 administration inhibited the formation of eosinophils significantly. However, there was no significant difference in the apoptosis levels of BmEos between IL-17 or IL-17 antibody-treated and control mice (Figures 1I and 1J). Similar results were observed at 12 hours after the last OVA challenge (Figure E3).

Taken together, these data demonstrate that IL-17 attenuates eosinophil differentiation independent of IL-3, IL-5, and GM-CSF, but has no appreciable effect on eosinophil apoptosis *in vivo*.

Figure 1. (Continued). of mRNA in NS mice was adjusted to 1 and served as the basal reference value. IL-3 (E), IL-5 (F), and granulocyte/macrophage colony-stimulating factor (GM-CSF) (G) levels in serum were evaluated by ELISA. Number of bone marrow eosinophil (SiglecF⁺ and F4/80[−]) (H) and their apoptotic levels (annexin⁺/4',6-diamidino-2-phenylindole [DAPI⁺]) (I and J). APC, allophycocyanin. Data are expressed as the mean $\pm$ SEM of individual groups of mice from three independent experiments (*n* = 8–10 mice per group). **P* < 0.05, ****P* < 0.001; n.s., not significant.

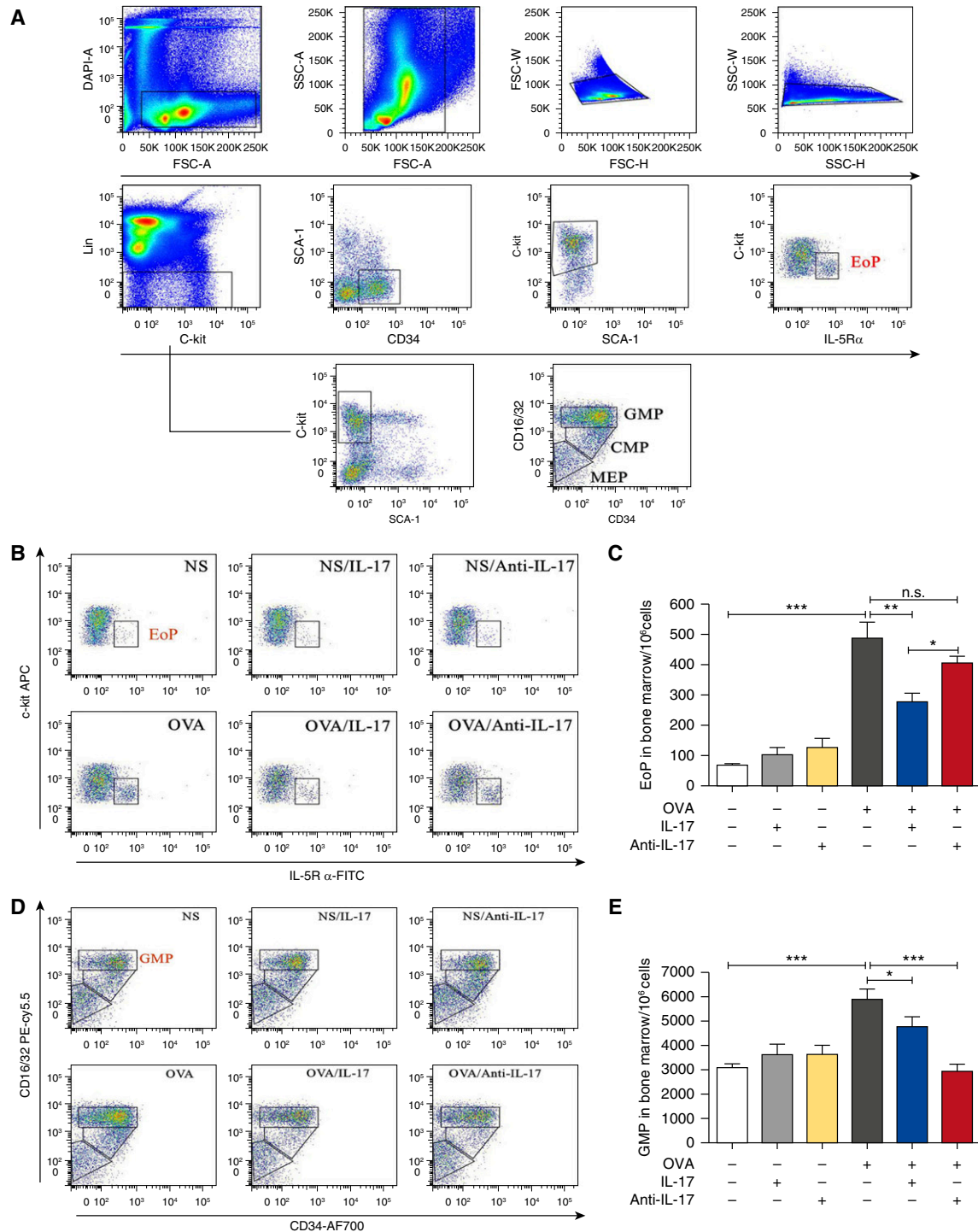


Figure 2. IL-17 inhibits the generation of bone marrow eosinophil (BmEos) progenitors *in vivo*. Experimental mice were sensitized and challenged with OVA and treated intratracheally with 40 μ l saline containing 100 ng IL-17 (OVA/IL-17) or 100 ng neutralizing IL-17 antibody (OVA/anti-IL-17) 1 hour before each OVA challenge. Control mice were challenged with saline alone and received IL-17 (NS/IL-17) or anti-IL-17 (NS/anti-IL-17) in an equal dosage and volume at each treatment time point. At 24 hours after the final OVA challenge, bone marrow cells were harvested for fluorescence-activated cell sorting (FACS) analysis. Gating strategies of the eosinophil progenitors (EoPs) and granulocyte/macrophage progenitors (GMPs) in bone marrow by FACS (A). Numbers of EoPs (B and C) and GMPs (D and E) in bone marrow were analyzed. Data are expressed as the mean $\pm$ SEM of individual groups of mice from three independent experiments ($n = 8-10$ mice per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n.s., not significant. APC, allophycocyanin; C-kit, v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog; CMP, common myeloid progenitor; FSC-A, forward scatter-area; FSC-H, forward scatter-height; FSC-W, forward scatter-width; Lin, lineage; MEP, megakaryocyte/erythrocyte progenitor; PE, phycoerythrin; SCA, stem cell antigen; SSC-A, side scatter-area; SSC-H, side scatter-height; SSC-W, side scatter-width.

IL-17 Inhibits the Generation of BmEos Progenitors *In Vivo*

