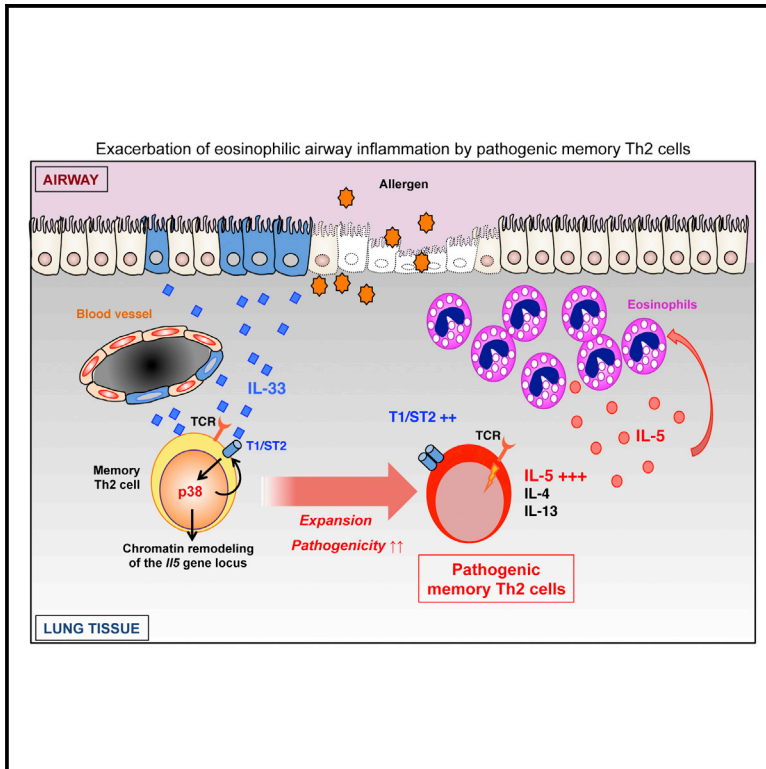


Immunity

The Interleukin-33-p38 Kinase Axis Confers Memory T Helper 2 Cell Pathogenicity in the Airway

Graphical Abstract



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In Brief

IL-33, a IL-1 family member identified as the ligand for the ST2 receptor, is deeply related to allergic inflammation. Nakayama and colleagues demonstrate that the IL-33-ST2-p38 axis is crucial for the induction of pathogenicity of memory Th2 cells in allergic airway inflammation in both mice and humans.

Highlights

- Memory Th2 cells are critical targets of IL-33 in allergic airway inflammation
- IL-33 selectively remodels chromatin of *IL5*, thereby licensing its expression
- Memory-Th2-cell-mediated airway inflammation depends on IL-33 and ST2
- p38 MAPK is a major downstream target of IL-33-ST2 signaling in memory Th2 cells



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The Interleukin-33-p38 Kinase Axis Confers Memory T Helper 2 Cell Pathogenicity in the Airway

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SUMMARY

Memory CD4⁺ T helper (Th) cells provide long-term protection against pathogens and are essential for the development of vaccines; however, some antigen-specific memory Th cells also drive immune-related pathology, including asthma. The mechanisms regulating the pathogenicity of memory Th cells remain poorly understood. We found that interleukin-33 (IL-33)-ST2 signals selectively licensed memory Th2 cells to induce allergic airway inflammation via production of IL-5 and that the p38 MAP kinase pathway was a central downstream target of IL-33-ST2 in memory Th2 cells. In addition, we found that IL-33 induced upregulation of IL-5 by memory CD4⁺ T cells isolated from nasal polyps of patients with eosinophilic chronic rhinosinusitis. Thus, IL-33-ST2-p38 signaling appears to directly instruct pathogenic memory Th2 cells to produce IL-5 and induce eosinophilic inflammation.

INTRODUCTION

The quality of adaptive immune responses depends on the number and function of antigen-specific memory T cells. Upon antigen recognition via the T cell receptor (TCR), naive CD4⁺ T cells undergo rapid clonal expansion, followed by differentiation into functionally distinct T helper (Th) cell subsets, such as Th1, Th2, and Th17 cells (O'Shea and Paul, 2010; Reiner, 2007). Some of these effector Th cells are maintained as memory Th cells for long times in vivo (Nakayama and Yamashita, 2008), and it is now becoming clear that these cells display functional heterogeneity (Sallusto and Lanzavecchia, 2009). Recent reports indicate that there are several distinct subsets of memory type Th2 cells that produce large amounts of

interleukin-5 (IL-5), IL-17, or interferon- γ (IFN- γ) in addition to IL-4 and IL-13 (Endo et al., 2014; Endo et al., 2011; Hegazy et al., 2010; Islam et al., 2011; Upadhyaya et al., 2011; Wang et al., 2010). In particular, IL-5-producing memory Th2 cell subsets appear to be crucial drivers of the pathology of allergic diseases in the airway and skin (Endo et al., 2011; Islam et al., 2011). However, environmental cues and signals that dictate how memory Th2 cells contribute to the pathogenicity of allergic diseases, including chronic airway inflammation, are poorly understood.

Asthma is a chronic lower-airway inflammatory disease characterized by recurrent airway obstruction and wheezing (Cohn et al., 2004). Allergic asthma is mainly driven by Th2-cell-type cytokines, such as IL-4, IL-5, and IL-13, and is characterized by the presence of elevated numbers of eosinophils in the lungs (Cohn et al., 2004). IL-5 regulates eosinophil development, recruitment to the lungs, and activation (Rosenberg et al., 2013). IL-13 plays an important role in the effector phase of asthma by inducing airway remodeling and airway hyperresponsiveness (AHR) as well as mucus hyperproduction (Ingram and Kraft, 2012). Th2 cells are the major source of IL-4, IL-5, and IL-13 in allergic asthma. Innate Th2 cell counterparts that also produce large amounts of IL-5 and IL-13 (Lin⁺CD127⁺ type 2 innate lymphoid cells [ILC2s]) have been identified (Furusawa et al., 2013; Lloyd, 2010; Price et al., 2010; Saenz et al., 2010). Recent research indicates that ILC2s play a critical role in eosinophilic airway inflammation in mice that lack the ability to mount adaptive immune responses (Chang et al., 2011; Halim et al., 2012; Scanlon and McKenzie, 2012).

Chronic rhinosinusitis (CRS) is a common chronic sinus inflammatory disease characterized by distinct cytokine production profiles and tissue-remodeling patterns (Hamilos, 2011; Van Bruaene et al., 2008; Zhang et al., 2008). CRS can be classified into two types of diseases according to the presence of nasal polyps. CRS with nasal polyps (CRSwNP) is often accompanied by Th2-cell-skewed eosinophilic inflammation, whereas CRS without nasal polyps (CRSSNP) is characterized by a predominantly Th1-cell-skewed response (Hamilos, 2011). IL-5 is more

abundant in the nasal mucosal tissues of CRSwNP than in those of CRSsNP (Van Bruaene et al., 2008). CRSwNP is further subdivided into two types of diseases on the basis of the extent of eosinophilic inflammation, particularly for people in East Asia (Zhang et al., 2008): eosinophilic CRS (ECRS) and non-eosinophilic rhinosinusitis (NECRS).

IL-33, a member of the IL-1 family, was newly identified as the ligand for the ST2 receptor (also known as IL-1RL1) (Liew et al., 2010; Schmitz et al., 2005). The major genome-wide association studies have reproducibly found significant associations between *IL33* and *IL1RL1* genetic variants and asthma in humans (Bønnelykke et al., 2014; Grotenboer et al., 2013; Torgerson et al., 2011). IL-33 expression is higher in asthmatic patients and in mouse models of asthma (Lloyd, 2010). Epithelial and airway smooth muscle cells appear to represent two major sources of IL-33 in asthmatics (Préfontaine et al., 2009). Previous reports showed that the depletion of IL-33 or ST2 attenuated murine ovalbumin (OVA)-induced airway inflammation (Kurowska-Stolarska et al., 2008; Oboki et al., 2010). ILC2s are characterized by their rapid production of IL-5 and IL-13 in response to IL-33 exposure (Scanlon and McKenzie, 2012). Therefore, understanding the mechanisms by which IL-33 induces allergic inflammation and differentiating between antigen-specific and antigen-independent functions of IL-33 are crucial for the effective design of therapeutics for patients with allergic inflammatory disorders such as chronic asthma.

We herein investigated the role of IL-33 in allergic airway inflammation induced by memory Th2 cells. We found that IL-33-ST2 signaling was crucial for the induction of pathogenicity of memory Th2 cells in allergic experimental asthma. Moreover, we found that like ILC2s, memory Th2 cells acquired the ability to produce IL-5 directly in response to IL-33; this property was not observed in effector Th2 cells. Genetic deletion of IL-33 or ST2 resulted in impaired memory-Th2-cell-dependent eosinophilic airway inflammation, and we identified p38 mitogen-activated protein kinase (MAPK) as the downstream target of IL-33-ST2 signaling in this cell type. Analysis of nasal polyps from patients with CRS showed that IL-33 could also directly enhance IL-5 production by human memory CD4⁺ T cells. Thus, we propose that the IL-33-ST2-p38 axis is crucial for the induction of pathogenicity of memory Th2 cells in eosinophilic airway inflammation in both mice and humans.

RESULTS

IL-33 Selectively Enhances IL-5 Production by Memory Th2 Cells

IL-33 is known to induce strong Th2-cell-type immune responses and eosinophilic inflammation in the lung and intestine (Lloyd, 2010). However, the types of cells on which IL-33 acts in these settings are still being defined. To explore the involvement of CD4⁺ T cells in IL-33-mediated inflammation, we assessed the expression of the IL-33 receptor (ST2) on naive CD4⁺ T cells, effector Th1 and Th2 cells generated in vitro, and memory Th1 and Th2 cells generated in vivo (Nakayama and Yamashita, 2008) (Figure S1A). Memory Th2 cells showed very high expression of IL-7 receptor- α chain (IL-7R α), and the majority also showed low expression of CD69 and IL-2R α (Figure S1B, upper panel). Expression patterns of these surface receptors

were quite different from that seen on the in-vitro-generated effector Th2 cells used in this study. We observed that compared to naive CD4⁺ T cells or memory Th1 cells, memory Th2 cells showed strongly increased expression of *Il1rl1* mRNA (Figure 1A). Compared to naive CD4⁺ T cells or effector Th1 cells, effector Th2 cells also showed significantly higher expression of *Il1rl1* ($p < 0.01$; Mann-Whitney U test), but this was not as pronounced as the expression observed in memory Th2 cells. The increased *Il1rl1* mRNA was also reflected by higher expression of ST2 on the surface of memory Th2 cells than on the surface of naive CD4⁺ T cells or effector Th2 cells (Figure 1B). Substantial ST2 expression was detected only on memory Th2 cells, and ST2 was specifically found on those cells with high expression of IL-7R α and low expression of CD69 and IL-2R α , strongly indicating that a distinct subset of ST2-expressing cells is induced in memory Th2 cells (Figure S1B, lower panel). In addition, exposure of memory Th2 cells to IL-33 dramatically enhanced ST2 expression (Figure 1C). Importantly, we found that stimulation with IL-33 for 5 days selectively induced IL-5 production by memory Th2 cells but not by effector Th2 cells (Figure 1D). In contrast, IL-33-induced upregulation of IL-4 expression was not observed in response to treatment with IL-33 (Figures 1D and 1F). IL-13 was slightly increased by cultivation of memory Th2 cells with IL-33 (Figures 1E and 1F). IL-33 supported the viability of memory Th2 cells as well as IL-2 and IL-7 (Figure S1C) without inducing significant proliferation (Figure S1D). We reported previously that memory Th2 cells can be subdivided into four distinct subpopulations according to the expression of CXCR3 and CD62L and that IL-5 production is normally restricted to a small number of cells in the CD62L^{lo}CXCR3^{lo} subpopulation (Endo et al., 2011) (Figure S1E). We also examined the effect of IL-33 on the four subpopulations (CXCR3 and CD62L) of memory Th2 cells. ST2 expression was detected on 10%–20% of all four subpopulations of freshly prepared memory Th2 cells (Figure S1F, left). IL-33 treatment enhanced ST2 expression on all four subpopulations (Figure S1F, right). Upon TCR stimulation, IL-5-producing cells were detected only in the CD62L^{lo}CXCR3^{lo} subpopulation of freshly prepared memory Th2 cells (Figure S1G, left), whereas after IL-33 cultivation, IL-5-producing cells were detected in all four subpopulations and showed their highest numbers in the CD62L^{lo}CXCR3^{lo} subpopulation (Figure S1G, right). IL-5 production was also assessed by ELISA, and similar results were obtained (Figure S1H). Thus, these results indicate that IL-33 upregulates ST2 expression and selectively enhances IL-5 expression and production by memory Th2 cells, but not effector Th2 cells.

IL-33 Induces Selective Remodeling of Chromatin at the *Il5* Locus in Memory Th2 Cells

Epigenetic chromatin modifications can control selective expression of genes that function in the immune system (Northrup and Zhao, 2011). We therefore explored whether IL-33 signaling could regulate the chromatin status of the Th2-cell-associated cytokine-encoding genetic loci in memory Th2 cells. We performed chromatin immunoprecipitation (ChIP) assays with antibodies specific to several histone modifications (Figure 2A). At the *Il5* locus, freshly prepared in-vivo-generated memory Th2 cells showed lower modifications associated with active

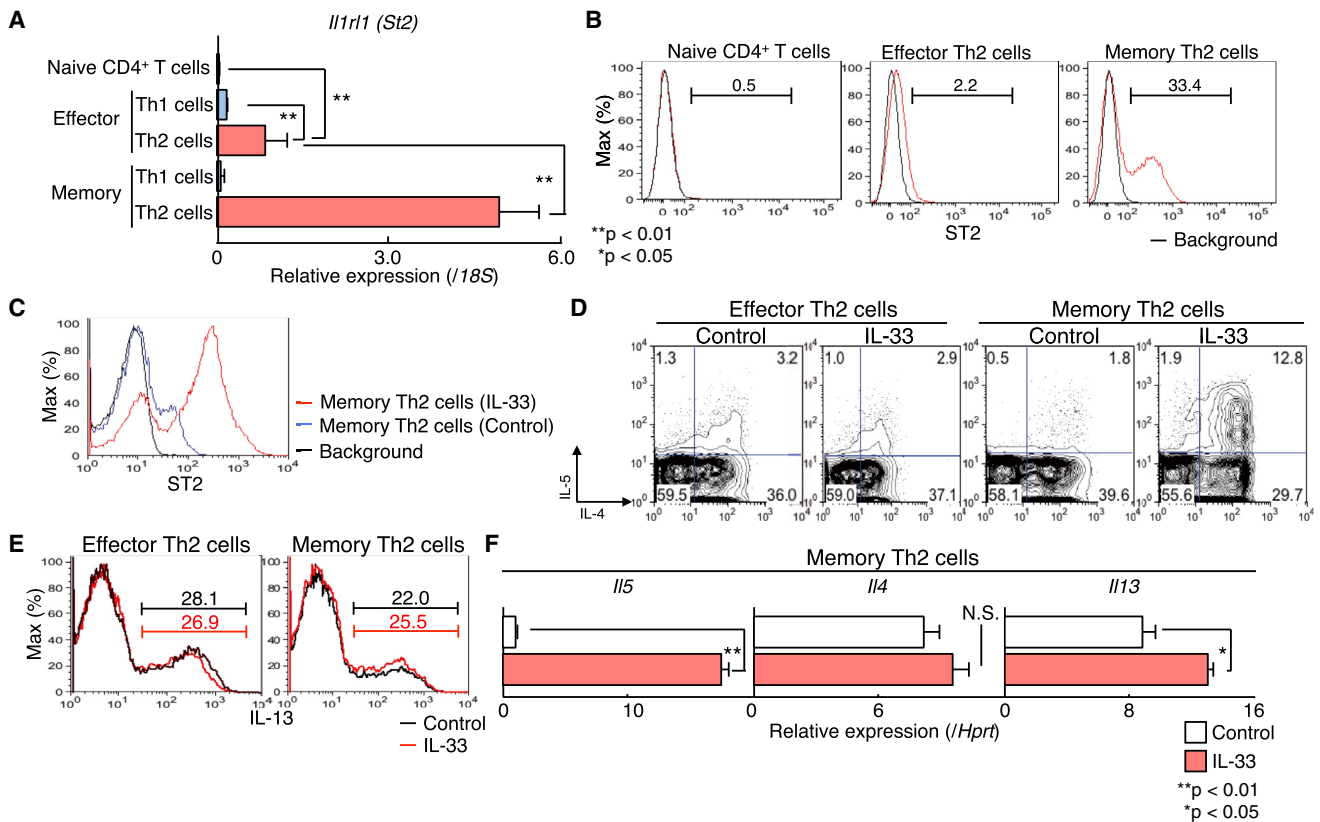


Figure 1. IL-33 Selectively Induces IL-5 Production by Memory Th2 Cells

(A) Quantitative RT-PCR analysis of *Il1r1* in the indicated cell types. Relative expression (normalized to *Hprt*) is shown with SDs.

(B) Expression profiles of ST2 on the indicated cell types.

(C) Expression profiles of ST2 on memory Th2 cells before (blue) and after (red) culture with IL-33.

(D) Memory and effector Th2 cells were cultured with IL-33 for 5 days. The cultured cells were stimulated with immobilized anti-TCR β for 6 hr. Intracellular-staining profiles of IL-5 and IL-4 are shown.

(E) Intracellular-staining profiles of IL-13 in effector and memory Th2 cells before and after culture with IL-33 are shown.

(F) Quantitative RT-PCR analysis of the indicated genes in memory Th2 cells before and after culture with IL-33.

More than five independent experiments were performed and showed similar results (***p* < 0.01; NS, not significant; Mann-Whitney U test; A–F). Three technical replicates were performed with quantitative RT-PCR (A and F). See also Figure S1.

transcription (H3-K4 trimethylation and H3-K9 acetylation) and higher modifications associated with genetic repression (H3-K27 trimethylation) than did effector Th2 cells (Figure 2B), indicating that these modifications were not efficiently maintained during the transition to the memory phase. However, after stimulation of memory Th2 cells with IL-33 (without TCR stimulation), H3-K4 trimethylation and H3-K9 acetylation increased and H3-K27 trimethylation decreased at the *Il5* locus, indicating that IL-33 could restore the chromatin-modification signature observed in IL-5-producing effector Th2 cells. In contrast to histone modifications at the *Il5* locus, those at the other Th2 cell cytokine-encoding loci (*Il4p*, *Il13p*, and *Va* enhancer) were not obviously affected by IL-33 stimulation (Figures S2A and S2B). We also observed increased H3-K4 trimethylation and H3-K9 acetylation at the *Il1r1* locus in IL-33-stimulated memory Th2 cells (Figures S2C and S2D). These changes in histone modifications at the *Il5* locus were specific to IL-33 because no obvious change was detected in memory Th2 cells treated with IL-2, IL-7, or IL-25 (Figure S2E). It has previously been shown that

like IL-33, a combination of IL-2 and IL-25 can induce IL-5 production in ILC2s (Furusawa et al., 2013). We examined the synergistic effect of IL-2 and IL-25 on IL-5 production by memory Th2 cells, and indeed cultivation with IL-2 and IL-25, similar to IL-33 cultivation, increased IL-5 production in memory Th2 cells (Figure S2F). Next, we performed ChIP assays to assess whether the chromatin remodeling at the *Il5* locus of memory Th2 cells was induced by the combination of IL-2 and IL-25. Active histone modifications were increased and repressive histone modifications were decreased at the *Il5* locus in memory Th2 cells after culture with both IL-2 and IL-25 (Figure S2G). The IL-33-induced histone modifications at the *Il5* locus were accompanied by enhanced recruitment of p300, a component of the histone acetyl transferase (HAT) complex, and RNA polymerase II (pol II) (Figure 2C). Stronger binding of pol II to the *Il5* locus, including the transcribed region in IL-33-cultured memory Th2 cells, was detected. These results indicate that IL-33, as well as the combination of IL-2 and IL-25, remodels chromatin structure to be permissive for transcription at the *Il5* locus in memory Th2 cells.

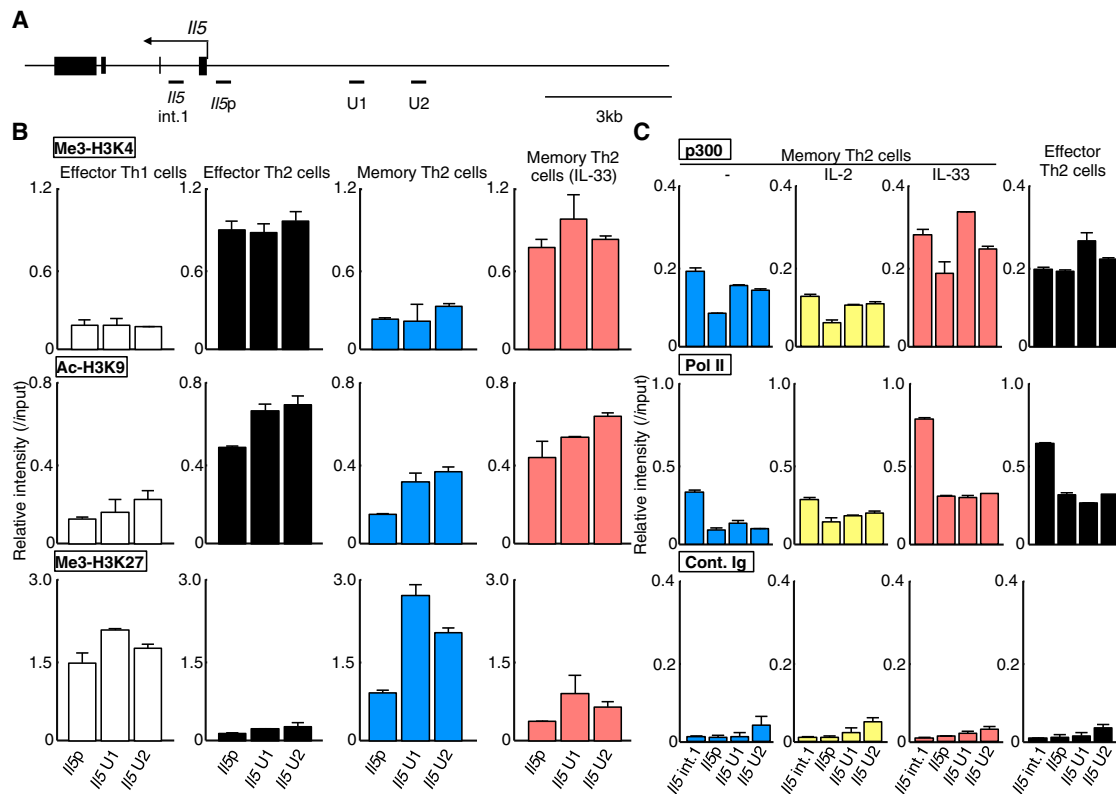


Figure 2. IL-33 Selectively Remodels Chromatin Structure at the *IL5* Locus in Memory Th2 Cells Independently of TCR Stimulation

(A) Schematic representation of the mouse *IL5* locus. The locations of primers and probes (upstream region 2 [U2] to *IL5* intron 1 [int.1]) and exons are indicated. (B) ChIP assays were performed with anti-trimethyl histone H3-K4, anti-acetyl histone H3K9, and anti-trimethyl histone H3K27 at the *IL5* locus from effector Th1, effector Th2, freshly prepared memory Th2, and memory Th2 cells cultured with IL-33 for 5 days. The relative intensities (relative to input DNA) of these modifications were determined by quantitative RT-PCR analysis. More than three independent experiments were performed and showed similar results. (C) The binding of p300 and pol II to the *IL5* locus in the indicated cell types was detected by ChIP with quantitative RT-PCR analysis. Two independent experiments were performed and showed similar results. Three technical replicates were performed with quantitative RT-PCR (B and C). See also Figure S2.

Because TCR stimulation was not included in this culture system, it appears as though the observed chromatin remodeling was solely induced by cytokine signaling.

The IL-33-ST2 Pathway Enhances IL-5 Production by Memory Th2 Cells In Vivo

We used *IL1rl1*-deficient (*IL1rl1*^{-/-}) mice in order to directly demonstrate the involvement of the IL-33-ST2 pathway in the generation of IL-5-producing memory Th2 cells. In the absence of ST2, effector Th2 cell differentiation was not impaired, and memory Th2 cells were generated normally in vivo (Figures S3A and S3B). As expected, IL-33-induced IL-5 augmentation was not observed in *IL1rl1*^{-/-} memory Th2 cells (Figure 3A). Next, we assessed the effect of IL-33 on the IL-5 production of memory Th2 cells in vivo, as illustrated in Figure 3B. Administration of IL-33 increased ST2 expression (Figure 3C) and IL-5 production (Figure 3D) of *IL1rl1*^{+/+} memory Th2 cells but did not substantially alter expression of ST2 or IL-5 production by *IL1rl1*^{-/-} memory Th2 cells. These results indicate that memory Th2 cells respond to IL-33 in vivo by increasing ST2 expression and producing IL-5, as was observed in memory Th2 cells after cultivation with IL-33 in vitro.

We also generated non-transgenic memory CD4⁺ T cells by using an OVA-alum (OVA with aluminum) immunization system in vivo, as depicted in Figure 3E. A substantial proportion of non-TCR transgenic memory CD4⁺ T cells expressed ST2 (Figure 3F and Figure S3C), and IL-33-induced IL-5 augmentation was detected in *IL1rl1*^{+/+} memory CD4⁺ T cells, but not in *IL1rl1*^{-/-} memory CD4⁺ T cells (Figure 3G). Thus, the IL-33-ST2 pathway is critical for the induction of ST2 expression and generation of IL-5-producing memory Th2 cells in vivo.

