Immunity
Article

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

Yusuke Endo,[1] Chiaki Iwamura,[1] Makoto Kuwahara,[1] Akane Suzuki,[1] Kaoru Sugaya,[1] Damon J. Tumes,[1] Koji Tokoyoda,[1] Hiroyuki Hosokawa,[1] Masakatsu Yamashita,[1] and Toshinori Nakayama[1,2,*]
[1]Department of Immunology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana Chuo-ku, Chiba 260-8670, Japan
[2]JST, CREST, 1-8-1 Inohana Chuo-ku, Chiba 260-8670, Japan
*Correspondence: tnakayama@faculty.chiba-u.jp
DOI 10.1016/j.immuni.2011.08.017

## 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-2Rβ$^{lo}$ 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-5$^+$ and IL-5$^-$ memory Th2 cells purified with an IL-5 secretion assay kit (Figure S2B). The purified IL-5$^+$ memory Th2 cells showed decreased levels of

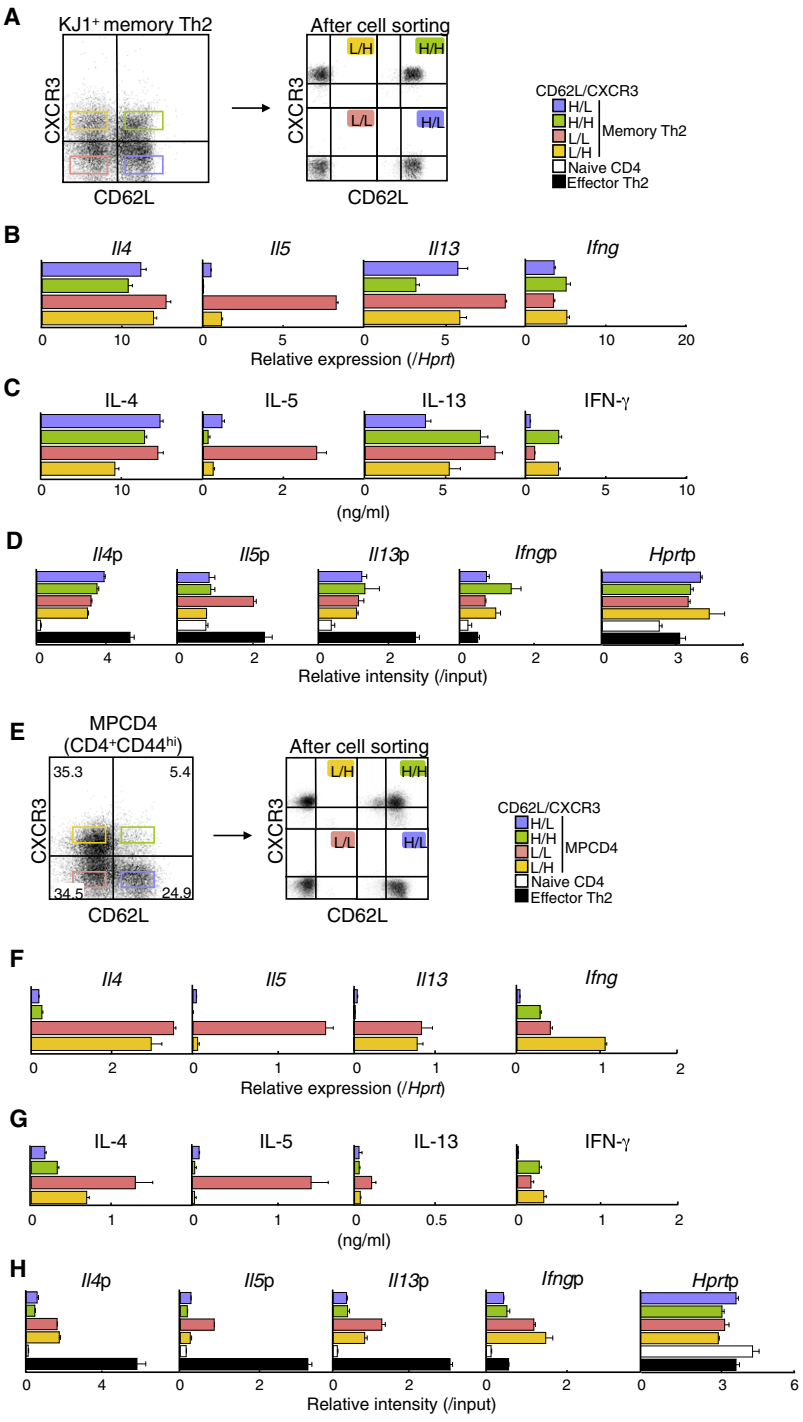


**Figure 1. IL-5 Production Is Selectively Detected in the CD62L$^{lo}$CXCR3$^{lo}$ Subpopulation of Memory Th2 Cells**

(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

