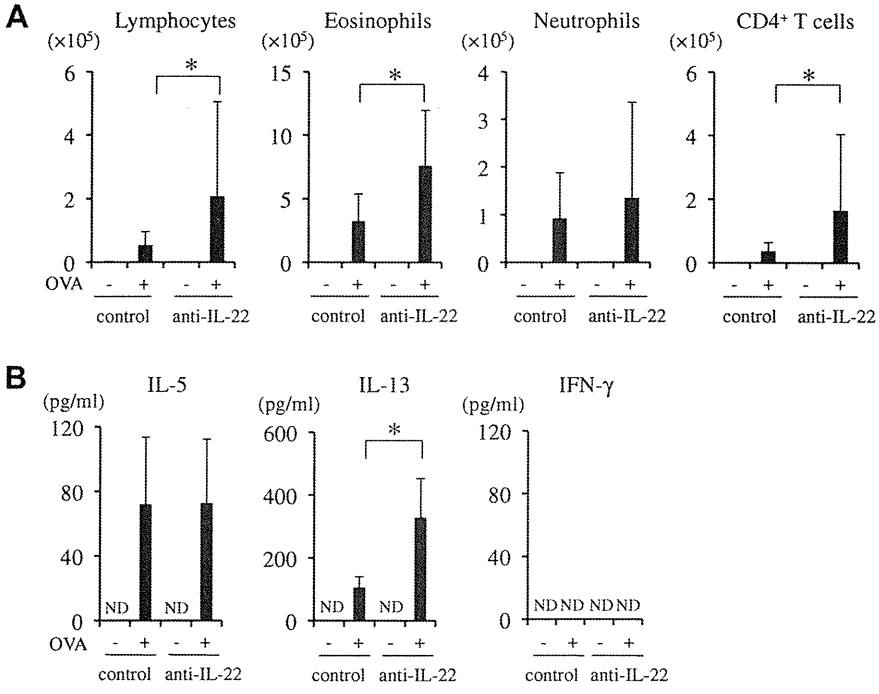

FIG 2. Anti–IL-22 antibody enhances antigen-induced eosinophil and $CD4^+$ T-cell recruitment into the airways. OVA-sensitized BALB/c mice were injected intraperitoneally with polyclonal anti–IL-22 antibody (20 μg per mouse) or control antibody. Twenty-four hours later, the mice were challenged with inhaled OVA or saline. **A**, Numbers of lymphocytes, eosinophils, neutrophils, and $CD4^+$ T cells in BALF were evaluated at 48 hours after OVA inhalation. Data are presented as means ± SDs for 10 mice in each group. $*P < .05$. **B**, Levels of IL-5, IL-13, and IFN-γ in BALF were evaluated by means of ELISA at 48 hours after OVA inhalation. Data are presented as means ± SDs for 10 mice in each group. *ND*, Not determined. $*P < .05$.

indicate that endogenously produced IL-22 inhibits the development of antigen-induced eosinophil and $CD4^+$ T-cell recruitment into the airways, goblet cell hyperplasia, and AHR.

Recombinant IL-22 inhibits antigen-induced eosinophil recruitment into the airways

We next examined the effect of IL-22 on the development of antigen-induced airway inflammation. Recombinant IL-22 (0.1 μg per mouse) or saline was administrated to OVA-sensitized mice intranasally at 48 hours and 2 hours before OVA inhalation, and airway inflammation was evaluated at 48 hours after OVA inhalation. As shown in Fig 4, *A*, the intranasal administration of IL-22 inhibited antigen-induced recruitment of eosinophils and $CD4^+$ T cells but not that of neutrophils into the airways. Although the difference did not reach statistical significance, intranasal administration of IL-22 also tended to inhibit antigen-induced IL-5 and IL-13 production in the airways (Fig 4, *B*) and airway responsiveness to acetylcholine (see Fig E2 in this article's Online Repository at www.jacionline.org). These results further indicate the inhibitory effects of IL-22 on the development of antigen-induced airway inflammation.

We also examined the effect of IL-22 on the resolution phase of allergic airway inflammation. In this experiment OVA-sensitized mice were challenged with OVA inhalation 3 times at a 48-hour interval, and IL-22 or saline was administered intranasally to the mice 3 times at 24, 48, and 72 hours after the last OVA inhalation. IL-22–treated mice exhibited significantly low numbers of eosinophils and lymphocytes and low levels of IL-13 in BALF (see Fig E3 in this article's Online Repository at www.jacionline.org). These results suggest that IL-22 is able to accelerate the resolution of allergic airway inflammation in mice.

Lung epithelial cells express functional IL-22 receptors

To address the mechanisms by which IL-22 attenuates antigen-induced airway inflammation, we searched possible IL-22–responding cells in patients with allergic airway inflammation. The functional IL-22 receptor is a heterodimer of IL-22R1 and IL-10R2,[12,13] and previous studies have shown that IL-10R2 is ubiquitously expressed in various cells but that the expression of IL-22R1 is restricted to nonimmune cells.[12,13,20] We first examined the expression of IL-22R1 in the lung by means of immunostaining with anti–IL-22R1 antibody. The expression of IL-22R1 was restricted to the luminal side of the epithelial cells in the lung (Fig 5, *A*). We also examined the expression of IL-22R1 and IL-10R2 mRNA in several cell types by means of qPCR analysis. IL-10R2 was expressed at moderate levels in $CD4^+$ T cells, $CD8^+$ T cells, and alveolar macrophages in the BALF and a lung epithelial cell line (MLE-15 cell), which shows the characteristics of the distal bronchiolar and alveolar epithelium,[34] and at high levels in BMDCs (Fig 5, *B*). In contrast, IL-22R1 was expressed in MLE-15 cells but not in $CD4^+$ T cells, $CD8^+$ T cells, or alveolar macrophages in BALF or BMDCs (Fig 5, *B*).

We further examined whether IL-22 transduced its signal in MLE-15 cells and found that IL-22, as well as IL-6, phosphorylated STAT3, a major signal transducer of IL-22,[43] in MLE-15 cells (Fig 5, *C*). IL-22 also induced the expression of suppressor of cytokine signaling 3 (SOCS3), one of the target genes of

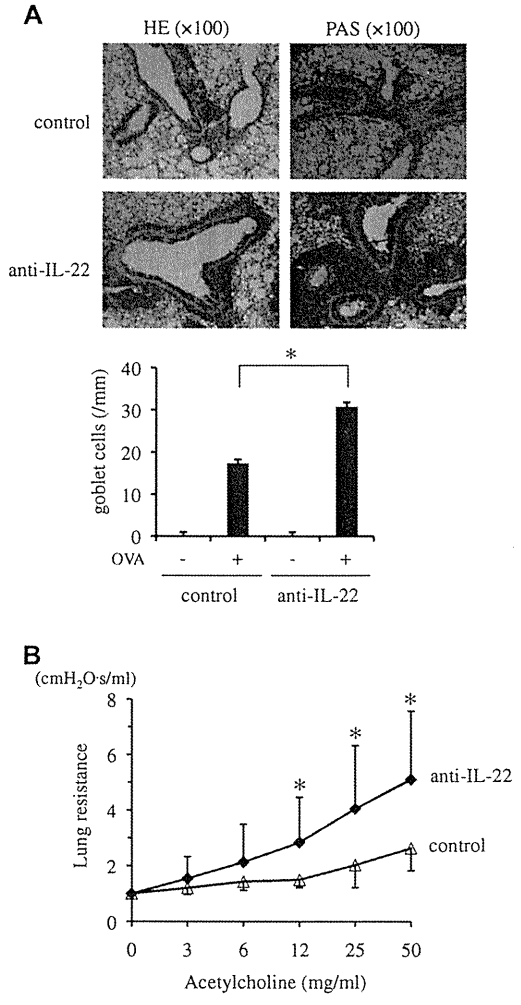

FIG 3. Anti–IL-22 antibody enhances antigen-induced goblet cell hyperplasia and AHR. OVA-sensitized BALB/c mice were injected intraperitoneally with polyclonal anti–IL-22 antibody or control antibody. Twenty-four hours later, the mice were challenged with inhaled OVA 3 times at a 48-hour interval. **A,** Histologic analysis of the lung was performed with hematoxylin and eosin *(HE)* and PAS staining at 48 hours after the final OVA inhalation. Representative photomicrographs of lung sections and the number of goblet cells in the airways are shown. Data are presented as means ± SDs for 5 mice in each group. $*P < .05$. **B,** Airway resistance to acetylcholine was measured at 24 hours after the final OVA inhalation by using the flexiVent system, as described in the Methods section. Data are presented as means ± SDs for 10 mice in each group. $*P < .05$.

STAT3 signaling, in MLE-15 cells (see Fig E4 in this article's Online Repository at www.jacionline.org), indicating that MLE-15 cells express functional IL-22 receptor complexes and signaling molecules. Although it is still possible that other cell types could respond to IL-22, these results suggest that the inhibitory effects of IL-22 on the development of antigen-induced allergic airway inflammation seem to be mediated by lung epithelial cells.

Anti–IL-22 antibody enhances antigen-induced IL-25 production in the airways

To further address the mechanisms of IL-22–mediated inhibition of antigen-induced airway inflammation, we examined the expression levels of chemokines in the lungs of OVA-sensitized mice after OVA or saline inhalation with or without IL-22 neutralization. However, the expression levels of eotaxin-1 and thymus and activation-regulated chemokine (TARC) in lung tissue, which seem to play important roles in allergic airway inflammation,[44,45] were not significantly enhanced in mice treated with anti–IL-22 antibody (Fig 6, *A*).

We then examined levels of epithelial cell–derived cytokines that promote T_H2-type immune responses, such as TSLP, IL-25, and IL-33,[46,47] in BALF of OVA-sensitized mice after OVA or saline inhalation with or without IL-22 neutralization. Importantly, the levels of IL-25 in BALF were significantly enhanced in mice treated with anti–IL-22 antibody ($n = 5$, $P < .05$; Fig 6, *B*). In contrast, the levels of TLSP and IL-33 were very low, even after OVA inhalation, and anti–IL-22 antibody did not significantly affect the levels of TLSP and IL-33 (Fig 6, *B*).

IL-22 inhibits IL-25 expression in MLE-15 cells

We also examined the effect of IL-22 on the expression of IL-25 in MLE-15 cells at mRNA levels. As shown in Fig 6, *C*, both IL-1β and LPS induced the expression of IL-25 in MLE-15 cells. IL-13 itself did not induce the expression of IL-25 but enhanced the expression of IL-25 in IL-1β– or LPS-stimulated MLE-15 cells (Fig 6, *C*). Importantly, although IL-22 did not significantly inhibit IL-1β– or LPS-induced expression of IL-25 in MLE-15 cells, IL-22 inhibited IL-13–mediated enhancement of IL-25 expression in IL-1β– or LPS-stimulated MLE-15 cells (Fig 6, *C*). On the other hand, although a previous report has shown that IL-17A reverses the protective effects of IL-22 on lung epithelial cells,[48] IL-17A did not significantly affect the inhibitory effect of IL-22 on the expression of IL-25 in MLE-15 cells (data not shown).

Anti–IL-25 antibody suppresses anti–IL-22 antibody–mediated enhancement of antigen-induced eosinophil recruitment into the airways

To determine whether IL-25 is involved in the enhancement of allergic airway inflammation in anti–IL-22 antibody–treated mice, we finally examined the effect of anti–IL-25 antibody on antigen-induced airway inflammation with or without administration of anti–IL-22 antibody. As shown in Fig 7, administration of anti–IL-25 antibody (20 μg per mouse) suppressed anti–IL-22 antibody–mediated enhancement of antigen-induced eosinophil and lymphocyte recruitment into the airways ($n = 5$, $P < .05$). Furthermore, although the difference did not reach significance, anti–IL-25 antibody tended to decrease anti–IL-22 antibody–mediated enhancement of IL-13 production in the airways (see Fig E5 in this article's Online Repository at www.jacionline.org). Taken together, these results suggest that IL-22 attenuates the development of antigen-induced allergic inflammation by inhibiting IL-25 production from lung epithelial cells in the T_H2 environment.