Memory-Th2-Cell-Dependent Eosinophilic Airway Inflammation and AHR Are Ameliorated by the Depletion of IL-33 or ST2

In order to assess the role of the IL-33-ST2 pathway in the pathological function of memory Th2 cells, we used memory-Th2-cell-dependent airway-inflammation models, as illustrated in Figure 4A. The expression of *IL33* in the lung was increased after OVA challenge by inhalation, whereas the expression of *IL33* in the spleen was not increased even after OVA challenge (Figure 4B). In this model, ST2-expressing cells were increased after OVA challenge in the lung, and highly ST2-expressing cells were found in the CXCR3⁺ memory Th2 cell population (indicated by

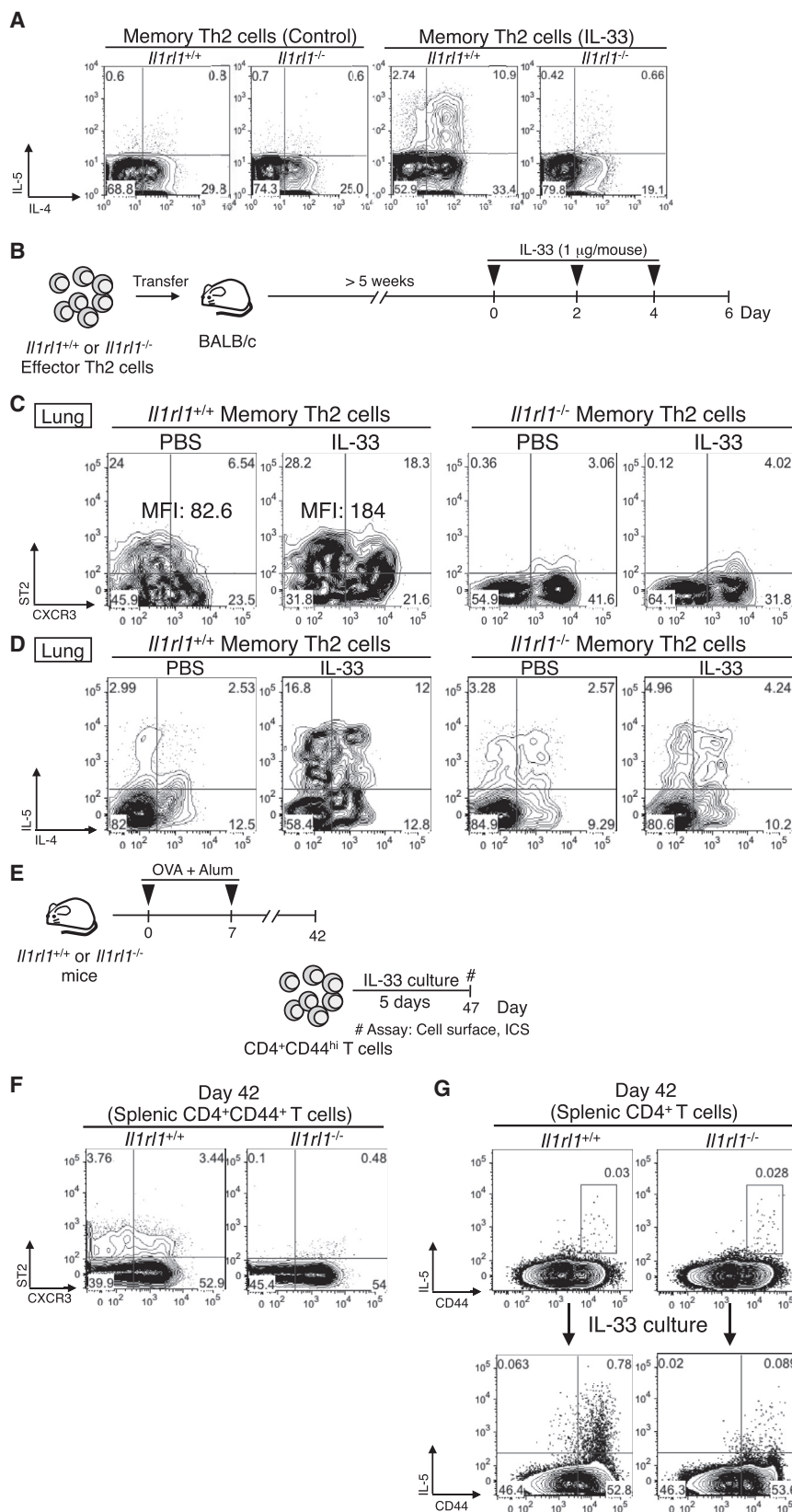


Figure 3. The IL-33-ST2 Pathway Enhances IL-5 Production by Memory Th2 Cells In Vivo

(A) Intracellular-staining profiles of IL-5 and IL-4 in *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory Th2 cells before (left, control) and after (right, IL-33) cultivation with IL-33. Five independent experiments were performed and showed similar results.

(B) Experimental protocols for the injection of IL-33 into the mice that received *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory Th2 cells.

(C) ST2 and CXCR3 expression profiles of KJ1⁺ memory Th2 cells recovered from the lungs of mice that received *Il1rl1*^{+/+} (left) or *Il1rl1*^{-/-} (right) memory Th2 cells.

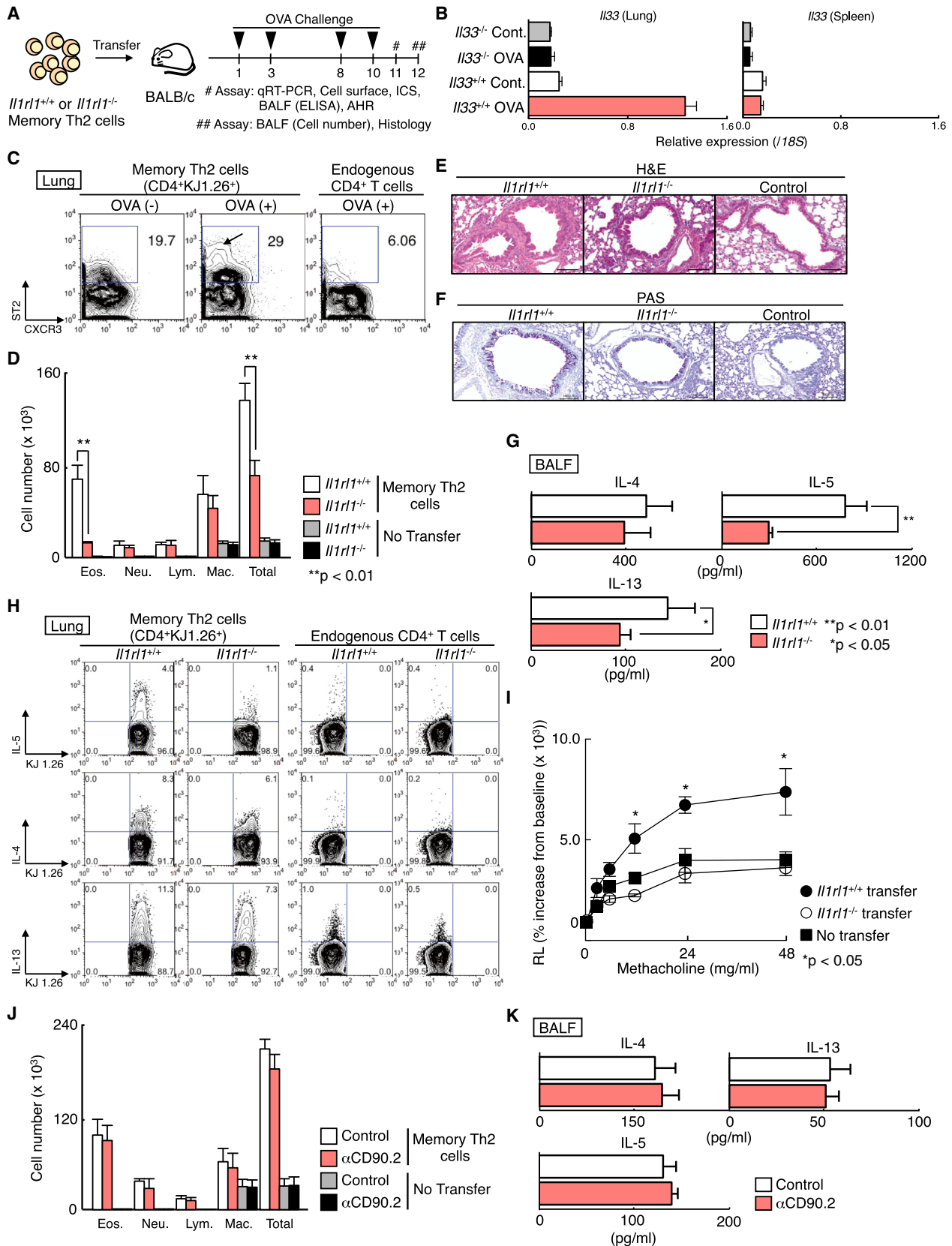
(D) KJ1⁺ memory Th2 cells shown in (C) were stimulated in vitro with immobilized anti-TCRβ for 6 hr. Intracellular-staining profiles of IL-5 and IL-4 are shown.

(E) Experimental protocols for the generation of non-TCR transgenic memory CD4⁺ T cells in vivo.

(F) Cell-surface-expression profiles of ST2 and CXCR3 on *Il1rl1*^{+/+} or *Il1rl1*^{-/-} CD44⁺ memory type CD4⁺ T cells are shown.

(G) *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory CD4⁺ T cells shown in (F) were stimulated with OVA-pulsed antigen-presenting cells for 24 hr before (upper) and after (lower) cultivation with IL-33. Intracellular-staining profiles of IL-5 and CD44 are shown with the percentage of cells in each area.

Two independent experiments were performed and showed similar results, and five technical replicates were included (C, D, F, and G). See also Figure S3.



(legend on next page)

an arrow, Figure 4C, middle). Only a small number of ST2-expressing cells were detected in endogenous lung CD4⁺ T cells even after OVA challenge (Figure 4C, right).

Next, OVA-specific *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory Th2 cells were transferred into wild-type BALB/c recipients, and their ability to induce airway inflammation was assessed. Compared to the *Il1rl1*^{+/+} group, the *Il1rl1*^{-/-} group showed a significant decrease ($p < 0.01$; Mann-Whitney U test) in the number of inflammatory eosinophils in the bronchoalveolar lavage (BAL) fluid (Figures 4D and S4A). No obvious changes in numbers of infiltrated cells were detected in OVA-challenged *Il1rl1*^{+/+} and *Il1rl1*^{-/-} mice that had not received memory Th2 cells. Histological analysis also revealed a similar reduction in mononuclear cell infiltration into the peribronchiolar regions of the lungs (Figure 4E). Periodic acid-Schiff (PAS) staining showed decreased production of mucus in the *Il1rl1*^{-/-} group (Figure 4F). Conversely, similar eosinophilic infiltration into the BAL fluid was observed from the mice that received *Il1rl1*^{+/+} and *Il1rl1*^{-/-} effector Th2 cells that were challenged with OVA on days 1 and 3 (Figures S4B and S4C). These results support the observation that effector Th2 cells possess little reactivity to IL-33 and that this pathway is less important for induction of eosinophilic inflammation by effector Th2 cells than for induction by memory Th2 cells. We also performed a set of in vivo kinetics experiments to better define the timing of acquisition of IL-33 responsiveness by memory Th2 cells. Eosinophilic airway inflammation, IL-5-producing Th2 cells in the lung, and IL-5 in the BAL fluid were assessed 6 or 35 days after the transfer of *Il1rl1*^{+/+} and *Il1rl1*^{-/-} effector Th2 cells (Figures S4D–S4K). Six days after cell transfer, no significant difference was detected in these three parameters between the *Il1rl1*^{-/-} and *Il1rl1*^{+/+} groups (Figures S4D–S4G). In contrast, significant decreases in eosinophilic infiltration ($p < 0.01$; Mann-Whitney U test), generation of IL-5-producing Th2 cells, and IL-5 production were observed in the day-35 *Il1rl1*^{-/-} group (Figures S4H–S4K). We also examined the OVA-induced airway inflammation in OVA-immunized *Il1rl1*^{+/+} and *Il1rl1*^{-/-} mice at different time points (12 or 45 days after OVA immunization) (Figures S4L–S4S). Significantly decreased infiltration of eosinophils into the BAL fluid ($p < 0.01$; Mann-Whitney U test), reduced IL-5-producing Th2 cells in the lung, and decreased IL-5 production in the BAL fluid were observed in the *Il1rl1*^{-/-} group 45 days after, but not 12 days after, the last immunization. These results indicate that ST2-mediated induction of IL-5 and exacerbation of eosinophilic airway inflammation were acquired by memory Th2 cells 35 days after effector Th2 cell transfer or 45 days after antigenic immunization, but not at the earlier time points analyzed.

The amount of IL-5 in the BAL fluid was significantly lower ($p < 0.01$; Mann-Whitney U test) in the *Il1rl1*^{-/-} memory Th2 cell group than in the *Il1rl1*^{+/+} group, whereas the amount of IL-4 was almost equivalent between the two groups (Figure 4G). We also detected a modest but significant reduction in IL-13 ($p < 0.05$; Mann-Whitney U test). Consistent with these observations, fewer IL-5-producing memory Th2 cells were detected in the lungs of mice from the *Il1rl1*^{-/-} group than in those from the *Il1rl1*^{+/+} group after OVA inhalation (Figure 4H, left), whereas decreases in IL-4- and IL-13-producing cells were less prominent. Th2-cell-associated cytokine production was not substantially different between endogenous *Il1rl1*^{+/+} and *Il1rl1*^{-/-} CD4⁺ T cells (Figure 4H, right). In addition, comparable proportions of Th2-cell-associated cytokine-producing memory Th2 cells were observed in the spleens of mice from the *Il1rl1*^{-/-} and *Il1rl1*^{+/+} groups (Figure S4T). These data are consistent with the absence of change in *Il33* expression in the spleen after OVA challenge (Figure 4B). We also analyzed the degree of AHR in the allergy-induced mice, which received *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory Th2 cells, by measuring methacholine-induced airflow obstruction with a mechanical ventilator. The degree of AHR in mice that received *Il1rl1*^{-/-} memory Th2 cells was lower than that of mice that received *Il1rl1*^{+/+} memory Th2 cells ($p < 0.05$; Mann-Whitney U test) (Figure 4I).

Finally, we assessed the relative contribution of memory Th2 cells to eosinophilic inflammation given that it is already known that ILC2s can respond to IL-33 by producing IL-5 in the lung (Halim et al., 2012). We examined the effect of the depletion of ILC2s on memory-Th2-cell-induced airway inflammation in the lung by using CD90.2 antibody. In brief, CD90.1⁺ memory Th2 cells were transferred into CD90.2 *Rag2*^{-/-} mice, and CD90.2⁺ ILC2s were depleted by the administration of CD90.2 antibody (Figures S4U and S4V). These mice were challenged with inhaled OVA four times. The number of infiltrated eosinophils and the concentration of IL-5 in the BAL fluid were found to be similar between control and ILC2-depleted groups (Figures 4J and 4K). Therefore, at least in this system, the contribution of ILC2s to eosinophilic inflammation is relatively minor.

***Il33*^{-/-} Mice Have Impaired Generation of IL-5-Producing Memory Th2 Cells and Reduced Eosinophilic Airway Inflammation**

We next assessed the function of IL-33 on memory Th2 cells by using *Il33*^{+/+} and *Il33*^{-/-} mice as recipients with or without OVA challenge (Figures S5A and S5B). First, we assessed the effect of endogenous IL-33 on memory Th2 cells in the steady state.

Figure 4. The Pathogenicity of Memory Th2 Cells Is Ameliorated by Loss of IL-33-ST2 Signaling

(A) Experimental protocols for memory-Th2-cell-dependent allergic inflammation.

(B–I) Mice were treated as described in (A). (B) Quantitative RT-PCR analysis of *Il33* in the spleen or lungs. (C) ST2 and CXCR3 expression profiles of lung KJ1⁺ memory Th2 cells from mice with no challenge (left) or OVA challenge (center) and endogenous CD4⁺ T cells from mice with OVA challenge (right). (D) The absolute cell numbers of eosinophils (Eos), neutrophils (Neu), lymphocytes (Lym), and macrophages (Mac) in the BAL fluid are shown. Mean values (five mice per group) are shown with SDs. (E and F) Lungs were fixed and stained with H&E or PAS. A representative staining pattern is shown. Scale bars represent 100 μ m. (G) ELISA of the indicated cytokines in the BAL fluid from the experimental groups shown in (D). (H) Intracellular-staining profiles of the indicated cytokines in *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory Th2 cells in the lung. (I) Lung resistance (RL) was assessed in response to increasing doses of methacholine. The mean values (five mice per group) are shown with SDs.

(J) The absolute cell numbers of leukocytes in the BAL fluid. Mean values (five mice per group) are shown with SDs.

(K) ELISA analysis of the indicated cytokines in the BAL fluid from the experimental groups with memory Th2 cell transfer shown in (J).

Three (B–H) or two (J and K) independent experiments were performed and showed similar results (** $p < 0.01$; * $p < 0.05$). Three technical replicates were included in quantitative RT-PCR analysis (B). Five technical replicates were included in ELISA on the BAL fluid samples (G and K). See also Figure S4.

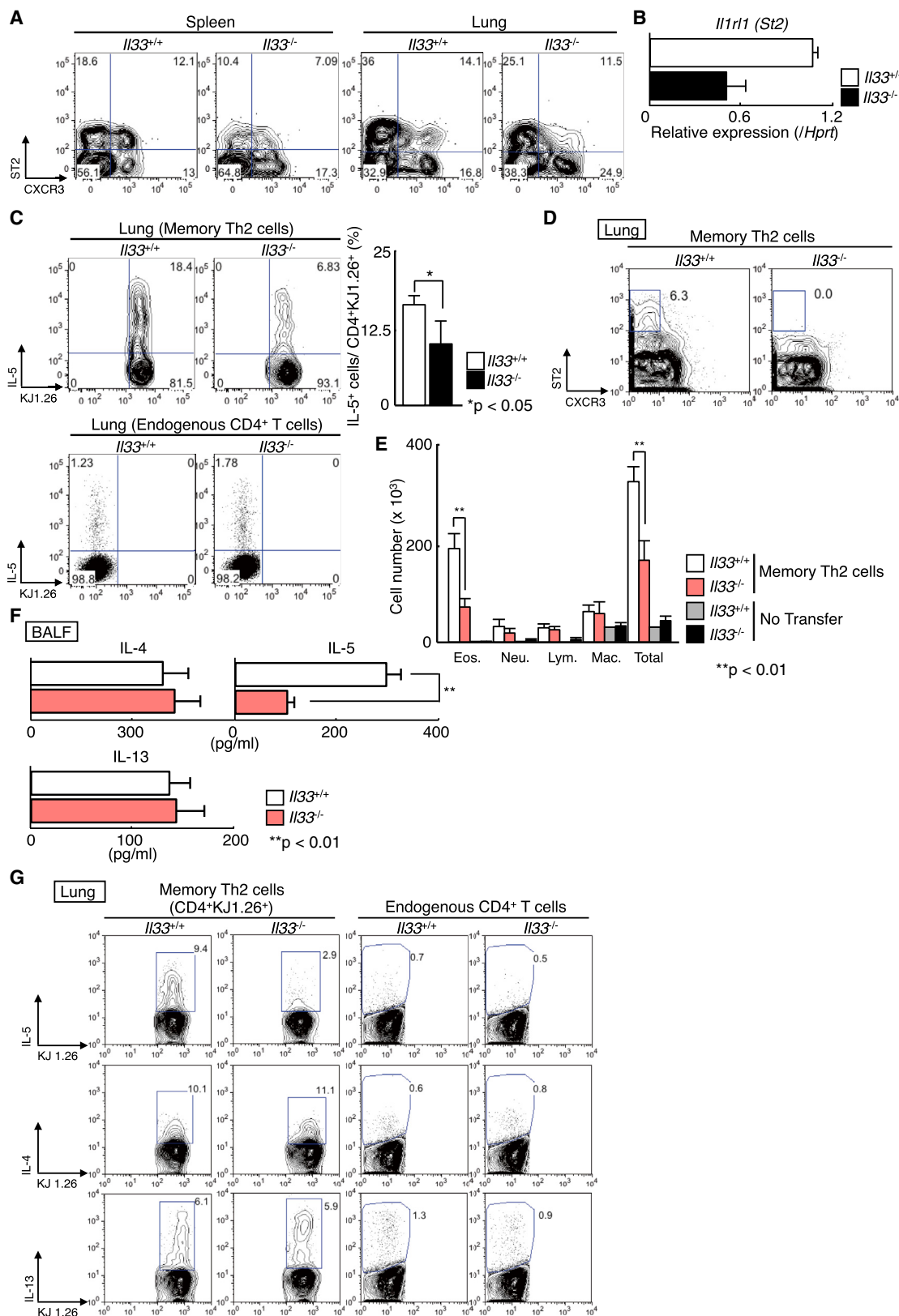


Figure 5. Reduced Memory-Th2-Cell-Dependent Airway Inflammation in *Il33*^{-/-} Mice

(A) ST2 and CXCR3 expression profiles of KJ1⁺ memory Th2 cells prepared from the spleens and lungs of *Il33*^{+/+} or *Il33*^{-/-} recipients.

(B) Quantitative RT-PCR analysis of *Il1rl1* in lung memory Th2 cells from *Il33*^{+/+} or *Il33*^{-/-} recipients. Relative expression (normalized to *Hprt*) is shown with SDs.

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Compared to memory Th2 cells generated in $Il33^{+/+}$ mice, memory Th2 cells generated in $Il33^{-/-}$ mice (without OVA inhalation) showed a moderate reduction of ST2 expression (Figures 5A and 5B) and significant reduction of IL-5-producing cells (Figure 5C) ($p < 0.05$; Mann-Whitney U test). We also examined memory-Th2-cell-dependent airway inflammation by using $Il33^{+/+}$ and $Il33^{-/-}$ mice as recipients (Figure S5B). The OVA-induced highly ST2-expressing CXCR3⁺ population was not detected when memory Th2 cells were transferred into $Il33^{-/-}$ mice (Figure 5D). Compared to the $Il33^{+/+}$ group, mice from the $Il33^{-/-}$ group displayed significantly decreased eosinophilic infiltration ($p < 0.01$; Mann-Whitney U test; Figure 5E), and the amount of IL-5 in the BAL fluid was significantly decreased ($p < 0.01$; Mann-Whitney U test; Figure 5F). No obvious changes in the numbers of infiltrated cells were detected in OVA-challenged $Il33^{+/+}$ and $Il33^{-/-}$ mice that had not received memory Th2 cells (Figure 5E). Consistent with these observations, fewer IL-5-producing memory Th2 cells were detected in the lungs of mice from the $Il33^{-/-}$ group than in those of mice from the $Il33^{+/+}$ group after OVA inhalation, whereas the percentages of IL-4- or IL-13-producing cells were equivalent (Figure 5G, left). No substantial changes in Th2-cell-associated cytokine production were detected between $Il33^{+/+}$ and $Il33^{-/-}$ endogenous CD4⁺ T cells (Figure 5G, right). Thus, endogenous IL-33 from the recipient mice appears to be critical for the induction of memory-Th2-cell-dependent eosinophilic airway inflammation.

p38 Is a Major Downstream Target of IL-33-Dependent IL-5 Augmentation in Memory Th2 Cells

Next, we sought to identify which signaling pathway downstream of the ST2 receptor was responsible for the IL-33-dependent augmentation of IL-5 production by memory Th2 cells. Given that IL-33 has been reported to induce MAPK activation and NF- κ B phosphorylation in mast cells (Schmitz et al., 2005), we first examined the effect of chemical inhibitors of several components of these two pathways. We assessed the p38 MAPK inhibitor SB203580, the MEK inhibitor U0126, the JNK inhibitor SP600125, and the PI3K inhibitor Wortmannin. SB203580 inhibited the IL-33-induced IL-5 augmentation in memory Th2 cells, whereas the other inhibitors had little effect on IL-5 production (Figure 6A). No change in IL-4 production was detected after treatment with SB203580. We also used SB203580 to assess whether the IL-2- and IL-25-induced IL-5 augmentation in memory Th2 cells was dependent on p38 MAPK pathway. As expected, SB203580 inhibited IL-2- and IL-25-induced IL-5 augmentation in memory Th2 cells (Figure S6). Phosphorylation of p38 was induced by IL-33 and was inhibited by SB203580 (Figure 6B). A very small increase in p38 phosphorylation was detected after culture with IL-2. Significant reductions in

mRNA expression of *Il5* and *Il1rl1* were detected in response to SB203580 treatment of memory Th2 cells stimulated with IL-33; however, no significant reduction was observed in *Il4*, *Il13*, or *Gata3* ($p < 0.01$; Mann-Whitney U test; Figure 6C). We also detected marked reduction of ST2 after SB203580 treatment of IL-33-stimulated memory Th2 cells (Figure 6D). We next analyzed the effect of SB203580 on histone modifications at the *Il5* locus in memory Th2 cells. Memory Th2 cells cultured with IL-33 in the presence of SB203580 showed reduced modifications of H3-K4 methylation and H3-K9 acetylation at the *Il5* locus, but not at the conserved GATA3 response element or Va enhancer regions, which are located near the *Il13* promoter and downstream of the *Il4* locus, respectively (Figure 6E). Specific reduction of *Il5* and *Il1rl1* expression in response to siRNA-mediated silenced p38 in memory Th2 cells was also detected, whereas silencing of p38 did not change IL-4 or IL-13 expression (Figure 6F). These results indicate that IL-33 selectively activates p38, increases the expression of ST2, and augments the production of IL-5 in memory Th2 cells.

Human IL-33 Enhances IL-5 Production in Memory CD4⁺ T Cells from the Polyps of Patients with ECRS

Finally, we sought to gain more insight into the possible pathophysiological effect of IL-33 on memory Th2 cells in human disease. We analyzed CD45RO⁺ memory CD4⁺ T cells from the nasal polyps of patients with ECRS, which is characterized by IL-5-dependent accumulation of large numbers of eosinophils (Gevaert et al., 2006). We included samples from ECRS and NECRS patients and categorized them by the number of eosinophils found in the nasal polyps (Zhang et al., 2008). All patients signed informed-consent forms, and the study was approved by the ethics committee of the Chiba University Graduate School of Medicine and each participating hospital (1006). The number of IL-33⁺ endothelial cells in the nasal polyps was comparable between ECRS and NECRS patients (data not shown). However, compared to nasal polyps from NECRS patients, those from ECRS patients showed significantly elevated numbers of IL-33⁺PECAM1⁺ endothelial cells ($p < 0.05$; Mann-Whitney U test; Figure 7A). In addition, the baseline expression of *IL4* and *IL5* (encoding Th2 cell cytokines), *IL1RL1*, and *GATA3* was higher in the ECRS polyps (Figure 7B). In contrast, the expression of *TBX21* was lower in the ECRS polyps. These results prompted us to assess the effect of IL-33 on Th2 cell cytokine production of memory CD4⁺ T cells from nasal polyps of these patients. As a control, we analyzed the effect of IL-33 on cytokine expression by the CD45RO⁺CD4⁺ T cell population of peripheral-blood mononuclear cells (PBMCs) from healthy donors. Very little expression of ST2 was detected on CD45RO⁺CD4⁺ T cells from PBMCs, and no obvious augmentation in

(C) Intracellular-staining profiles of IL-5 and KJ1.26 in lung memory Th2 cells and endogenous CD4⁺ T cells from $Il33^{+/+}$ or $Il33^{-/-}$ recipients.

(D) ST2 and CXCR3 expression profiles of lung KJ1⁺ memory Th2 cells in $Il33^{+/+}$ or $Il33^{-/-}$ recipients after OVA challenge.

(E) The absolute numbers of leukocytes in BAL fluid from $Il33^{+/+}$ or $Il33^{-/-}$ mice transferred with or without memory Th2 cells are shown. Samples were collected 2 days after the last OVA challenge. The mean values (five mice per group) are shown with SDs.

(F) ELISA of the indicated cytokines in the BAL fluid from each experimental group shown in (E).

(G) Intracellular-staining profiles of the indicated cytokines in lung memory Th2 cells and endogenous CD4⁺ T cells from $Il33^{+/+}$ or $Il33^{-/-}$ mice after OVA challenging four times.

Two (A–C) or three (D–G) independent experiments were performed and showed similar results (** $p < 0.01$; * $p < 0.05$). Three technical replicates were performed with quantitative RT-PCR (B). Five technical replicates were performed with ELISA in BAL fluid (F). See also Figure S5.