The decreased eosinophil differentiation by IL-17 in OVA-treated mice might be due either to decreased formation of EoPs, or to attenuated differentiation of these progenitors to mature eosinophils. To clarify this, the numbers of EoPs among bone marrow cells were evaluated. The entire gating strategy of the EoPs and GMPs is shown in Figure 2A. Interestingly, bone marrow cells from OVA-challenged plus IL-17-treated mice showed decreased numbers of EoPs compared with the only OVA-sensitized and-challenged mice (Figures 2B and 2C, *bar 5* versus 4). In addition, the number of GMPs, which can differentiate into EoPs, was also decreased after IL-17 administration (Figures 2D and 2E, *bar 5* versus 4). Notably, IL-17 antibody markedly reduced the number of GMPs compared with that in OVA-only-sensitized and -challenged mice (Figure 2E, *bar 6* versus 4), suggesting a correlation between IL-17 and GM-CSF. These data, taken together, demonstrate that the decreased differentiation of eosinophils *in vivo* is likely, at least in part, due to the attenuated formation of EoPs.

IL-17 Decreases Eosinophil Production in IL-5-Transgenic Mice

To further clarify the direct inhibitory role of IL-17 in eosinophil differentiation *in vivo*, N.J.1638 mice were treated with IL-17. These mice are IL-5-transgenic, and constitutively express high levels of IL-5 in plasma and profound eosinophil populations in bone marrow and peripheral blood (23). Mice treated with IL-17 showed significantly lower levels of eosinophils in bone marrow and in peripheral blood than those in mice treated with saline (Figures 3A and 3B). More interestingly, IL-17 administration induced higher expression of serum IL-3, IL-5, and GM-CSF, with no effect on eotaxin/CCL11 (Figures 3C–3F), again suggesting that IL-17 inhibition of eosinophil production *in vivo* was independent of these hematopoietic factors. In addition, the total inflammatory cell and neutrophil counts in bronchoalveolar lavage fluid (BALF), as well as the percentage of neutrophils in peripheral blood, showed no difference between the saline- and IL-17-treated mice, but IL-17 induced the production of

more mature neutrophil in bone marrow (Figure E4).

IL-17 Inhibits the Differentiation without Changing the Viability and Function of BmEos *In Vitro*

To further demonstrate a direct inhibitory effect of IL-17 on eosinophil differentiation, we used *in vitro* liquid culture and CFU assays. We first found that the IL-17 receptor A was highly expressed in bone marrow cells, which was comparable to its expression in lung tissues (Figure E5). Bone marrow cells were isolated and cultured in liquid medium with SCF and IL-5, with or without IL-17. The increased proportion of mature eosinophils induced by IL-5 was always lower after treatment with IL-17 (Figure 4A). Similarly, IL-17 significantly reduced the eosinophil CFUs induced by IL-5 (Figure 4B). In addition, we measured the change of mRNA encoding *Ccr3*, *Gata-1*, and *Gata-2* after treatment with IL-17. As expected, these factors were significantly increased during eosinophil differentiation, and all were remarkably attenuated by IL-17 (Figures 4C–4E). Interestingly, there was no significant difference in the percentage of BmEos between IL-17-deficient mice and wild-type controls, and the numbers of eosinophil CFUs also showed no difference (Figure E6), indicating that endogenous IL-17 has no effect on eosinophil differentiation, at least under basal conditions.