DISCUSSION

In this study we show that IL-22 is produced by $CD4^+$ T cells in the effector phase of allergic airway inflammation and attenuates T_H2 cell–mediated airway inflammation, possibly by inhibiting IL-25 production from lung epithelial cells.

We show that $CD4^+$ T cells are major IL-22–producing cells at the site of allergic airway inflammation (Fig 1). Consistent with previous studies showing that IL-22 is detected in lungs on

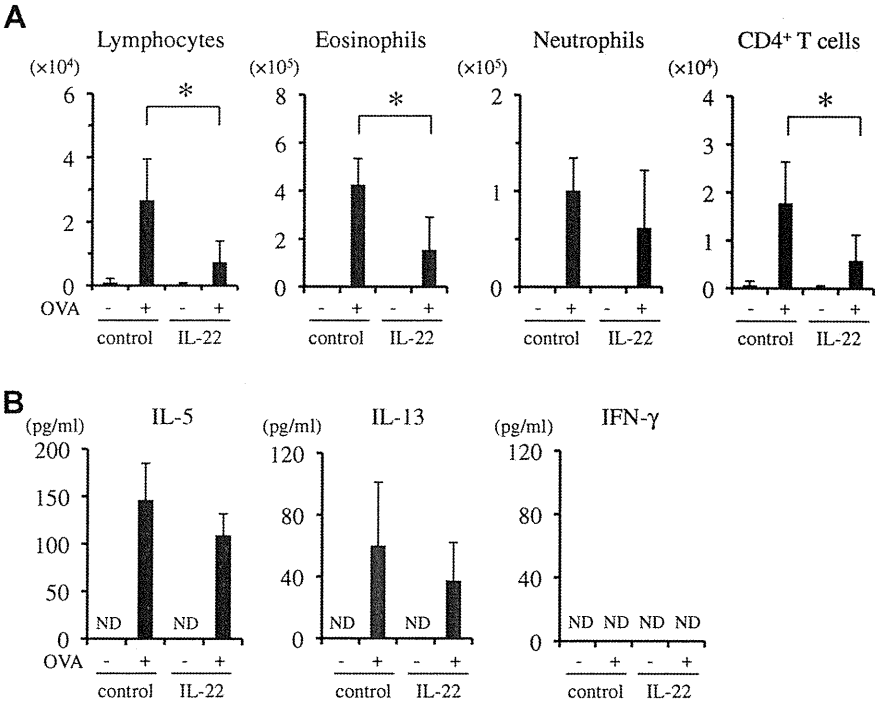

FIG 4. Intranasal administration of IL-22 attenuates antigen-induced eosinophil recruitment into the airways. Recombinant IL-22 (0.1 μg per mouse) or saline was administrated intranasally to OVA-sensitized BALB/c mice twice at 48 hours and 2 hours before the inhaled OVA challenge. Mice were challenged with OVA or saline inhalation, and at 48 hours after inhalation, numbers of lymphocytes, eosinophils, neutrophils, and $CD4^+$ T cells **(A)** and levels of IL-5, IL-13, and IFN-γ **(B)** in BALF were evaluated. Data are presented as means ± SDs for 4 mice in each group. *ND*, Not determined. $*P < .05$.

antigen challenge,[10,11,29] we found that $CD4^+$ T cells recovered from the airways of sensitized mice after antigen inhalation produced IL-22 on stimulation (Fig 1, *B*). In addition, we found that although the frequency of cells expressing IL-22 was very low in the airways, approximately 80% of signals with anti–IL-22 staining were colocalized with $CD4^+$ cells (Fig 1, *E*). On the other hand, although previous reports have shown that NK,[17,26,41] $CD11c^+$ myeloid,[40] and LTi-like cells[19] produce IL-22, we found that $DX5^+$, $CD11c^+$, or $CD4^+CD3\epsilon^-$ cells in the lung did not express IL-22 in allergic airway inflammation (see Fig E1). Taken together, these results suggest that $CD4^+$ T cells are a major source for IL-22 in patients with allergic airway inflammation.

We also show that T_H17 cells could produce IL-22 in allergic airway inflammation (Fig 1, *C*). Among $CD4^+$ T-cell subsets, T_H1 and T_H17 cells have been shown to be potent IL-22–producing cells.[9,12,13] In addition, recent studies have shown that skin-homing $CCR10^+$ IL-22–producing T cells (T_H22 cells) show a stable and distinct phenotype from T_H1, T_H2, and T_H17 cells and play a role in chronic inflammatory skin disease in human subjects.[14-16,23] In a murine model of asthma we found that one third of IL-22–producing $CD4^+$ T cells in the airways were also positive for intracellular IL-17A staining (Fig 1, *C*) and that approximately 20% of IL-22–producing $CD4^+$ T cells expressed CCR6 (Fig 1, *D*), suggesting that part of lung-infiltrating T_H17 cells have a potential to produce IL-22 on stimulation. In contrast, IL-22–producing $CD4^+$ T cells did not express IFN-γ (Fig 1, *C*), CCR5, or CCR10 (Fig 1, *D*), suggesting that T_H1 cells and T_H22 cells are not responsible for IL-22 production in patients with allergic airway inflammation.

We show that IL-22 inhibits allergic airway inflammation in the effector phase. We found that intranasal administration of IL-22 attenuated antigen-induced eosinophil and $CD4^+$ T-cell recruitment into the airways (Fig 4). We also found that the neutralization of IL-22 in the effector phase enhanced antigen-induced eosinophil and $CD4^+$ T-cell recruitment and IL-13 production in the airways (Fig 2) and AHR (Fig 3), which is consistent with the results of recent studies.[10,29] These results indicate that endogenously produced IL-22 inhibits antigen-induced allergic inflammation in the effector phase. Importantly, we also found that the administration of IL-22 accelerated the resolution of antigen-induced eosinophilic inflammation in the airways (see Fig E3), suggesting that inhaled IL-22 might have the therapeutic potential for asthma.

Regarding the mechanisms by which IL-22 inhibits allergic airway inflammation, it has been shown that the alteration of dendritic cell function by IL-22 is responsible for the inhibition of allergic inflammation.[10] On the other hand, consistent with a previous report,[20] we found that IL-22R1 was expressed on lung epithelial cells (Fig 5, *A* and *B*) but not on immune cells, including BMDCs (Fig 5, *B*). We also found that IL-22 phosphorylated STAT3 in a lung epithelial cell line (MLE-15 cells; Fig 5, *C*) but not in BMDCs (data not shown), suggesting that dendritic cells might not be a direct target of IL-22 in allergic airway inflammation.

Importantly, we found that anti–IL-22 antibody enhanced antigen-induced production of IL-25, an epithelial cell–derived cytokine that enhances T_H2-type immune responses,[30-32,46,47] in the airways (Fig 6, *B*). In addition, we found that IL-22 inhibited

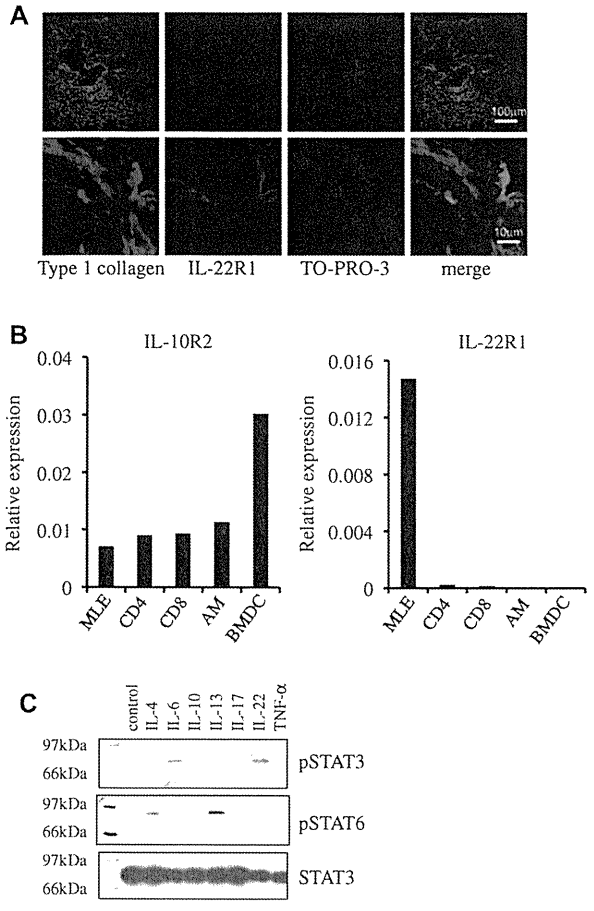

FIG 5. Lung epithelial cells express functional IL-22 receptors. **A**, Lung sections of OVA-sensitized, OVA-inhaled BALB/c mice were stained with anti–IL-22R1 antibody and anti–type 1 collagen antibody. The nuclei were counterstained with TO-PRO-3 iodide. Representative photomicrographs from 3 independent experiments are shown. **B**, $CD4^+$ T cells, $CD8^+$ T cells, and alveolar macrophages *(AM)* were purified from BALF at 48 hours after OVA inhalation. BMDCs were prepared as described in the Methods section. cDNA was prepared from $CD4^+$ T cells, $CD8^+$ T cells, alveolar macrophages, BMDCs, and MLE-15 cells, and expression levels of IL-10R2, and IL-22R1 mRNA were analyzed by means of qPCR analysis. Shown are representative data of 4 independent experiments. **C**, MLE-15 cells were stimulated with indicated cytokines (20 ng/mL) for 15 minutes, and cell lysates were subjected to immunoblotting with anti-phospho-STAT3 *(pSTAT3)* antibody, anti-phospho-STAT6 *(pSTAT6)* antibody, or anti-STAT3 antibody. Shown are representative data of 3 independent experiments.

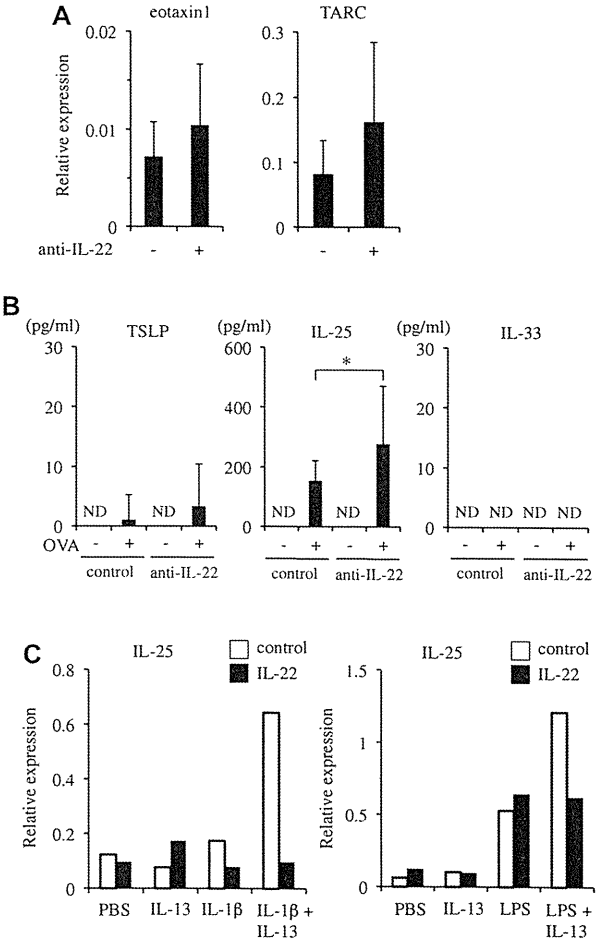

FIG 6. Anti–IL-22 antibody enhances antigen-induced IL-25 production in the airways. **A** and **B**, OVA-sensitized BALB/c mice were injected intraperitoneally with anti–IL-22 antibody or control antibody. Twenty-four hours later, the mice were challenged with inhaled OVA or saline. Fig 6, *A*, Expression of eotaxin-1 and TARC mRNA in the lung tissue was evaluated at 48 hours after OVA inhalation determined by mean of qPCR analysis. Data are presented as means ± SDs for 5 mice in each group. Fig 6, *B*, Levels of TSLP, IL-25, and IL-33 in BALF were evaluated by means of ELISA at 48 hours after OVA inhalation. Data are presented as means ± SDs for 5 mice in each group. *ND*, Not determined. $*P < .05$. **C**, MLE-15 cells were stimulated with IL-1β (20 ng/mL), LPS (100 ng/mL), and/or IL-13 (20 ng/mL) in the presence or absence of IL-22 (20 ng/mL) for 6 hours. The expression of IL-25 mRNA was evaluated by means of qPCR. Data shown are representative of 4 independent experiments.