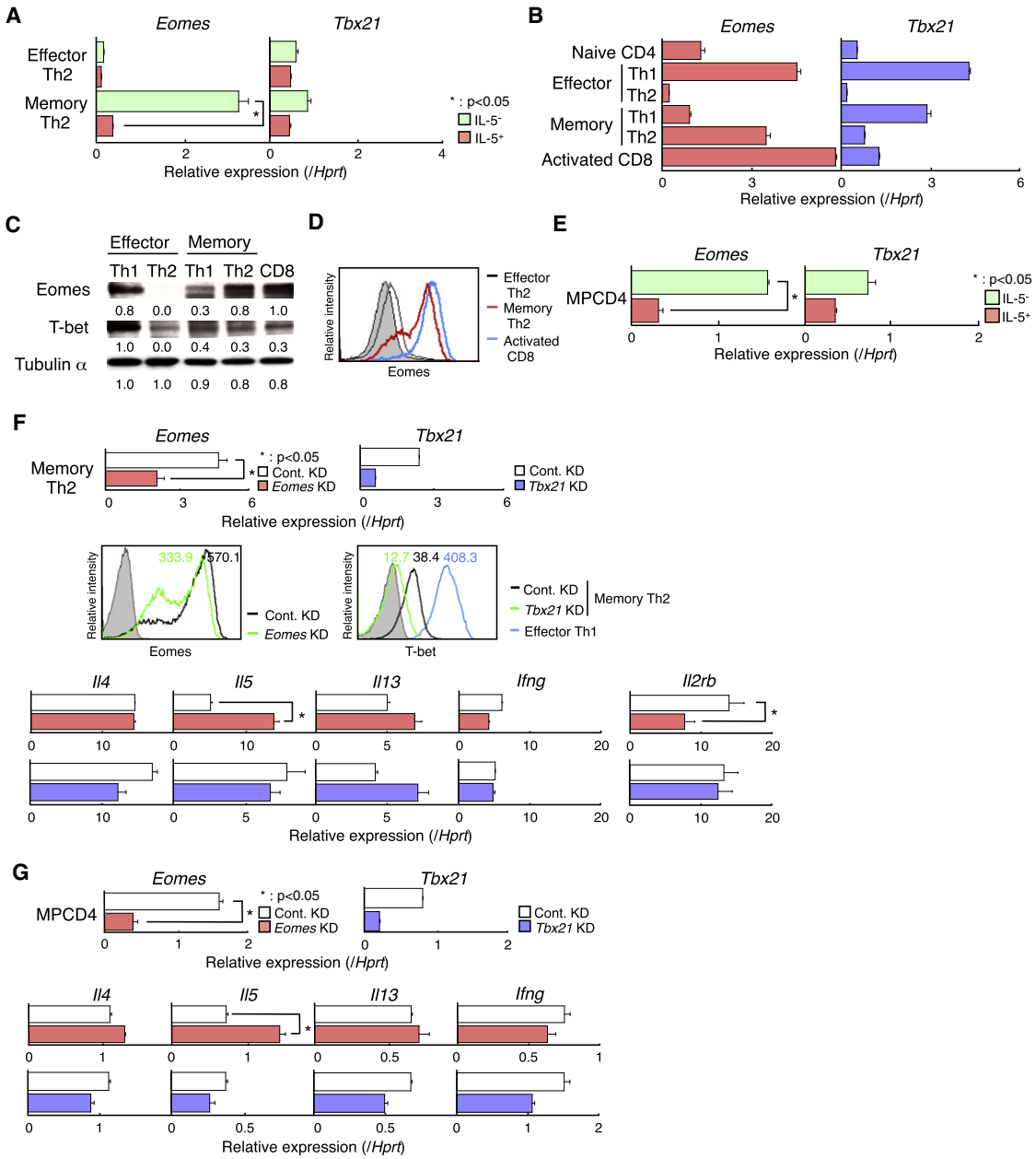


**Figure 2. Decreased Expression of *Eomes*, but Not *Tbx21*, Enhanced IL-5 Production by Memory Th2 Cells**

(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

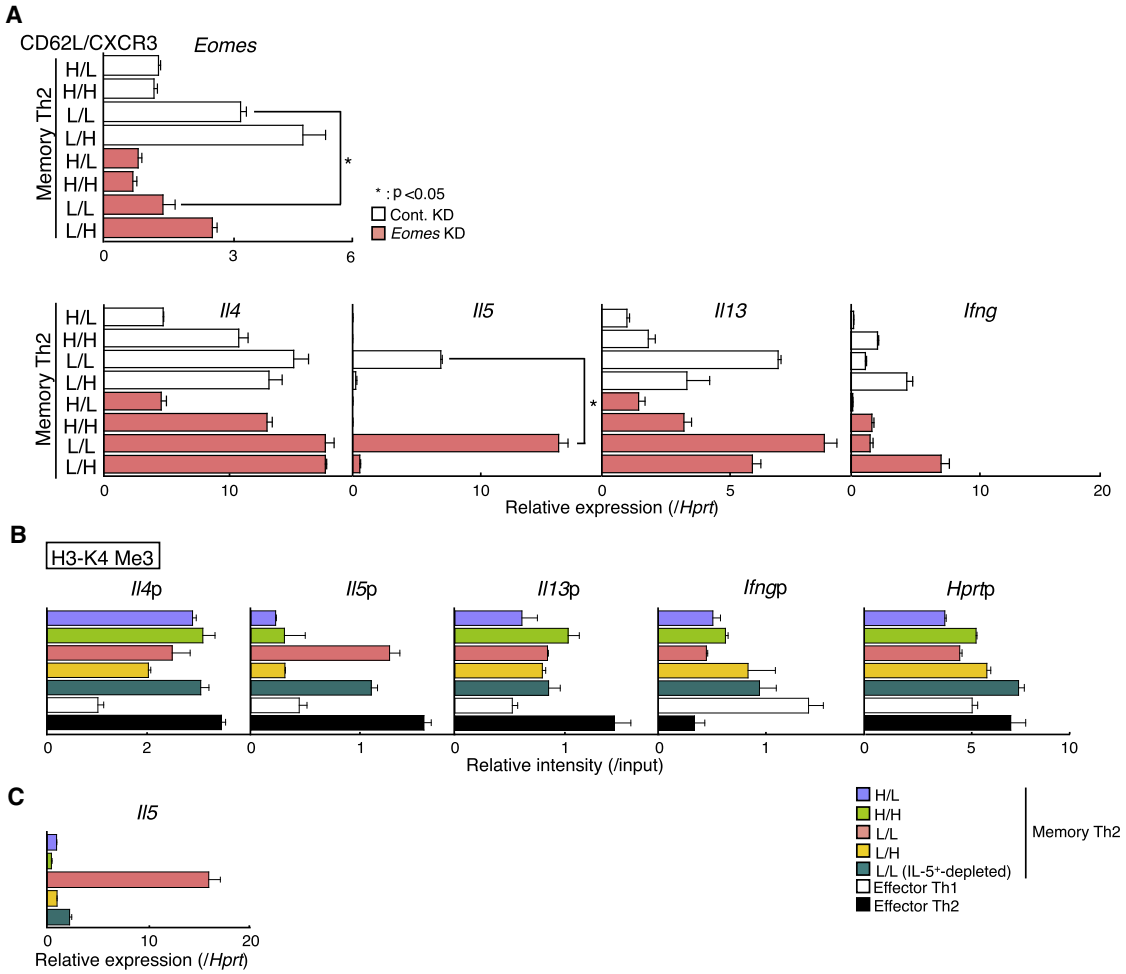


**Figure 3. Eomes Limits the Production of IL-5 in the CD62L$^{lo}$CXCR3$^{lo}$ Population of Memory Th2 Cells**

(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

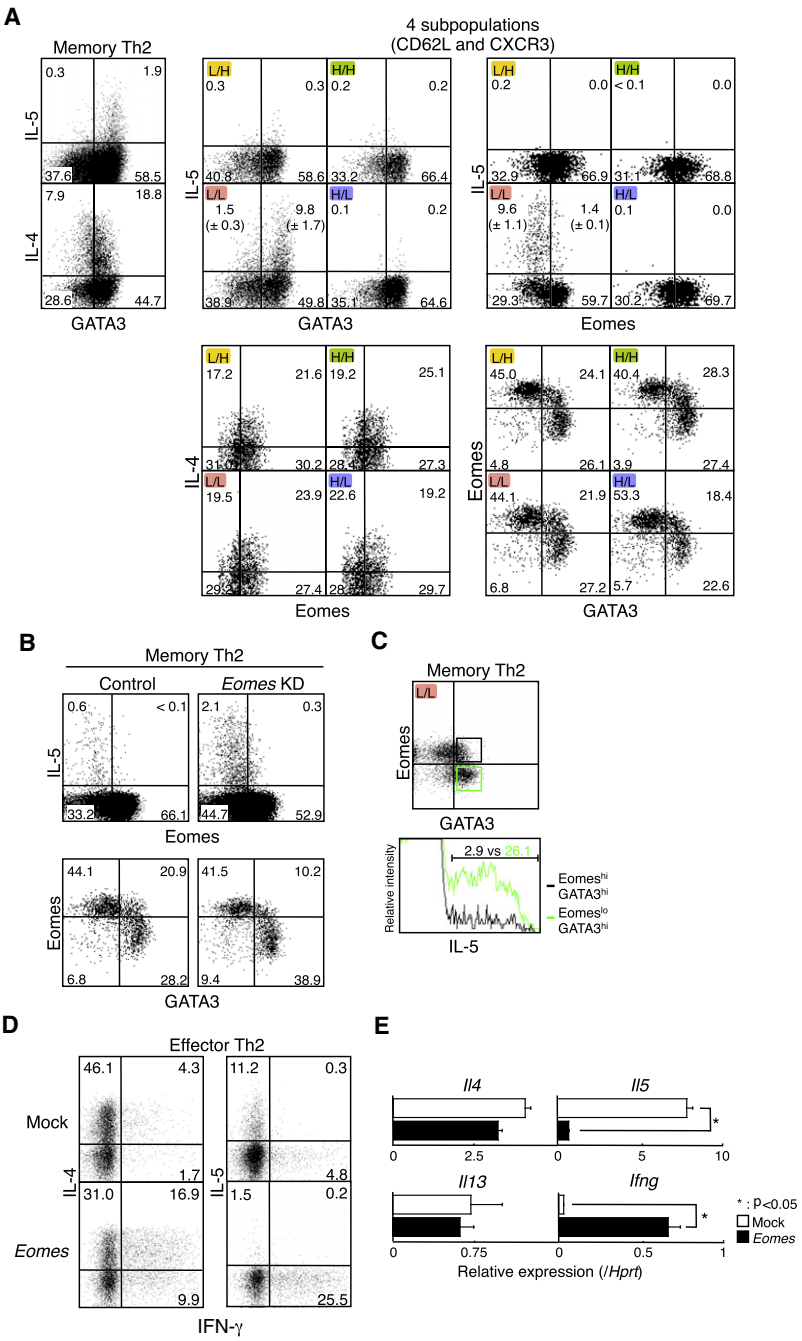