We next investigated the role of IL-17 on their viability and functions. Eosinophils were induced from bone marrow cells for 10 days, as previously illustrated (24), and were treated with IL-17 or vehicle. As indicated by the results of AnnexinV/PI staining (Figures 4G and 4H), IL-17 again failed to affect eosinophil viability. Mature eosinophils from IL-5-transgenic mice that were treated with IL-17 exerted no considerable effects on the levels of IL-4, IL-13, IFN- γ , or IL-10 in supernatant (Figure E7), suggesting that IL-17 has no influence on eosinophil secretion.

We next sought to investigate the possible links between IL-17 and the pathways involved in eosinophil differentiation, such as Janus kinase 2, p38 mitogen-activated protein kinase, and v-src-1 Yanaguchi sarcoma viral related oncogene homology kinase pathways (27–29). Interestingly, there was no synergistic inhibitory effect of IL-17 and these signaling pathways inhibitors in eosinophil differentiation (Figure E8), suggesting that

IL-17 exerts inhibitory actions partially through these pathways.

Exogenous IL-17 Reduces Eosinophilic Inflammation in Asthma

Finally, we assessed the effects of exogenous IL-17 on systemic and local airway inflammation in the mouse model of asthma. IL-17 given together with OVA-sensitized and -challenged sensitization and challenge, significantly reduced the AHR in comparison to allergic mice without treatment. In asthmatic mice, treatment with IL-17 neutralizing antibody induced a slight increase of airway resistance compared with the OVA-challenged mice (Figure E9), which suggests that endogenous IL-17 may play a limited, but not a crucial, role in airway hyperresponsiveness. Differential cell counts in BALF and blood were performed to further confirm the role of IL-17 in allergic inflammation. IL-17 administration significantly decreased the total cells (Figure 5A) and eosinophil counts (Figure 5B) in BALF, and the eosinophil ratio in peripheral blood (Figure 5C), but had no effect on the amount of macrophage or neutrophil in BALF or on the neutrophil ratio in blood (Figure E10). It was noted that neutralizing IL-17 antibody exerted the prominent proeosinophilic inflammation in contrast to OVA-only-sensitized and -challenged mice (Figures 5A and 5B, *bar 6* versus 4), demonstrating that endogenous IL-17 contributes to eosinophil infiltration in some way. In addition, as indicated in Figures 5D–5F, OVA challenge induced a significant increase of eotaxin/CCL11, IL-22, and IL-25 in serum compared with that of control mice (*bar 4* versus 1). IL-17 inhibited eotaxin/CCL11, but without affecting IL-22 or IL-25 expression (*bar 5* versus 4), suggesting that exogenous IL-17 has some specific inhibitory action in eosinophilic inflammation. We also found the elevated IL-22 could be significantly reversed by IL-17 antibody (Figure 5E, *bar 6* versus 0.4).

The inflammation levels and mucus hypersecretion in airways were estimated according to hematoxylin and eosin and periodic acid–Schiff staining. Compared with the OVA group, mice receiving exogenous IL-17 showed significantly reduced infiltration of inflammatory cells around pulmonary blood vessels and airways (Figures 5G and 5H), and the OVA-induced goblet cell hyperplasia