IL-13–mediated enhancement of IL-25 expression in IL-1β– or LPS-stimulated MLE-15 cells (Fig 6, *C*). Moreover, we found that IL-22 induced SOCS3, which could inhibit IL-13 signaling,[49] in MLE-15 cells (see Fig E4). Furthermore, we found that anti–IL-25 antibody suppressed anti–IL-22 antibody–mediated enhancement of antigen-induced eosinophil recruitment into the airways (Fig 7). Therefore it is suggested that IL-22 attenuates T_H2 cell–mediated airway inflammation by inhibiting IL-25 production from lung epithelial cells.

Our results indicate that T_H17 cells play both promotional and inhibitory roles in the induction of allergic airway inflammation. We show here that part of T_H17 cells produce IL-22 in the airways and that IL-22 attenuates allergic airway inflammation (Figs 1 and 4 and see Fig E3). On the other hand, we have previously shown that by using adoptive transfer experiments, T_H17 cells enhance T_H2 cell–mediated allergic airway inflammation.[7] In relation to these observations, it has recently been demonstrated that IL-22 is pathological in bleomycin-induced airway inflammation in the presence of IL-17A but is tissue protective in the absence of IL-17A.[48] These findings suggest that although an overall effect of T_H17 cells on T_H2 cell–mediated allergic airway inflammation seems to be enhancing,[7] T_H17 cell–derived IL-22 might play a role in fine tuning of allergic airway inflammation.

It is also possible that the role of IL-22 in patients with allergic airway inflammation depends on the phase of immune responses. Interestingly, we found that when anti–IL-22 antibody was given to sensitized mice at 48 hours after inhaled antigen challenge, anti–IL-22 antibody did not significantly enhance allergic airway inflammation (data not shown), suggesting that endogenously produced IL-22 functions as a negative regulator of allergic

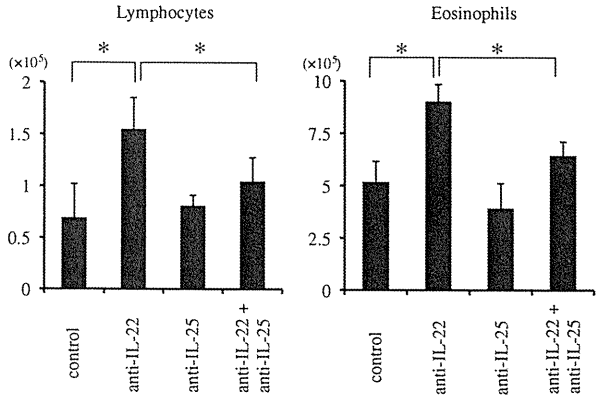

FIG 7. Anti–IL-25 antibody reverses the enhancing effect of anti–IL-22 antibody on antigen-induced airway inflammation. OVA-sensitized BALB/c mice were injected intraperitoneally with anti–IL-22 antibody, anti–IL-25 antibody, or both (20 μg per mouse) at 24 hours before OVA inhalation. Numbers of eosinophils and lymphocytes in BALF were evaluated at 48 hours after OVA inhalation. Data are presented as means ± SD for 5 mice in each group. $*P < .05$.

airway inflammation at the early stage but not at the late stage of the effector phase. On the other hand, it has recently been demonstrated that IL-22 is required for mounting allergic airway inflammation during the sensitization phase by using IL-22 knockout mice.[29] Further studies with conditional IL-22 knockout mice and IL-22 knock-in mice seem beneficial to address the complicated role of IL-22 in allergic airway inflammation.

In summary, we have shown that IL-22 is expressed by $CD4^+$ T cells at the site of allergic airway inflammation and attenuates eosinophilic airway inflammation, possibly by inhibiting IL-25 production from lung epithelial cells. Although additional studies are required for further elucidation of molecular mechanisms, our results raise the possibility that IL-22 could be used as a novel therapeutic approach for asthma.

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Key message

- **Endogenously produced IL-22 inhibits T_H2 cell–mediated allergic airway inflammation by acting on lung epithelial cells.**

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METHODS

Reagents

A murine lung epithelial cell line (MLE-15 cells, a kind gift from Dr Jeffrey Whitsett, University of Cincinnati), which was produced from lung tumors generated in transgenic mice expressing the viral oncogene SV40 large T antigen under the control of a promoter region from the human surfactant protein C gene, was grown in HITES medium, as described previously.[E1] MLE-15 cells show the characteristics of the distal bronchiolar and alveolar epithelium.[E1]

Antigen-induced allergic inflammation in the airways

BALB/c mice (aged 6-8 weeks) were immunized intraperitoneally twice with 10 μg of OVA in 4 mg of aluminum hydroxide (alum) at a 2-week interval, and 2 weeks after the second immunization, the sensitized mice were administered aerosolized OVA (50 mg/mL) dissolved in 0.9% saline for 20 minutes through a DeVilbiss 646 nebulizer (DeVilbiss Corp, Somerset, Pa).[E2]

Effect of IL-22 on antigen-induced allergic inflammation in the resolution phase

OVA-sensitized BALB/c mice were challenged with inhaled OVA 3 times at a 48-hour interval. Recombinant IL-22 (0.1 μg per mouse) was administered intranasally to the mice 3 times at 24, 48, and 72 hours after the last OVA inhalation. Numbers of eosinophils and lymphocytes and cytokine levels in BALF were evaluated at 4 hours after the last IL-22 administration.

Cytokine assay

Amounts of IL-13, IL-22, IL-33, IFN-γ, and TSLP in BALF were determined by means of ELISA, according to the manufacturer's instructions (R&D Systems). Levels of IL-5 and IL-25 in BALF were determined by using ELISA kits from BD Biosciences and BioLegend, respectively. The detection limits of these assays were 15 pg/mL for IL-5 and TSLP; 30 pg/mL for IFN-γ, IL-22, IL-25, and IL-33; and 60 pg/mL for IL-13.

Cytokine production and chemokine receptors of $CD4^+$ T cells

$CD4^+$ T cells were isolated from BALF cells, inguinal lymph node cells, or lung homogenates by means of magnetic cell sorting,[E3] according to the manufacturer's instructions (Miltenyi Biotec, Auburn, Calif). For intracellular cytokine analysis, $CD4^+$ T cells were stimulated with phorbol 12-myristate 13-acetate (20 ng/mL, Calbiochem) plus ionomycin (1 μg/mL, Calbiochem) at 37°C for 4 hours in the presence of brefeldin A (10 μmol/L, BD Bioscience). Cells were then stained with antibodies to CD4 and either CCR3 (BioLegend), CCR5 (eBioscience, San Diego, Calif), CCR6 (BioLegend), or CCR10 (eBioscience). After surface staining, cells were fixed, permeabilized, and stained by Alexa Fluor 488–conjugated anti–IL-22 antibody together with allophycocyanin-conjugated anti–IFN-γ, anti–IL-4, or anti–IL-17A antibody, as described previously.[E4] Anti–IL-22 antibody (clone MH22B2) was labeled with Monoclonal Antibody Labeling Kit (Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions.

qPCR analysis

Total RNA was prepared with ISOGEN solution (Nippon GENE, Tokyo, Japan), and reverse transcription was carried out with an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, Calif). Expression of IL-10R2, IL-22R1, SOCS3, eotaxin-1, TARC, IL-25, IL-23p19, and IL-12/IL-23p40 was determined by means of qPCR with a standard protocol on an ABI PRISM7300 instruments (Applied Biosystems, Foster City, Calif) by using a SYBER green reagent (Power SYBER Green PCR Master Mix, Applied Bioscience). Expression of IL-22 was determined by means of quantitative Taqman PCR with a standard protocol on the ABI PRISM 7300 instrument. The levels of each expression were normalized to the levels of β-actin. The sequences of PCR primers and fluorogenic probes are as follows: IL-23 p19—forward primer, ATCCAGTGTGAAGATGGTTGTGA; reverse primer, GCAAGCAGAAC TGGCTGTTG; IL-12/IL-23 p40—forward primer, TGGTTTGCCATCGTT TTGCTG; reverse primer, ACAGGTGAGGTTCACTGTTTCT; IL-10R2—forward primer, GGACGTCTCTTCCACAGCAC; reverse primer, CTGCT TGCTGCCTTCAGACT; IL-22R1—forward primer, GCTCGCTGCAGCAC ACTACCAT; reverse primer, TGAGTGTGGGGTGGACCAGCAT; SOCS3—forward primer, CCTTCAGCTCCAAAAGCCAG; reverse primer, GCTCT CCTGCAGCTTGCG; eotaxin-1—forward primer; TGTCTCCCTCCAC CATGCA; reverse primer, GATCTTCTTACTGGTCATGATAAAGCA; TARC—forward primer, TTGTGTTCGCCTGTAGTGCATA; reverse primer, CAGGAAGTTGGTGAGCTGGTATA; IL-25—forward primer, CGGAGG AGTGGCTGAAGTGGAG; reverse primer, ATGGGTACCTTCCTCGC CATG; IL-22—forward primer, TCCGAGGAGTCAGTGCTAAA; reverse primer, AGAACGTCTTCCAGGGTGAA; probe, TGAGCACCTGCTTCAT CAGGTAGCA; β-actin—forward primer, GCTCTGGCTCCTAGCACCAT; reverse primer, GCCACCGATCCACACAGAGT; probe, TCAAGATCAT TGCTCCTCCTGAGCGC.

Histologic and immunohistologic analysis

Lung sections (3 μm thick) were stained with hematoxylin and eosin and PAS according to standard protocols. The number of goblet cells was counted on PAS-stained lung sections, as described elsewhere.[E5] Immunostaining of cryosections was performed as described previously.[E6] The following antibodies were used: anti-CD4 antibody (Clone GK1.5, eBioscience), anti–IL-22 antibody (Clone 140301, R&D Systems), anti–type 1 collagen antibody (COSMO Bio, Tokyo, Japan), and anti–IL-22 receptor 1 antibody (Clone 496514, R&D Systems). The nuclei were counterstained with TO-PRO-3 iodide (Invitrogen).

Preparation of BMDCs

BMDCs were prepared as described previously.[E7] In brief, a single-cell suspension of bone marrow cells was obtained from 8-week-old BALB/c mice and cultured in complete DMEM medium containing GM-CSF (50 ng/mL) and IL-4 (20 ng/mL) for 9 days, with replacement of the medium containing cytokines every 3 days. $CD11c^+$ cells were purified by using an isolation kit from Miltenyi Biotech. The purity of collected cells was determined by means of flow cytometry, and these were routinely greater than 95% $CD11c^+$ cells.