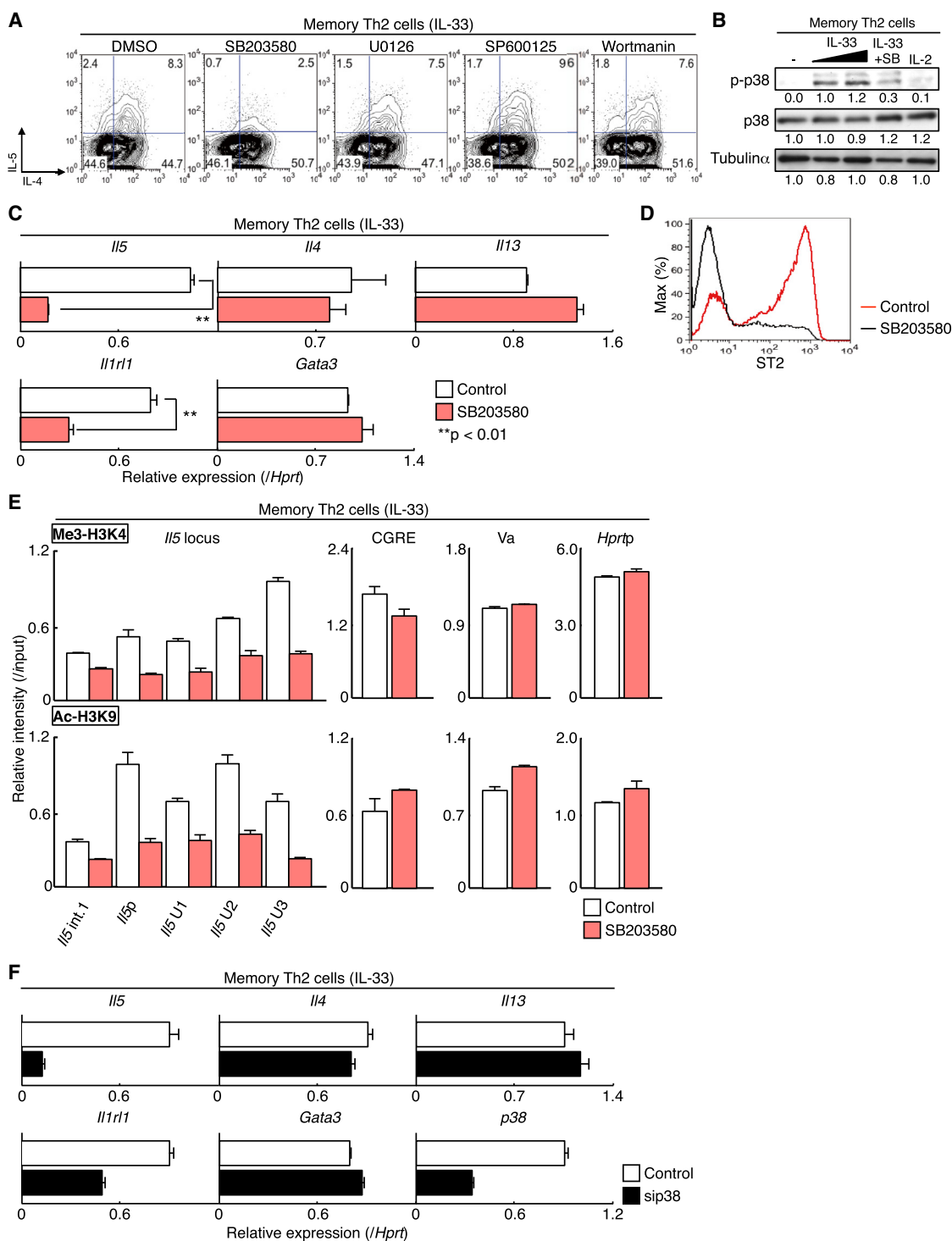


Figure 6. p38 Signaling Is Crucial for IL-33-Dependent IL-5 Induction by Memory Th2 Cells

(A) Intracellular-staining profiles of IL-5 and IL-4 in IL-33-treated memory Th2 cells with the indicated chemical inhibitors.

(B) The phosphorylated p38 (p-p38) and total p38 (p38) in freshly prepared memory Th2 cells stimulated with IL-2 or graded amounts of IL-33 with or without SB203580.

(C) Quantitative RT-PCR analysis of the indicated genes in memory Th2 cells cultured with IL-33 with or without SB203580. Four independent experiments were performed and showed similar results (** $p < 0.01$).

(D) Expression profiles of ST2 on memory Th2 cells treated with IL-33 with or without SB203580.

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IL-5 expression or production was detected after IL-33 treatment (Figures S7A–S7C). However, IL-33 dramatically enhanced *IL5* expression in CD45RO⁺CD4⁺ T cells from nasal polyps of ECRS patients ($p < 0.01$; Mann-Whitney U test; Figure 7B). Modest enhancement of *IL13* expression was also detected ($p < 0.05$; Mann-Whitney U test). In addition, the expression of *IL1RL1* was enhanced dramatically after IL-33 treatment, which supported the results obtained from analysis of murine memory Th2 cells (Figure 1). In contrast, no obvious effect of IL-33 on the expression of *IL5* or *IL13* was detected in CD45RO⁺CD4⁺ T cells from nasal polyps of NECRS patients. Furthermore, the expression and production of IL-5 and the *IL1RL1* expression that was enhanced by IL-33 in CD45RO⁺CD4⁺ T cells from nasal polyps of ECRS patients were selectively inhibited by SB203580 treatment ($p < 0.01$; Mann-Whitney U test; Figures 7C and 7D). These results indicate that IL-33 specifically induces enhanced expression of *IL1RL1* and IL-5 in CD45RO⁺CD4⁺ T cells from nasal polyps of ECRS patients and that p38 activation is required for IL-33-induced IL-5 augmentation. Thus, IL-33-ST2-p38 signaling might play an important role in the induction of pathogenicity in tissue-infiltrating memory CD4⁺ T cells in human chronic allergic inflammatory diseases such as ECRS.

DISCUSSION

Herein, we have identified memory Th2 cells as a critical target of IL-33 in the pathogenesis of allergic airway inflammation in both the murine and the human immune systems. IL-33 induced increased expression of ST2 on memory Th2 cells and also enhanced production of IL-5 both in vitro and in vivo. IL-33 exposure induced chromatin remodeling at the *IL5* locus. We found that p38 MAPK was a downstream target of IL-33-ST2 signaling in memory Th2 cells. In vivo, the depletion of IL-33 or ST2 attenuated memory-Th2-cell-mediated allergic responses in the airway. Memory CD4⁺ T cells in the nasal polyps of ECRS patients showed increased IL-5 expression after stimulation with IL-33, and IL-33 was highly expressed in the chronic inflammatory polyps of ECRS patients. Thus, the IL-33-ST2-p38 pathway could be a potential therapeutic target for treatment of chronic allergic inflammation induced by memory Th2 cells.

IL-33-mediated production of IL-5 and IL-13 in ILC2s depends on the phosphorylation of p38 (Furusawa et al., 2013), and IL-33 increases phosphorylation of p38 in mast cells (Liew et al., 2010). In human T cells, IL-5 production induced by TCR stimulation is dependent on p38 activity (Maneechotesuwan et al., 2007; Mori et al., 1999). Likewise, we found that the p38-mediated signaling pathway is critical for IL-33-induction of IL-5 expression in both murine and human memory T cells. Thus, the p38 MAPK pathway appears to be critical for the production of IL-5 in various types of cells, including memory Th2 cells.

Chromatin remodeling of *IL4* is dependent on the calcineurin-NFAT pathway (Ansel et al., 2006), whereas that of IL-5 and IL-13 appears to be more dependent on NF- κ B, AP-1, or other

transcription factors rather than NFAT (Guo et al., 2009; Wang et al., 2006). In the current study, we found that the p38 MAPK pathway was activated by IL-33 and was responsible for the induction of chromatin remodeling of the *IL5* locus and thus left the chromatin signature at the *IL4* and *IL13* loci almost unchanged. Therefore, this might represent a connection between the IL-33-p38 MAPK pathway and selective chromatin remodeling of the *IL5* locus.

An important finding from the present study is that IL-33-induced chromatin remodeling at the *IL5* locus in memory Th2 cells occurs independently from TCR stimulation. TCR stimulation is indispensable for the induction of chromatin remodeling at the Th1 and Th2 cell cytokine-encoding loci during differentiation of naive CD4⁺ T cells into effector Th1 and Th2 cells (Nakayama and Yamashita, 2010). However, we have shown that the cytokine IL-33 alone is capable of selectively inducing chromatin remodeling at the *IL5* locus in memory Th2 cells. These results might indicate that IL-33 possesses a unique ability to induce chromatin remodeling at the *IL5* locus if the IL-33 receptor ST2 is expressed. Indeed, IL-2, IL-7, or IL-25 did not induce chromatin remodeling at the *IL5* locus in memory Th2 cells, although their receptors were expressed on memory Th2 cells. The combination of IL-2 and IL-25 also induced chromatin remodeling at the *IL5* locus in memory Th2 cells, as shown previously in ILC2s (Halim et al., 2012), suggesting that similar mechanisms might operate cytokine-dependent IL-5 induction in memory Th2 cells and ILC2s. In ILC2s, IL-33 is reported to be more potent than IL-25 in inducing IL-13 production and AHR (Barlow et al., 2013). At the *IL5* locus, we detected permissive histone marks accompanied by binding of p300 and pol II in memory Th2 cells stimulated with IL-33. Therefore, the activation of the IL-33-ST2-p38 axis might induce the formation of a chromatin-remodeling complex, including the HAT complex, and recruit it to the *IL5* locus in memory Th2 cells.

We have demonstrated that memory Th2 cells are an important target of IL-33 in the pathogenesis of airway inflammation. We detected increased expression of *IL33* mRNA in the lung after OVA challenge, which is consistent with the notion that tissue damage leads to the release of IL-33 from structural cells, such as epithelial cells and endothelial cells in the lung (Préfontaine et al., 2009; Préfontaine et al., 2010), and that IL-33 is expressed higher in asthmatic patients (Préfontaine et al., 2009). IL-33 secreted around the inflammatory airways might increase ST2 expression and IL-5 production by memory Th2 cells that are either resident in or migrating through the lung tissue. Therefore, IL-33 might enhance allergic airway responses through the induction of our proposed IL-5-producing pathogenic memory Th2 cells in the airway (Endo et al., 2014; Endo et al., 2011; Hegazy et al., 2010; Islam et al., 2011; Upadhyaya et al., 2011; Wang et al., 2010). Although it is already known that ILC2s respond to IL-33 to produce IL-5 in the lung, the depletion of ILC2s in *Rag2*^{−/−} mice by anti-CD90.2 antibody treatment did not affect memory-Th2-cell-dependent airway inflammation.

(E) ChIP assays were performed as shown in Figure 2B. Histone modifications at the *IL5* locus in memory Th2 cells treated with IL-33 with or without SB203580 were measured by quantitative RT-PCR analysis.

(F) Effect of silenced *p38* on *IL5* expression in IL-33-cultured memory Th2 cells. Memory Th2 cells were introduced to control *p38* siRNA and cultured with IL-33 for 5 days, and quantitative RT-PCR analysis of the indicated molecules after 4 hr stimulation with immobilized anti-TCR β is shown. At least three (A–D) or two (E and F) independent experiments were performed and showed similar results. See also Figure S6.

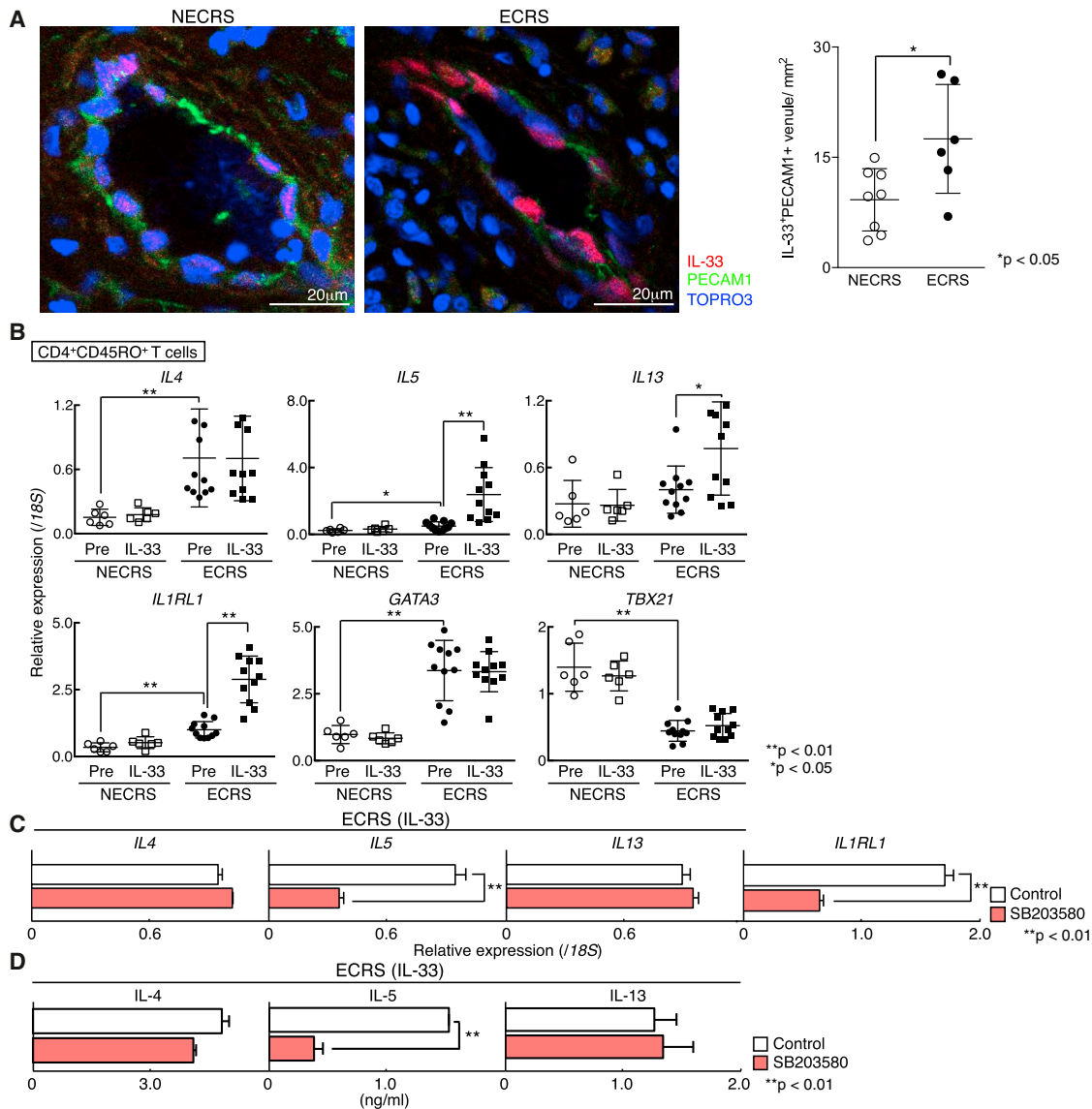


Figure 7. Human IL-33 Enhances IL-5 Production in Memory CD4⁺ T Cells Prepared from Nasal Polyps of ECRS Patients

(A) Immunofluorescent analysis (with staining for IL-33 [red], PECAM [green], and TOPRO3 [blue]) shows a representative section of nasal polyps from NECRS (left) or ECRS (right) patients. The frequency of IL-33⁺PECAM1⁺ cells among PECAM⁺ cells of the nasal polyps from patients is shown (mean $\pm$ SD; $n = 6$ for ECRS and $n = 8$ for NECRS).

(B) Quantitative RT-PCR analysis of relative expression of the indicated genes in human memory CD4⁺ T cells from nasal polyps ($n = 11$ for ECRS, $n = 6$ for NECRS) was performed after 4 hr stimulation with PMA plus ionomycin. Relative expression (normalized to 18S) with SD is shown. Mean values and SDs are shown.

(C) Human nasal memory CD4⁺ T cells were cultured with IL-33 with or without SB203580 for 5 days. Quantitative RT-PCR analysis of the indicated cytokines in these cells after 4 hr stimulation with PMA plus ionomycin is shown.

(D) ELISA ($n = 8$ for ECRS and $n = 5$ for NECRS) of the indicated cytokines secreted by IL-33-cultured memory CD4⁺ T cells stimulated with PMA plus ionomycin for 16 hr. The mean values of triplicate cultures with SDs are shown.

More than five independent experiments in each group were performed and showed similar results (** $p < 0.01$; * $p < 0.05$; C and D). Three technical replicates were performed with quantitative RT-PCR and ELISA (B–D). See also Figure S7.

Thus, according to the experimental systems we used, IL-33 appears to act mainly on memory Th2 cells to increase their ability to produce IL-5 and exacerbate eosinophilic inflammation. It remains unknown whether IL-33 induces recruitment of memory Th2 cells to the inflamed lung, given that IL-33 can also act as a chemoattractant for Th2 cells (Komai-Koma et al., 2007).

ECRS is a chronic inflammatory disease characterized by prominent accumulation of eosinophils in the sinuses and nasal polyp tissue (Gevaert et al., 2006). Functionally distinct populations of memory CD4⁺ T cells might be present within the nasal polyps of ECRS and NECRS (Th1 cell type for NECRS and Th2 cell type for ECRS). We detected more IL-33-producing cells in

nasal polyps from ECRS patients than in those from NECRS patients. Consistent with our findings in murine CD4⁺ T cells, we demonstrated that IL-33 induced augmentation of *IL5* and *IL1RL1* expression in memory CD4⁺ T cells from ECRS patients. IL-33 induced expression of not only *IL5* but also *IL1RL1*, suggesting that a positive-feedback mechanism resulted in a greater responsiveness to IL-33 in memory CD4⁺ T cells from ECRS patients. In addition, IL-33-induced IL-5 augmentation also depended on the activation of p38. Our study clearly demonstrates the function and pathophysiological role of IL-33 in ECRS, a chronic inflammatory human disease.

In summary, our study has identified memory Th2 cells as an important target of IL-33 in the pathogenesis of airway inflammation. The p38-mediated signaling pathway is critical for TCR-independent IL-33-induced IL-5 expression in both murine and human memory Th2 cells. Further detailed studies focused on the IL-33-ST2-p38-axis in pathogenic memory Th2 cells might lead to the discovery of potential therapeutic targets for the treatment of chronic allergic diseases.

EXPERIMENTAL PROCEDURES

Mice

The animals used in this study were backcrossed to BALB/c or C57BL/6 mice ten times. Anti-OVA-specific TCR- $\alpha\beta$ (DO11.10) transgenic (Tg) mice were provided by Dr. D. Loh (Washington University School of Medicine, St. Louis) (Murphy et al., 1990). *Il33*^{-/-} mice were generated as previously described (Oboki et al., 2010). *Il1rl1*^{-/-} mice were kindly provided by Dr. Andrew N.J. McKenzie (Medical Research Council, Cambridge) (Townsend et al., 2000). Ly5.1 mice were purchased from Sankyo Laboratory. All mice were used at 6–8 weeks old and were maintained under specific-pathogen-free conditions. BALB/c, BALB/c *nu/nu*, and *Rag2*^{-/-} mice were purchased from CLEA Japan. Animal care was conducted in accordance with the guidelines of Chiba University.

The Generation and Culture of Effector and Memory Th2 Cells

Splenic CD62L⁺CD44⁻KJ1⁺CD4⁺ T cells from DO11.10 OVA-specific TCR Tg mice were stimulated with an OVA peptide (Loh15, 1 μ M) plus antigen-presenting cells (irradiated splenocytes) under Th2-cell-culture conditions (25 U/ml IL-2, 10 U/ml IL-4, anti-IL-12 monoclonal antibody [mAb], and anti-IFN- γ mAb) for 6 days in vitro. The effector Th2 cells (3×10^6) were transferred intravenously into BALB/c *nu/nu* or BALB/c recipient mice. Five weeks after cell transfer, KJ1⁺CD4⁺ T cells in the spleen were purified by autoMACS (Miltenyi Biotec) and cell sorting (BD Aria II) and were then used as memory Th2 cells.

Assessment of Memory Th2 Cell Function In Vivo

Memory Th2 cells were purified by fluorescence-activated cell sorting and transferred (3×10^6 /mouse) again into *Il33*^{+/+} or *Il33*^{-/-} mice. The mice were exposed to aerosolized 1% OVA four times on days 1, 3, 9, and 11. For depletion of ILC2s, *Rag2*^{-/-} mice were injected intraperitoneally with anti-CD90.2 (BioX Cell) antibody at a dose of 200 μ g per day on days 2, 5, and 9. BAL fluid for the analysis of cytokine production by ELISA was collected 12 hr after the last challenge, and BAL fluid for the assessment of inflammatory cell infiltration was collected on day 13. Intracellular-staining analysis was performed 12 hr after the last inhalation. Lung histology was assessed on day 13. AHR was assessed on day 12.

Quantitative Real-Time PCR

Total RNA was isolated with the TRIzol reagent (Invitrogen). cDNA was synthesized with an oligo (dT) primer and Superscript II RT (Invitrogen). Quantitative real-time PCR was performed with the ABI PRISM 7500 Sequence Detection System as described previously (Endo et al., 2011). Primers and TaqMan probes were purchased from Applied Biosystems. Primers and Roche Universal probes were purchased from Sigma and Roche, respectively. Gene

expression was normalized with the *Hprt* mRNA signal or the 18S ribosomal RNA signal.

siRNA Analysis of Gene Targeting

siRNA was introduced into memory Th2 cells by electroporation with a mouse T cell Nucleofector Kit and Nucleofector I (Amaxa). Memory Th2 cells were transfected with 675 pmol of control random siRNA or siRNA for *p38* (Applied Biosystems) and cultured for 5 days with IL-33.

Nasal Polyp Mononuclear Cells and Homogenate Preparation

Nasal polyp mononuclear cells (NPMCs) were obtained as previously described (Yamamoto et al., 2007). In brief, freshly obtained nasal polyps were immediately minced and incubated in RPMI 1640 medium containing 1 mg/ml collagenase, 0.5 mg/ml hyaluronidase, and 0.2 mg/ml DNase I (Sigma-Aldrich). After incubation, NPMCs were obtained by the Ficoll-Hypaque technique. A volume of 1 ml of PBS was added for every 100 mg of tissue and was supplemented with aprotinin and leupeptin (Roche).

Statistical Analysis

Data were analyzed with GraphPad Prism software (version 6). Comparisons of two groups were calculated with non-parametric Mann-Whitney U tests. Differences with *p* values below 0.05 or 0.01 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2015.01.016>.

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Immunity

Supplemental Information

The Interleukin-33-p38 Kinase Axis Confers Memory T Helper 2 Cell Pathogenicity in the Airway

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Figure S1 related to main Figure 1

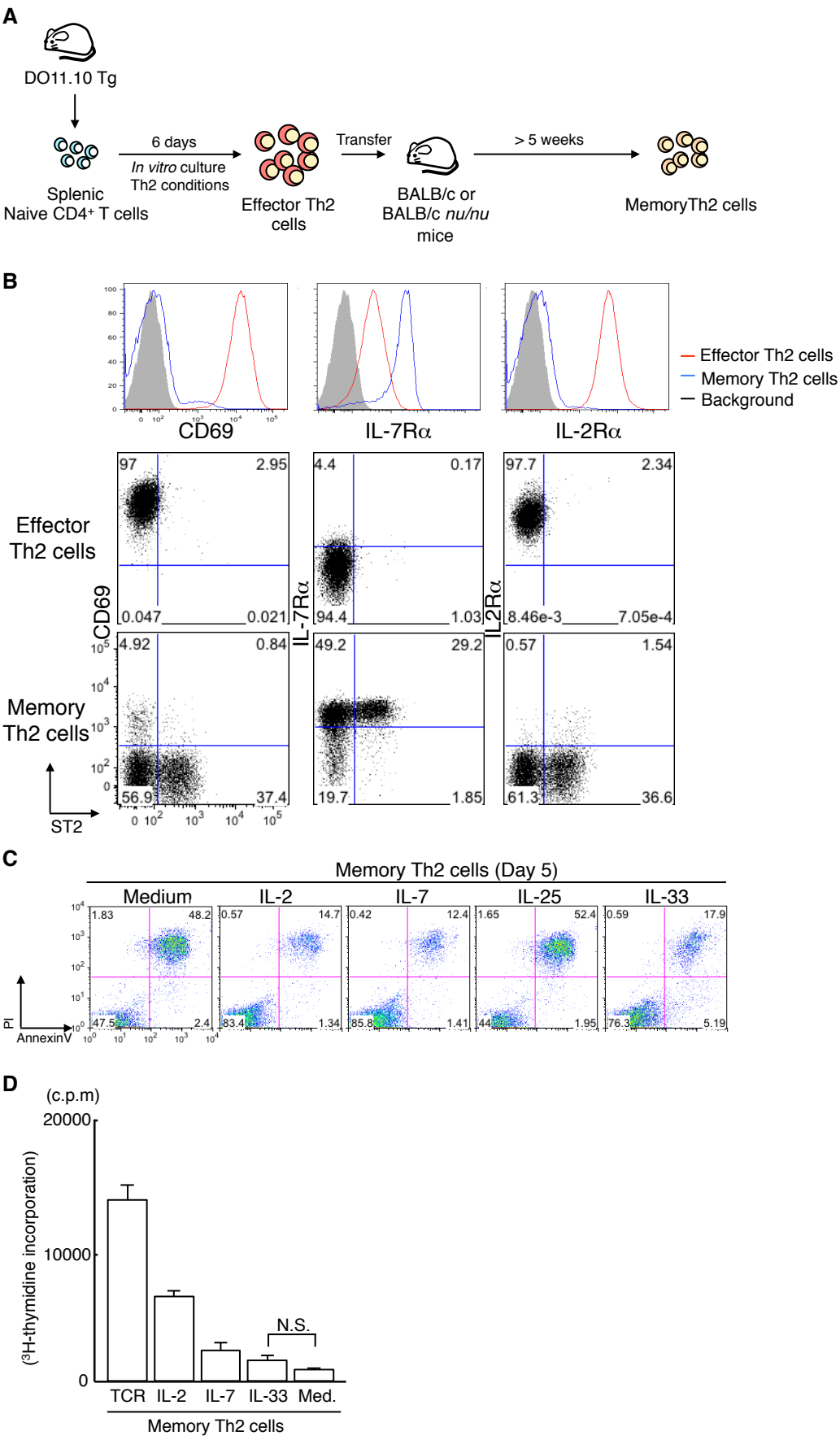


Figure S1 (continued) related to main Figure 1

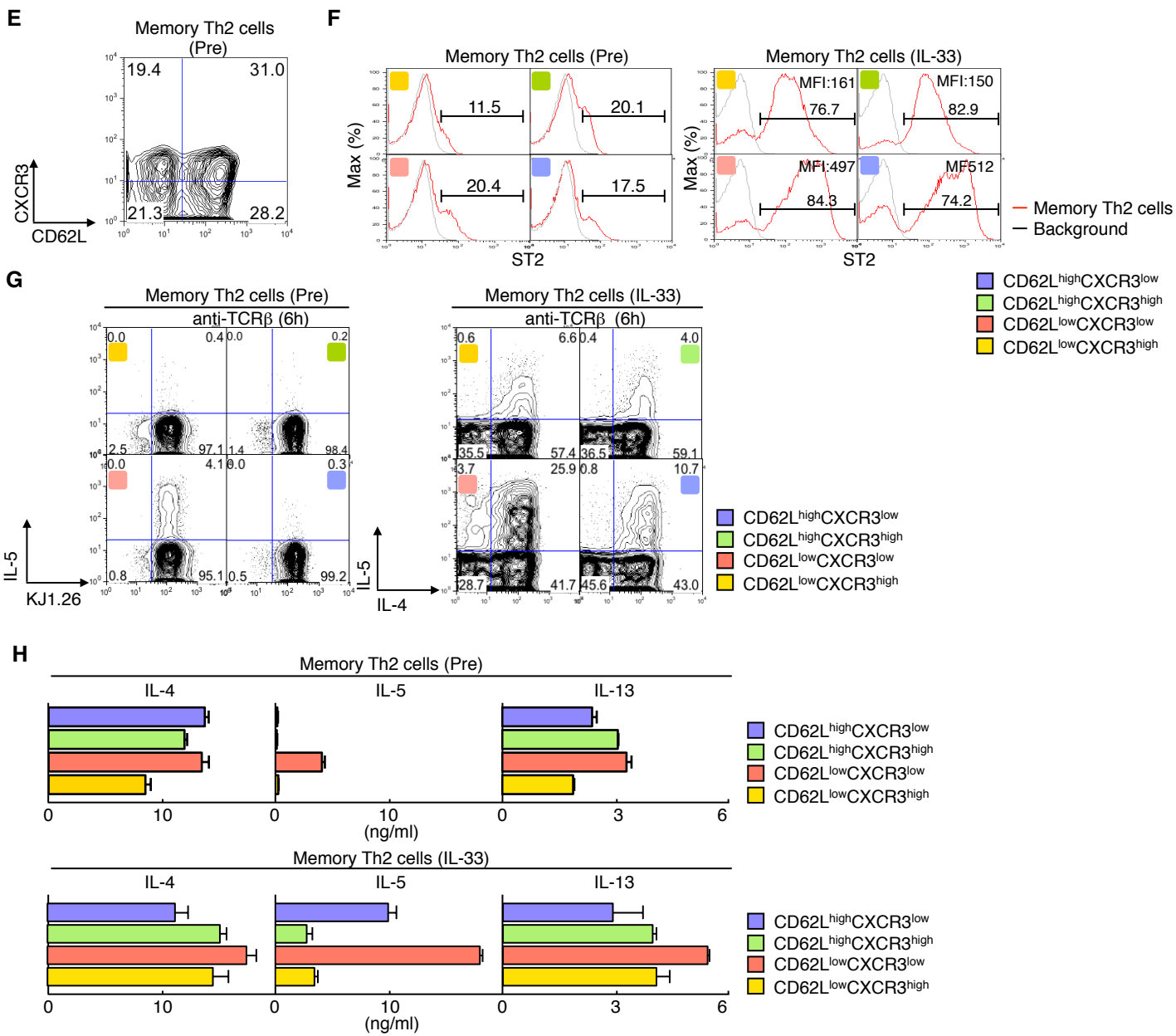


Figure S1. Cell surface expression profiles of effector and memory Th2 cells (related to Figure 1).