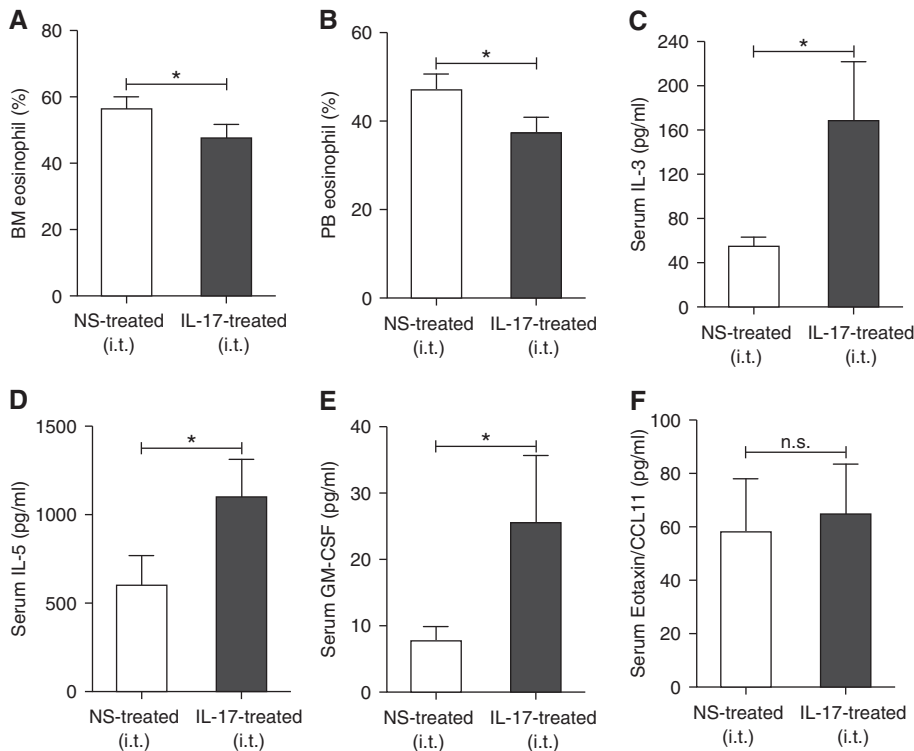


Figure 3. IL-17 decreases eosinophil production in IL-5-transgenic mice after intranasal IL-17 treatment. IL-5-transgenic mice (NJ.1638) were given intratracheal (i.t.) saline (40 μ l) with or without 100 ng IL-17 (NS versus NS/IL-17) on Days 0, 2, and 4. The mice were killed 48 hours after the last treatment. Percentages of eosinophil in bone marrow (A) and in peripheral blood (B) were assessed after Wright-Giemsa staining. Concentration of IL-3 (C), IL-5 (D), GM-CSF (E), and eotaxin/CCL11 (F) in serum, as detected by ELISA. Data are expressed as the mean $\pm$ SEM of individual groups of mice from two independent experiments ($n = 6$ mice per group). * $P < 0.05$; n.s., not significant. BM, bone marrow; PB, peripheral blood.

was also inhibited by IL-17 (Figures 5I and 5J). The OVA-challenged mice treated with IL-17 antibody exhibited a more serious inflammatory response compared with the OVA group (Figure 5H, bar 6 versus 4).

Taken together, these results indicate that IL-17 inhibition of eosinophil differentiation in bone marrow likely contributes to its overall protection of systemic and local airway inflammation and goblet cell hyperplasia.

Discussion

The roles of IL-17 in allergic asthma are controversial, and the mechanisms underlying these functions are incompletely understood. IL-17 is known to play important roles in mediating AHR and airway inflammation. Levels of IL-17 were significantly increased in the asthmatic patients with bronchial hyperreactivity compared to healthy subjects (30), but the

results in animal models are not always consistent. In one study (21), although IL-17 is essential during antigen sensitization to establish allergic asthma, in sensitized mice it attenuates the allergic response possibly by inhibiting dendritic cells and chemokine synthesis. The effect of IL-17 in the modulation of airway inflammation is also deemed to be dependent on the dosage (22). In this study, we clearly demonstrated that IL-17 inhibited eosinophil differentiation from bone marrow precursors, accompanied by decreased expression of *Gata-1*, *Gata-2*, and *Ccr3*, but not via modulation of the levels of IL-3, IL-5, and GM-CSF. We also found that IL-17 decreased the formation of EoPs and GMPs in bone marrow without affecting the viability of BmEos *in vivo*. In addition, IL-17 had no effect on the survival and secretory function of eosinophils *in vitro*. Thus, the present study provides a heretofore uncharacterized mechanism of IL-17 in the suppression of eosinophil differentiation with respect to its overall

protective effect in the mouse model of allergic asthma.

In our study, IL-17 significantly reduced the generation of eosinophils *in vivo* and *in vitro*. The transcription factors, *Gata-1* and *Gata-2*, are known to play important roles in the regulation of eosinophil differentiation. Targeted deletion of a high-affinity GATA-binding site in the *Gata-1* promoter leads to selective loss of the eosinophil lineage *in vivo* (31), and mice lacking the ability to produce eosinophils show significant declines of airway remodeling and AHR in chronic asthma (32). *Gata-1* expression exclusively promotes development and terminal maturation of eosinophils, and *Gata-2* also has a beneficial effect comparable to *Gata-1* *in vitro*, and efficiently compensates for *Gata-1* deficiency in terms of eosinophil development *in vivo*, clearly indicating that *Gata-1* and *Gata-2* are both critical in the regulation of eosinophil differentiation and function (4). In this study, we found that IL-17 significantly inhibited the expression of *Gata-1* and *Gata-2* in bone marrow from OVA-treated mice *in vivo*, and in liquid cultures *in vitro*. We have also recently shown that eotaxin binding to *Ccr3* triggers a positive signal to enhance and promote the differentiation of CD34⁺ progenitors to eosinophils in the context of allergic asthma (9), indicating that *Ccr3* signaling and up-regulation of *Ccr3* expression are not only crucial for the migration, but also for the eosinophil differentiation in asthmatic mice. Apparently, the protective effect of IL-17 against allergic airway inflammation and the inhibition of eosinophil differentiation by exogenous IL-17 may be due, in part, to decreased *Gata-1*, *Gata-2*, and *Ccr3* expression. However, these data cannot rule out other possibilities, such as that IL-17 regulates eosinophil differentiation through other pathways that eventually results in decreased numbers of eosinophils, and the decreased *Gata* and *Ccr3* expression is just a marker of down-regulated eosinophils in this case. Nonetheless, the detailed molecular pathways that mediate the IL-17 function in eosinophil differentiation warrant further study.