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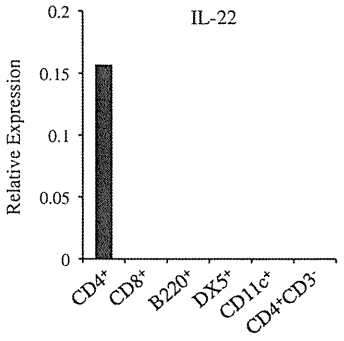

FIG E1. Lung-infiltrating $CD4^+$ cells but not $CD4^+CD3\epsilon^-$ cells express IL-22 mRNA in allergic airway inflammation. OVA-sensitized BALB/c mice were challenged with inhaled OVA. Forty-eight hours after inhalation, $CD4^+$, $CD8^+$, $B220^+$, $DX5^+$, $CD11c^+$, and $CD4^+CD3\epsilon^-$ cells were isolated from lung homogenates by means of magnetic cell sorting, and qPCR analysis for IL-22 was performed. Representative data from 4 independent experiments are shown.

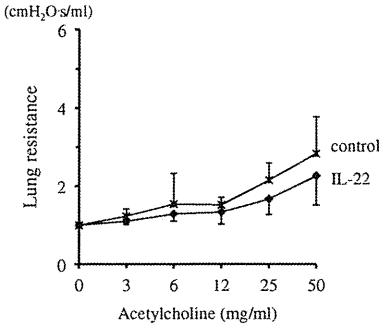

FIG E2. Intranasal administration of IL-22 tends to attenuate airway hyperreactivity. OVA-sensitized BALB/c mice were challenged with inhaled OVA 3 times at a 48-hour interval. Recombinant IL-22 (0.1 μg per mouse) or saline was administrated intranasally to the mice 2 hours before each OVA inhalation. Twenty-four hours after the last OVA inhalation, airway resistance to acetylcholine was evaluated. Data are presented as means ± SDs for 4 mice in each group.

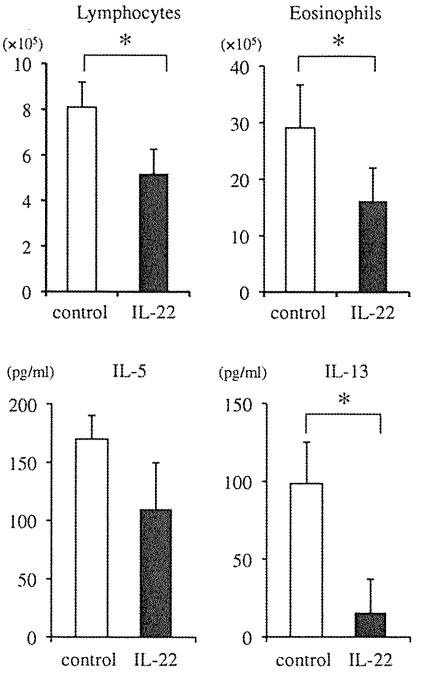

FIG E3. Intranasal administration of IL-22 accelerates the resolution of allergic airway inflammation. OVA-sensitized BALB/c mice were challenged with inhaled OVA 3 times at a 48-hour interval. IL-22 (0.1 μg per mouse) or saline was administered intranasally to the mice 3 times at 24, 48, and 72 hours after the last OVA inhalation. Four hours alter the last IL-22 administration, numbers of eosinophils and lymphocytes and levels of IL-5 and IL-13 in BALF were evaluated. Data are presented as means ± SDs (n = 5 each). *$P < .05$.

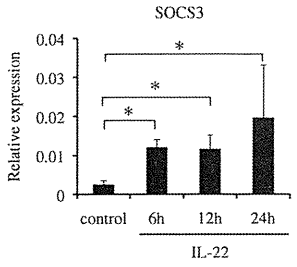

FIG E4. IL-22 induces SOCS3 mRNA in MLE-15 cells. MLE-15 cells were stimulated with IL-22 (20 ng/mL) for indicated time periods, and the expression of SOCS3 mRNA was evaluated by means of qPCR analysis. Data are presented as means ± SDs (n = 4 in each group). $*P < .05$.

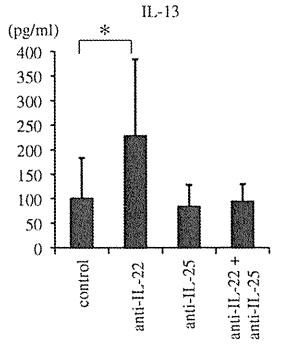

FIG E5. Anti–IL-25 antibody tends to reverse the enhancing effect of anti–IL-22 antibody on antigen-induced IL-13 production in the airways. OVA-sensitized BALB/c mice were injected intraperitoneally with anti–IL-22 antibody (20 μg per mouse), anti–IL-25 antibody (20 μg per mouse), or both at 24 hours before OVA inhalation. Levels of IL-13 in BALF were evaluated at 48 hours after OVA inhalation. Data are presented as means ± SDs (n = 5 mice per group). *$P < .05$.

Immunity
Article

Eomesodermin Controls Interleukin-5 Production in Memory T Helper 2 Cells through Inhibition of Activity of the Transcription Factor GATA3

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SUMMARY

The regulation of memory $CD4^+$ helper T (Th) cell function, such as polarized cytokine production, remains unclear. Here we show that memory T helper 2 (Th2) cells are divided into four subpopulations by CD62L and CXCR3 expression. All four subpopulations produced interleukin-4 (IL-4) and IL-13, whereas only the $CD62L^{lo}CXCR3^{lo}$ population produced IL-5 accompanied by increased H3-K4 methylation at the *Il5* gene locus. The transcription factor Eomesodermin (encoded by *Eomes*) was highly expressed in memory Th2 cells, whereas its expression was selectively downregulated in the IL-5-producing cells. *Il5* expression was enhanced in *Eomes*-deficient cells, and Eomesodermin was shown to interact with the transcription factor GATA3, preventing GATA3 binding to the *Il5* promoter. Memory Th2 cell-dependent airway inflammation was attenuated in the absence of the $CD62L^{lo}CXCR3^{lo}$ population but was enhanced by *Eomes*-deficient memory Th2 cells. Thus, IL-5 production in memory Th2 cells is regulated by Eomesodermin via the inhibition of GATA3 activity.

INTRODUCTION

Effector helper T (Th) cells can be categorized into at least three subsets: T helper 1 (Th1), Th2, and Th17 cells (O'Shea and Paul, 2010; Reiner, 2007; Zhu et al., 2010). Th1 cells produce large amounts of interferon-γ (IFN-γ) and direct cell-mediated immunity against intracellular pathogens. Th2 cells produce interleukin-4 (IL-4), IL-5, and IL-13 and are involved in humoral immunity and allergic reactions. The recently identified subset Th17 cells produce IL-17A, IL-17F, and IL-22 and are thought to contribute to certain autoimmune diseases (Dong, 2008; Korn et al., 2009).

Several transcription factors that control the differentiation and function of these Th cell subsets have been identified. Among them, GATA3 appears to be a critical transcription factor for Th2 cell differentiation (Ho et al., 2009; Zheng and Flavell, 1997), T-bet for Th1 (Szabo et al., 2003), and RORγt for Th17 (Ivanov et al., 2006). GATA3 induces chromatin remodeling at Th2 cytokine gene loci in developing Th2 cells (Ansel et al., 2006; Wilson et al., 2009) and plays an essential role in the establishment of "Th2 cell identity:" that is, the ability to produce large amounts of Th2 cytokines upon antigenic restimulation (Nakayama and Yamashita, 2008). GATA3 is also known to act as a transcriptional activator for Th2 cytokine genes, particularly for IL-5 and IL-13 (Klein-Hessling et al., 2008; Siegel et al., 1995). Th2 cell identity is maintained in memory Th2 cells for long periods in vivo (Nakayama and Yamashita, 2008). Memory Th2 cells maintain the cardinal features of Th2 cells, such as the selective production of Th2 cytokines, high-level expression of *Gata3*, and histone modifications at the Th2 cytokine gene loci via the expression of the nuclear factor mixed-lineage leukemia (MLL), a mammalian homolog of the *Drosophila* trithorax (Yamashita et al., 2006). However, the precise mechanism governing the selective production of each cytokine (IL-4, IL-13, and IL-5) in memory Th2 cells remains unclear.

Immunological memory is a hallmark of acquired immunity (Kalia et al., 2006; Lefrançois, 2006; Stockinger et al., 2006; Williams and Bevan, 2007). Two major subsets of memory $CD8^+$ T cells have been described: central memory T (Tcm) cells and effector memory T (Tem) cells (Kaech and Wherry, 2007; Sallusto et al., 2004). Tcm cells preferentially express CD62L (L-selectin), which allows recirculation through lymph nodes. Tem cells lack CD62L and yet express other homing receptors needed for migration into nonlymphoid organs and upon restimulation with antigen Tem cells are immediately capable of effector cytokine production, whereas Tcm cells proliferate to produce new effector cells, which then acquire these functions (Seder and Ahmed, 2003). Chemokine receptors have been instrumental in the characterization of memory T cell subsets with distinct migratory capacity and effector functions (Woodland and Kohlmeier, 2009). For example, the chemokine receptor CCR7 discriminates between lymph node-homing central memory T cells and tissue-homing effector memory T cells, whereas expression of the B cell follicle-homing receptor CXCR5 identifies follicular helper T cells (King, 2009). In addition, CXCR3 is preferentially expressed on Th1 cells, whereas CCR4 is expressed on Th2 cells (Song et al., 2005). The ligands for these receptors are inflammatory chemokines and chemoattractants, which are expressed in inflammatory tissues and mediate the selective recruitment of different types of effector cells

(Acosta-Rodriguez et al., 2007; Trifari et al., 2009). Heterogeneity in cytokine production potential is suggested in memory $CD4^+$ T cells (MacLeod et al., 2009; McKinstry et al., 2010; Pepper et al., 2010; Sallusto and Lanzavecchia, 2009; van Leeuwen et al., 2009). However, the functional distinctions among memory $CD4^+$ T cell subpopulations are poorly understood. A greater understanding of functional memory T cell subpopulations and their regulation of cytokine production may lead to the design of better vaccine and immune-targeted therapies (Seder et al., 2008).

In this study, we show that IL-5-producing memory $CD4^+$ T cells exist selectively in the $CD62L^{lo}CXCR3^{lo}$ subpopulation and have investigated the molecular mechanism underlying the regulation of IL-5 expression in these cells. IL-5 production in memory Th2 cells was uniquely regulated by the expression of Eomesodermin (Eomes) and was associated with histone H3-K4 methylation marks at the *Il5* promoter (*Il5*p). Eomes interacted with GATA3 and prevented GATA3 binding to the *Il5*p. Furthermore, *Eomes*-deficient memory Th2 cells showed increased production of IL-5 and induced enhanced allergic airway inflammation, indicating a role for Eomes in memory Th2 cell responses in vivo.