(A) Experimental protocols for the generation of effector and memory Th2 cells. Naive CD4⁺ T cells from DO11.10 OVA-specific TCR Tg mice were stimulated with an OVA peptide plus spleen-derived antigen presenting cells under Th2-culture conditions (IL-2 (25U/ml), IL-4 (10U/ml) and anti-IFN γ mAb) for 6 days *in vitro*. We used these cells as effector Th2 cells. Effector Th2 cells were then transferred intravenously into syngeneic BALB/c or BALB/c *nu/nu* mice. More than 5 weeks after cell transfer, KJ1⁺CD4⁺ cells in the spleen or lung were purified by auto-MACS and cell sorting (using a FACS Aria II) and then were used as memory Th2 cells. **(B)** Expression profiles of CD69, IL-7R α , IL-2R α and ST2 on effector and memory Th2 cells. **(C)** The susceptibility for apoptosis of memory Th2 cells cultured with medium alone (Medium), IL-2, IL-7, IL-25, or IL-33 for 5 days were investigated by staining of Annexin V and propidium iodide (PI). Three independent experiments were performed with similar results. **(D)** [³H]-thymidine incorporation in memory Th2 cells stimulated with immobilized anti-TCR β , or with indicated cytokines or medium alone (Med) is shown with standard deviations (N.S., not significant). Two independent experiments were performed with similar results. **(E)** CD62L and CXCR3 profiles of memory Th2 cells. **(F)** Expression profiles of ST2 on the four subpopulations subdivided by the expression of CD62L and CXCR3 of memory Th2 cells before (Left) and after (Right) cultivation with IL-33 for 5 days. Mean fluorescence intensity (MFI) is shown for each population. **(G)** Four subpopulations subdivided by the expression of CD62L and CXCR3 of memory Th2 cells before and after culture with IL-33 were stimulated *in vitro* with immobilized anti-TCR β for 6 hours. Intracellular staining profiles of IL-5 and IL-4 are shown with the percentage of cells in each area. **(H)** ELISA analysis of IL-4, IL-5, and IL-13 secreted by the four subpopulations

subdivided by the expression of CD62L and CXCR3 of memory Th2 cells before (Top) or after (Bottom) cultivation with IL-33. Cells were stimulated with immobilized anti-TCR β for 24 hours. The mean values and standard deviations are shown. Three independent experiments were performed with similar results. Three technical replicates were included in ELISA (**H**).

Figure S2 related to main Figure 2

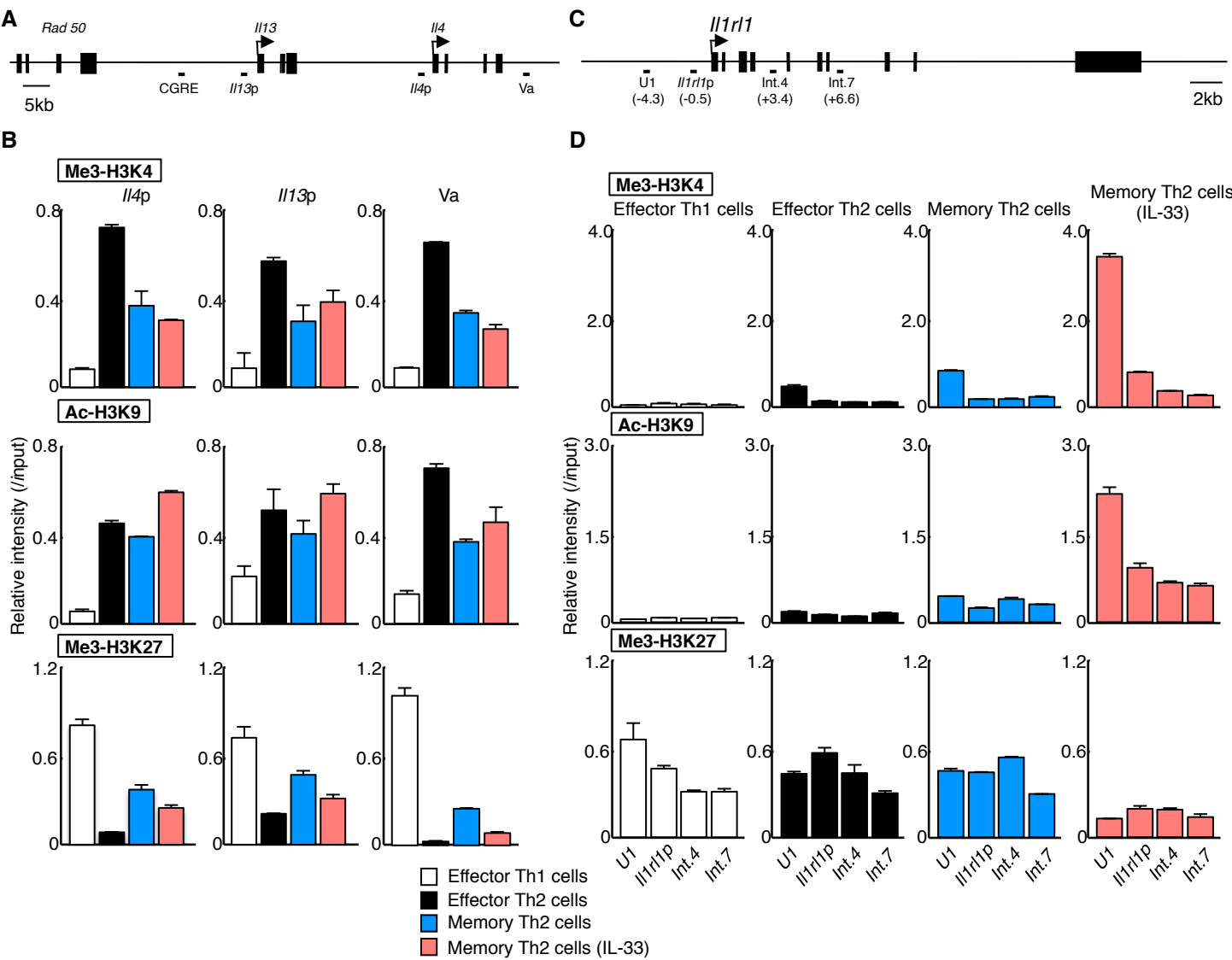


Figure S2 (continued) related to main Figure 2

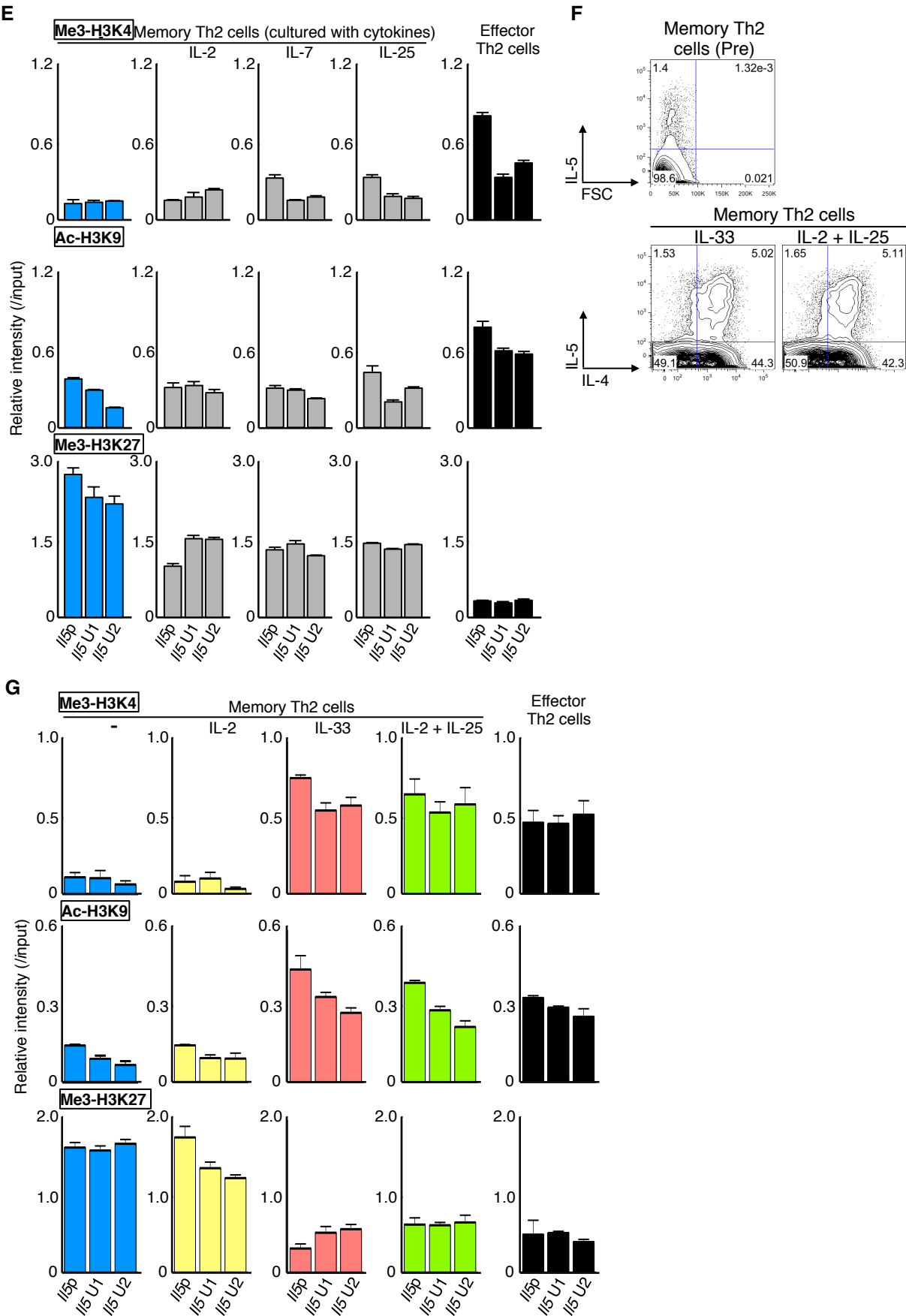


Figure S2. Effect of IL-33 cultivation on histone modifications at the *Il4* and *Il13* gene loci and *Il1rl1* locus in memory Th2 cells (related to Figure 2).

(A) Schematic representation of the mouse Th2 cytokine gene loci. The location of primers and probes (CGRE to Va) and exons are indicated. (B) ChIP assays were performed with anti-acetyl histone H3K9, anti-trimethyl histone H3-K4, and anti-trimethyl histone H3K27 at the Th2 cytokine gene loci from effector Th1, effector Th2, freshly prepared memory Th2, and IL-33 cultured memory Th2 cells (IL-33) for 5 days. The relative intensities (relative to input DNA) of these modifications were determined by quantitative RT-PCR analysis. More than three independent experiments were performed with similar results. (C) Schematic representation of the mouse *Il1rl1* gene locus. The location of primers and probes and exons are indicated. (D) ChIP assays were performed as in (B). The histone modifications at the *Il1rl1* locus in the indicated samples were determined by quantitative RT-PCR analysis. Three independent experiments were performed with similar results. (E) ChIP assays were performed as in (B). The histone modifications at the *Il5* locus in effector Th2, freshly prepared memory Th2, and memory Th2 cells cultured with the indicated cytokines for 5 days were determined by quantitative RT-PCR analysis. Three independent experiments were performed with similar results. (F) Memory Th2 cells were cultured with IL-33 or IL-2 and IL-25 for 5 days. The cultured cells were stimulated with immobilized anti-TCR β for 6 hours. Intracellular staining profiles of IL-5 and IL-4 are shown. Three independent experiments were performed with similar results. (G) ChIP assays were performed as in (B). The histone modifications at the *Il5* locus in effector Th2, freshly prepared memory Th2, and memory Th2 cells cultured with the indicated cytokines for 5 days were determined by quantitative RT-PCR analysis. Two independent experiments were performed with similar

results. Two independent experiments were performed with similar results. Three technical replicates were performed with quantitative RT-PCR (**B**, **D**, **E** and **G**).

Figure S3 related to main Figure 3

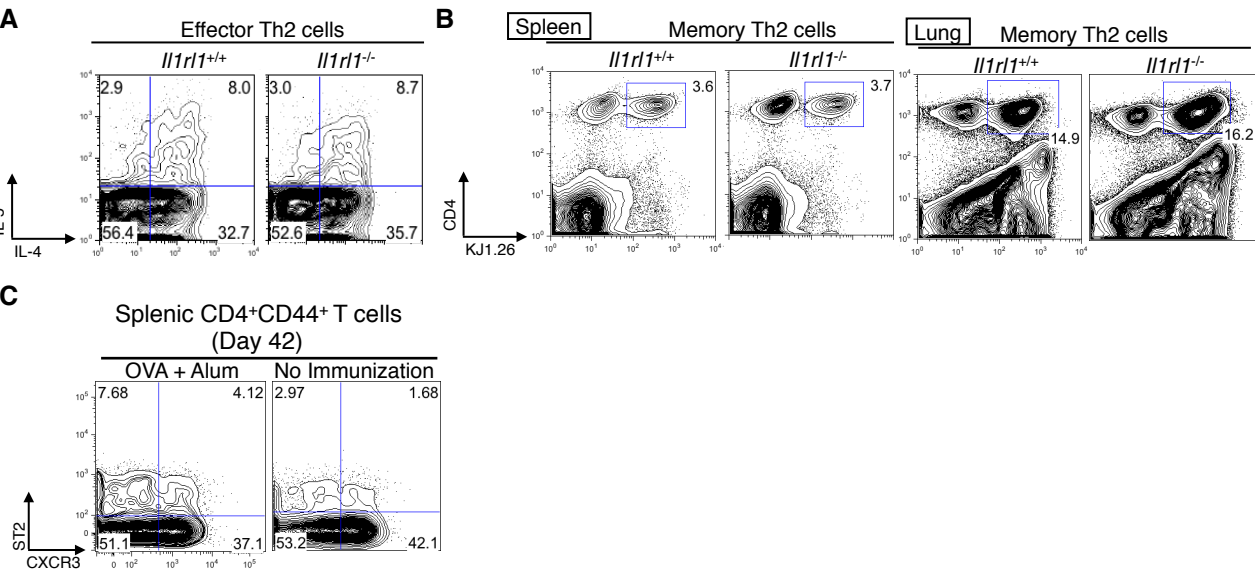


Figure S3. Normal effector Th2 differentiation and normal memory Th2 cell generation from *Il1rl1*^{+/+} or *Il1rl1*^{-/-} CD4⁺ T cells (related to Figure 3).

(A) Intracellular staining profiles of IL-5 and IL-4 in *Il1rl1*^{+/+} or *Il1rl1*^{-/-} effector Th2 cells. (B)

The generation of *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory Th2 cells in the spleen and the lung. (C) Cell

surface expression profiles of ST2 and CXCR3 on CD4⁺CD44⁺ T cells recovered from the

mice that were not immunized (Right) or immunized with OVA and Alum. (Left) are shown.

Three independent experiments were performed with similar results.

Figure S4 related to main Figure 4

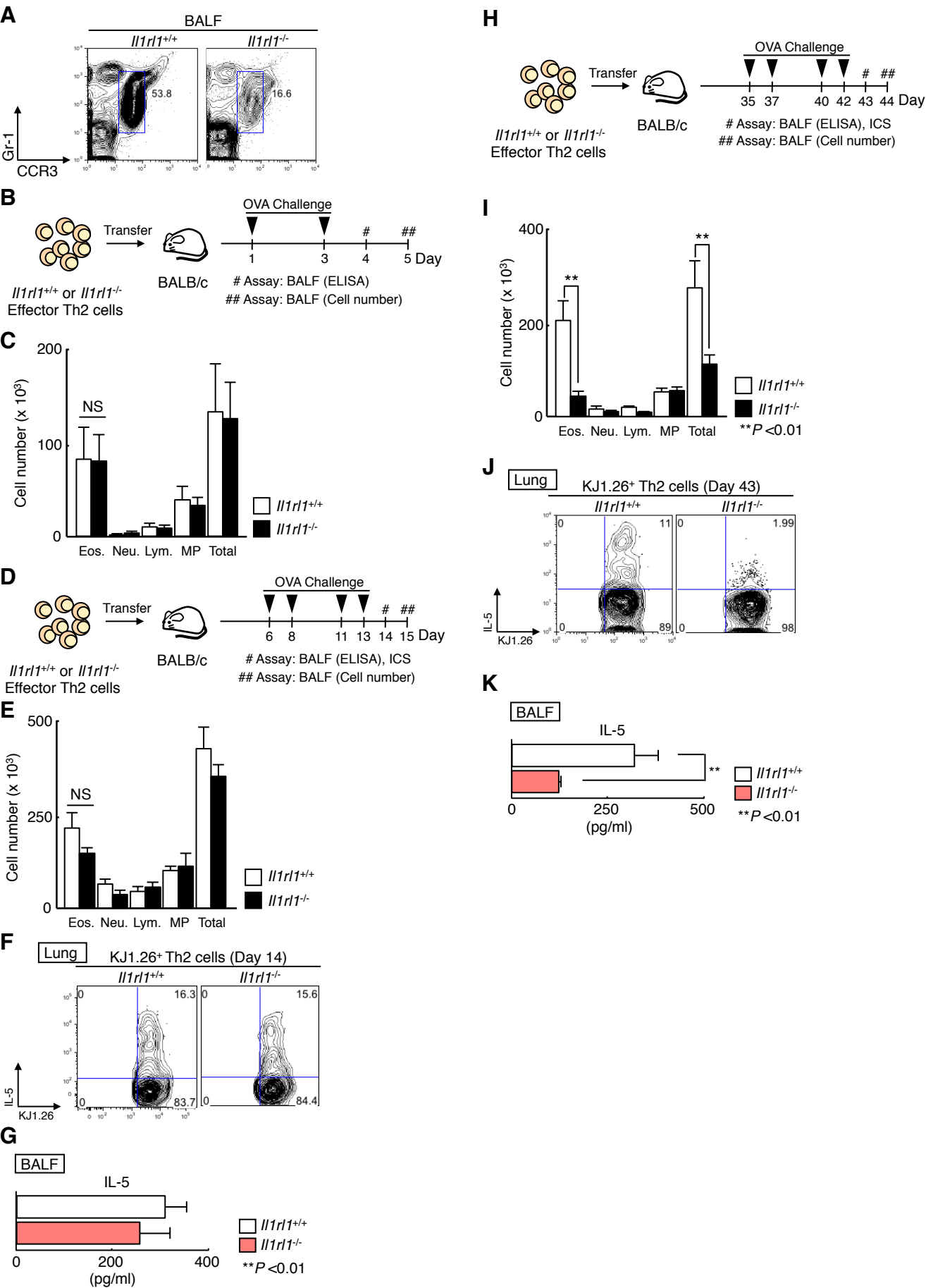


Figure S4 (continued) related to main Figure 4

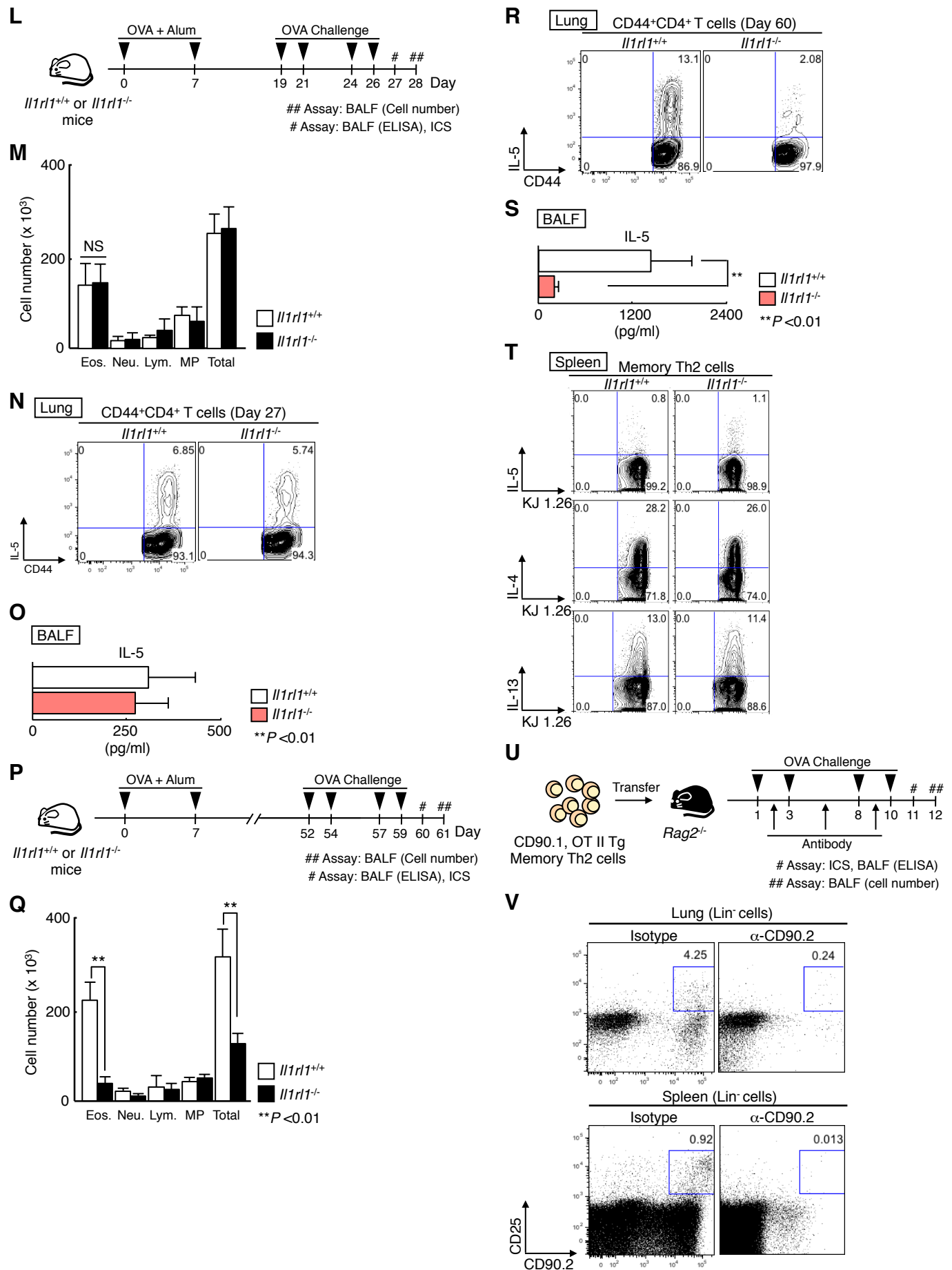


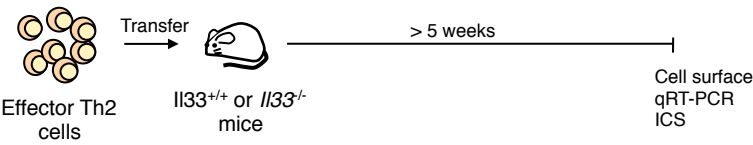
Figure S4. Airway inflammation model induced by OVA and Alum immunization of *Il1rl1*^{+/+} or *Il1rl1*^{-/-} mice (related to Figure 4).

(A) Cell surface expression profiles of Gr-1 and CCR3 on leukocytes collected from the BAL fluid of *Il1rl1*^{+/+} or *Il1rl1*^{-/-} groups. Experimental protocols are shown in **Figure 4A**. The eosinophil population is CCR3⁺Gr-1^{int}. (B) Experimental protocols for effector Th2 cell-dependent allergic inflammation mouse models using *Il1rl1*^{+/+} or *Il1rl1*^{-/-} donor cells. (C) The absolute cell numbers of eosinophils (Eos.), neutrophils (Neu.), lymphocytes (Lym.), and macrophages (Mac.) in the BAL fluid are shown as described in **Figure 4D**. Mean values (5 mice per group) are shown with standard deviations. Two independent experiments were performed with similar results. MS: not significant (D) Experimental protocols for Th2 cell-dependent allergic inflammation mouse models (6 days after cell transfer) using *Il1rl1*^{+/+} or *Il1rl1*^{-/-} donor cells. (E) The absolute cell numbers of eosinophils (Eos.), neutrophils (Neu.), lymphocytes (Lym.), and macrophages (Mac.) in the BAL fluid are shown. Mean values (5 mice per group) are shown with standard deviations. (F) Intracellular staining profiles of IL-5 and KJ1.26 in the lung KJ1.26⁺ Th2 cells (on day 14). (G) ELISA analysis of IL-5 in the BAL fluid from the experimental groups with *Il1rl1*^{+/+} or *Il1rl1*^{-/-} Th2 cell transfer shown in panel D. Two independent experiments were performed with similar results. (H) Experimental protocols for Th2 cell-dependent allergic inflammation mouse models (35 days after cell transfer) using *Il1rl1*^{+/+} or *Il1rl1*^{-/-} donor cells. (I) The absolute cell numbers of eosinophils (Eos.), neutrophils (Neu.), lymphocytes (Lym.), and macrophages (Mac.) in the BAL fluid are shown. Mean values (5 mice per group) are shown with standard deviations (**: $P < 0.01$). (J) Intracellular staining profiles of IL-5 and KJ1.26 in the lung KJ1.26⁺ Th2 cells (on day 43). (K) ELISA analysis of IL-5 in the BAL fluid from the experimental groups

with *Il1rl1*^{+/+} or *Il1rl1*^{-/-} Th2 cell transfer shown in panel H. Two independent experiments were performed with similar results (**: $P < 0.01$). (L) Experimental protocols for an OVA and Alum immunization-induced allergic inflammation mouse model (12 days after the last immunization) using *Il1rl1*^{+/+} or *Il1rl1*^{-/-} mice. (M) The absolute cell numbers of eosinophils (Eos.), neutrophils (Neu.), lymphocytes (Lym.), and macrophages (Mac.) in the BAL fluid are shown. Mean values (5 mice per group) are shown with standard deviations. NS: not significant (N) Intracellular staining profiles of IL-5 and CD44 in the lung CD44⁺CD4⁺ T cells (on day 27). (O) ELISA analysis of IL-5 in the BAL fluid from the experimental groups with *Il1rl1*^{+/+} or *Il1rl1*^{-/-} mice in panel M. (P) Experimental protocols for OVA and Alum immunization-induced allergic inflammation mouse model (45 days after immunization) using *Il1rl1*^{+/+} or *Il1rl1*^{-/-} mice. (Q) The absolute cell number of eosinophils (Eos.), neutrophils (Neu.), lymphocytes (Lym.), and macrophages (Mac.) in the BAL fluid. Mean values (5 mice per group) are shown with standard deviations (**: $P < 0.01$). (R) Intracellular staining profiles of IL-5 and CD44 in the lung CD44⁺CD4⁺ T cells (on day 60). (S) ELISA analysis of IL-5 in the BAL fluid from the experimental groups with *Il1rl1*^{+/+} or *Il1rl1*^{-/-} mice in panel Q. Two independent experiments were performed with similar results (**: $P < 0.01$). (T) Intracellular staining profiles of IL-4, IL-5, and IL-13 in the *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory Th2 cells in the spleen after challenge with OVA 4 times. Three independent experiments were performed with similar results. (U) Experimental protocols for the depletion of ILC2 cells in memory Th2 cell-dependent allergic inflammation mouse models using CD90.2 antibody. (V) Cell surface expression profiles of CD90.2 and CD25 on Lin⁻ cells (Monticelli et al., 2011; Roediger et al., 2013) from the lung and the spleen of isotype control or anti-CD90.2 groups. Two independent experiments were performed with similar results.