**Figure 4. Eomes Negatively Regulates the Production of IL-5 in Memory Th2 Cells**

(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).

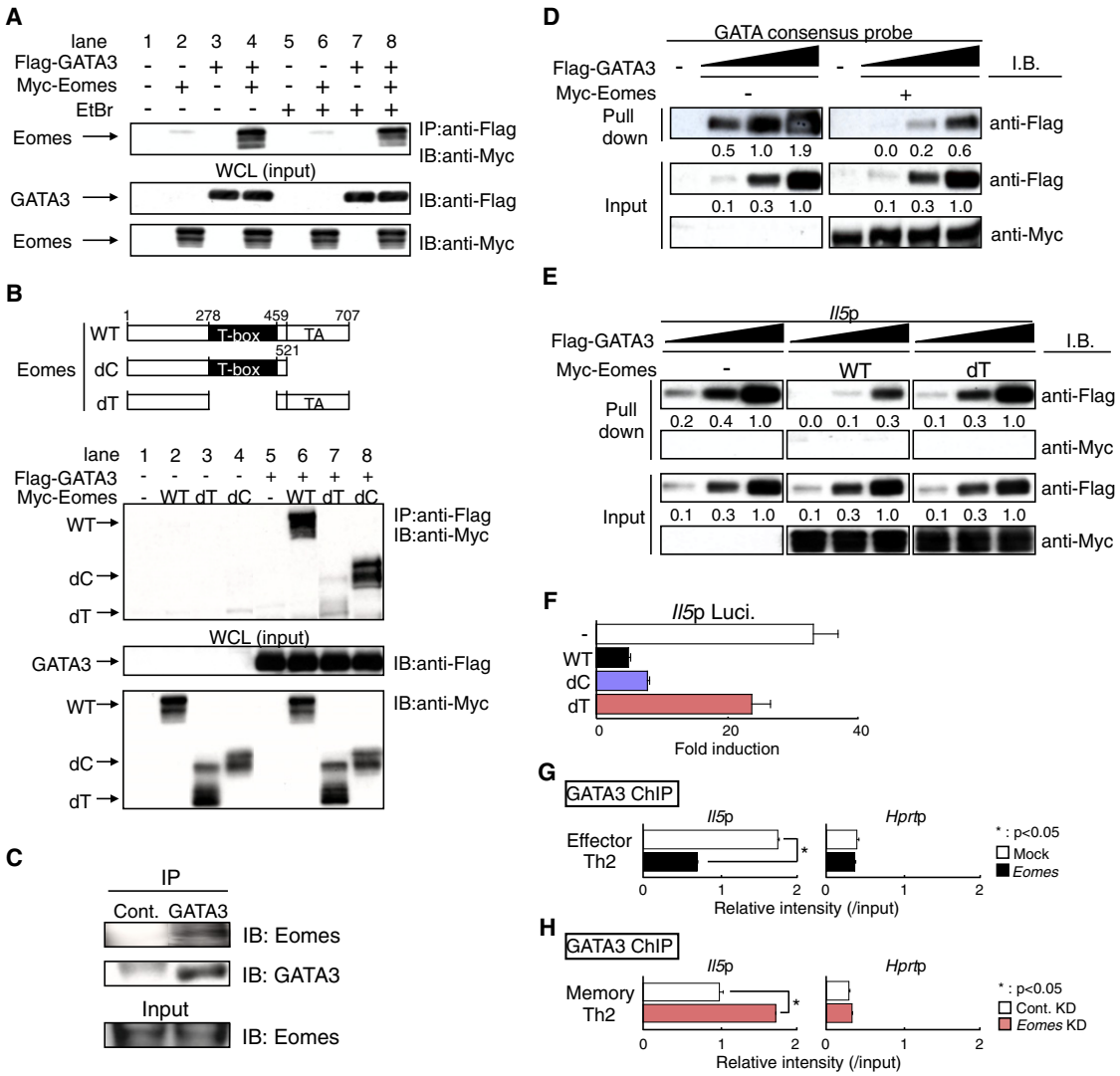


**Figure 5. Eomes Suppresses the Transcriptional Activity of GATA3 via the Inhibition of Binding to the *Il5* Promoter**

(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 ($1 \times 10^8$ 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

inflammation was exacerbated by downregulation of Eomes in memory Th2 cells.