RESULTS

IL-5 Is Selectively Produced by the $CD62L^{lo}CXCR3^{lo}$ Subpopulation of Memory Th2 Cells

Antigen-specific functional memory Th1 and Th2 cells are efficiently generated in vivo by adoptive transfer of effector Th1 or Th2 cells (Figure S1A available online; Nakayama and Yamashita, 2009). To identify functionally distinct subpopulations of memory Th1 and Th2 cells, we examined the expression of cell surface marker antigens, including CXCR3, IL-2Rβ, DX5, CD69, IL-7Rα, IL-4Rα, PD1, CD61, CCR4, and CD62L on memory Th2 cells. Memory Th2 cells were divided into at least four distinct subpopulations according to their expression of CXCR3 and CD62L, IL-2Rβ and CD62L, or DX5 and CD62L (Figures 1A and S1B). Effector Th2 cells showed a $CD62L^{lo}$ $CXCR3^{lo}$ phenotype (Figure S1C). Interestingly, a substantial proportion of in-vivo-generated memory Th2 cells expressed CXCR3, a well-known marker for Th1 cells. The transfer of sorted $CXCR3^{lo}$ effector Th2 cells also generated four subpopulations ($CD62L^{lo}CXCR3^{lo}$, $CD62L^{lo}CXCR3^{hi}$, $CD62L^{hi}CXCR3^{lo}$, and $CD62L^{hi}CXCR3^{hi}$) of memory Th2 cells, the same as that observed for unsorted Th2 cells (Figure S1D). We assessed expression of Th1 and Th2 cytokines in these four subpopulations after anti-TCR stimulation (Figures 1B, 1C, S1E, and S1F). As shown in Figures 1B and 1C, all four subpopulations expressed a large amount of IL-4 and IL-13, whereas only the $CD62L^{lo}CXCR3^{lo}$ subpopulation expressed IL-5. The production of IFN-γ by memory Th2 cells tended to be higher in the $CXCR3^{hi}$ population, although the amount was still very low relative to Th1 cells. Selective expression of *Il5* was also detected in the $CD62L^{lo}IL\text{-}2R\beta^{lo}$ subpopulation (Figure S1E), but no difference was observed for *Il5* expression between the DX5 high or low populations (Figure S1F). Memory Th1 cells were also subdivided into at least four subpopulations according to their expression of CD62L and CXCR3 (Figure S1G). However, no expression of Th2 cytokines (IL-4, IL-5, or IL-13) was observed and the expression of IFN-γ in memory Th1 cells was higher in the $CXCR3^{hi}$ subpopulation (Figures S1H and S1I). The proportion of $CD62L^{hi}$ memory Th2 cells was increased in the lymph nodes and decreased in the lung and liver as compared to spleen (Figure S1J).

Covalent histone modifications, such as histone H3-K4 methylation and histone H3-K9 acetylation, are typically associated with transcriptionally active chromatin. Particularly, histone H3-K4 methylation is a marker for the maintenance of the permissive conformation of chromatin (Ruthenburg et al., 2007). The degree of histone H3-K4 methylation at the *Il5*p was selectively higher in the $CD62L^{lo}CXCR3^{lo}$ population as compared to the other three subpopulations, and the degree was equivalent to effector Th2 cells (Figure 1D). As a control, a total histone H3 ChIP assay was performed and equivalent levels of histone were detected (Figure S1L). Similar patterns of H3-K4 methylation were observed at other regions around the *Il5* gene locus (*Il5* exon3 and *Il5* U1) (Figures S1K and S1M). These results indicate that the $CD62L^{lo}CXCR3^{lo}$ subpopulation of memory Th2 cells selectively produces IL-5 accompanied by histone H3-K4 methylation marks at the *Il5*p.

Naturally existing $CD44^{hi}$ memory phenotype $CD4^+$ (MPCD4) T cells are considered to be nearly indistinguishable from memory cells generated in response to defined antigen (Boyman et al., 2009). We found that spleen $MPCD4^+$ T cells could also be divided into four distinct subpopulations according to their expression of CXCR3 and CD62L, although the proportion of $CD62L^{hi}CXCR3^{hi}$ population was relatively small as compared to memory Th2 cells generated by effector Th2 cell transfer (Figures 1E and S1N). Upon restimulation, selective *Il5* mRNA and IL-5 protein expression by the $CD62L^{lo}CXCR3^{lo}$ population of $MPCD4^+$ T cells was detected (Figures 1F and 1G). Expression of IL-4, IL-13, and IFN-γ was observed in the $CD62L^{lo}$ subpopulation, as reported previously (Sallusto et al., 2004). Furthermore, in the $CD62L^{lo}CXCR3^{lo}$ population of $MPCD4^+$ T cells, the degree of H3-K4 methylation was highest at the *Il5*p after anti-TCR stimulation (Figure 1H). These results indicate that IL-5-producing cells were detected selectively in the $CD62L^{lo}CXCR3^{lo}$ population of $MPCD4^+$ T cells.

Decreased Expression of *Eomes*, but Not *Tbx21*, Enhanced IL-5 Production by Memory Th2 Cells

We next analyzed the expression of genes previously shown to be involved in the regulation of *Il5* transcription (*Gata3, Cebpa, Maf, Nfat1, Nfat2, Rela, Jun, Junb, Jund*, and *Fra2*) in the four subpopulations ($CD62L^{lo}CXCR3^{lo}$, $CD62L^{lo}CXCR3^{hi}$, $CD62L^{hi}CXCR3^{lo}$, and $CD62L^{hi}CXCR3^{hi}$) of memory Th2 cells, but none of these genes were specifically expressed in the $CD62L^{lo}CXCR3^{lo}$ population (data not shown). Intracellular cytokine staining of IL-4, IL-5, and IL-13 showed that only a fraction of the $CD62L^{lo}CXCR3^{lo}$ Th2 memory cells produced IL-5 (about 10%), although the IL-5-producing cells existed selectively in this population (Figure S2A). In contrast, IL-4- and IL-13-producing cells were almost equivalent (around 20%–25%) among these four subpopulations. To identify genes that may control the expression of *Il5* in memory Th2 cells, a DNA microarray analysis was performed on $IL\text{-}5^+$ and $IL\text{-}5^-$ memory Th2 cells purified with an IL-5 secretion assay kit (Figure S2B). The purified $IL\text{-}5^+$ memory Th2 cells showed decreased levels of

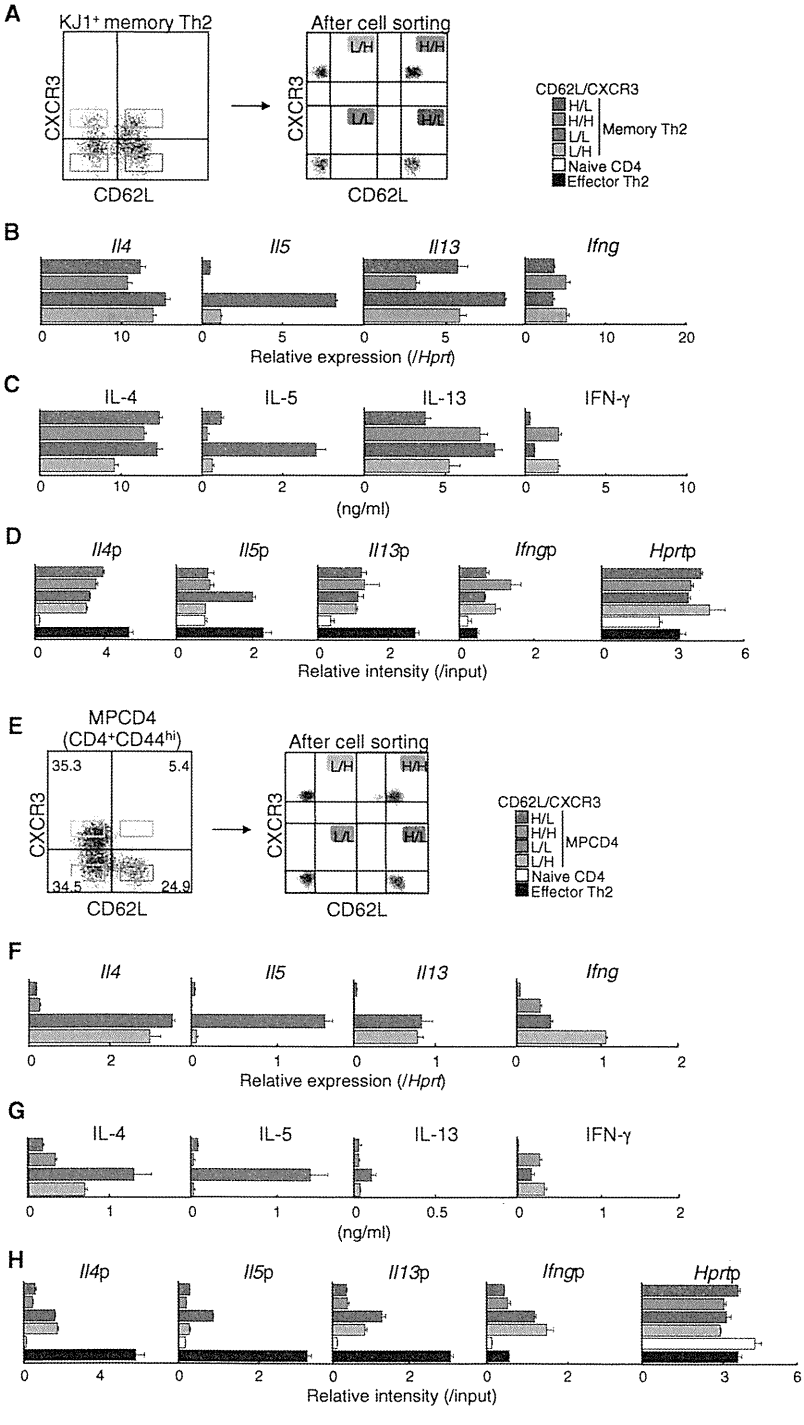

Figure 1. IL-5 Production Is Selectively Detected in the CD62L^{lo}CXCR3^{lo} Subpopulation of Memory Th2 Cells

(A) Five weeks after transfer of DO11.10 TCR Tg Th2 cells into BALB/c *nu/nu* mice, donor-derived KJ1$^+$ memory Th2 cells in the spleen were stained with CD62L and CXCR3 mAbs. Four subpopulations (CD62L^{lo}CXCR3^{lo}, CD62L^{lo}CXCR3^{hi}, CD62L^{hi}CXCR3^{lo}, and CD62L^{hi}CXCR3^{hi}) of memory Th2 cells were sorted by fluorescence activated cell sorting (FACS).

(B) Quantitative RT-PCR analysis of *Il4*, *Il5*, *Il13*, and *Ifng* in the four subpopulations of memory Th2 cells after 4 hr stimulation with immobilized anti-TCRβ.

(C) ELISA analysis of IL-4, IL-5, IL-13, and IFN-γ secreted by the four subpopulations of memory Th2 cells after 24 hr stimulation with immobilized anti-TCRβ.

(D) A ChIP assay was performed with anti-trimethylhistone H3-K4 at the Th2 cytokines gene loci and *Hprt* promoter (*Hprt*p) from naive CD4$^+$ T, effector Th2, and the four subpopulations of memory Th2 cells after 4 hr stimulation with immobilized anti-TCRβ. The degree of this modification was determined by quantitative RT-PCR.

Five independent experiments (B) and three independent experiments (C and D) were performed with similar results.

(E) CD44^{hi} memory phenotype CD4$^+$ (MPCD4$^+$) T cells from the spleen were stained with CD62L and CXCR3 mAbs. Four subpopulations were sorted by FACS.

(F–H) Quantitative RT-PCR (F), ELISA (G), and ChIP assay (H) were performed as described in (B)–(D). Three independent experiments were performed with similar results. The mean values with standard deviations (SD) are shown (B–D, F–H).