The elevated concentrations of endogenous IL-17 induced by allergen have been found in the serum, sputum, and BALF of patients with asthma, and the IL-17 levels at these sites positively correlates with asthma severity in these patients (16, 33).

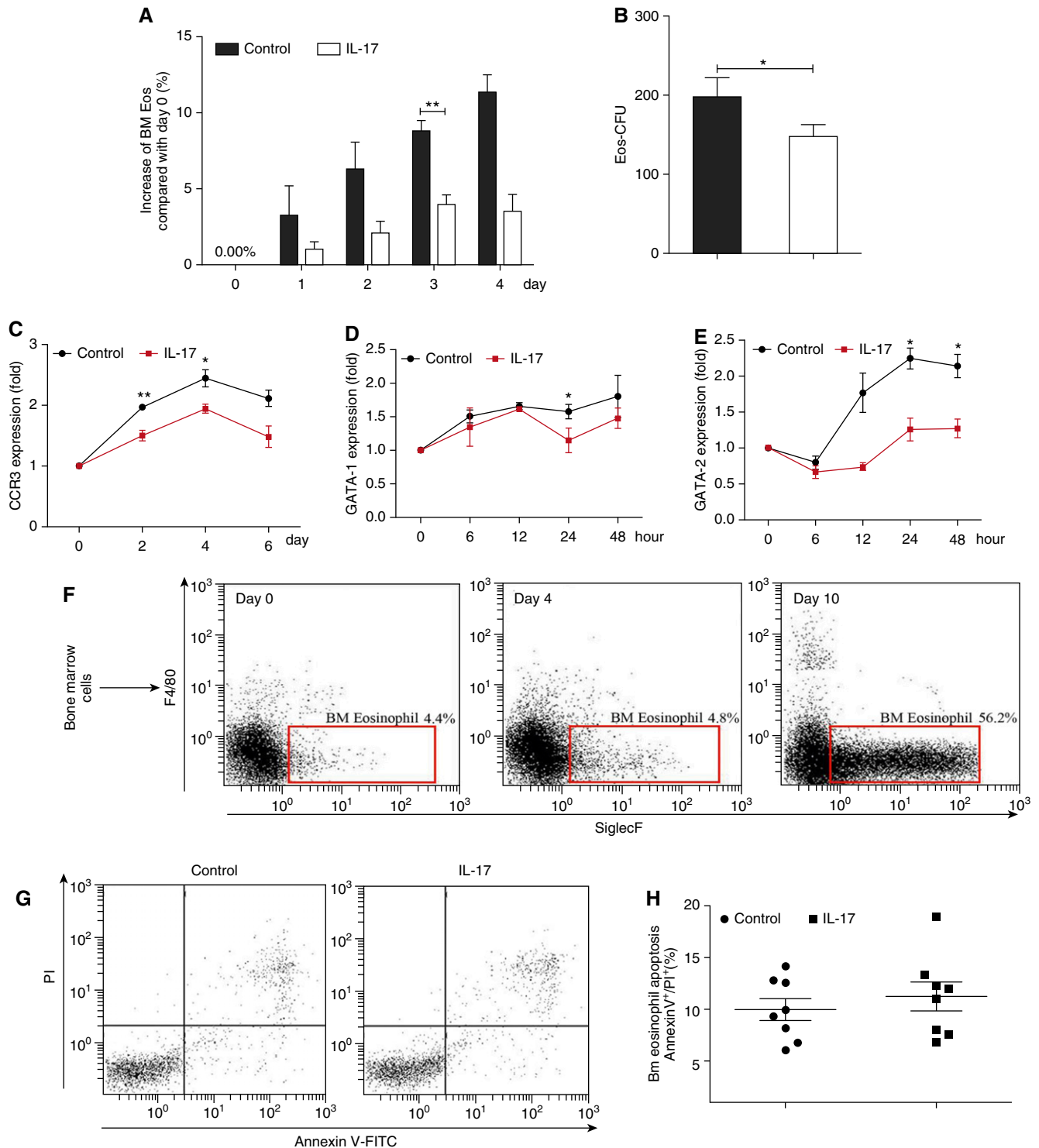


Figure 4. IL-17 inhibits the differentiation without modifying the viability and function of bone marrow eosinophils *in vitro*. Eosinophils were generated from bone marrow cells *in vitro* as shown in MATERIALS AND METHODS. (A) Percentages of eosinophil among 400 cells on Days 0, 1, 2, 3, and 4 in culture. (B) Numbers of eosinophil colony-forming units (Eos-CFUs) on Day 7 in culture. (C–E) Expression of mRNA encoding *Ccr3* (C), *Gata-1* (D), and *Gata-2* (E) were assessed by qRT-PCR. The mRNA levels in untreated cells (Day 0) were adjusted to 1 and served as the reference values. (F) Eosinophils (SiglecF⁺ and F4/80⁺) generated from bone marrow. (G and H) Viability analyzed by FACS (annexin V-FITC/propidium iodide [PI] staining) based on gating of eosinophils. Data are mean $\pm$ SEM ($n = 8$ per group). * $P < 0.05$, ** $P < 0.01$.