## DISCUSSION

We have identified functionally relevant IL-5-producing memory Th2 cells in the CD62L$^{lo}$CXCR3$^{lo}$ subpopulation, which are required for the induction of Th2 cell-dependent eosinophilic airway inflammation. The possible molecular mechanisms that control IL-5 production in memory Th2 cells are depicted in the graphical abstract. Upon TCR stimulation, all four CD62L$^{lo}$CXCR3$^{lo}$, CD62L$^{lo}$CXCR3$^{hi}$, CD62L$^{hi}$CXCR3$^{lo}$, and CD62L$^{hi}$CXCR3$^{hi}$ subpopulations can produce a large amount of both IL-4 and IL-13. However, only a fraction of the CD62L$^{lo}$CXCR3$^{lo}$ subpopulation can produce IL-5. In this population, the expression of Eomes is limited, and high H3-K4 methylation at the *Il5*p was detected. The IL-5 nonproducing CD62L$^{lo}$CXCR3$^{lo}$ subpopulation also shows high H3-K4 methylation at the *Il5*p, but this subpopulation contains two types of cells: one expresses high amounts of both Eomes and GATA3, and the other expresses high amounts of Eomes but low amounts of GATA3. The other three subpopulations do not produce IL-5 and show low H3-K4 methylation at the *Il5*p. These three populations contain roughly the same proportion of GATA3$^{hi}$Eomes$^{lo}$, GATA3$^{hi}$Eomes$^{hi}$, and GATA3$^{lo}$Eomes$^{hi}$ cells as compared to the CD62L$^{lo}$CXCR3$^{lo}$ population, indicating that Eomes may not play an important role in the expression of IL-5 in these populations. Moreover, GATA3 expression itself appears insufficient for the expression of IL-5 in the majority of the memory Th2 cells because very few GATA3$^{hi}$ cells express IL-5 even in the absence of high-level Eomes expression. Therefore, other unknown factors appear to repress the expression of IL-5 or prevent the activation of IL-5 transcription in these populations, regardless of the expression of GATA3. Although the mechanisms that regulate histone modification at the *Il5* locus in memory Th2 cells remain unknown, H3-K4 methylation appears to be associated with the ability to produce IL-5.

The regulatory mechanism governing expression of Eomes in CD4$^+$ T cells has not been well established. Although previous reports suggest that IFN-γ upregulates the expression of Eomes in CD4$^+$ T cells (Suto et al., 2006), under some conditions, IL-4 appears to induce the expression of Eomes in antigen-stimulated CD8$^+$ and CD4$^+$ T cells (Takemoto et al., 2006; Weinreich et al., 2009). Based on these findings and the results of a DNA microarray analysis via IL-5$^-$ and IL-5$^+$ memory Th2 cells, we have defined several candidate molecules that may participate in control of Eomes expression in the IL-5-producing CD62L$^{lo}$CXCR3$^{lo}$ subpopulation. Reduced mRNA expression of the IFN-γ receptor component *Ifngr2*, the IFN-γ downstream signaling molecule *Stat1*, and also the IL-4 receptor α chain (*Il4ra*) were detected in IL-5$^+$ memory Th2 cells as compared to IL-5$^-$ memory Th2 cells, and therefore, reduced expression of these molecules may contribute to the low expression of Eomes in IL-5$^+$ memory Th2 cells. A recent report showed that Eomes can be upregulated when GATA3 expression ceases in Th2 cells (Yagi et al., 2010). We observed a reciprocal expression profile of GATA3 and Eomes in all four subpopulations (CD62L$^{lo}$CXCR3$^{lo}$, CD62L$^{lo}$CXCR3$^{hi}$, CD62L$^{hi}$CXCR3$^{lo}$, and CD62L$^{hi}$CXCR3$^{hi}$) of memory Th2 cells. Therefore, the counterregulation of expression of GATA3 and Eomes may exist in CD4$^+$ T cells. Eomes-mediated IL-5 suppression probably occurs predominantly in GATA3 and Eomes double-expressing cells in the CD62L$^{lo}$CXCR3$^{lo}$ population. However, it is also possible that other Eomes-dependent and -independent mechanisms operate in this population. Eomes may mediate indirect control of *Il5* expression by altering expression and/or function of other transcription factors or work in concert with other factors to ultimately suppress IL-5 production. The expression of Eomes was downregulated in memory Th2 cells after secondary challenge with antigen for 6 days (data not shown). In addition, the upregulation of Eomes was detected also in memory CD4$^+$ T cells induced by the immunization of antigen in vivo (data not shown).