CD62L and CXCR3 (Figure S2C). A summary of the differentially expressed genes is shown in Table S1. We focused especially on nuclear factors and confirmed their expression via quantitative RT-PCR. mRNA levels of *Eomes* and *Tbx21* were significantly lower, and those of *Rora* and *Pparg* were significantly higher in IL-5$^+$ memory Th2 cells as compared to IL-5$^-$ memory Th2 cells (Figures 2A and S2D). Other nuclear factors listed in Table S1 were either not detected substantially or not expressed differentially between IL-5$^+$ and IL-5$^-$ memory Th2 cells when measured by RT-PCR (data not shown). Among the four subpopulations of memory Th2 cells, no significant difference was observed in the expression of *Eomes* or *Tbx21*, although the expression of these two genes tends to be higher in the CD62L^{lo}CXCR3^{hi} population (Figure S2E). The expression of *Rora* and *Pparg* were highest in the CD62L^{lo}CXCR3^{lo} subpopulation.

We next analyzed expression of *Eomes*, *Tbx21*, *Rora*, and *Pparg* mRNA (Figures 2B and S2F) and Eomes and T-bet protein (Figure 2C) in naive CD4$^+$ T cells, stimulated effector Th1 and Th2 cells, memory Th1 and Th2 cells, and activated CD8$^+$ T cells. The expression of *Eomes* in memory Th2 cells was almost equivalent to stimulated effector Th1 cells and was slightly lower than activated CD8$^+$ T cells. In contrast, the expression of *Tbx21* in memory Th2 cells was considerably lower than that of effector or memory Th1 cells. Moreover, intracellular staining of Eomes revealed that the majority of memory Th2 cells expressed substantial amounts of Eomes protein (Figure 2D). Again, marginal expression was detected in effector Th2 cells. In addition, both MPCD4$^+$ T cells and MPCD8$^+$ T cells expressed substantial amounts of Eomes protein as compared to naive CD4$^+$ T cells, although the expression was lower in MPCD4$^+$ T cells (Figure S2G). These

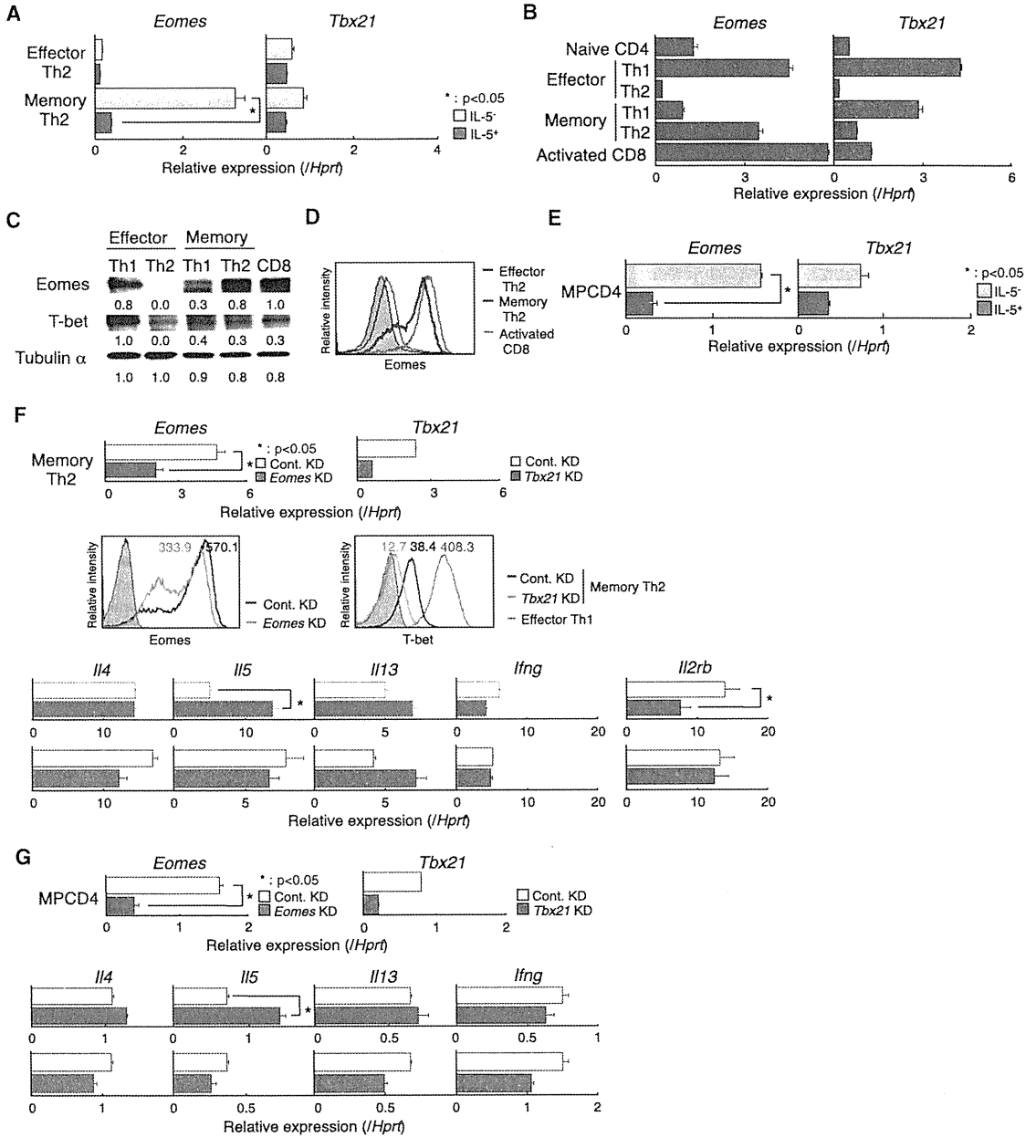

Figure 2. Decreased Expression of *Eomes*, but Not *Tbx21*, Enhanced IL-5 Production by Memory Th2 Cells

(A and B) Quantitative RT-PCR analysis of *Eomes* and *Tbx21* in IL-5$^+$ and IL-5$^-$ effector or memory Th2 cells (A) and naive CD4$^+$ T, stimulated effector Th1 and Th2, memory Th1 and Th2, and activated CD8$^+$ T cells (B).

(C) Protein expression of Eomes and T-bet in stimulated effector Th1 and Th2, memory Th1 and Th2, and activated CD8$^+$ T cells. Band intensities were measured with a densitometer and arbitrary densitometric units are shown.

(D) Intracellular staining profiles of Eomes in stimulated effector, memory Th2, and activated CD8$^+$ T cells are shown. Gray filled histogram shows isotype control staining.

(E) Quantitative RT-PCR analysis of *Eomes* and *Tbx21* in IL-5$^+$ and IL-5$^-$ MPCD4$^+$ T cells.

(F) Effect of siRNA gene targeting of *Eomes* and *Tbx21* on *Il5* expression in memory Th2 cells. Memory Th2 cells were introduced with control (Cont. KD), *Eomes* (*Eomes* KD), or *Tbx21* siRNA (*Tbx21* KD) and cultured with medium for 24 hr. Representative expression of *Eomes* and *Tbx21* in *Eomes* and *Tbx21* siRNA gene-targeted memory Th2 cells (top). The number in the histogram represents mean fluorescent units. Gray filled histogram shows isotype control staining. Quantitative RT-PCR analysis of indicated molecules in these cells after 6 hr stimulation with immobilized anti-TCRβ are shown (bottom).

(G) siRNA gene targeting analysis in MPCD4$^+$ T cells.

The mean values with standard deviations (SD) are shown (A, B, E–G). At least three independent experiments (A–G) were performed with similar results. *$p < 0.05$.

results indicate that the expression of Eomes was increased in memory Th2 cells and that mRNA expression of *Eomes* was very low in IL-5$^+$ memory Th2 cells. Similar results were obtained from assessment of the expression of these genes in IL-5$^+$ and IL-5$^-$ MPCD4$^+$ T cells (Figure 2E). Therefore, downregulation of Eomes may be required for IL-5 production in memory Th2 and MPCD4$^+$ T cells.

To assess the role of *Eomes* and *Tbx21* in *Il5* expression in memory Th2 cells, a transient siRNA gene targeting system was established. *Eomes* or *Tbx21* siRNA gene targeting in memory Th2 cells resulted in decreased *Eomes* and *Tbx21* mRNA and protein expression of Eomes and T-bet, respectively (Figure 2F, top). As shown in Figure 2F (bottom), siRNA gene targeting of *Eomes* induced increased expression of *Il5* but no substantial effect was observed in the expression of *Il4* and *Il13*. In contrast, decreased expression of *Tbx21* by siRNA gene targeting did not affect *Il5* expression in memory Th2 cells. Similar results were obtained in the experiments with MPCD4$^+$ T cells (Figure 2G). *Eomes* siRNA gene targeting induced a decreased expression of *Il2rb* in memory Th2 cells (Figure 2F; Intlekofer et al., 2005). Again, no effect was observed after *Tbx21* siRNA gene targeting, although the expression levels of *Tbx21* were decreased substantially. The expression of *Rora* and *Pparg* was increased in IL-5$^+$ memory Th2 cells (Table S1 and Figure S2D), but siRNA gene targeting of these genes had no effect on the expression of *Il5* (Figure S2H). These results indicate that Eomes but not T-bet plays an important role in the regulation of *Il5* expression in memory Th2 and MPCD4$^+$ T cells.

Eomes Limits the Production of IL-5 in the CD62L^{lo}CXCR3^{lo} Population of Memory Th2 Cells

Next, we performed *Eomes* siRNA experiments on each of the four subpopulations (CD62L^{lo}CXCR3^{lo}, CD62L^{lo}CXCR3^{hi}, CD62L^{hi}CXCR3^{lo}, and CD62L^{hi}CXCR3^{hi}) of memory Th2 cells. More than a 2-fold increase in the expression of *Il5* was detected in the CD62L^{lo}CXCR3^{lo} subpopulation (Figure 3A), whereas no obvious increase in *Il5* expression was detected in the other three subpopulations. Next, the degree of H3-K4 methylation at the *Il5*p was assessed in the four subpopulations in addition to the CD62L^{lo}CXCR3^{lo} population depleted of IL-5$^+$ cells. Histone H3-K4 methylation at the *Il5*p region in the CD62L^{lo}CXCR3^{lo} population depleted of IL-5$^+$ cells was almost equivalent to that of the whole CD62L^{lo}CXCR3^{lo} population (Figure 3B). The other three subpopulations showed a low level of histone H3-K4 methylation similar to that observed in effector Th1 cells. We confirmed reduced *Il5* mRNA expression in the CD62L^{lo}CXCR3^{lo} population depleted of IL-5$^+$ cells (Figure 3C). These results indicate that the level of histone H3-K4 methylation at the *Il5*p region in the CD62L^{lo}CXCR3^{lo} population is high even in the absence of IL-5-producing cells. Therefore, Eomes appears to limit the transcription of *Il5* but does not control the histone H3-K4 methylation in the CD62L^{lo}CXCR3^{lo} population (Figure S3, top and middle). Eomes-dependent and -independent mechanisms may operate in the other three subpopulations (Figure S3, bottom).