Figure S5 related to main Figure 5

A



B

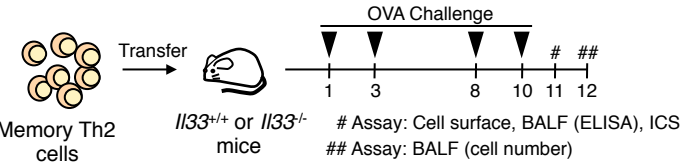


Figure S5. Experimental protocols for the generation and in vivo function of memory Th2 cells using $Il33^{-/-}$ mice (related to Figure 5).

(A) Experimental protocols for the generation of memory Th2 cells using $Il33^{+/+}$ or $Il33^{-/-}$ mice as recipients. (B) Experimental protocols for memory Th2 cell-dependent allergic inflammation models using $Il33^{+/+}$ or $Il33^{-/-}$ recipient mice.

Figure S6 related to main Figure 6

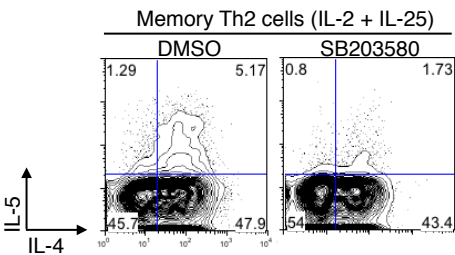


Figure S6. The p38 signaling is required for IL-2 and IL-25-dependent IL-5 induction by memory Th2 cells (related to Figure 6).

Intracellular staining profiles of IL-5 and IL-4 in IL-2- and IL-25-treated memory Th2 cells with DMSO and p38 inhibitor (SB203580; 10 μ M). Two independent experiments were performed with similar results.

Figure S7 related to main Figure 7

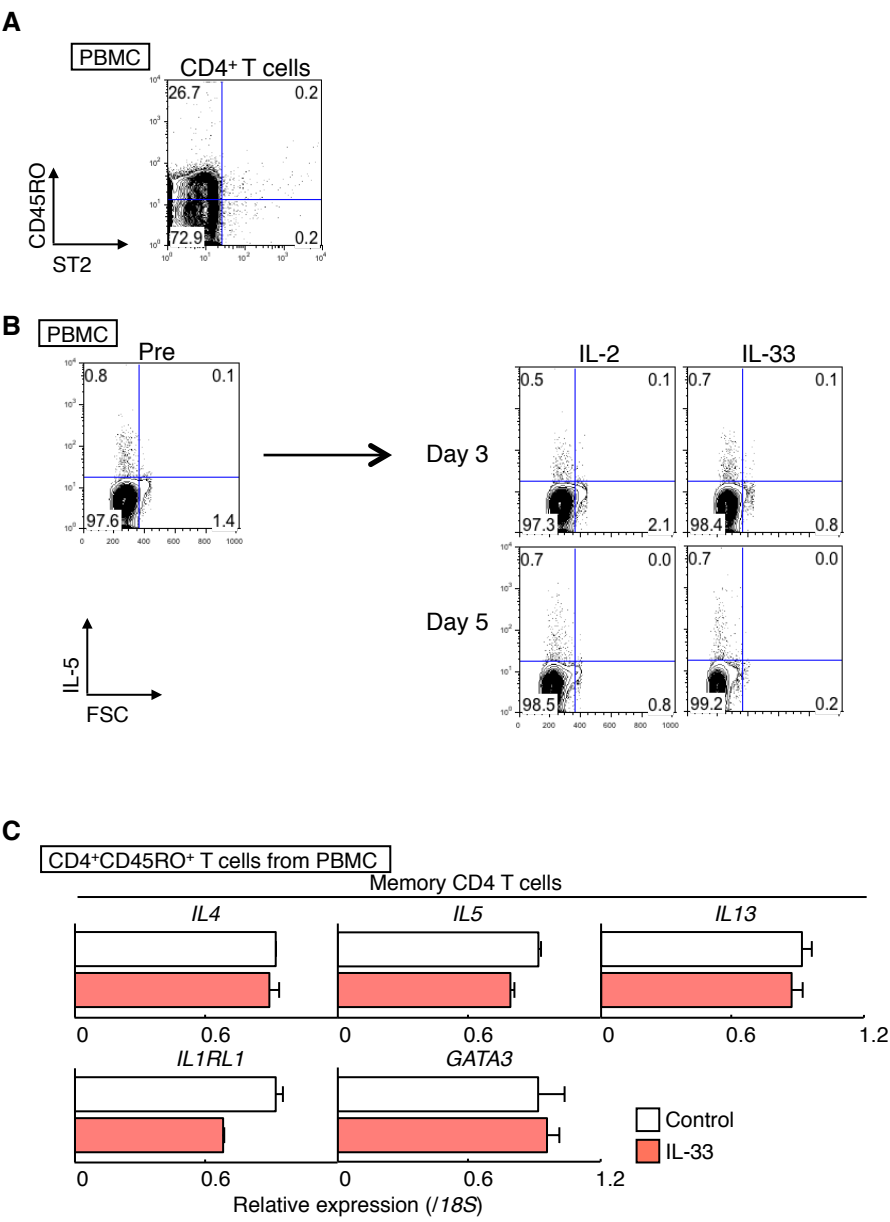


Figure S7. IL-33 showed no obvious effect on the induction of ST2 and Th2 cytokines in CD45RO⁺ memory CD4⁺ T cells collected from PBMC of healthy donors (related to Figure 7).

(A) Cell surface expression profiles of CD45RO and ST2 on CD4⁺ T cells from PBMC of human healthy donors. (B) Intracellular staining profiles of IL-5 in IL-2 or IL-33 cultured CD45RO⁺ memory CD4⁺ T cells from PBMC of human healthy donors are shown. (C) Quantitative RT-PCR analysis of *IL4*, *IL5*, *IL13*, *IL1RL1*, or *GATA3* in CD45RO⁺ memory CD4⁺ T cells before and after cultured with IL-33 for 5 days are shown. Three independent experiments were performed with similar results.

Supplemental Experimental Procedures

Reagents.

The reagents used in this study are as follows: FITC- or APC-conjugated anti-CD4, FITC-, PE-, APC-conjugated KJ1-26 (anti-clonotypic for DO11.10 TCR), PE-, APC-conjugated anti-CD62L, PE/Cy7-conjugated anti-CXCR3, and PE-conjugated Gr-1 were purchased from BD-PharMingen, San Diego, CA. PE-, APC-conjugated anti-CXCR3, APC-conjugated anti-CCR3, and APC-conjugated anti-ST2 were purchased from R&D Biotech. Anti-FcR γ II and III (2.4G2) and unconjugated anti-IL-4 (11B11), anti-IL-12 (C17.8) and anti-IFN γ (R4-6A2) were used as culture supernatants. Recombinant mouse IL-4 was from TOYOBO, Osaka, Japan. Recombinant mouse and human IL-33 and mouse IL-25 were purchased from R&D Biotech. The OVA peptide (residues #323-339; ISQAVHAAHAEINEAGR) was synthesized by BEX Corporation, Tokyo, Japan. For inhibition of cytokine-mediated signals, SB203580 (Merck), U0126 (Merck), SP600125 (Merck), and Wortmannin (Merck) were used.

Detection of apoptotic cells.

For the detection of apoptotic cells, Annexin V^{FITC} apoptosis detection kit II (BD Biosciences) was used according to the manufacturer's protocol.

Proliferation assay.

For the analysis of memory Th2-cell proliferation in response to cytokines, [³H]-thymidine (37 kBq per well) was added to the culture for the last 16 hours, and the incorporated radioactivity was measured on a β plate.

ELISA for the measurement of cytokine concentration.

Cells were stimulated with immobilized anti-TCR β (3 μ g/ml) in 48-well flat bottom plates (1.0 x 10⁵ cells/well) for 16 hours at 37°C. The production of IL-4, IL-5 and IFN γ was assessed by ELISA as described previously (Yamashita et al., 2006). The production of IL-13 was evaluated with a mouse IL-13 ELISA kit (R&D systems) according to the manufacturer's protocol.

Chromatin Immunoprecipitation (ChIP) assay.

ChIP assay was performed as described previously (Yamashita et al., 2002). The antibodies using ChIP assay as follows; anti-trimethyl histone H3-K4 (LP Bio), anti-acethyl histone H3-K9 (Millipore), anti-trimethyl histone H3K27 (Millipore), anti-RNA pol II (Abcam), anti-p300 (Santa Cruz), and normal rabbit IgG (Santa Cruz). The specific primers used in ChIP assay as follows;

//4p FW: 5'-AAGGCAGACTTTCTTGATATT-3'

//4p RV: 5'-GGCCTTTCAGACTAATCTT-3'

//5 int.1 FW: 5'-TGGCCTTGAACCTCACAGAGA-3'

//5 int.1 RV: 5'-TTGGCCTTTAATCCCAGAAC-3'

//5p FW: 5'-AAGTCTAGCTACCGCCAATA-3'

//5p RV: 5'-AGCAAAGGTGAGTTCAATCT-3'

//5 U1 FW: 5'-CGGCAGATTTGGGGAGATA-3'

//5 U1 RV: 5'-TTGTGAGTTTCACATCATGCAC-3'

//5 U2 FW: 5'-CATGGTGTTGAATCACTCTGAAA-3'

//5 U2 RV: 5'-TCAGGGGAATTGCCAAGTAA-3'

Il5 U3 FW: 5'-TGCACATAACCACACACATACATATAA-3'

Il5 U3 RV: 5'-GTAATAAATCTTAAAGAAGGCGGAGA-3'

Il13p FW: 5'-GGTCAGCGGGTGAATTAC-3'

Il13p RV: 5'-AAAATCCAGTGGCCGCTAAA-3'

CGRE FW: 5'-GGCTGGACCGCTGGATCAGT-3'

CGRE RV: 5'-CCTCTCCTGGTGGCGTGTTG-3'

V_α FW: 5'-TCCATACAGTTCCTGCAGTAGCTG-3'

V_α RV: 5'-CATCTCCCCAACCCCAAGATATA-3'

Il1rl1 int.4 FW: 5'-TGGAGAGCTGTGAACAGTGG-3'

Il1rl1 int.4 RV: 5'-TCAATATATCCATGACAAAGACGTG-3'

Il1rl1 int.7 FW: 5'-AATTCCCTTGCTTGGTCCTT-3'

Il1rl1 int.7 RV: 5'-ATCGGATCAGGTTGGTCATC-3'

Il1rl1p FW: 5'-TGGGGGAACAGTCTTGGA-3'

Il1rl1p RV: 5'-CCAGTACTCAVAGATAGGCATCA-3'

Il1rl1 U1 FW: 5'-GTATTTGGCAACTTTCGCTTG-3'

Il1rl1 U1 RV: 5'-TCATTTCAAAGTGAACGAGCA-3'

Hprtp FW: 5'-TCCTCCTCAGACCGCTTTT-3'

Hprtp RV: 5'-TCTGCTGGAGTCCCCTTG-3'

Immunoblotting assay.

Immunoblotting was performed as described previously (Yamashita et al., 2006). Briefly, cytoplasmic extracts and nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Fisher Scientific). The antibodies used for the

immunoblot analysis were anti-Phospho-p38 (Cell signaling), anti-p38 (Cell signaling), and anti-Tub α (NewMarkers).

The culture conditions of effector and memory Th2 cells.

In some experiments, effector and memory Th2 cells were cultured with cytokines (IL-33, IL-2, IL-7, or IL-25) for a further 5 days. For the administration of inhibitors (SB203580; 10 μ M, U0126; 10 μ M, SP600125; 20 μ M and Wortmannin; 1 μ M), memory Th2 cells were cultured with IL-33 and limited amounts of IL-2 in the presence or absence of inhibitors. For the administration of IL-33, *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory Th2 mice were injected with recombinant IL-33 at a dose of 1 μ g per mouse on days 0, 2 and 4, then were sacrificed and the organs collected on day 6. For the generation of non-TCR transgenic memory CD4⁺ T cells *in vivo*, BALB/c mice were immunized with OVA+Alum twice. 42 days after the immunization, CD4⁺CD44⁺ T cells prepared from spleen were cultured with IL-33 for 5 days *in vitro*.

The purification of Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were purified from whole blood. Human CD45RO⁺ memory CD4⁺ T cells were purified from mononuclear cells using a Memory CD4⁺ T cell isolation kit (Miltenyi Biotec.).

Patients

Patients with chronic rhinosinusitis with nasal polyps (CRSwNP) were recruited from Chiba University Hospital, Kimitsu Chuo Hospital and the Chiba Otolaryngology Surgical Center. All patients met the criteria for CRSwNP defined in the European position paper on

rhinosinusitis and nasal polyps (NPs). All patients signed informed consent forms and the study was approved by the Ethics Committee of the Chiba University Graduate School of Medicine and each participating hospital (#1006). NP tissues were obtained during endoscopic sinus surgery. None of the patients received systemic steroids for at least 4 weeks before surgery. CRSwNP was classified as ECRS when tissue eosinophils were >70/ high power field (HPF). The number of tissue eosinophils was counted under a HPF (HPF x 400) in a blinded manner by two observers.

Immunofluorescent staining and confocal microscopy.

For immunofluorescent staining, samples (n=6 for ECRS, n=8 for NECRS) were fixed in 4% paraformaldehyde and equilibrated in 30% sucrose/PBS solution. Cryostat sections of adult femurs were stained and mounted with fluorescent mounting medium (DakoCytomation). Histological analysis was carried out with a confocal laser microscope (LSM710; Carl Zeiss). The antibodies used for the staining were anti-IL-33 (SIGMA), anti-PECAM1 (Santa Cruz), or TO-PRO3 (Invitrogen).

Supplemental References

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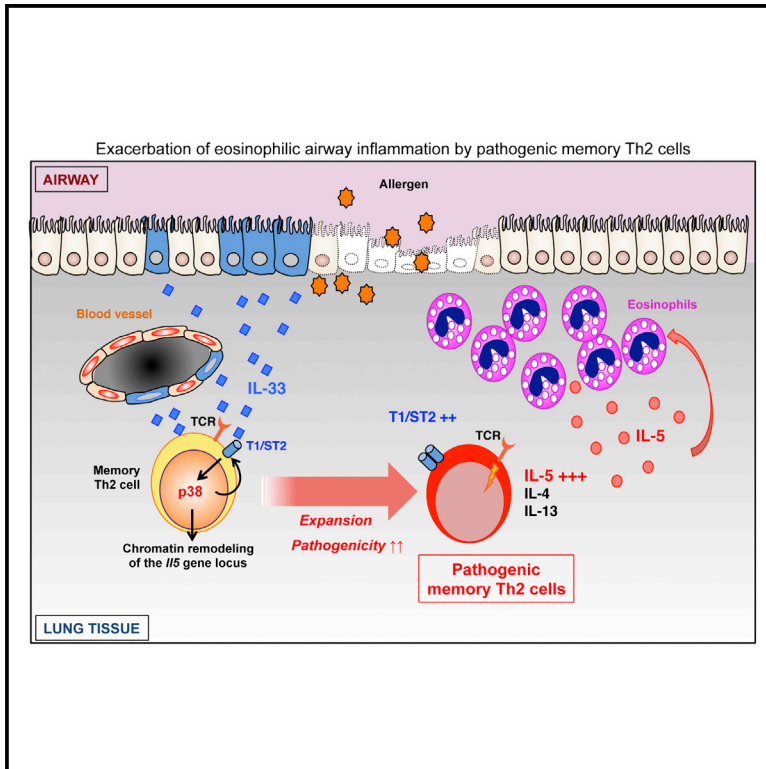
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Immunity

The Interleukin-33-p38 Kinase Axis Confers Memory T Helper 2 Cell Pathogenicity in the Airway

Graphical Abstract



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In Brief

IL-33, a IL-1 family member identified as the ligand for the ST2 receptor, is deeply related to allergic inflammation. Nakayama and colleagues demonstrate that the IL-33-ST2-p38 axis is crucial for the induction of pathogenicity of memory Th2 cells in allergic airway inflammation in both mice and humans.

Highlights

- Memory Th2 cells are critical targets of IL-33 in allergic airway inflammation
- IL-33 selectively remodels chromatin of *IL5*, thereby licensing its expression
- Memory-Th2-cell-mediated airway inflammation depends on IL-33 and ST2
- p38 MAPK is a major downstream target of IL-33-ST2 signaling in memory Th2 cells



The Interleukin-33-p38 Kinase Axis Confers Memory T Helper 2 Cell Pathogenicity in the Airway

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SUMMARY

Memory CD4⁺ T helper (Th) cells provide long-term protection against pathogens and are essential for the development of vaccines; however, some antigen-specific memory Th cells also drive immune-related pathology, including asthma. The mechanisms regulating the pathogenicity of memory Th cells remain poorly understood. We found that interleukin-33 (IL-33)-ST2 signals selectively licensed memory Th2 cells to induce allergic airway inflammation via production of IL-5 and that the p38 MAP kinase pathway was a central downstream target of IL-33-ST2 in memory Th2 cells. In addition, we found that IL-33 induced upregulation of IL-5 by memory CD4⁺ T cells isolated from nasal polyps of patients with eosinophilic chronic rhinosinusitis. Thus, IL-33-ST2-p38 signaling appears to directly instruct pathogenic memory Th2 cells to produce IL-5 and induce eosinophilic inflammation.

INTRODUCTION

The quality of adaptive immune responses depends on the number and function of antigen-specific memory T cells. Upon antigen recognition via the T cell receptor (TCR), naive CD4⁺ T cells undergo rapid clonal expansion, followed by differentiation into functionally distinct T helper (Th) cell subsets, such as Th1, Th2, and Th17 cells (O'Shea and Paul, 2010; Reiner, 2007). Some of these effector Th cells are maintained as memory Th cells for long times in vivo (Nakayama and Yamashita, 2008), and it is now becoming clear that these cells display functional heterogeneity (Sallusto and Lanzavecchia, 2009). Recent reports indicate that there are several distinct subsets of memory type Th2 cells that produce large amounts of

interleukin-5 (IL-5), IL-17, or interferon- γ (IFN- γ) in addition to IL-4 and IL-13 (Endo et al., 2014; Endo et al., 2011; Hegazy et al., 2010; Islam et al., 2011; Upadhyaya et al., 2011; Wang et al., 2010). In particular, IL-5-producing memory Th2 cell subsets appear to be crucial drivers of the pathology of allergic diseases in the airway and skin (Endo et al., 2011; Islam et al., 2011). However, environmental cues and signals that dictate how memory Th2 cells contribute to the pathogenicity of allergic diseases, including chronic airway inflammation, are poorly understood.

Asthma is a chronic lower-airway inflammatory disease characterized by recurrent airway obstruction and wheezing (Cohn et al., 2004). Allergic asthma is mainly driven by Th2-cell-type cytokines, such as IL-4, IL-5, and IL-13, and is characterized by the presence of elevated numbers of eosinophils in the lungs (Cohn et al., 2004). IL-5 regulates eosinophil development, recruitment to the lungs, and activation (Rosenberg et al., 2013). IL-13 plays an important role in the effector phase of asthma by inducing airway remodeling and airway hyperresponsiveness (AHR) as well as mucus hyperproduction (Ingram and Kraft, 2012). Th2 cells are the major source of IL-4, IL-5, and IL-13 in allergic asthma. Innate Th2 cell counterparts that also produce large amounts of IL-5 and IL-13 (Lin⁺CD127⁺ type 2 innate lymphoid cells [ILC2s]) have been identified (Furusawa et al., 2013; Lloyd, 2010; Price et al., 2010; Saenz et al., 2010). Recent research indicates that ILC2s play a critical role in eosinophilic airway inflammation in mice that lack the ability to mount adaptive immune responses (Chang et al., 2011; Halim et al., 2012; Scanlon and McKenzie, 2012).

Chronic rhinosinusitis (CRS) is a common chronic sinus inflammatory disease characterized by distinct cytokine production profiles and tissue-remodeling patterns (Hamilos, 2011; Van Bruaene et al., 2008; Zhang et al., 2008). CRS can be classified into two types of diseases according to the presence of nasal polyps. CRS with nasal polyps (CRSwNP) is often accompanied by Th2-cell-skewed eosinophilic inflammation, whereas CRS without nasal polyps (CRSSNP) is characterized by a predominantly Th1-cell-skewed response (Hamilos, 2011). IL-5 is more

abundant in the nasal mucosal tissues of CRSwNP than in those of CRSsNP (Van Bruaene et al., 2008). CRSwNP is further subdivided into two types of diseases on the basis of the extent of eosinophilic inflammation, particularly for people in East Asia (Zhang et al., 2008): eosinophilic CRS (ECRS) and non-eosinophilic rhinosinusitis (NECRS).

IL-33, a member of the IL-1 family, was newly identified as the ligand for the ST2 receptor (also known as IL-1RL1) (Liew et al., 2010; Schmitz et al., 2005). The major genome-wide association studies have reproducibly found significant associations between *IL33* and *IL1RL1* genetic variants and asthma in humans (Bønnelykke et al., 2014; Grotenboer et al., 2013; Torgerson et al., 2011). IL-33 expression is higher in asthmatic patients and in mouse models of asthma (Lloyd, 2010). Epithelial and airway smooth muscle cells appear to represent two major sources of IL-33 in asthmatics (Préfontaine et al., 2009). Previous reports showed that the depletion of IL-33 or ST2 attenuated murine ovalbumin (OVA)-induced airway inflammation (Kurowska-Stolarska et al., 2008; Oboki et al., 2010). ILC2s are characterized by their rapid production of IL-5 and IL-13 in response to IL-33 exposure (Scanlon and McKenzie, 2012). Therefore, understanding the mechanisms by which IL-33 induces allergic inflammation and differentiating between antigen-specific and antigen-independent functions of IL-33 are crucial for the effective design of therapeutics for patients with allergic inflammatory disorders such as chronic asthma.

We herein investigated the role of IL-33 in allergic airway inflammation induced by memory Th2 cells. We found that IL-33-ST2 signaling was crucial for the induction of pathogenicity of memory Th2 cells in allergic experimental asthma. Moreover, we found that like ILC2s, memory Th2 cells acquired the ability to produce IL-5 directly in response to IL-33; this property was not observed in effector Th2 cells. Genetic deletion of IL-33 or ST2 resulted in impaired memory-Th2-cell-dependent eosinophilic airway inflammation, and we identified p38 mitogen-activated protein kinase (MAPK) as the downstream target of IL-33-ST2 signaling in this cell type. Analysis of nasal polyps from patients with CRS showed that IL-33 could also directly enhance IL-5 production by human memory CD4⁺ T cells. Thus, we propose that the IL-33-ST2-p38 axis is crucial for the induction of pathogenicity of memory Th2 cells in eosinophilic airway inflammation in both mice and humans.

RESULTS

IL-33 Selectively Enhances IL-5 Production by Memory Th2 Cells

IL-33 is known to induce strong Th2-cell-type immune responses and eosinophilic inflammation in the lung and intestine (Lloyd, 2010). However, the types of cells on which IL-33 acts in these settings are still being defined. To explore the involvement of CD4⁺ T cells in IL-33-mediated inflammation, we assessed the expression of the IL-33 receptor (ST2) on naive CD4⁺ T cells, effector Th1 and Th2 cells generated in vitro, and memory Th1 and Th2 cells generated in vivo (Nakayama and Yamashita, 2008) (Figure S1A). Memory Th2 cells showed very high expression of IL-7 receptor- α chain (IL-7R α), and the majority also showed low expression of CD69 and IL-2R α (Figure S1B, upper panel). Expression patterns of these surface receptors

were quite different from that seen on the in-vitro-generated effector Th2 cells used in this study. We observed that compared to naive CD4⁺ T cells or memory Th1 cells, memory Th2 cells showed strongly increased expression of *Il1rl1* mRNA (Figure 1A). Compared to naive CD4⁺ T cells or effector Th1 cells, effector Th2 cells also showed significantly higher expression of *Il1rl1* ($p < 0.01$; Mann-Whitney U test), but this was not as pronounced as the expression observed in memory Th2 cells. The increased *Il1rl1* mRNA was also reflected by higher expression of ST2 on the surface of memory Th2 cells than on the surface of naive CD4⁺ T cells or effector Th2 cells (Figure 1B). Substantial ST2 expression was detected only on memory Th2 cells, and ST2 was specifically found on those cells with high expression of IL-7R α and low expression of CD69 and IL-2R α , strongly indicating that a distinct subset of ST2-expressing cells is induced in memory Th2 cells (Figure S1B, lower panel). In addition, exposure of memory Th2 cells to IL-33 dramatically enhanced ST2 expression (Figure 1C). Importantly, we found that stimulation with IL-33 for 5 days selectively induced IL-5 production by memory Th2 cells but not by effector Th2 cells (Figure 1D). In contrast, IL-33-induced upregulation of IL-4 expression was not observed in response to treatment with IL-33 (Figures 1D and 1F). IL-13 was slightly increased by cultivation of memory Th2 cells with IL-33 (Figures 1E and 1F). IL-33 supported the viability of memory Th2 cells as well as IL-2 and IL-7 (Figure S1C) without inducing significant proliferation (Figure S1D). We reported previously that memory Th2 cells can be subdivided into four distinct subpopulations according to the expression of CXCR3 and CD62L and that IL-5 production is normally restricted to a small number of cells in the CD62L^{lo}CXCR3^{lo} subpopulation (Endo et al., 2011) (Figure S1E). We also examined the effect of IL-33 on the four subpopulations (CXCR3 and CD62L) of memory Th2 cells. ST2 expression was detected on 10%–20% of all four subpopulations of freshly prepared memory Th2 cells (Figure S1F, left). IL-33 treatment enhanced ST2 expression on all four subpopulations (Figure S1F, right). Upon TCR stimulation, IL-5-producing cells were detected only in the CD62L^{lo}CXCR3^{lo} subpopulation of freshly prepared memory Th2 cells (Figure S1G, left), whereas after IL-33 cultivation, IL-5-producing cells were detected in all four subpopulations and showed their highest numbers in the CD62L^{lo}CXCR3^{lo} subpopulation (Figure S1G, right). IL-5 production was also assessed by ELISA, and similar results were obtained (Figure S1H). Thus, these results indicate that IL-33 upregulates ST2 expression and selectively enhances IL-5 expression and production by memory Th2 cells, but not effector Th2 cells.