Eomes siRNA gene targeting experiments revealed that the expression of *Il5* is more dependent on Eomes expression as compared to *Il4* and *Il13* in both memory Th2 and MPCD4$^+$ T cells. Furthermore, enforced expression of *Eomes* suppressed the expression of IL-5 but not IL-4 and IL-13 in in vitro developing effector Th2 cells. Both GATA3$^{hi}$ and GATA3$^{lo}$ memory Th2 cells produced substantial amounts of IL-4, but only GATA3$^{hi}$ cells produced IL-5. Indeed, IL-5 expression is known to be more dependent on the expression levels of GATA3 as compared to IL-4 (Inami et al., 2004). Eomes was found to interact with GATA3 in memory Th2 cells and suppress GATA3 DNA binding to the *Il5*p. These results may explain the preferential effect of Eomes on *Il5* expression as compared to *Il4* and *Il13*.

The current study indicates that the IL-5-producing CD62L$^{lo}$CXCR3$^{lo}$ population of memory Th2 cells is essential for the induction of allergic eosinophilic inflammation and AHR and that Eomes plays an important role in suppression of IL-5 and the induction of eosinophilic inflammation. Better defining the IL-5-producing CD4$^+$ T cells in allergic disorders may help to identify appropriate targets for intervention and the analysis of downstream target molecules of Eomes may be of interest. The DNA microarray analysis identified several potentially functional cell surface molecules upregulated on IL-5$^+$ memory Th2 cells. Interestingly, *Il1rl1* (*ST2*) is upregulated in IL-5$^+$ memory Th2 cells. IL-1RL1 is also the receptor for IL-33, a member of the IL-1 family, and IL-33- and ST2-mediated signaling triggers the activation of NF-κB leading to the production of Th2 cytokines (Kurowska-Stolarska et al., 2008; Schmitz et al., 2005). Therefore, the IL-5-producing CD62L$^{lo}$CXCR3$^{lo}$ population of memory Th2 cells identified in this study could be critical in the pathogenesis of chronic type 2 inflammation. Although preliminary, human IL-5-producing CD45RO$^+$ memory CD4$^+$ T cells in the peripheral blood showed decreased *EOMES* expression as compared to IL-5 nonproducing memory CD4$^+$ T cells, and siRNA gene targeting of *EOMES* enhanced *IL5* expression in the CD45RO$^+$ memory CD4$^+$ T cells (data not shown). Thus, further detailed studies focused on the IL-5-producing memory Th2 cells in chronic asthma models may lead to the discovery of novel therapeutic targets for the treatment of asthma.

In summary, we have identified IL-5-producing functionally relevant memory Th2 cells in the CD62L$^{lo}$CXCR3$^{lo}$ subpopulation. *Il5* expression is uniquely regulated by the expression of Eomes in memory Th2 cells. Eomes plays an important role in