Eomes Negatively Regulates the Production of IL-5 in Memory Th2 Cells

GATA3 is known to bind the *Il5*p directly and controls its promoter activity in Th2 cells (Klein-Hessling et al., 2008). First, intracellular staining of IL-4, IL-5, GATA3, and Eomes was performed on the four subpopulations after anti-TCR restimulation. Most of the IL-5-producing cells expressed high amounts of GATA3 protein in both the whole (Figure 4A, far left) and the CD62L^{lo}CXCR3^{lo} population of memory Th2 cells (Figure 4A, GATA3 versus IL-5 profile). Conversely, the majority of IL-5-producing memory Th2 cells expressed lower levels of Eomes protein (Figure 4A, Eomes versus IL-5 profile). In contrast, IL-4-producing cells were detected in both Eomes^{hi} and Eomes^{lo} or GATA3^{hi} and GATA3^{lo} populations (Figure 4A, GATA3 versus IL-4 and Eomes versus IL-4 profiles). We detected GATA3 and Eomes double expressing cells in all four subpopulations (approximately 20% to 30%) but also a reciprocal expression profile of GATA3 and Eomes was noted in the four subpopulations of memory Th2 cells (Figure 4A, GATA3 versus Eomes profiles). *Eomes* siRNA gene targeting resulted in an increase in the percentage of IL-5-producing cells in the Eomes^{lo} population (0.6% versus 2.1%) and decreased proportion of GATA3^{hi}Eomes^{hi} cells (20.9% versus 10.2%) with increased GATA3^{hi}Eomes^{lo} cells (28.2% versus 38.9%) (Figure 4B). We also examined IL-5, Eomes, and GATA3 staining in the CD62L^{lo}CXCR3^{lo} population, and IL-5-producing cells were predominantly detected in the GATA3^{hi} Eomes^{lo} populations (Figure 4C). *Eomes* siRNA gene targeting resulted in an increase in the percentage of IL-5-producing cells in the GATA3^{hi}Eomes^{lo} population (Figure S4). Furthermore, enforced expression of *Eomes* in effector Th2 cells suppressed the production of IL-5 but not IL-4 or IL-13 (Figures 4D and 4E). These results indicate that Eomes negatively regulates the production of IL-5 in memory Th2 cells and also effector Th2 cells if Eomes is expressed.

Eomes Suppresses the Transcriptional Activity of GATA3 via Inhibition of GATA3 Binding to the *Il5* Promoter

To identify the molecular mechanism by which Eomes downregulates the expression of *Il5* in memory Th2 cells, we sought to demonstrate the possible physical association of Eomes with GATA3. Eomes protein associated with GATA3 was easily detected in the precipitates even in the presence of ethidium bromide (Figure 5A, lanes 4 and 8). Having shown that Eomes and GATA3 can associate, several Myc-tagged Eomes mutants were generated to determine which domains of Eomes are important for its association with GATA3 (Figure 5B, top). The association between the dC mutant (C-terminal region including transactivation domain deleted; Figure 5A, lane 8) with GATA3 was equivalent to the wild-type (WT) Eomes (Figure 5A, compare lanes 6 and 8), but the association of dT mutant (T-box region deleted; Figure 5A, lane 7) with GATA3 was very weak (Figure 5B, middle). The amount of each protein was estimated by immunoblotting with anti-Myc or anti-Flag (Figure 5B, bottom). The association of Eomes with GATA3 was detected in memory Th2 cells (Figure 5C). These results indicate that Eomes associates with GATA3 in memory Th2 cells and that the T-box region is critical for this association.

Next, to assess the effect of Eomes on the DNA-binding activity of GATA3, a pull-down assay was performed as described in the Experimental Procedures. The binding of GATA3 to the consensus GATA sequence was substantially decreased in the presence of Eomes (Figure 5D, top). The

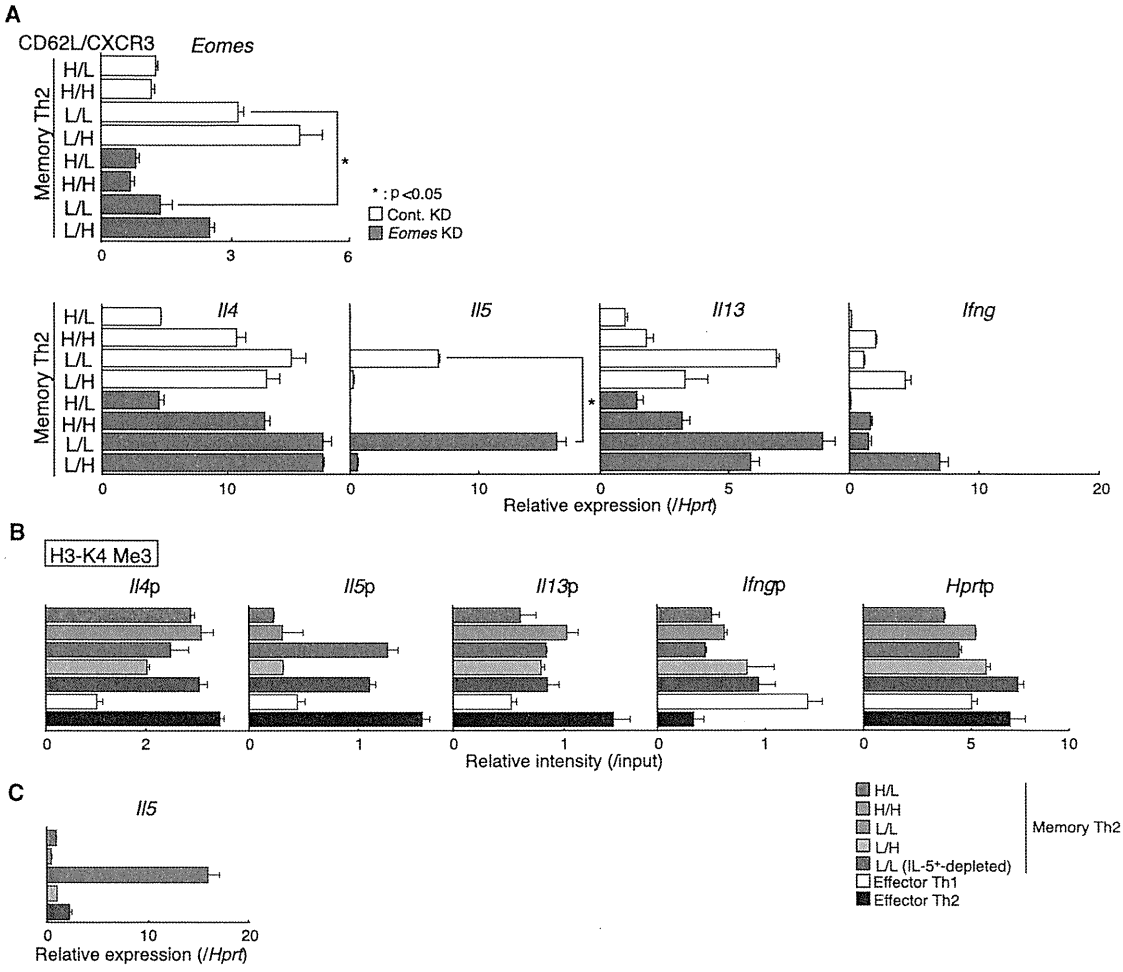

Figure 3. Eomes Limits the Production of IL-5 in the CD62L^{lo}CXCR3^{lo} Population of Memory Th2 Cells

(A) Memory cells were introduced with siRNA and cytokine expression was detected by quantitative RT-PCR as described in Figure 2F.

(B) A ChIP assay was performed with anti-trimethylhistone H3-K4 Ab at the Th2 cytokine gene loci and *Hprt*p from effector Th1, effector Th2 cells, the four subpopulations (CD62L^{lo}CXCR3^{lo}, CD62L^{lo}CXCR3^{hi}, CD62L^{hi}CXCR3^{lo}, and CD62L^{hi}CXCR3^{hi}) of memory Th2 cells and the CD62L^{lo}CXCR3^{lo} population depleted of IL-5^{+} memory Th2 cells.

(C) Quantitative RT-PCR analysis of *Il5* in the four subpopulations of memory Th2 cells and the CD62L^{lo}CXCR3^{lo} population depleted of IL-5^{+} memory Th2 cells. The mean values with standard deviations (SD) are shown. Three independent experiments were performed with similar results. *p < 0.05.

quantity of input Flag-tagged GATA3 and Myc-tagged Eomes protein were also assessed (Figure 5D, middle and bottom). We also performed a pull-down assay with the *Il5*p sequence in the presence of Eomes WT or dT mutant. The binding of GATA3 to the *Il5*p was substantially decreased in the presence of Eomes WT. This suppressive effect was not observed by the Eomes dT mutant (Figure 5E, top). The binding of Eomes to the *Il5*p was not detected (Figure 5E, second panel). The quantity of input Flag-tagged GATA3 and Myc-tagged Eomes protein was also assessed (Figure 5E, bottom). Next, the suppressive effect of Eomes on *Il5*p activity was assessed, and as expected both WT and dC mutants efficiently suppressed *Il5* activity whereas the Eomes dT mutant did not (Figure 5F). To test the effects of Eomes on GATA3 binding to the *Il5*p in Th2 cells, we performed a ChIP assay with *Eomes*-overexpressing effector Th2 cells and *Eomes* siRNA gene-targeted memory Th2 cells (Figures 5G and 5H). The binding of GATA3 to the *Il5*p was reduced by enforced overexpression of Eomes in effector Th2 cells and was enhanced by the reduction of *Eomes* expression in memory Th2 cells. These results indicate that Eomes suppresses the transcriptional activity of GATA3 via inhibition of GATA3 DNA binding to the *Il5*p in memory Th2 cells.

Memory Th2 Cell-Dependent Airway Inflammation Is Ameliorated after CD62L^{lo}CXCR3^{lo} Cell Depletion

Finally, we assessed the function of IL-5-producing memory Th2 cells in the CD62L^{lo}CXCR3^{lo} subpopulation with a memory Th2 cell-dependent allergic airway inflammation model (Yamashita et al., 2006). OVA-specific memory Th2 cells were first generated in vivo (Nakayama and Yamashita, 2009), and whole memory Th2 cells or memory Th2 cells depleted of the CD62L^{lo}CXCR3^{lo} population (Δ L/L) were transferred into BALB/c or BALB/c *nu/nu* mice, and then these mice were then challenged twice by inhalation with OVA. A no cell transfer group