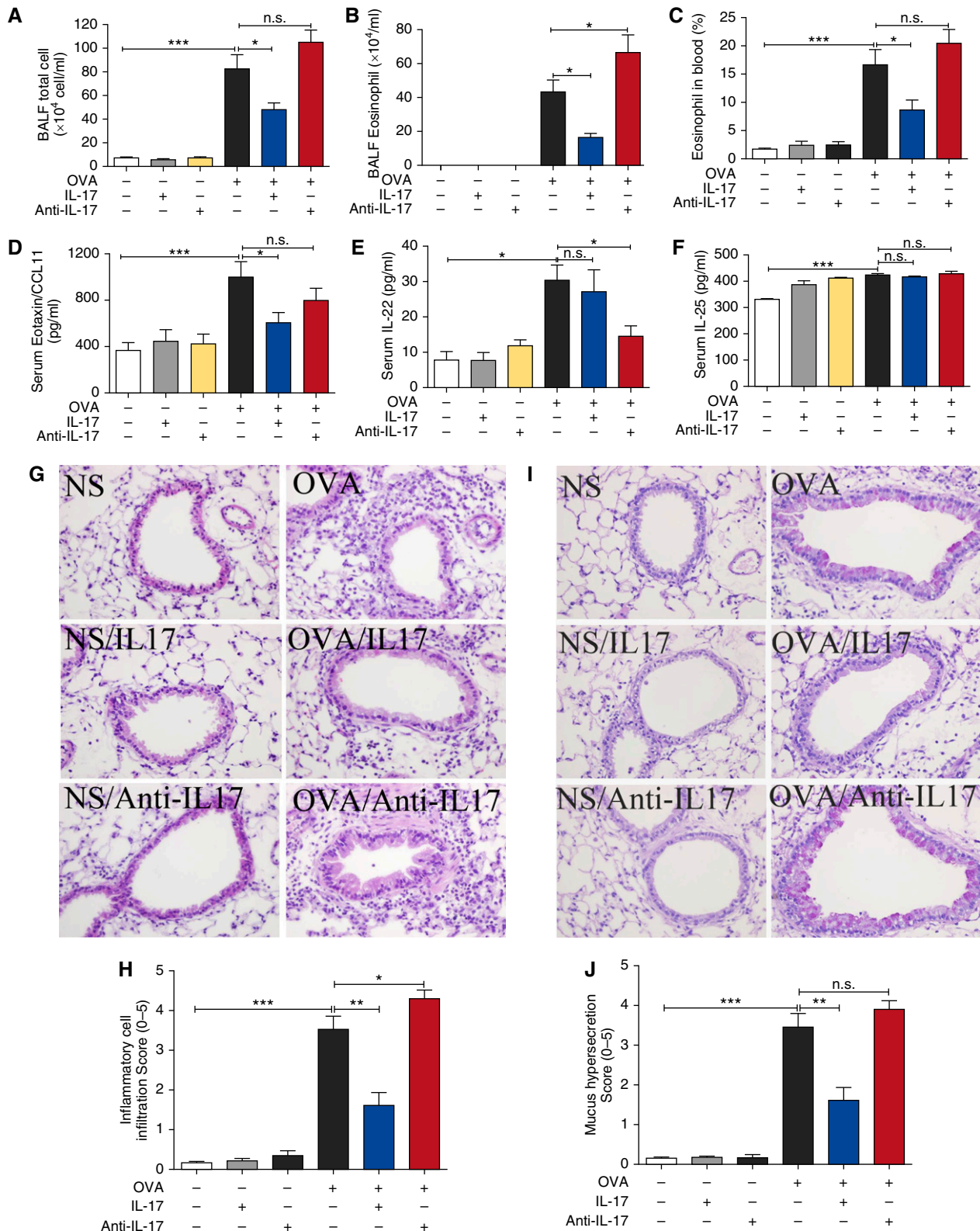


Figure 5. Exogenous IL-17 reduces eosinophilic inflammation in asthmatic mice. Experimental mice were sensitized and challenged with OVA and treated intratracheally with 40 μ l saline containing 100 ng IL-17 (OVA/IL-17) or 100 ng neutralizing IL-17 antibody (OVA/anti-IL-17) 1 hour before each OVA challenge. Control mice were challenged with saline alone and received IL-17 (NS/IL-17) or anti-IL-17 (NS/anti-IL-17) in an equal dosage and volume at each treatment time point. At 24 hours after the final OVA challenge, bronchoalveolar lavage fluid (BALF) cells, sera, and lung sections were harvested. Total BALF cells (A), BALF

In a mouse model of allergic asthma, overexpression of IL-17 was also confirmed to promote allergic inflammation (34). These findings indicate that endogenous IL-17 exerts specific proinflammatory effects. In our studies, however, IL-17 neutralization resulted in little difference in BmEos generation and *Gata* and *Ccr3* expression, indicating the limited effect of endogenous IL-17 in eosinophil differentiation.