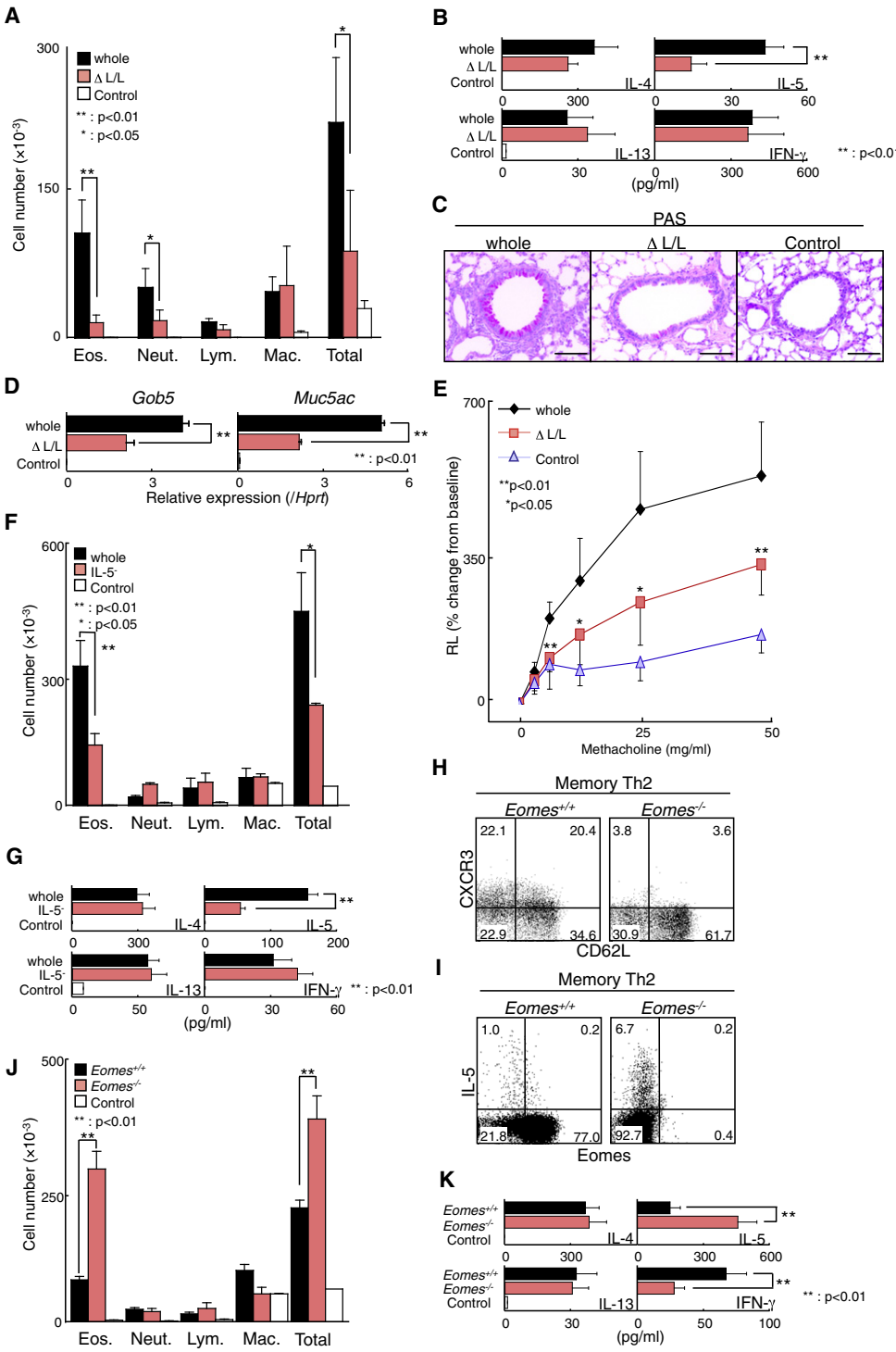


**Figure 6. Memory Th2 Cell-Dependent Airway Inflammation Is Ameliorated after Depletion of the CD62L$^{lo}$CXCR3$^{lo}$ Population**

(A–E) OVA-specific memory Th2 cells were sorted into KJ1$^{+}$ whole memory Th2 cells (whole) and memory Th2 cells depleted of the CD62L$^{lo}$CXCR3$^{lo}$ population (ΔL/L) by FACS. These cells were intravenously transferred into BALB/c (A–D) or BALB/c *nu/nu* (E) mice. No cell transfer was performed in the control group (Control). Airway inflammation was induced with OVA challenge.

(A) The absolute cell numbers of eosinophils (Eos.), neutrophils (Neu.), lymphocytes (Lym.), and macrophages (Mac.) in the BAL fluid are shown. The results were calculated with the percentages of the different cell types, the total cell number per milliliter, and the volume of the BAL fluid recovered. Samples were collected 2 days after the last OVA challenge. The mean values (five mice per group) are shown with SD.

(B) ELISA analysis of IL-4, IL-5, IL-13, and IFN-γ in the BAL fluid. Samples were collected 12 hr after the last OVA challenge. The mean values (five mice per group) are shown with SD. **p < 0.01.

the development of memory Th2 cell-dependent allergic airway inflammation demonstrating a role for Eomes in the regulation of polarized function of $CD4^+$ T cells.

## EXPERIMENTAL PROCEDURES

### Mice

The animals used in this study were backcrossed to BALB/c or C57BL/6 mice 10 times. Anti-OVA-specific TCR-αβ (DO11.10) transgenic (Tg) mice were provided by D. Loh (Washington University School of Medicine, St. Louis) (Murphy et al., 1990). *Eomes*$^{fl/fl}$ mice were kindly provided by S. Reiner (Pennsylvania University) (Intlekofer et al., 2008). Ly5.1 mice were purchased from Sankyo Laboratory. CD4-Cre mice were purchased from Taconic Farms (Germantown, NY). All mice were used at 6–8 weeks old and were maintained under SPF conditions. BALB/c and BALB/c *nu/nu* mice were purchased from Clea Inc. (Tokyo). Animal care was conducted in accordance with the guidelines of Chiba University.

### The Generation of Effector and Memory Th1 and Th2 Cells

Effector and memory Th1 and Th2 cells were generated as previously described (Inami et al., 2004; Yamashita et al., 2006). The detailed protocols are described in the Supplemental Experimental Procedures.