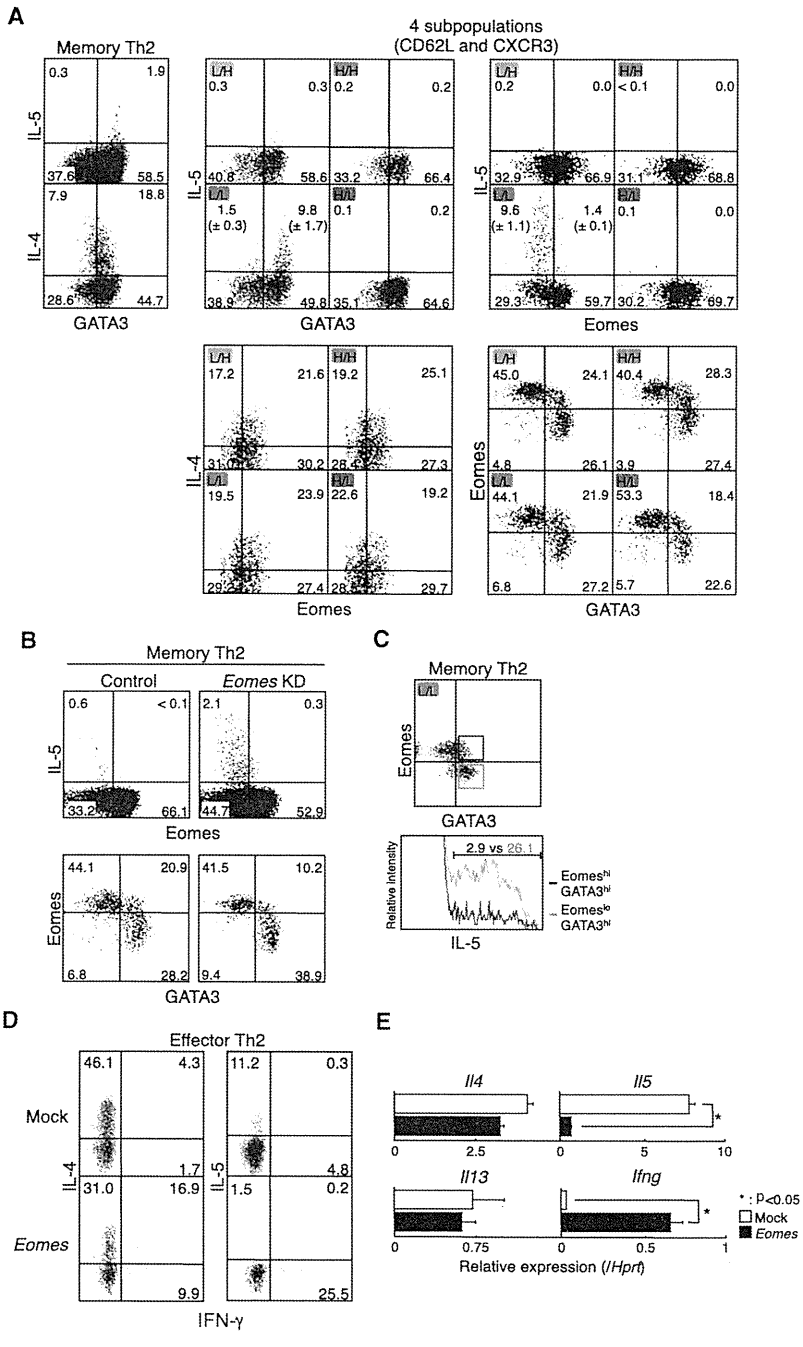

Figure 4. Eomes Negatively Regulates the Production of IL-5 in Memory Th2 Cells

(A) Whole memory Th2 and the four subpopulations ($CD62L^{lo}CXCR3^{lo}$, $CD62L^{lo}CXCR3^{hi}$, $CD62L^{hi}CXCR3^{lo}$, and $CD62L^{hi}CXCR3^{hi}$) of memory Th2 cells were stimulated in vitro with immobilized anti-TCRβ for 6 hr, and intracellular staining profiles of Eomes, GATA3, IL-5, and IL-4 are shown with the percentage of cells in each area. The numbers in parentheses represent standard deviations.

(B) Control or *Eomes* siRNA gene-targeted memory Th2 cells were generated as described in Figure 2F and these cells were stimulated in vitro with immobilized anti-TCRβ for 6 hr; intracellular staining profiles of Eomes versus IL-5, and GATA3 versus Eomes are shown with the percentage of cells in each area.

(C) Intracellular staining profiles of IL-5 in $Eomes^{hi}GATA3^{hi}$ and $Eomes^{lo}GATA3^{hi}$ cells in the $CD62L^{lo}CXCR3^{lo}$ population are shown.

(D and E) Naive $CD4^+$ T cells were stimulated under Th2 cell culture conditions for 2 days, and then the cells were infected with an *Eomes*-IRES-hNGFR-containing retrovirus. Three days after infection, IFN-γ versus IL-4 and IFN-γ versus IL-5 staining profiles of *Eomes*-infected cells ($hNGFR^+$) were determined by intracellular staining (D). The hNGFR-positive infected cells were enriched by magnetic cell sorting. Quantitative RT-PCR analysis of the relative expression of each cytokine in infected cells was performed 4 hr after stimulation with immobilized anti-TCRβ (E).

The mean values with standard deviations (SD) are shown. *$p < 0.05$. Three (A and B) and two (C–E) independent experiments were performed with similar results.

was included as a negative control (Control). A dramatic decrease in the number of inflammatory cells, including eosinophils in the bronchoalveolar lavage (BAL) fluid, was observed in the Δ L/L group as compared to the undepleted group (Figure 6A). A similar reduction was observed after histological analysis of the lung (Figure S5A). The concentration of IL-5 in the BAL fluid was decreased in the Δ L/L group in comparison to the undepleted group, whereas the concentration of IL-4, IL-13, and IFN-γ was almost equivalent between the two (Figure 6B). Periodic acid-Schiff (PAS) staining and the measurement of *Gob5* and *Muc5ac* mRNA expression in the lung tissue indicated decreased production of mucus in the Δ L/L group (Figures 6C and 6D). Furthermore, methacholine-induced airway hyperresponsiveness (AHR) was significantly decreased in the Δ L/L group (Figure 6E). In order to specifically address the role of $IL\text{-}5^+$ memory Th2 cells, $IL\text{-}5^+$ cell-depleted memory Th2 cells ($IL\text{-}5^-$) were transferred. As expected, the infiltration of eosinophils was decreased in the $IL\text{-}5^-$ group as compared to the whole group (Figure 6F). The concentration of IL-5 in the BAL fluid was decreased in the $IL\text{-}5^-$ group whereas the concentration of IL-4, IL-13, and IFN-γ was almost equivalent (Figure 6G). These results indicate that allergic airway inflammation was attenuated after depletion of the $CD62L^{lo}CXCR3^{lo}$ population or the IL-5-producing population of memory Th2 cells.

To assess a role for Eomes in allergic airway inflammation and IL-5 production in vivo more directly, we generated memory Th2 cells from Eomes-deficient ($Eomes^{-/-}$) effector Th2 cells. IL-5 production by $Eomes^{-/-}$ effector Th2 cells was equivalent to that detected in $Eomes^{+/+}$ cells (Figure S5B). $Eomes^{-/-}$ memory Th2 cells showed reduced surface expression of CXCR3 as compared to control ($Eomes^{+/+}$) (Figure 6H). As expected, IL-5 production was dramatically increased in $Eomes^{-/-}$ memory Th2 cells as compared to control (Figure 6I).

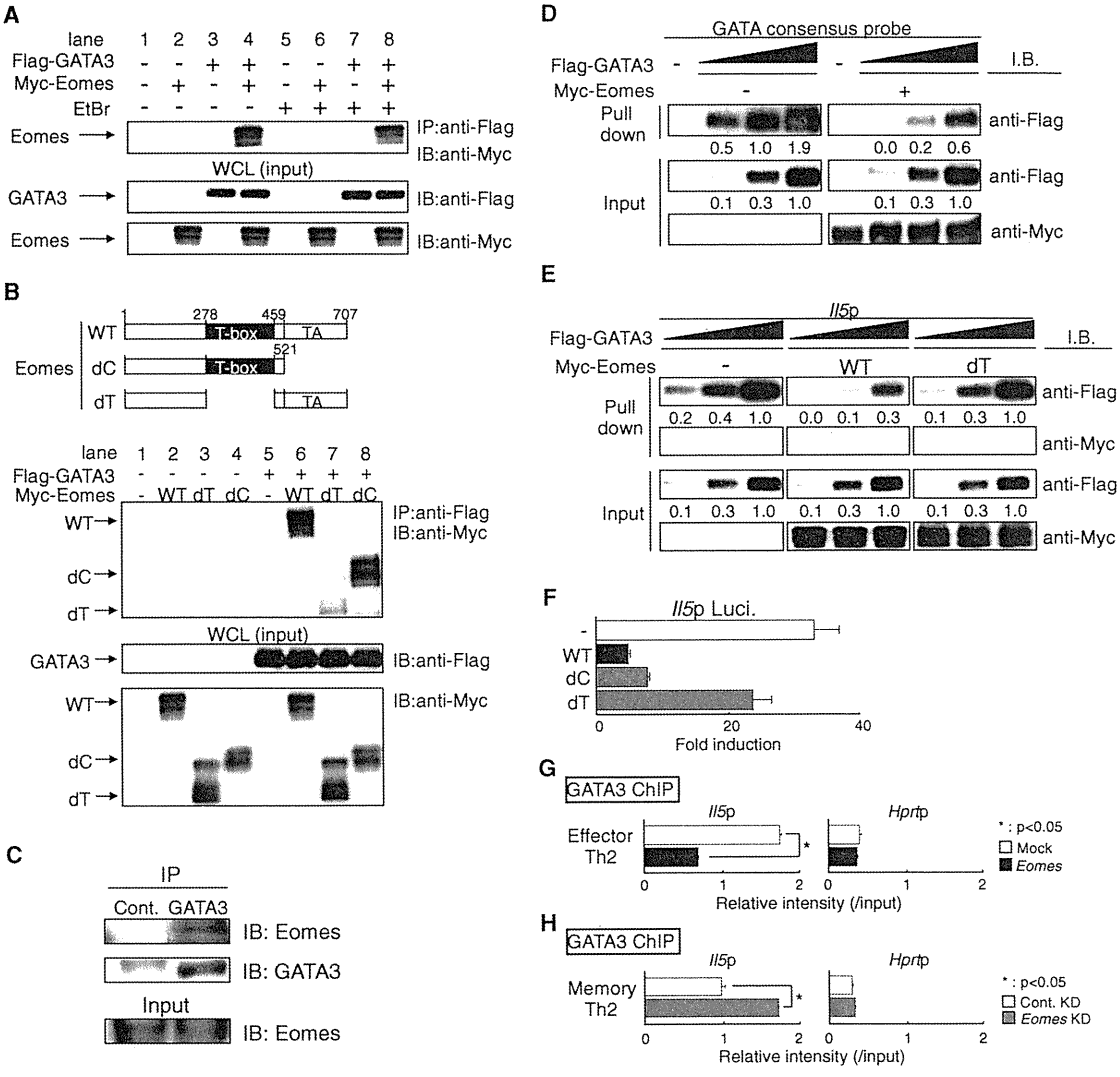

Figure 5. Eomes Suppresses the Transcriptional Activity of GATA3 via the Inhibition of Binding to the *Il5* Promoter

(A) 293T cells were transfected with Myc-tagged Eomes or Flag-tagged GATA3, and immunoprecipitation assay was performed with anti-Flag in the absence or presence of ethidium bromide. Immunoblotting of whole cell lysates (WCL) is also shown as a control (input).

(B) Schematic representation of Myc-tagged Eomes mutants; wild-type Eomes (WT), dC mutant with deletion of the transactivation domain (TA), and dT mutant with deletion of T-box region (top). 293T cells were transfected with Myc-tagged WT or mutant Eomes and Flag-tagged GATA3. A coimmunoprecipitation analysis was performed.

(C) The association of Eomes with GATA3 detected in memory Th2 cells. Coimmunoprecipitation assay with anti-GATA3 was performed with memory Th2 cells (1×10^8 cells) after stimulation with anti-TCRβ for 6 hr.

(D and E) A pull-down assay was performed as described in Experimental Procedures. Immunoblotting of total cell lysates is also shown (Input).

(F) Eomes interacted with GATA3 and suppressed GATA3-induced transcriptional activation of the *Il5*p. Reporter assays with the *Il5*p were performed with the D10G4.1 Th2 cell line. The mean values with standard deviations of relative luciferase activity of three different experiments are shown. Stimulation was done with PMA (30 ng/ml) plus dbcAMP (100 μM).

(G and H) A ChIP assay was performed with anti-GATA3 at the *Il5*p and *Hprt*p in *Eomes*-overexpressing effector Th2 cells shown in Figure 4C (G), and memory Th2 cells with control (Cont. KD) or *Eomes* siRNA (*Eomes* KD) shown in Figure 2F (H).

Three independent experiments were performed with similar results.

We also examined memory Th2 cell-dependent airway inflammation by using *Eomes*$^{-/-}$ memory Th2 cells. The infiltration of inflammatory cells, mainly eosinophils, was significantly increased in the group transferred with *Eomes*$^{-/-}$ memory Th2 cells as compared to the *Eomes*$^{+/+}$ group (Figure 6J). Consistent with the increased eosinophilic infiltration, the concentration of IL-5 in the BAL fluid was increased in the *Eomes*$^{-/-}$ group, whereas the concentration of IL-4 and IL-13 was almost equivalent between the two groups (Figure 6K). Decreased levels of IFN-γ in the BAL fluid of *Eomes*$^{-/-}$ memory Th2 cell transferred mice were also observed. These in vivo results indicate that memory Th2 cell-dependent allergic airway