IL-33 Induces Selective Remodeling of Chromatin at the *Il5* Locus in Memory Th2 Cells

Epigenetic chromatin modifications can control selective expression of genes that function in the immune system (Northrup and Zhao, 2011). We therefore explored whether IL-33 signaling could regulate the chromatin status of the Th2-cell-associated cytokine-encoding genetic loci in memory Th2 cells. We performed chromatin immunoprecipitation (ChIP) assays with antibodies specific to several histone modifications (Figure 2A). At the *Il5* locus, freshly prepared in-vivo-generated memory Th2 cells showed lower modifications associated with active

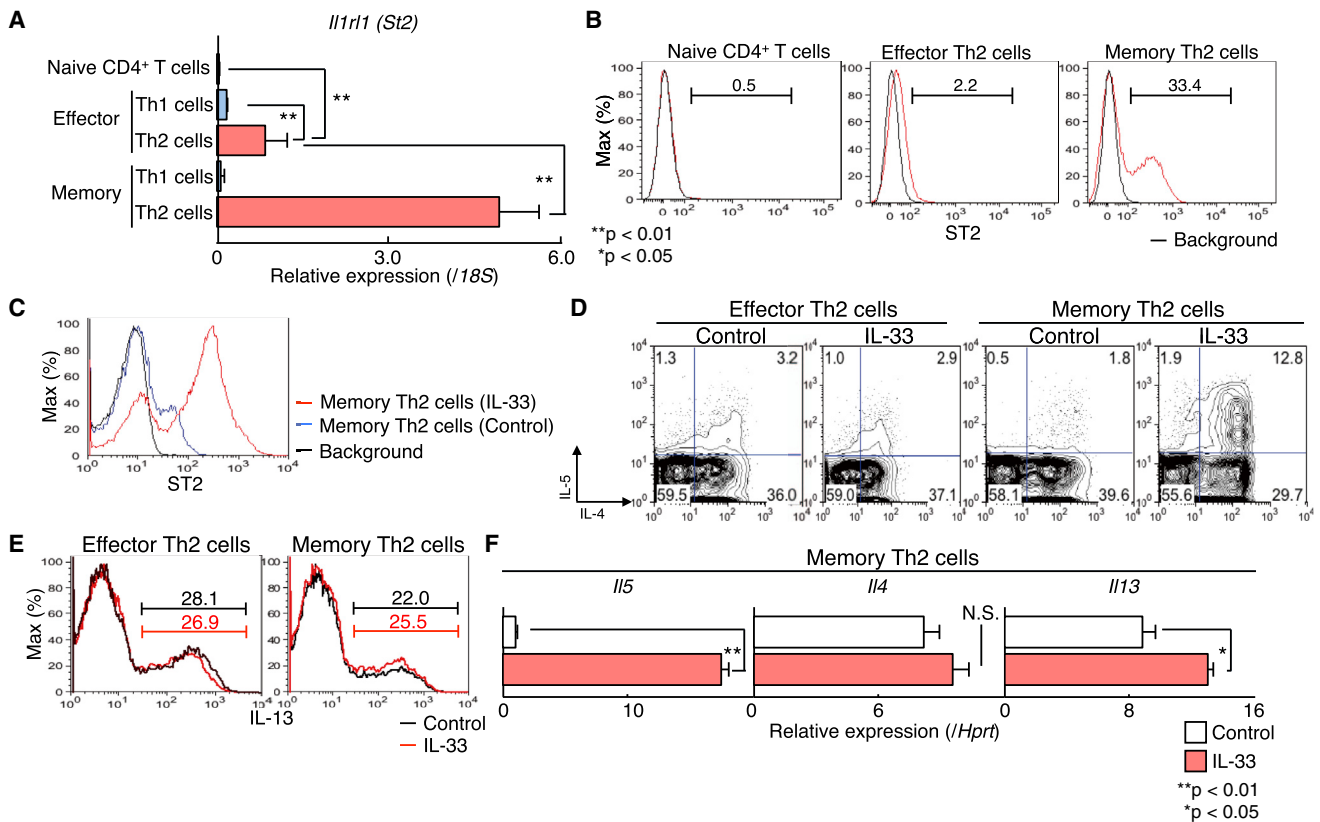


Figure 1. IL-33 Selectively Induces IL-5 Production by Memory Th2 Cells

(A) Quantitative RT-PCR analysis of *Il1r1* in the indicated cell types. Relative expression (normalized to *Hprt*) is shown with SDs.

(B) Expression profiles of ST2 on the indicated cell types.

(C) Expression profiles of ST2 on memory Th2 cells before (blue) and after (red) culture with IL-33.

(D) Memory and effector Th2 cells were cultured with IL-33 for 5 days. The cultured cells were stimulated with immobilized anti-TCR β for 6 hr. Intracellular-staining profiles of IL-5 and IL-4 are shown.

(E) Intracellular-staining profiles of IL-13 in effector and memory Th2 cells before and after culture with IL-33 are shown.

(F) Quantitative RT-PCR analysis of the indicated genes in memory Th2 cells before and after culture with IL-33.

More than five independent experiments were performed and showed similar results (***p* < 0.01; NS, not significant; Mann-Whitney U test; A–F). Three technical replicates were performed with quantitative RT-PCR (A and F). See also Figure S1.

transcription (H3-K4 trimethylation and H3-K9 acetylation) and higher modifications associated with genetic repression (H3-K27 trimethylation) than did effector Th2 cells (Figure 2B), indicating that these modifications were not efficiently maintained during the transition to the memory phase. However, after stimulation of memory Th2 cells with IL-33 (without TCR stimulation), H3-K4 trimethylation and H3-K9 acetylation increased and H3-K27 trimethylation decreased at the *Il5* locus, indicating that IL-33 could restore the chromatin-modification signature observed in IL-5-producing effector Th2 cells. In contrast to histone modifications at the *Il5* locus, those at the other Th2 cell cytokine-encoding loci (*Il4p*, *Il13p*, and Va enhancer) were not obviously affected by IL-33 stimulation (Figures S2A and S2B). We also observed increased H3-K4 trimethylation and H3-K9 acetylation at the *Il1r1* locus in IL-33-stimulated memory Th2 cells (Figures S2C and S2D). These changes in histone modifications at the *Il5* locus were specific to IL-33 because no obvious change was detected in memory Th2 cells treated with IL-2, IL-7, or IL-25 (Figure S2E). It has previously been shown that

like IL-33, a combination of IL-2 and IL-25 can induce IL-5 production in ILC2s (Furusawa et al., 2013). We examined the synergistic effect of IL-2 and IL-25 on IL-5 production by memory Th2 cells, and indeed cultivation with IL-2 and IL-25, similar to IL-33 cultivation, increased IL-5 production in memory Th2 cells (Figure S2F). Next, we performed ChIP assays to assess whether the chromatin remodeling at the *Il5* locus of memory Th2 cells was induced by the combination of IL-2 and IL-25. Active histone modifications were increased and repressive histone modifications were decreased at the *Il5* locus in memory Th2 cells after culture with both IL-2 and IL-25 (Figure S2G). The IL-33-induced histone modifications at the *Il5* locus were accompanied by enhanced recruitment of p300, a component of the histone acetyl transferase (HAT) complex, and RNA polymerase II (pol II) (Figure 2C). Stronger binding of pol II to the *Il5* locus, including the transcribed region in IL-33-cultured memory Th2 cells, was detected. These results indicate that IL-33, as well as the combination of IL-2 and IL-25, remodels chromatin structure to be permissive for transcription at the *Il5* locus in memory Th2 cells.

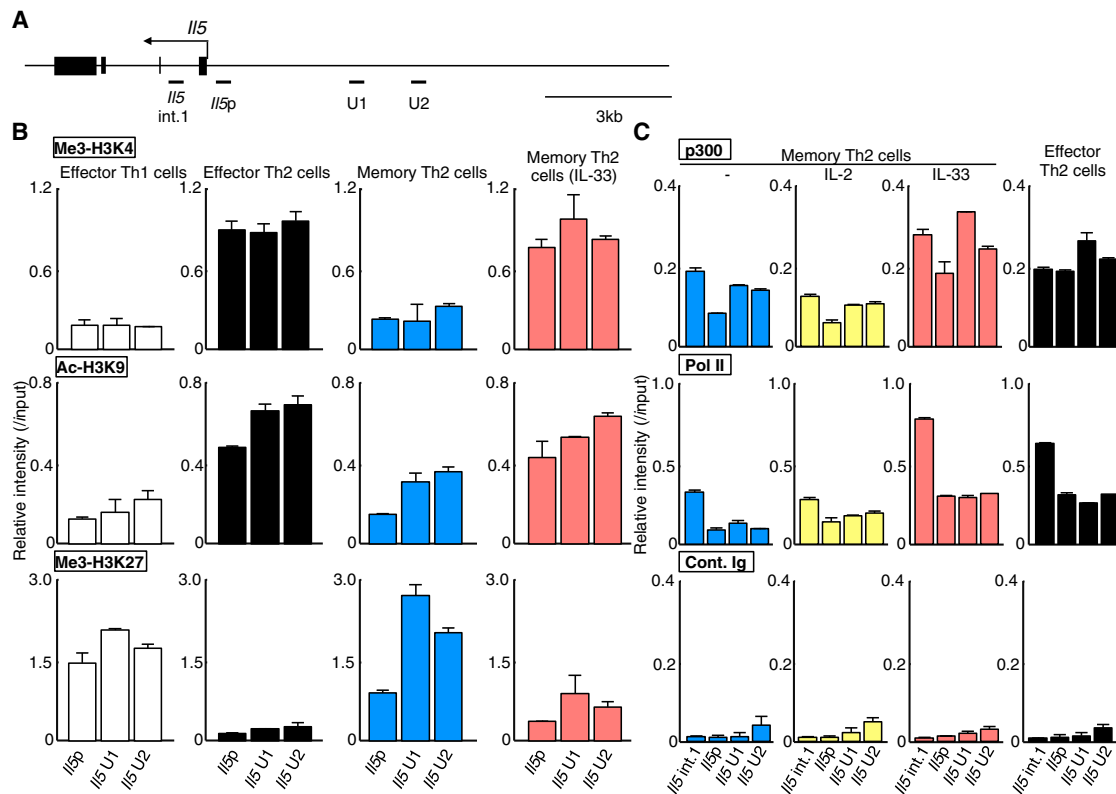


Figure 2. IL-33 Selectively Remodels Chromatin Structure at the *IL5* Locus in Memory Th2 Cells Independently of TCR Stimulation

(A) Schematic representation of the mouse *IL5* locus. The locations of primers and probes (upstream region 2 [U2] to *IL5* intron 1 [int.1]) and exons are indicated. (B) ChIP assays were performed with anti-trimethyl histone H3-K4, anti-acetyl histone H3K9, and anti-trimethyl histone H3K27 at the *IL5* locus from effector Th1, effector Th2, freshly prepared memory Th2, and memory Th2 cells cultured with IL-33 for 5 days. The relative intensities (relative to input DNA) of these modifications were determined by quantitative RT-PCR analysis. More than three independent experiments were performed and showed similar results. (C) The binding of p300 and pol II to the *IL5* locus in the indicated cell types was detected by ChIP with quantitative RT-PCR analysis. Two independent experiments were performed and showed similar results. Three technical replicates were performed with quantitative RT-PCR (B and C). See also Figure S2.

Because TCR stimulation was not included in this culture system, it appears as though the observed chromatin remodeling was solely induced by cytokine signaling.

The IL-33-ST2 Pathway Enhances IL-5 Production by Memory Th2 Cells In Vivo

We used *IL1rl1*-deficient (*IL1rl1*^{-/-}) mice in order to directly demonstrate the involvement of the IL-33-ST2 pathway in the generation of IL-5-producing memory Th2 cells. In the absence of ST2, effector Th2 cell differentiation was not impaired, and memory Th2 cells were generated normally in vivo (Figures S3A and S3B). As expected, IL-33-induced IL-5 augmentation was not observed in *IL1rl1*^{-/-} memory Th2 cells (Figure 3A). Next, we assessed the effect of IL-33 on the IL-5 production of memory Th2 cells in vivo, as illustrated in Figure 3B. Administration of IL-33 increased ST2 expression (Figure 3C) and IL-5 production (Figure 3D) of *IL1rl1*^{+/+} memory Th2 cells but did not substantially alter expression of ST2 or IL-5 production by *IL1rl1*^{-/-} memory Th2 cells. These results indicate that memory Th2 cells respond to IL-33 in vivo by increasing ST2 expression and producing IL-5, as was observed in memory Th2 cells after cultivation with IL-33 in vitro.

We also generated non-transgenic memory CD4⁺ T cells by using an OVA-alum (OVA with aluminum) immunization system in vivo, as depicted in Figure 3E. A substantial proportion of non-TCR transgenic memory CD4⁺ T cells expressed ST2 (Figure 3F and Figure S3C), and IL-33-induced IL-5 augmentation was detected in *IL1rl1*^{+/+} memory CD4⁺ T cells, but not in *IL1rl1*^{-/-} memory CD4⁺ T cells (Figure 3G). Thus, the IL-33-ST2 pathway is critical for the induction of ST2 expression and generation of IL-5-producing memory Th2 cells in vivo.

Memory-Th2-Cell-Dependent Eosinophilic Airway Inflammation and AHR Are Ameliorated by the Depletion of IL-33 or ST2

In order to assess the role of the IL-33-ST2 pathway in the pathological function of memory Th2 cells, we used memory-Th2-cell-dependent airway-inflammation models, as illustrated in Figure 4A. The expression of *IL33* in the lung was increased after OVA challenge by inhalation, whereas the expression of *IL33* in the spleen was not increased even after OVA challenge (Figure 4B). In this model, ST2-expressing cells were increased after OVA challenge in the lung, and highly ST2-expressing cells were found in the CXCR3⁺ memory Th2 cell population (indicated by

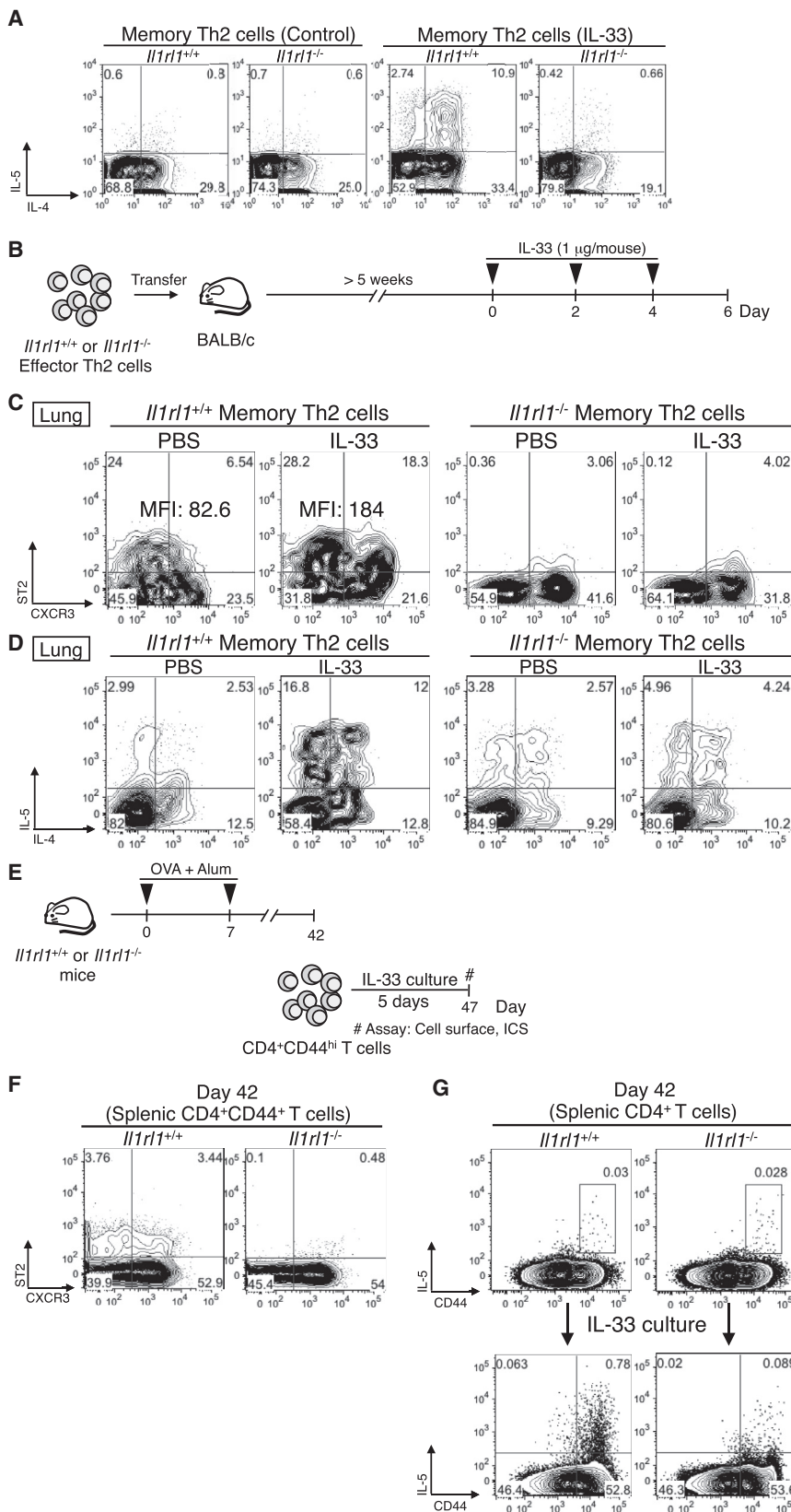


Figure 3. The IL-33-ST2 Pathway Enhances IL-5 Production by Memory Th2 Cells In Vivo

(A) Intracellular-staining profiles of IL-5 and IL-4 in *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory Th2 cells before (left, control) and after (right, IL-33) cultivation with IL-33. Five independent experiments were performed and showed similar results.

(B) Experimental protocols for the injection of IL-33 into the mice that received *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory Th2 cells.

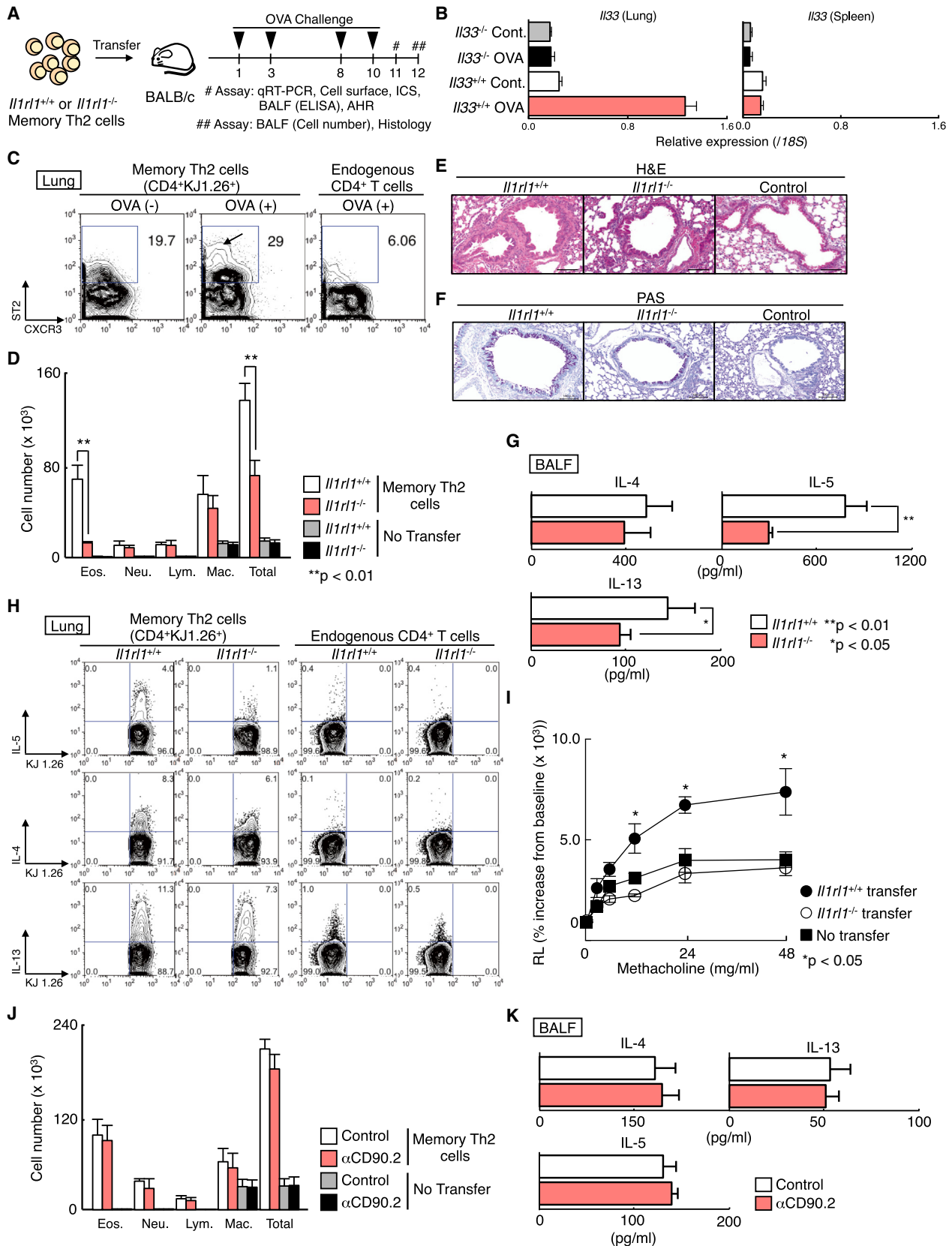
(C) ST2 and CXCR3 expression profiles of KJ1⁺ memory Th2 cells recovered from the lungs of mice that received *Il1rl1*^{+/+} (left) or *Il1rl1*^{-/-} (right) memory Th2 cells.

(D) KJ1⁺ memory Th2 cells shown in (C) were stimulated in vitro with immobilized anti-TCR β for 6 hr. Intracellular-staining profiles of IL-5 and IL-4 are shown.

(E) Experimental protocols for the generation of non-TCR transgenic memory CD4⁺ T cells in vivo. (F) Cell-surface-expression profiles of ST2 and CXCR3 on *Il1rl1*^{+/+} or *Il1rl1*^{-/-} CD44⁺ memory type CD4⁺ T cells are shown.

(G) *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory CD4⁺ T cells shown in (F) were stimulated with OVA-pulsed antigen-presenting cells for 24 hr before (upper) and after (lower) cultivation with IL-33. Intracellular-staining profiles of IL-5 and CD44 are shown with the percentage of cells in each area.

Two independent experiments were performed and showed similar results, and five technical replicates were included (C, D, F, and G). See also Figure S3.



(legend on next page)

an arrow, Figure 4C, middle). Only a small number of ST2-expressing cells were detected in endogenous lung CD4⁺ T cells even after OVA challenge (Figure 4C, right).

Next, OVA-specific *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory Th2 cells were transferred into wild-type BALB/c recipients, and their ability to induce airway inflammation was assessed. Compared to the *Il1rl1*^{+/+} group, the *Il1rl1*^{-/-} group showed a significant decrease ($p < 0.01$; Mann-Whitney U test) in the number of inflammatory eosinophils in the bronchoalveolar lavage (BAL) fluid (Figures 4D and S4A). No obvious changes in numbers of infiltrated cells were detected in OVA-challenged *Il1rl1*^{+/+} and *Il1rl1*^{-/-} mice that had not received memory Th2 cells. Histological analysis also revealed a similar reduction in mononuclear cell infiltration into the peribronchiolar regions of the lungs (Figure 4E). Periodic acid-Schiff (PAS) staining showed decreased production of mucus in the *Il1rl1*^{-/-} group (Figure 4F). Conversely, similar eosinophilic infiltration into the BAL fluid was observed from the mice that received *Il1rl1*^{+/+} and *Il1rl1*^{-/-} effector Th2 cells that were challenged with OVA on days 1 and 3 (Figures S4B and S4C). These results support the observation that effector Th2 cells possess little reactivity to IL-33 and that this pathway is less important for induction of eosinophilic inflammation by effector Th2 cells than for induction by memory Th2 cells. We also performed a set of in vivo kinetics experiments to better define the timing of acquisition of IL-33 responsiveness by memory Th2 cells. Eosinophilic airway inflammation, IL-5-producing Th2 cells in the lung, and IL-5 in the BAL fluid were assessed 6 or 35 days after the transfer of *Il1rl1*^{+/+} and *Il1rl1*^{-/-} effector Th2 cells (Figures S4D–S4K). Six days after cell transfer, no significant difference was detected in these three parameters between the *Il1rl1*^{-/-} and *Il1rl1*^{+/+} groups (Figures S4D–S4G). In contrast, significant decreases in eosinophilic infiltration ($p < 0.01$; Mann-Whitney U test), generation of IL-5-producing Th2 cells, and IL-5 production were observed in the day-35 *Il1rl1*^{-/-} group (Figures S4H–S4K). We also examined the OVA-induced airway inflammation in OVA-immunized *Il1rl1*^{+/+} and *Il1rl1*^{-/-} mice at different time points (12 or 45 days after OVA immunization) (Figures S4L–S4S). Significantly decreased infiltration of eosinophils into the BAL fluid ($p < 0.01$; Mann-Whitney U test), reduced IL-5-producing Th2 cells in the lung, and decreased IL-5 production in the BAL fluid were observed in the *Il1rl1*^{-/-} group 45 days after, but not 12 days after, the last immunization. These results indicate that ST2-mediated induction of IL-5 and exacerbation of eosinophilic airway inflammation were acquired by memory Th2 cells 35 days after effector Th2 cell transfer or 45 days after antigenic immunization, but not at the earlier time points analyzed.

The amount of IL-5 in the BAL fluid was significantly lower ($p < 0.01$; Mann-Whitney U test) in the *Il1rl1*^{-/-} memory Th2 cell group than in the *Il1rl1*^{+/+} group, whereas the amount of IL-4 was almost equivalent between the two groups (Figure 4G). We also detected a modest but significant reduction in IL-13 ($p < 0.05$; Mann-Whitney U test). Consistent with these observations, fewer IL-5-producing memory Th2 cells were detected in the lungs of mice from the *Il1rl1*^{-/-} group than in those from the *Il1rl1*^{+/+} group after OVA inhalation (Figure 4H, left), whereas decreases in IL-4- and IL-13-producing cells were less prominent. Th2-cell-associated cytokine production was not substantially different between endogenous *Il1rl1*^{+/+} and *Il1rl1*^{-/-} CD4⁺ T cells (Figure 4H, right). In addition, comparable proportions of Th2-cell-associated cytokine-producing memory Th2 cells were observed in the spleens of mice from the *Il1rl1*^{-/-} and *Il1rl1*^{+/+} groups (Figure S4T). These data are consistent with the absence of change in *Il33* expression in the spleen after OVA challenge (Figure 4B). We also analyzed the degree of AHR in the allergy-induced mice, which received *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory Th2 cells, by measuring methacholine-induced airflow obstruction with a mechanical ventilator. The degree of AHR in mice that received *Il1rl1*^{-/-} memory Th2 cells was lower than that of mice that received *Il1rl1*^{+/+} memory Th2 cells ($p < 0.05$; Mann-Whitney U test) (Figure 4I).

Finally, we assessed the relative contribution of memory Th2 cells to eosinophilic inflammation given that it is already known that ILC2s can respond to IL-33 by producing IL-5 in the lung (Halim et al., 2012). We examined the effect of the depletion of ILC2s on memory-Th2-cell-induced airway inflammation in the lung by using CD90.2 antibody. In brief, CD90.1⁺ memory Th2 cells were transferred into CD90.2 *Rag2*^{-/-} mice, and CD90.2⁺ ILC2s were depleted by the administration of CD90.2 antibody (Figures S4U and S4V). These mice were challenged with inhaled OVA four times. The number of infiltrated eosinophils and the concentration of IL-5 in the BAL fluid were found to be similar between control and ILC2-depleted groups (Figures 4J and 4K). Therefore, at least in this system, the contribution of ILC2s to eosinophilic inflammation is relatively minor.

IL33^{-/-} Mice Have Impaired Generation of IL-5-Producing Memory Th2 Cells and Reduced Eosinophilic Airway Inflammation

We next assessed the function of IL-33 on memory Th2 cells by using *Il33*^{+/+} and *Il33*^{-/-} mice as recipients with or without OVA challenge (Figures S5A and S5B). First, we assessed the effect of endogenous IL-33 on memory Th2 cells in the steady state.

Figure 4. The Pathogenicity of Memory Th2 Cells Is Ameliorated by Loss of IL-33-ST2 Signaling

(A) Experimental protocols for memory-Th2-cell-dependent allergic inflammation.

(B–I) Mice were treated as described in (A). (B) Quantitative RT-PCR analysis of *Il33* in the spleen or lungs. (C) ST2 and CXCR3 expression profiles of lung KJ1⁺ memory Th2 cells from mice with no challenge (left) or OVA challenge (center) and endogenous CD4⁺ T cells from mice with OVA challenge (right). (D) The absolute cell numbers of eosinophils (Eos), neutrophils (Neu), lymphocytes (Lym), and macrophages (Mac) in the BAL fluid are shown. Mean values (five mice per group) are shown with SDs. (E and F) Lungs were fixed and stained with H&E or PAS. A representative staining pattern is shown. Scale bars represent 100 μ m. (G) ELISA of the indicated cytokines in the BAL fluid from the experimental groups shown in (D). (H) Intracellular-staining profiles of the indicated cytokines in *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory Th2 cells in the lung. (I) Lung resistance (RL) was assessed in response to increasing doses of methacholine. The mean values (five mice per group) are shown with SDs.