### Flow Cytometry and Sorting

Memory Th2 cells were stained with anti-CD62L-APC and anti-CXCR3-PE, and four subpopulations ($CD62L^{lo}CXCR3^{lo}$, $CD62L^{lo}CXCR3^{hi}$, $CD62L^{hi}CXCR3^{lo}$, and $CD62L^{hi}CXCR3^{hi}$) were purified by FACS. Memory Th2 cells were stimulated with immobilized anti-TCRβ for 6 hr, and IL-$5^+$ and IL-$5^-$ cells were purified with an IL-5 secretion assay kit (130-091-175, Miltenyi Biotec.) and FACS. The other reagents used in flow cytometry are listed in the Supplemental Experimental Procedures.

### Quantitative Real-Time PCR and ELISA for the Measurement of Cytokine Expression

Quantitative RT-PCR and ELISA were performed as described previously (Yamashita et al., 2006).

### Chromatin Immunoprecipitation Assay

ChIP assays were performed as described previously (Yamashita et al., 2002). The antibodies and primer pairs used in the ChIP assays are listed in the Supplemental Experimental Procedures.

### siRNA Gene Targeting Analysis

siRNA was introduced into memory Th2 or $MPCD4^+$ T cells by electroporation with a mouse T cell Nucleofector Kit and Nucleofector I (Amaxa). Memory Th2 cells and $MPCD4^+$ T cells were transfected with 675 pmole of control random siRNA or siRNA for *Eomes* and *Tbx21* (Applied Biosystems) and cultured for 24 hr.

### Immunoprecipitation, Immunoblotting, and Pull-Down Assay

The detailed protocol is described in the Supplemental Experimental Procedures.

### Assessment of Memory Th2 Cell Function In Vivo

OVA-specific memory Th2 cells were first generated in vivo (Nakayama and Yamashita, 2009). AHR was assessed on day 4 as described previously (Yamashita et al., 2008). The mRNA expression of *Gob5* and *Muc5ac* in the lung was assessed on day 5 (Yamashita et al., 2006). BAL fluid for the analysis of cytokine production by ELISA was collected 12 hr after the last inhalation and that for the assessment of inflammatory cell infiltration was collected on day 5. Lung histology was assessed on day 5.

### Statistical Analysis

Student's t test was used for all comparisons, data represented as mean ± SD.

## ACCESSION NUMBERS

The microarray data are available in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/gds) under the accession number GSE33516.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at doi:10.1016/j.immuni.2011.08.017.

## ACKNOWLEDGMENTS

The authors are grateful to R. Kubo for his helpful comments and constructive criticisms in the preparation of the manuscript. We thank H. Asou, M. Kato, and T. Ito for their excellent technical assistance. This work was supported by Global COE Program (Global Center for Education and Research in Immune System Regulation and Treatment), City Area Program (Kazusa/Chiba Area) MEXT (Japan), and by grants from the Ministry of Education, Culture, Sports, Science and Technology (Japan) (Grants-in-Aid: for Scientific Research on Priority Areas #17016010, #20060003, #22021011; Scientific Research [B] #21390147, Young Scientists [B] #22790452, and [JSPS fellows] #21.09747).

Received: September 30, 2010
Revised: June 6, 2011
Accepted: August 23, 2011
Published online: November 23, 2011

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(C) Two days after the last OVA challenge, the lungs were fixed and stained with periodic-acid-Schiff (PAS). A representative staining pattern is shown. Scale bars represent 100 μm.
(D) Quantitative RT-PCR analysis of *Gob5* and *Muc5ac* from the lung tissue 2 days after the last OVA challenge. $^{**}p < 0.01$.
(E) One day after the last OVA inhalation, changes in lung resistance (RL) were assessed. The mean values (five mice per group) are shown with standard deviations.
The experiments were performed twice with similar results (A, B, D, and E).
(F) The number of infiltrated leukocytes in the BAL fluid from whole or IL-$5^-$ memory Th2 cell transferred group are shown as in (A).
(G) ELISA analysis of IL-4, IL-5, IL-13, and IFN-γ in the BAL fluid from each experimental group is shown in (F).
(H and I) *Eomes*$^{+/+}$ or *Eomes*$^{-/-}$ memory Th2 cells were generated by transferring OT-II Tg-CD4-Cre-$CD45.1^+$ or OT-II Tg-CD4-Cre-*Eomes*$^{fl/fl}$-$CD45.1^+$ effector Th2 cells into *TCRbd*$^{-/-}$ mice.
(H) *Eomes*$^{+/+}$ or *Eomes*$^{-/-}$ memory Th2 cells were stained with CD62L and CXCR3 mAbs.
(I) *Eomes*$^{+/+}$ or *Eomes*$^{-/-}$ memory Th2 cells were stimulated in vitro with immobilized anti-TCRβ for 6 hr. Intracellular staining profiles of Eomes and IL-5 are shown with the percentage of cells in each area.
(J) The absolute cell numbers of leukocytes recovered in the BAL fluid of *Eomes*$^{+/+}$ or *Eomes*$^{-/-}$ memory Th2 cell transferred groups are shown as in (A).
(K) ELISA analysis of IL-4, IL-5, IL-13, and IFN-γ in the BAL fluid of the experiments shown in (J).
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