IL-17 acts on a broad range of cells to induce the expression of proinflammatory cytokines, such as GM-CSF or granulocyte colony-stimulating factor, and these two cytokines are considered to be closely associated with neutrophils formation (35). It has also been apparent that IL-17 and GM-CSF favor recruitment, activation, and prolonged survival of neutrophils at inflammatory sites (36). Besides, evidence indicates that, in response to overexpressed IL-17, there is a neutrophilic influx into the lung, which subsequently contributes to the inflammatory responses and bronchial hyperresponsiveness in asthma (36, 37). In our current study, neutralization of IL-17 strongly inhibited GM-CSF expression, confirming the links between IL-17 and GM-CSF in inflammatory response. However, this could not explain why exogenous IL-17 suppresses eosinophil differentiation. These studies, taken together, indicate that IL-17 could target different inflammatory cells, and may cause various effects; for example, IL-17 is likely to promote inflammation in the neutrophilic context.

Cytokines indispensable for the survival, expansion, and terminal differentiation of eosinophils from EoPs include IL-3, IL-5, and GM-CSF, which bind to receptors that share a common β chain and have unique α chains (38). IL-3 is a major stimulus for mast cell and basophil production and activation (39), and GM-CSF was first described as a hematopoietic cytokine promoting proliferation and differentiation of macrophages, granulocytes, and dendritic cells from precursors (40). However, IL-3, IL-5, and GM-CSF have been discovered to be the only cytokines known to stimulate eosinophil differentiation. Of these cytokines, IL-5 plays the most critical role,

and is involved in the differentiation, migration, activation, and survival of eosinophils (41). As for differentiation, IL-5 is responsible for selective activity, and this has been clearly demonstrated in animal models. IL-5 does not induce commitment to the eosinophil lineage, but rather is an enhancer of the differentiation and proliferation of EoPs (42). It is noteworthy that IL-17 inhibited the differentiation of eosinophils, but without significant influence on serum IL-3, IL-5, and GM-CSF expression in asthmatic mice, and it even slightly increased the levels of these cytokines in NJ.1638 mice, clearly suggesting that the inhibitory effect of IL-17 in allergic inflammation is independent of IL-3, IL-5, and GM-CSF expression. We have also previously shown that budesonide inhibits allergen-induced eosinophil production and mobilization from the bone marrow and subsequent trafficking through peripheral blood into the airways, but has no appreciable effect on the local and systemic levels of IL-5 (43). Together, these data strongly suggested that targeting the inhibition of eosinophil differentiation independent of IL-5 might be an effective approach to controlling allergic airway inflammation.

Like other blood cells, eosinophils are derived from hematopoietic stem cells, which are pluripotent and give rise to all blood cell types from myeloid and lymphoid lineages. EoPs differentiate from GMPs in response to IL-3, IL-5, and GM-CSF (44). Here, we found that exogenous IL-17 decreased the number of EoPs and GMPs, which may partially explain the fewer mature BmEos and the reduced eosinophil accumulation in blood and inflamed tissue. However, neutralization of endogenous IL-17 resulted in a significant loss of GMPs, which may indicate that endogenous, but not exogenous, IL-17 is involved in the formation of cell types downstream of GMPs, and the different sources of IL-17 brought about the different effects.

Recruitment of inflammatory cells into tissue is orchestrated by the coordinated induction of chemokines. Increased eotaxin/CCL11 mRNA expression has been found both in bronchial biopsies and in cells from BALF of subjects with asthma, and this

chemokine also exerts a functional role in promoting airway inflammation in an animal model (45). Unlike other CC chemokines, eotaxin/CCL11 selectively recruits eosinophils and other CCR3 receptor-bearing cells, which potentially makes it a central chemokine in asthma pathogenesis. We suggest that exogenous IL-17 reduced serum eotaxin/CCL11, and that this might partially contribute to attenuated eosinophilic inflammation. However, IL-17 has been reported to induce the synthesis and release of eotaxin/CCL11 in human primary airway smooth muscle cells (45), and the mechanisms mediating these different observations remain unknown currently. The different models (airway smooth muscle cells versus mice) may be partially responsible for the opposite results.

Aside from the inhibition of differentiation, up-regulation of eosinophil apoptosis by IL-17 could contribute to the decreased eosinophils in bone marrow. However, our findings indicate that IL-17 had no effect on the survival of BmEos *in vivo* and *in vitro*, which further supports the hypothesis that the inhibition of eosinophil formation by IL-17 is due to inhibition of eosinophil differentiation, but not to an increase of cell death. Our findings are consistent with the conclusions of Cheung and colleagues (19), who reported that IL-17 has no significant influence on eosinophil viability in human blood-derived eosinophils.