(J) The absolute cell numbers of leukocytes in the BAL fluid. Mean values (five mice per group) are shown with SDs.

(K) ELISA analysis of the indicated cytokines in the BAL fluid from the experimental groups with memory Th2 cell transfer shown in (J).

Three (B–H) or two (J and K) independent experiments were performed and showed similar results (** $p < 0.01$; * $p < 0.05$). Three technical replicates were included in quantitative RT-PCR analysis (B). Five technical replicates were included in ELISA on the BAL fluid samples (G and K). See also Figure S4.

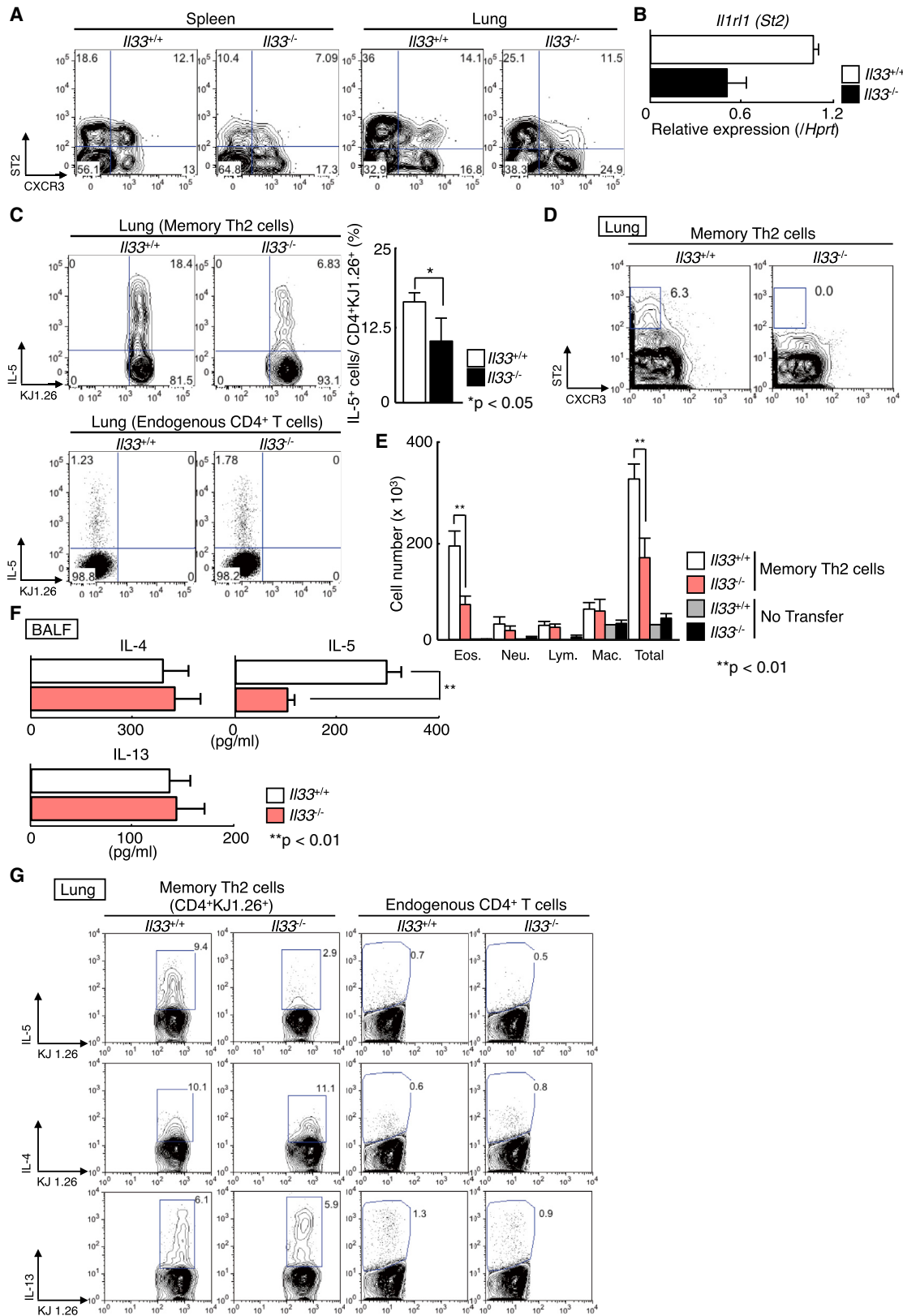


Figure 5. Reduced Memory-Th2-Cell-Dependent Airway Inflammation in *Il33^{-/-}* Mice

(A) ST2 and CXCR3 expression profiles of KJ1⁺ memory Th2 cells prepared from the spleens and lungs of *Il33^{+/+}* or *Il33^{-/-}* recipients.

(B) Quantitative RT-PCR analysis of *Il1r1* in lung memory Th2 cells from *Il33^{+/+}* or *Il33^{-/-}* recipients. Relative expression (normalized to *Hprt*) is shown with SDs.

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Compared to memory Th2 cells generated in $Il33^{+/+}$ mice, memory Th2 cells generated in $Il33^{-/-}$ mice (without OVA inhalation) showed a moderate reduction of ST2 expression (Figures 5A and 5B) and significant reduction of IL-5-producing cells (Figure 5C) ($p < 0.05$; Mann-Whitney U test). We also examined memory-Th2-cell-dependent airway inflammation by using $Il33^{+/+}$ and $Il33^{-/-}$ mice as recipients (Figure S5B). The OVA-induced highly ST2-expressing CXCR3⁺ population was not detected when memory Th2 cells were transferred into $Il33^{-/-}$ mice (Figure 5D). Compared to the $Il33^{+/+}$ group, mice from the $Il33^{-/-}$ group displayed significantly decreased eosinophilic infiltration ($p < 0.01$; Mann-Whitney U test; Figure 5E), and the amount of IL-5 in the BAL fluid was significantly decreased ($p < 0.01$; Mann-Whitney U test; Figure 5F). No obvious changes in the numbers of infiltrated cells were detected in OVA-challenged $Il33^{+/+}$ and $Il33^{-/-}$ mice that had not received memory Th2 cells (Figure 5E). Consistent with these observations, fewer IL-5-producing memory Th2 cells were detected in the lungs of mice from the $Il33^{-/-}$ group than in those of mice from the $Il33^{+/+}$ group after OVA inhalation, whereas the percentages of IL-4- or IL-13-producing cells were equivalent (Figure 5G, left). No substantial changes in Th2-cell-associated cytokine production were detected between $Il33^{+/+}$ and $Il33^{-/-}$ endogenous CD4⁺ T cells (Figure 5G, right). Thus, endogenous IL-33 from the recipient mice appears to be critical for the induction of memory-Th2-cell-dependent eosinophilic airway inflammation.

p38 Is a Major Downstream Target of IL-33-Dependent IL-5 Augmentation in Memory Th2 Cells

Next, we sought to identify which signaling pathway downstream of the ST2 receptor was responsible for the IL-33-dependent augmentation of IL-5 production by memory Th2 cells. Given that IL-33 has been reported to induce MAPK activation and NF- κ B phosphorylation in mast cells (Schmitz et al., 2005), we first examined the effect of chemical inhibitors of several components of these two pathways. We assessed the p38 MAPK inhibitor SB203580, the MEK inhibitor U0126, the JNK inhibitor SP600125, and the PI3K inhibitor Wortmannin. SB203580 inhibited the IL-33-induced IL-5 augmentation in memory Th2 cells, whereas the other inhibitors had little effect on IL-5 production (Figure 6A). No change in IL-4 production was detected after treatment with SB203580. We also used SB203580 to assess whether the IL-2- and IL-25-induced IL-5 augmentation in memory Th2 cells was dependent on p38 MAPK pathway. As expected, SB203580 inhibited IL-2- and IL-25-induced IL-5 augmentation in memory Th2 cells (Figure S6). Phosphorylation of p38 was induced by IL-33 and was inhibited by SB203580 (Figure 6B). A very small increase in p38 phosphorylation was detected after culture with IL-2. Significant reductions in

mRNA expression of *Il5* and *Il1rl1* were detected in response to SB203580 treatment of memory Th2 cells stimulated with IL-33; however, no significant reduction was observed in *Il4*, *Il13*, or *Gata3* ($p < 0.01$; Mann-Whitney U test; Figure 6C). We also detected marked reduction of ST2 after SB203580 treatment of IL-33-stimulated memory Th2 cells (Figure 6D). We next analyzed the effect of SB203580 on histone modifications at the *Il5* locus in memory Th2 cells. Memory Th2 cells cultured with IL-33 in the presence of SB203580 showed reduced modifications of H3-K4 methylation and H3-K9 acetylation at the *Il5* locus, but not at the conserved GATA3 response element or Va enhancer regions, which are located near the *Il13* promoter and downstream of the *Il4* locus, respectively (Figure 6E). Specific reduction of *Il5* and *Il1rl1* expression in response to siRNA-mediated silenced p38 in memory Th2 cells was also detected, whereas silencing of p38 did not change IL-4 or IL-13 expression (Figure 6F). These results indicate that IL-33 selectively activates p38, increases the expression of ST2, and augments the production of IL-5 in memory Th2 cells.

Human IL-33 Enhances IL-5 Production in Memory CD4⁺ T Cells from the Polyps of Patients with ECRS

Finally, we sought to gain more insight into the possible pathophysiological effect of IL-33 on memory Th2 cells in human disease. We analyzed CD45RO⁺ memory CD4⁺ T cells from the nasal polyps of patients with ECRS, which is characterized by IL-5-dependent accumulation of large numbers of eosinophils (Gevaert et al., 2006). We included samples from ECRS and NECRS patients and categorized them by the number of eosinophils found in the nasal polyps (Zhang et al., 2008). All patients signed informed-consent forms, and the study was approved by the ethics committee of the Chiba University Graduate School of Medicine and each participating hospital (1006). The number of IL-33⁺ endothelial cells in the nasal polyps was comparable between ECRS and NECRS patients (data not shown). However, compared to nasal polyps from NECRS patients, those from ECRS patients showed significantly elevated numbers of IL-33⁺PECAM1⁺ endothelial cells ($p < 0.05$; Mann-Whitney U test; Figure 7A). In addition, the baseline expression of *IL4* and *IL5* (encoding Th2 cell cytokines), *IL1RL1*, and *GATA3* was higher in the ECRS polyps (Figure 7B). In contrast, the expression of *TBX21* was lower in the ECRS polyps. These results prompted us to assess the effect of IL-33 on Th2 cell cytokine production of memory CD4⁺ T cells from nasal polyps of these patients. As a control, we analyzed the effect of IL-33 on cytokine expression by the CD45RO⁺CD4⁺ T cell population of peripheral-blood mononuclear cells (PBMCs) from healthy donors. Very little expression of ST2 was detected on CD45RO⁺CD4⁺ T cells from PBMCs, and no obvious augmentation in

(C) Intracellular-staining profiles of IL-5 and KJ1.26 in lung memory Th2 cells and endogenous CD4⁺ T cells from $Il33^{+/+}$ or $Il33^{-/-}$ recipients.

(D) ST2 and CXCR3 expression profiles of lung KJ1⁺ memory Th2 cells in $Il33^{+/+}$ or $Il33^{-/-}$ recipients after OVA challenge.

(E) The absolute numbers of leukocytes in BAL fluid from $Il33^{+/+}$ or $Il33^{-/-}$ mice transferred with or without memory Th2 cells are shown. Samples were collected 2 days after the last OVA challenge. The mean values (five mice per group) are shown with SDs.

(F) ELISA of the indicated cytokines in the BAL fluid from each experimental group shown in (E).

(G) Intracellular-staining profiles of the indicated cytokines in lung memory Th2 cells and endogenous CD4⁺ T cells from $Il33^{+/+}$ or $Il33^{-/-}$ mice after OVA challenging four times.

Two (A–C) or three (D–G) independent experiments were performed and showed similar results (** $p < 0.01$; * $p < 0.05$). Three technical replicates were performed with quantitative RT-PCR (B). Five technical replicates were performed with ELISA in BAL fluid (F). See also Figure S5.

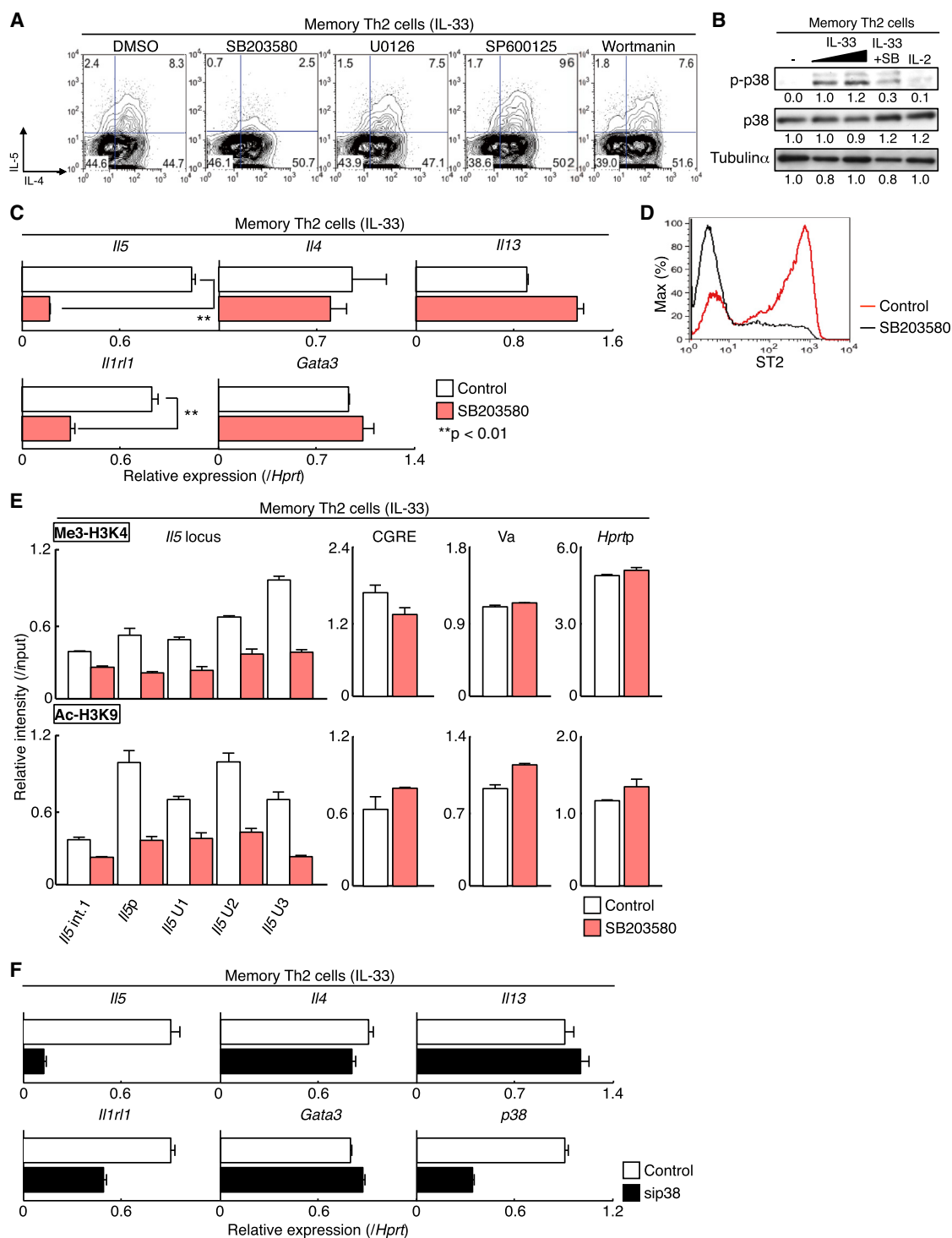


Figure 6. p38 Signaling Is Crucial for IL-33-Dependent IL-5 Induction by Memory Th2 Cells

(A) Intracellular-staining profiles of IL-5 and IL-4 in IL-33-treated memory Th2 cells with the indicated chemical inhibitors.

(B) The phosphorylated p38 (p-p38) and total p38 (p38) in freshly prepared memory Th2 cells stimulated with IL-2 or graded amounts of IL-33 with or without SB203580.

(C) Quantitative RT-PCR analysis of the indicated genes in memory Th2 cells cultured with IL-33 with or without SB203580. Four independent experiments were performed and showed similar results (** $p < 0.01$).

(D) Expression profiles of ST2 on memory Th2 cells treated with IL-33 with or without SB203580.

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IL-5 expression or production was detected after IL-33 treatment (Figures S7A–S7C). However, IL-33 dramatically enhanced *IL5* expression in CD45RO⁺CD4⁺ T cells from nasal polyps of ECRS patients ($p < 0.01$; Mann-Whitney U test; Figure 7B). Modest enhancement of *IL13* expression was also detected ($p < 0.05$; Mann-Whitney U test). In addition, the expression of *IL1RL1* was enhanced dramatically after IL-33 treatment, which supported the results obtained from analysis of murine memory Th2 cells (Figure 1). In contrast, no obvious effect of IL-33 on the expression of *IL5* or *IL13* was detected in CD45RO⁺CD4⁺ T cells from nasal polyps of NECRS patients. Furthermore, the expression and production of IL-5 and the *IL1RL1* expression that was enhanced by IL-33 in CD45RO⁺CD4⁺ T cells from nasal polyps of ECRS patients were selectively inhibited by SB203580 treatment ($p < 0.01$; Mann-Whitney U test; Figures 7C and 7D). These results indicate that IL-33 specifically induces enhanced expression of *IL1RL1* and IL-5 in CD45RO⁺CD4⁺ T cells from nasal polyps of ECRS patients and that p38 activation is required for IL-33-induced IL-5 augmentation. Thus, IL-33-ST2-p38 signaling might play an important role in the induction of pathogenicity in tissue-infiltrating memory CD4⁺ T cells in human chronic allergic inflammatory diseases such as ECRS.

DISCUSSION

Herein, we have identified memory Th2 cells as a critical target of IL-33 in the pathogenesis of allergic airway inflammation in both the murine and the human immune systems. IL-33 induced increased expression of ST2 on memory Th2 cells and also enhanced production of IL-5 both in vitro and in vivo. IL-33 exposure induced chromatin remodeling at the *IL5* locus. We found that p38 MAPK was a downstream target of IL-33-ST2 signaling in memory Th2 cells. In vivo, the depletion of IL-33 or ST2 attenuated memory-Th2-cell-mediated allergic responses in the airway. Memory CD4⁺ T cells in the nasal polyps of ECRS patients showed increased IL-5 expression after stimulation with IL-33, and IL-33 was highly expressed in the chronic inflammatory polyps of ECRS patients. Thus, the IL-33-ST2-p38 pathway could be a potential therapeutic target for treatment of chronic allergic inflammation induced by memory Th2 cells.

IL-33-mediated production of IL-5 and IL-13 in ILC2s depends on the phosphorylation of p38 (Furusawa et al., 2013), and IL-33 increases phosphorylation of p38 in mast cells (Liew et al., 2010). In human T cells, IL-5 production induced by TCR stimulation is dependent on p38 activity (Maneechotesuwan et al., 2007; Mori et al., 1999). Likewise, we found that the p38-mediated signaling pathway is critical for IL-33-induction of IL-5 expression in both murine and human memory T cells. Thus, the p38 MAPK pathway appears to be critical for the production of IL-5 in various types of cells, including memory Th2 cells.

Chromatin remodeling of *IL4* is dependent on the calcineurin-NFAT pathway (Ansel et al., 2006), whereas that of IL-5 and IL-13 appears to be more dependent on NF- κ B, AP-1, or other

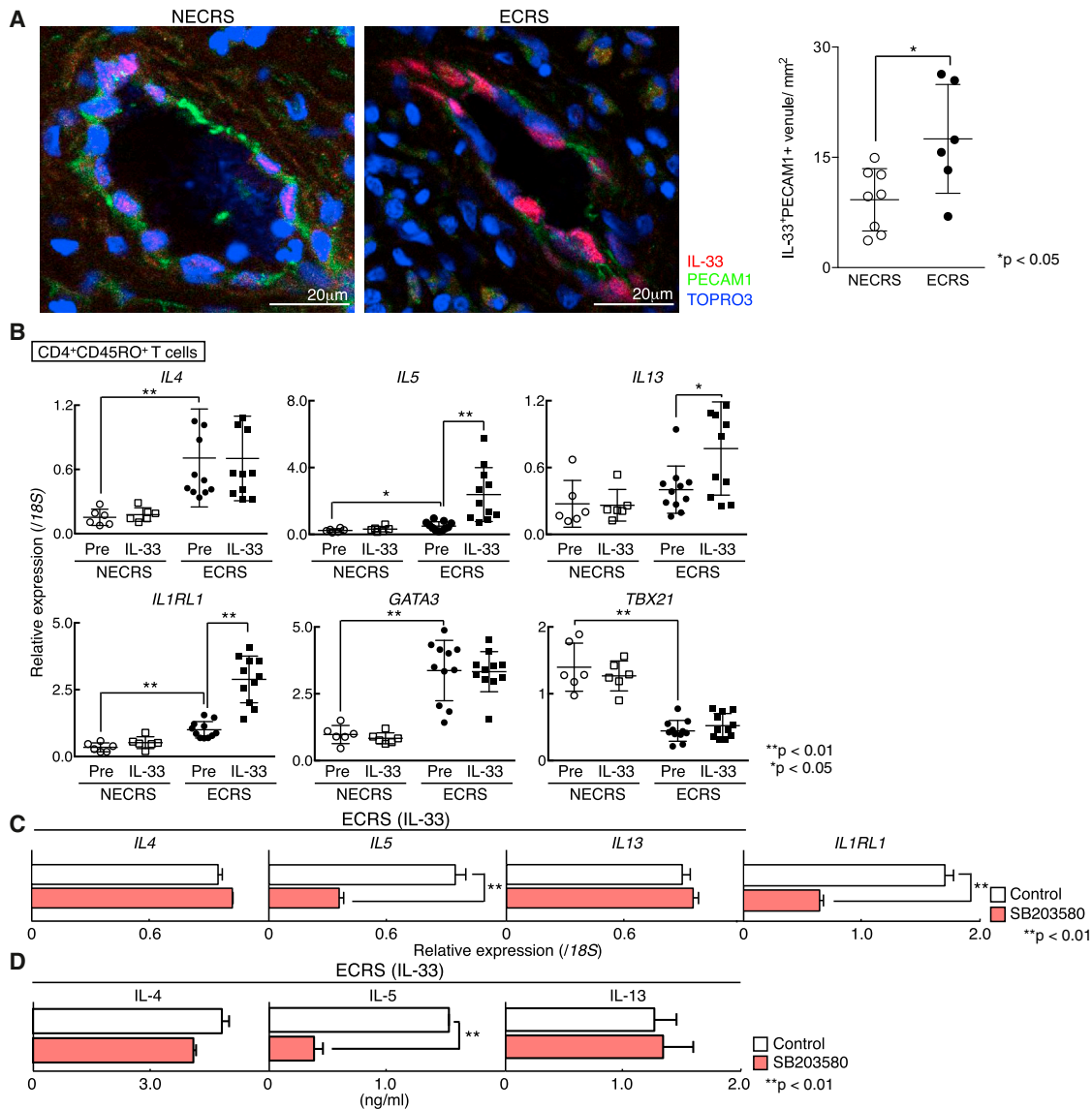
transcription factors rather than NFAT (Guo et al., 2009; Wang et al., 2006). In the current study, we found that the p38 MAPK pathway was activated by IL-33 and was responsible for the induction of chromatin remodeling of the *IL5* locus and thus left the chromatin signature at the *IL4* and *IL13* loci almost unchanged. Therefore, this might represent a connection between the IL-33-p38 MAPK pathway and selective chromatin remodeling of the *IL5* locus.

An important finding from the present study is that IL-33-induced chromatin remodeling at the *IL5* locus in memory Th2 cells occurs independently from TCR stimulation. TCR stimulation is indispensable for the induction of chromatin remodeling at the Th1 and Th2 cell cytokine-encoding loci during differentiation of naive CD4⁺ T cells into effector Th1 and Th2 cells (Nakayama and Yamashita, 2010). However, we have shown that the cytokine IL-33 alone is capable of selectively inducing chromatin remodeling at the *IL5* locus in memory Th2 cells. These results might indicate that IL-33 possesses a unique ability to induce chromatin remodeling at the *IL5* locus if the IL-33 receptor ST2 is expressed. Indeed, IL-2, IL-7, or IL-25 did not induce chromatin remodeling at the *IL5* locus in memory Th2 cells, although their receptors were expressed on memory Th2 cells. The combination of IL-2 and IL-25 also induced chromatin remodeling at the *IL5* locus in memory Th2 cells, as shown previously in ILC2s (Halim et al., 2012), suggesting that similar mechanisms might operate cytokine-dependent IL-5 induction in memory Th2 cells and ILC2s. In ILC2s, IL-33 is reported to be more potent than IL-25 in inducing IL-13 production and AHR (Barlow et al., 2013). At the *IL5* locus, we detected permissive histone marks accompanied by binding of p300 and pol II in memory Th2 cells stimulated with IL-33. Therefore, the activation of the IL-33-ST2-p38 axis might induce the formation of a chromatin-remodeling complex, including the HAT complex, and recruit it to the *IL5* locus in memory Th2 cells.

We have demonstrated that memory Th2 cells are an important target of IL-33 in the pathogenesis of airway inflammation. We detected increased expression of *IL33* mRNA in the lung after OVA challenge, which is consistent with the notion that tissue damage leads to the release of IL-33 from structural cells, such as epithelial cells and endothelial cells in the lung (Préfontaine et al., 2009; Préfontaine et al., 2010), and that IL-33 is expressed higher in asthmatic patients (Préfontaine et al., 2009). IL-33 secreted around the inflammatory airways might increase ST2 expression and IL-5 production by memory Th2 cells that are either resident in or migrating through the lung tissue. Therefore, IL-33 might enhance allergic airway responses through the induction of our proposed IL-5-producing pathogenic memory Th2 cells in the airway (Endo et al., 2014; Endo et al., 2011; Hegazy et al., 2010; Islam et al., 2011; Upadhyaya et al., 2011; Wang et al., 2010). Although it is already known that ILC2s respond to IL-33 to produce IL-5 in the lung, the depletion of ILC2s in *Rag2*^{−/−} mice by anti-CD90.2 antibody treatment did not affect memory-Th2-cell-dependent airway inflammation.

(E) ChIP assays were performed as shown in Figure 2B. Histone modifications at the *IL5* locus in memory Th2 cells treated with IL-33 with or without SB203580 were measured by quantitative RT-PCR analysis.

(F) Effect of silenced *p38* on *IL5* expression in IL-33-cultured memory Th2 cells. Memory Th2 cells were introduced to control *p38* siRNA and cultured with IL-33 for 5 days, and quantitative RT-PCR analysis of the indicated molecules after 4 hr stimulation with immobilized anti-TCR β is shown. At least three (A–D) or two (E and F) independent experiments were performed and showed similar results. See also Figure S6.



Thus, according to the experimental systems we used, IL-33 appears to act mainly on memory Th2 cells to increase their ability to produce IL-5 and exacerbate eosinophilic inflammation. It remains unknown whether IL-33 induces recruitment of memory Th2 cells to the inflamed lung, given that IL-33 can also act as a chemoattractant for Th2 cells (Komai-Koma et al., 2007).