Eosinophils are known to produce and release a variety of cytokines, chemokines, and cytotoxic proteins to exert proinflammatory effects, including up-regulation of adhesion systems, modulation of cellular trafficking and activation, and regulation of vascular permeability, mucus secretion, and smooth muscle constriction (46). In this study, we found that, in the presence of IL-17, the secretion of IL-4, IL-13, IFN- γ , and IL-10 by eosinophils did not change, further indicating that the protective role of IL-17 in airway inflammation is not dependent on restraining eosinophil functions, or at least the function of cytokine secretion.

Finally, we investigated the overall effect of IL-17 on allergen-induced eosinophilic airway inflammation, and have

Figure 5. (Continued). eosinophils (B), and percentage of peripheral blood eosinophils (C) were assessed according to Wright-Giemsa staining. Concentration of eotaxin/CCL11 (C), IL-22 (D), and IL-25 (F) in serum, as detected by ELISA. (G and I) Representative photomicrographs showing lung inflammation (hematoxylin and eosin and periodic acid–Schiff staining), respectively. Scores for inflammatory cell infiltration (H) and mucus hypersecretion (J). Data are expressed as the mean $\pm$ SEM of individual groups of mice from three independent experiments ($n = 8$ –10 mice per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n.s., not significant.

indicated the protective role in AHR, inflammatory response, and mucus production. Similarly, the studies using IL-17 neutralization conducted by Barlow and colleagues (47) show that the protective effects of IL-17 in inflammation and AHR are likely dependent on the suppression of IL-25 and, consequently, IL-13. However, they failed to observe the obvious changes of thymus and activation-regulated chemokine/CCL17 after IL-17 neutralization, which is supposed by Schnyder-Candrian and colleagues (21) to be the possible mechanism involved in the protective role of IL-17 in established asthma. Besides, at lower doses, IL-17 promotes recruitment of numerous eosinophils and augments airway inflammation; at higher doses, IL-17 negatively regulates AHR, airway inflammation, and chemokine expression (22). IL-17 produced by CD4⁺ T cells positively regulates Th2-driven AHR and airway eosinophilia (12), and that produced by $\alpha\beta$ T cells could drive AHR in mice and enhance mouse and human airway smooth muscle contraction (48). However, one study also indicates that adoptive transfer of IL-17-producing $\gamma\delta$ T cells into mice at the peak of acute allergic responses ameliorates AHR and enhances the resolution of eosinophil infiltration (49). These findings indicate that IL-17, which from the different sources or in the different inflammatory conditions, could cause the various effects. In addition, the confused effects of IL-17 could possibly

attribute to the mouse strains or the specific method of antigen sensitization (13, 50).

Neutralization of endogenous IL-17 increases BAL eosinophil count, suggesting that endogenous IL-17 also performs specific roles in BAL eosinophil infiltration.

We observed overexpression of IL-22 and IL-25 in airway inflammation, which is consistent with previous findings (51, 52). The systemic administration of IL-25 results in eosinophilia through the production of IL-5 (53), and administration of anti-IL-25 monoclonal antibody during the sensitization phase leads to significantly reduced inflammatory response, mucus production, and AHR (54). Mechanically, IL-25 enhances antigen-induced allergic airway inflammation by amplifying a Th2 cell-dependent pathway (51). In this study, the treatment on asthmatic mice with IL-17 or IL-17 neutralizing antibody exerts no influence on IL-25 level, suggesting little relevance between IL-17 and IL-25 in the current study. In line with increased levels of IL-17 in the lungs of subjects with severe asthma, the plasma concentrations of IL-17 and IL-22 tended to increase with disease severity (55). IL-22 has both proinflammatory and protective properties, but the dual effects are not yet fully understood. IL-22, which is produced by Th17 cells during allergic airway inflammation, inhibits allergic airway inflammation in the effector course by altering the function of dendritic cells and inhibiting IL-25 production from lung epithelial cells, subsequently performs as

a protective role (52). We found that neutralization of endogenous IL-17, but not the treatment of exogenous IL-17, reduced the IL-22 level, suggesting that endogenous IL-17 performs as the upstream proinflammatory factor for IL-22. This also confirms the favorable effect of IL-22 in allergic airway inflammation, because of the more serious inflammation in IL-17 antibody-treated, OVA-challenged mice.

In summary, the present study represents an initial effort to demonstrate that exogenous IL-17 attenuates airway hyperresponsiveness and airway inflammation in allergic asthma, most likely through inhibition of eosinophil differentiation followed by decreased eosinophil recruitment to inflamed tissue, rather than down-regulation of the survival and cytokine secretion of eosinophils. This study also re-emphasizes the importance of targeting the inhibition of eosinophil differentiation in allergic inflammation, which may lead to new therapeutic targets for asthma. ■

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