ECRS is a chronic inflammatory disease characterized by prominent accumulation of eosinophils in the sinuses and nasal polyp tissue (Gevaert et al., 2006). Functionally distinct populations of memory CD4⁺ T cells might be present within the nasal polyps of ECRS and NECRS (Th1 cell type for NECRS and Th2 cell type for ECRS). We detected more IL-33-producing cells in

nasal polyps from ECRS patients than in those from NECRS patients. Consistent with our findings in murine CD4⁺ T cells, we demonstrated that IL-33 induced augmentation of *IL5* and *IL1RL1* expression in memory CD4⁺ T cells from ECRS patients. IL-33 induced expression of not only *IL5* but also *IL1RL1*, suggesting that a positive-feedback mechanism resulted in a greater responsiveness to IL-33 in memory CD4⁺ T cells from ECRS patients. In addition, IL-33-induced IL-5 augmentation also depended on the activation of p38. Our study clearly demonstrates the function and pathophysiological role of IL-33 in ECRS, a chronic inflammatory human disease.

In summary, our study has identified memory Th2 cells as an important target of IL-33 in the pathogenesis of airway inflammation. The p38-mediated signaling pathway is critical for TCR-independent IL-33-induced IL-5 expression in both murine and human memory Th2 cells. Further detailed studies focused on the IL-33-ST2-p38-axis in pathogenic memory Th2 cells might lead to the discovery of potential therapeutic targets for the treatment of chronic allergic diseases.

EXPERIMENTAL PROCEDURES

Mice

The animals used in this study were backcrossed to BALB/c or C57BL/6 mice ten times. Anti-OVA-specific TCR- $\alpha\beta$ (DO11.10) transgenic (Tg) mice were provided by Dr. D. Loh (Washington University School of Medicine, St. Louis) (Murphy et al., 1990). *IL33*^{-/-} mice were generated as previously described (Oboki et al., 2010). *IL1rl1*^{-/-} mice were kindly provided by Dr. Andrew N.J. McKenzie (Medical Research Council, Cambridge) (Townsend et al., 2000). Ly5.1 mice were purchased from Sankyo Laboratory. All mice were used at 6–8 weeks old and were maintained under specific-pathogen-free conditions. BALB/c, BALB/c *nu/nu*, and *Rag2*^{-/-} mice were purchased from CLEA Japan. Animal care was conducted in accordance with the guidelines of Chiba University.

The Generation and Culture of Effector and Memory Th2 Cells

Splenic CD62L⁺CD44⁻KJ1⁺CD4⁺ T cells from DO11.10 OVA-specific TCR Tg mice were stimulated with an OVA peptide (Loh15, 1 μ M) plus antigen-presenting cells (irradiated splenocytes) under Th2-cell-culture conditions (25 U/ml IL-2, 10 U/ml IL-4, anti-IL-12 monoclonal antibody [mAb], and anti-IFN- γ mAb) for 6 days in vitro. The effector Th2 cells (3×10^6) were transferred intravenously into BALB/c *nu/nu* or BALB/c recipient mice. Five weeks after cell transfer, KJ1⁺CD4⁺ T cells in the spleen were purified by autoMACS (Miltenyi Biotec) and cell sorting (BD Aria II) and were then used as memory Th2 cells.

Assessment of Memory Th2 Cell Function In Vivo

Memory Th2 cells were purified by fluorescence-activated cell sorting and transferred (3×10^6 /mouse) again into *IL33*^{+/+} or *IL33*^{-/-} mice. The mice were exposed to aerosolized 1% OVA four times on days 1, 3, 9, and 11. For depletion of ILC2s, *Rag2*^{-/-} mice were injected intraperitoneally with anti-CD90.2 (BioX Cell) antibody at a dose of 200 μ g per day on days 2, 5, and 9. BAL fluid for the analysis of cytokine production by ELISA was collected 12 hr after the last challenge, and BAL fluid for the assessment of inflammatory cell infiltration was collected on day 13. Intracellular-staining analysis was performed 12 hr after the last inhalation. Lung histology was assessed on day 13. AHR was assessed on day 12.

Quantitative Real-Time PCR

Total RNA was isolated with the TRIzol reagent (Invitrogen). cDNA was synthesized with an oligo (dT) primer and Superscript II RT (Invitrogen). Quantitative real-time PCR was performed with the ABI PRISM 7500 Sequence Detection System as described previously (Endo et al., 2011). Primers and TaqMan probes were purchased from Applied Biosystems. Primers and Roche Universal probes were purchased from Sigma and Roche, respectively. Gene

expression was normalized with the *Hprt* mRNA signal or the 18S ribosomal RNA signal.

siRNA Analysis of Gene Targeting

siRNA was introduced into memory Th2 cells by electroporation with a mouse T cell Nucleofector Kit and Nucleofector I (Amaxa). Memory Th2 cells were transfected with 675 pmol of control random siRNA or siRNA for *p38* (Applied Biosystems) and cultured for 5 days with IL-33.

Nasal Polyp Mononuclear Cells and Homogenate Preparation

Nasal polyp mononuclear cells (NPMCs) were obtained as previously described (Yamamoto et al., 2007). In brief, freshly obtained nasal polyps were immediately minced and incubated in RPMI 1640 medium containing 1 mg/ml collagenase, 0.5 mg/ml hyaluronidase, and 0.2 mg/ml DNase I (Sigma-Aldrich). After incubation, NPMCs were obtained by the Ficoll-Hypaque technique. A volume of 1 ml of PBS was added for every 100 mg of tissue and was supplemented with aprotinin and leupeptin (Roche).

Statistical Analysis

Data were analyzed with GraphPad Prism software (version 6). Comparisons of two groups were calculated with non-parametric Mann-Whitney U tests. Differences with *p* values below 0.05 or 0.01 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2015.01.016>.

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Immunity

Supplemental Information

The Interleukin-33-p38 Kinase Axis Confers Memory T Helper 2 Cell Pathogenicity in the Airway

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Figure S1 related to main Figure 1

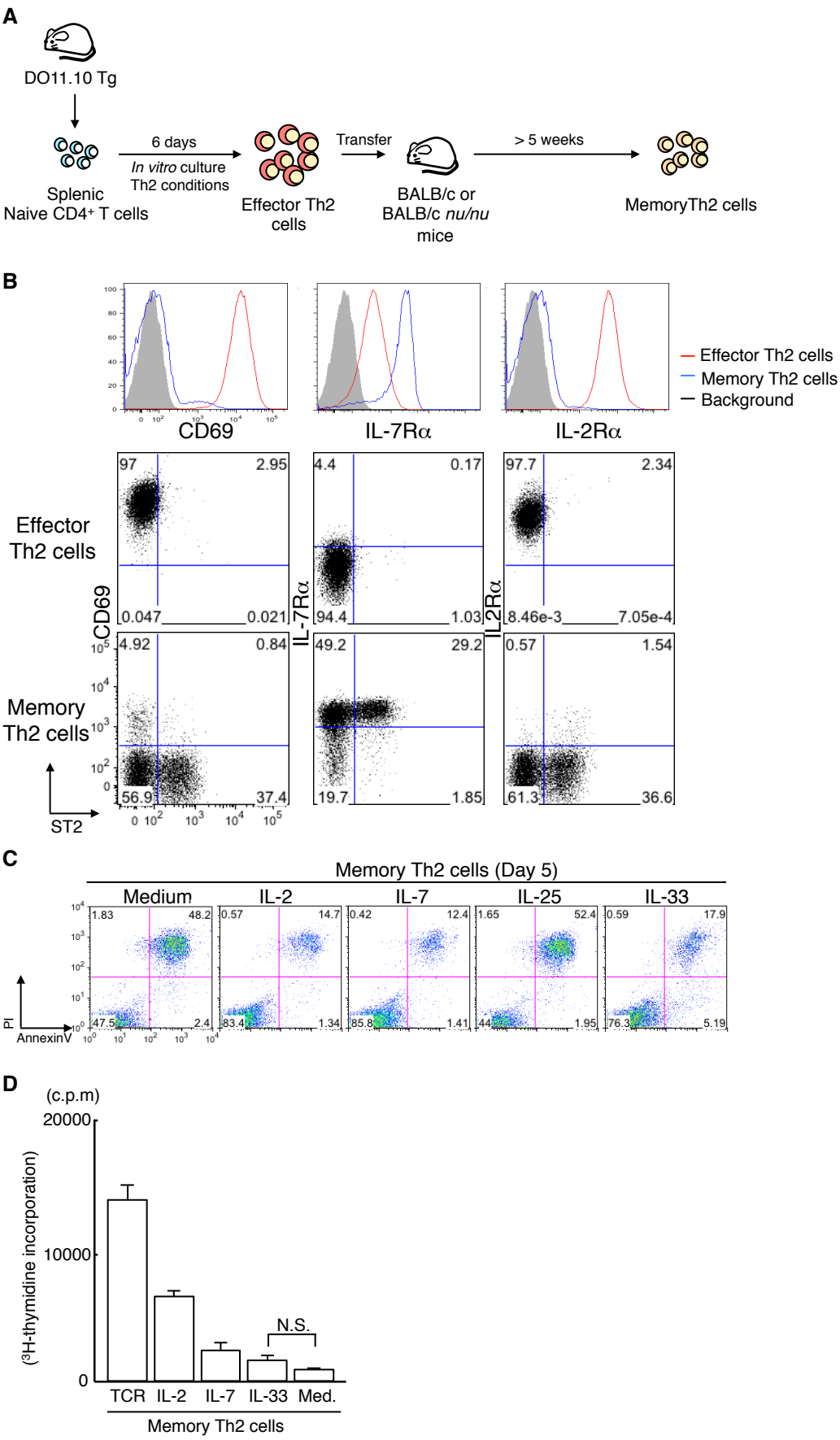


Figure S1 (continued) related to main Figure 1

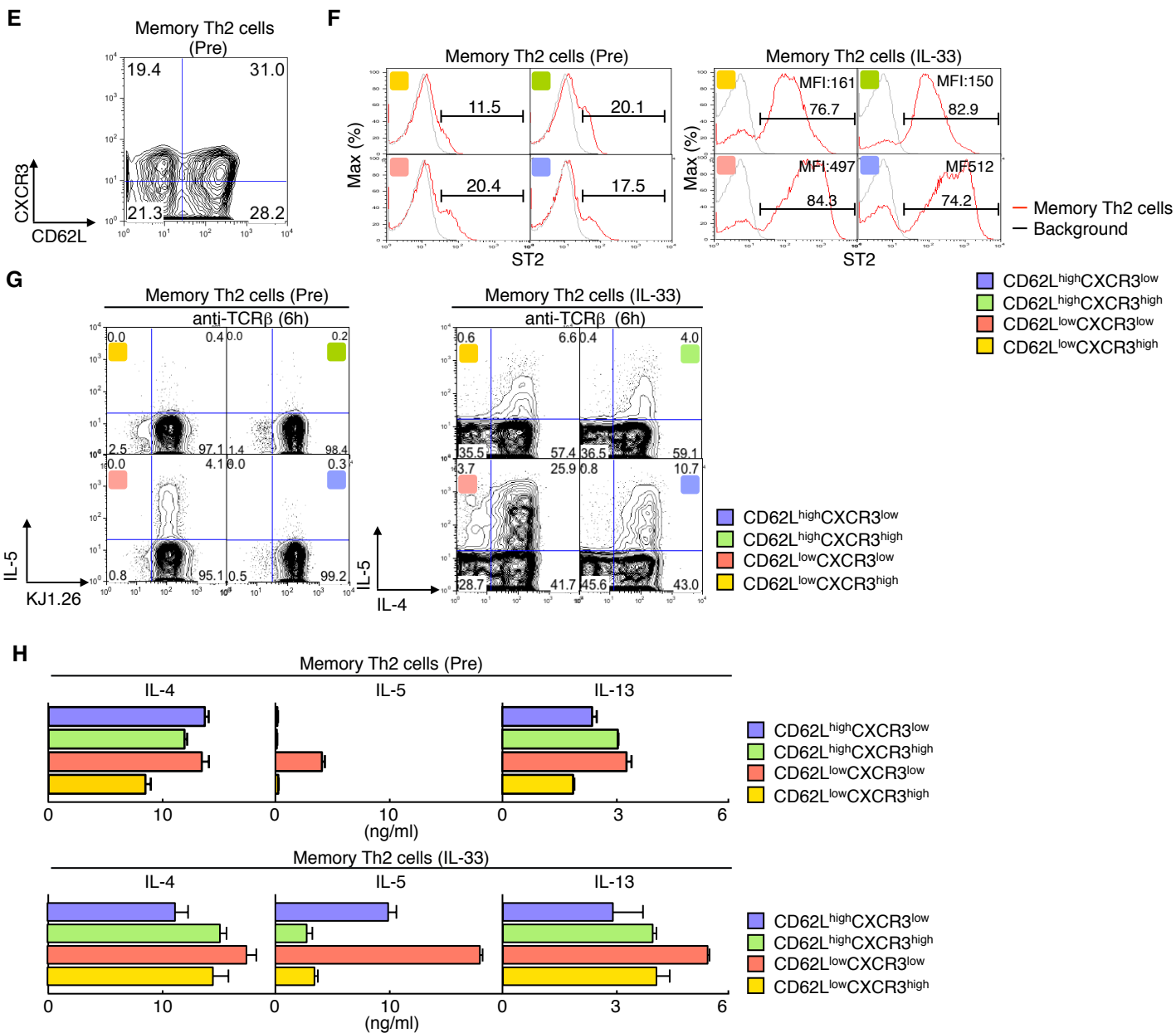


Figure S1. Cell surface expression profiles of effector and memory Th2 cells (related to Figure 1).

(A) Experimental protocols for the generation of effector and memory Th2 cells. Naive CD4⁺ T cells from DO11.10 OVA-specific TCR Tg mice were stimulated with an OVA peptide plus spleen-derived antigen presenting cells under Th2-culture conditions (IL-2 (25U/ml), IL-4 (10U/ml) and anti-IFN γ mAb) for 6 days *in vitro*. We used these cells as effector Th2 cells. Effector Th2 cells were then transferred intravenously into syngeneic BALB/c or BALB/c *nu/nu* mice. More than 5 weeks after cell transfer, KJ1⁺CD4⁺ cells in the spleen or lung were purified by auto-MACS and cell sorting (using a FACS Aria II) and then were used as memory Th2 cells. **(B)** Expression profiles of CD69, IL-7R α , IL-2R α and ST2 on effector and memory Th2 cells. **(C)** The susceptibility for apoptosis of memory Th2 cells cultured with medium alone (Medium), IL-2, IL-7, IL-25, or IL-33 for 5 days were investigated by staining of Annexin V and propidium iodide (PI). Three independent experiments were performed with similar results. **(D)** [³H]-thymidine incorporation in memory Th2 cells stimulated with immobilized anti-TCR β , or with indicated cytokines or medium alone (Med) is shown with standard deviations (N.S., not significant). Two independent experiments were performed with similar results. **(E)** CD62L and CXCR3 profiles of memory Th2 cells. **(F)** Expression profiles of ST2 on the four subpopulations subdivided by the expression of CD62L and CXCR3 of memory Th2 cells before (Left) and after (Right) cultivation with IL-33 for 5 days. Mean fluorescence intensity (MFI) is shown for each population. **(G)** Four subpopulations subdivided by the expression of CD62L and CXCR3 of memory Th2 cells before and after culture with IL-33 were stimulated *in vitro* with immobilized anti-TCR β for 6 hours. Intracellular staining profiles of IL-5 and IL-4 are shown with the percentage of cells in each area. **(H)** ELISA analysis of IL-4, IL-5, and IL-13 secreted by the four subpopulations

subdivided by the expression of CD62L and CXCR3 of memory Th2 cells before (Top) or after (Bottom) cultivation with IL-33. Cells were stimulated with immobilized anti-TCR β for 24 hours. The mean values and standard deviations are shown. Three independent experiments were performed with similar results. Three technical replicates were included in ELISA (**H**).

Figure S2 related to main Figure 2

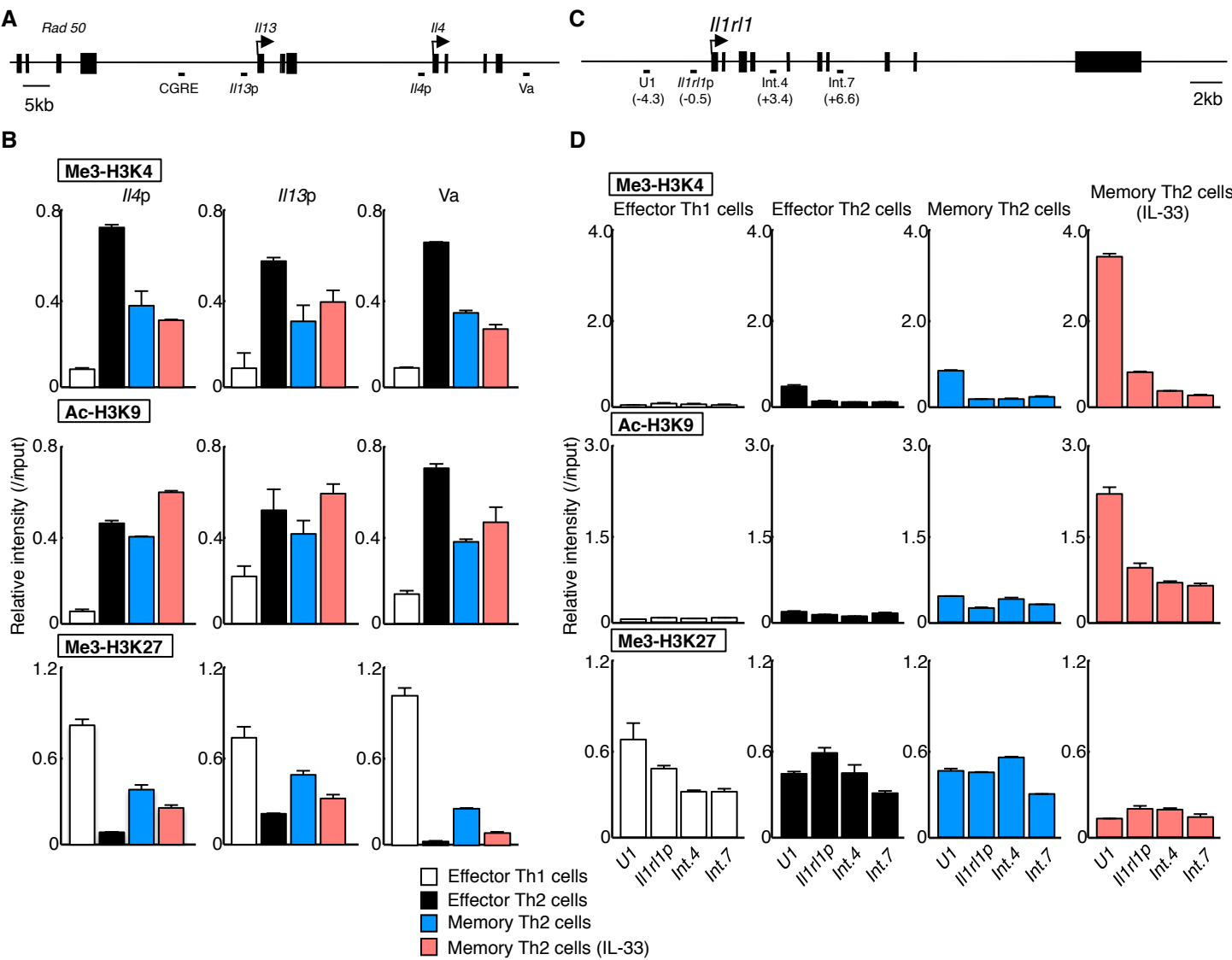


Figure S2 (continued) related to main Figure 2

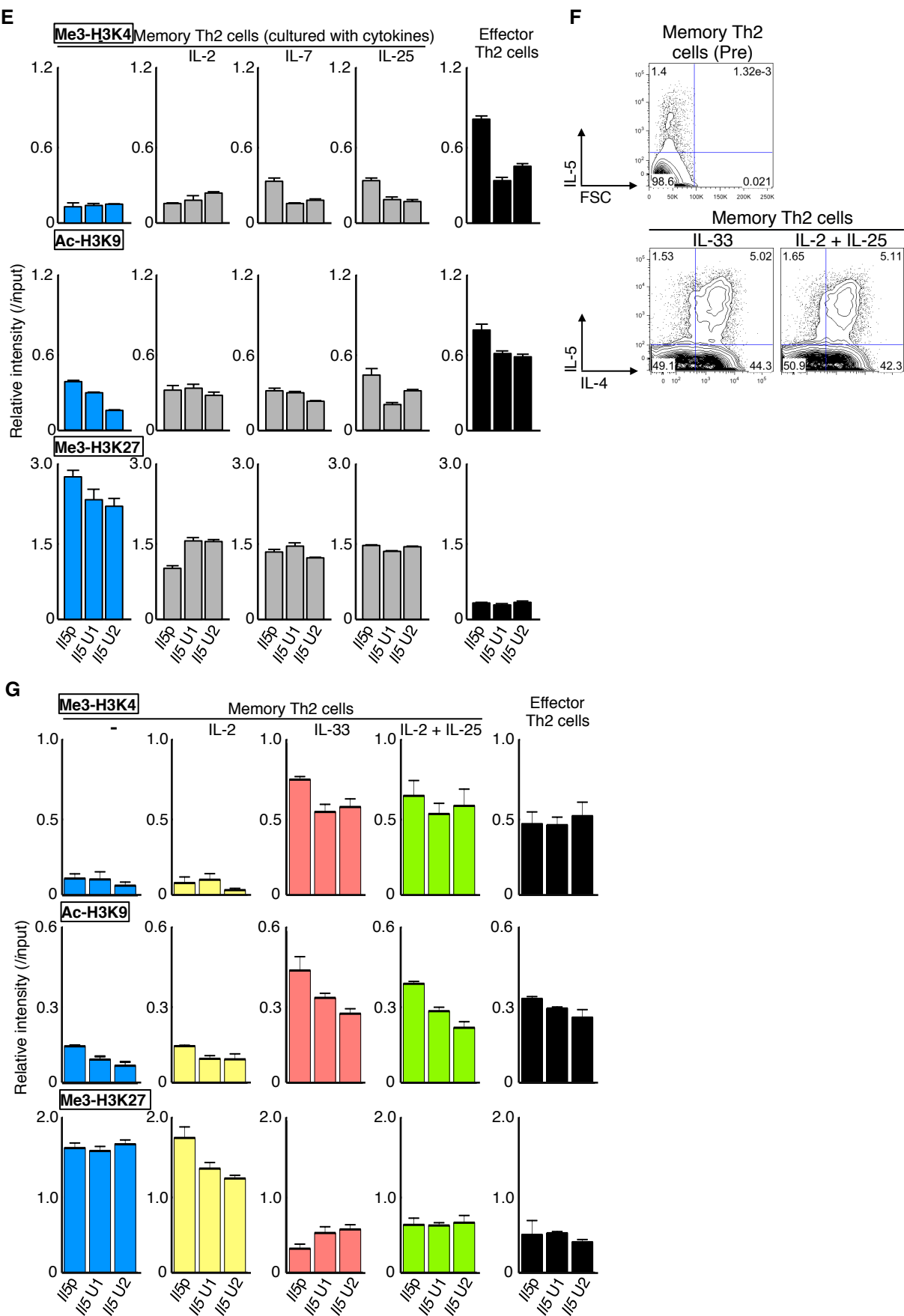


Figure S2. Effect of IL-33 cultivation on histone modifications at the *Il4* and *Il13* gene loci and *Il1rl1* locus in memory Th2 cells (related to Figure 2).

(A) Schematic representation of the mouse Th2 cytokine gene loci. The location of primers and probes (CGRE to Va) and exons are indicated. (B) ChIP assays were performed with anti-acetyl histone H3K9, anti-trimethyl histone H3-K4, and anti-trimethyl histone H3K27 at the Th2 cytokine gene loci from effector Th1, effector Th2, freshly prepared memory Th2, and IL-33 cultured memory Th2 cells (IL-33) for 5 days. The relative intensities (relative to input DNA) of these modifications were determined by quantitative RT-PCR analysis. More than three independent experiments were performed with similar results. (C) Schematic representation of the mouse *Il1rl1* gene locus. The location of primers and probes and exons are indicated. (D) ChIP assays were performed as in (B). The histone modifications at the *Il1rl1* locus in the indicated samples were determined by quantitative RT-PCR analysis. Three independent experiments were performed with similar results. (E) ChIP assays were performed as in (B). The histone modifications at the *Il5* locus in effector Th2, freshly prepared memory Th2, and memory Th2 cells cultured with the indicated cytokines for 5 days were determined by quantitative RT-PCR analysis. Three independent experiments were performed with similar results. (F) Memory Th2 cells were cultured with IL-33 or IL-2 and IL-25 for 5 days. The cultured cells were stimulated with immobilized anti-TCR β for 6 hours. Intracellular staining profiles of IL-5 and IL-4 are shown. Three independent experiments were performed with similar results. (G) ChIP assays were performed as in (B). The histone modifications at the *Il5* locus in effector Th2, freshly prepared memory Th2, and memory Th2 cells cultured with the indicated cytokines for 5 days were determined by quantitative RT-PCR analysis. Two independent experiments were performed with similar

results. Two independent experiments were performed with similar results. Three technical replicates were performed with quantitative RT-PCR (**B**, **D**, **E** and **G**).

Figure S3 related to main Figure 3

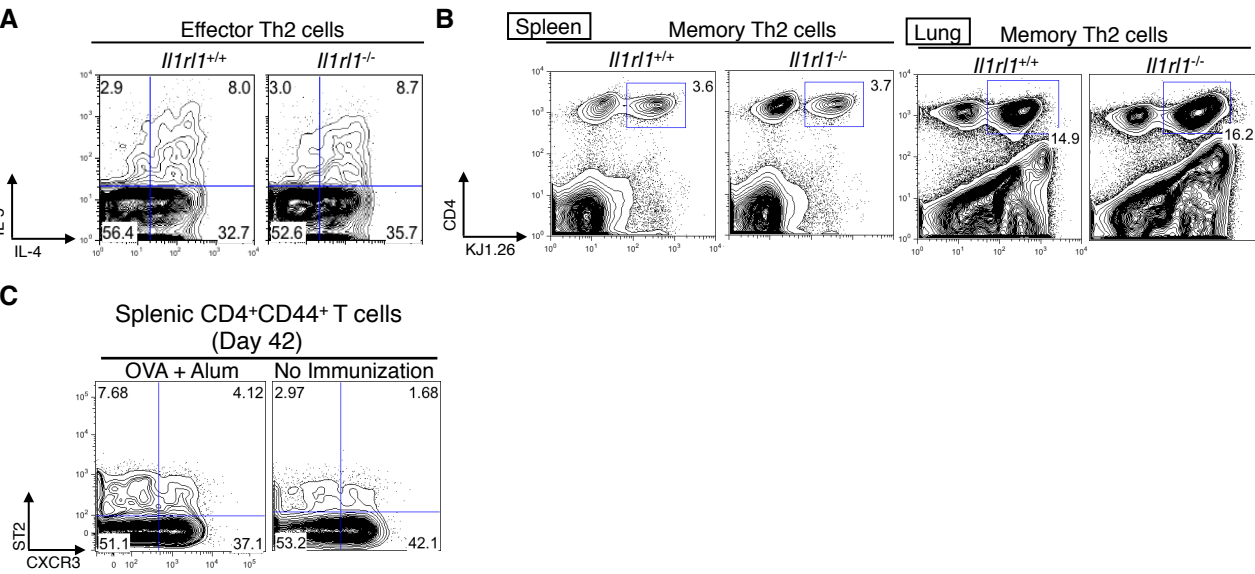


Figure S3. Normal effector Th2 differentiation and normal memory Th2 cell generation from *Il1rl1*^{+/+} or *Il1rl1*^{-/-} CD4⁺ T cells (related to Figure 3).

(A) Intracellular staining profiles of IL-5 and IL-4 in *Il1rl1*^{+/+} or *Il1rl1*^{-/-} effector Th2 cells. (B)

The generation of *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory Th2 cells in the spleen and the lung. (C) Cell

surface expression profiles of ST2 and CXCR3 on CD4⁺CD44⁺ T cells recovered from the

mice that were not immunized (Right) or immunized with OVA and Alum. (Left) are shown.

Three independent experiments were performed with similar results.

Figure S4 related to main Figure 4

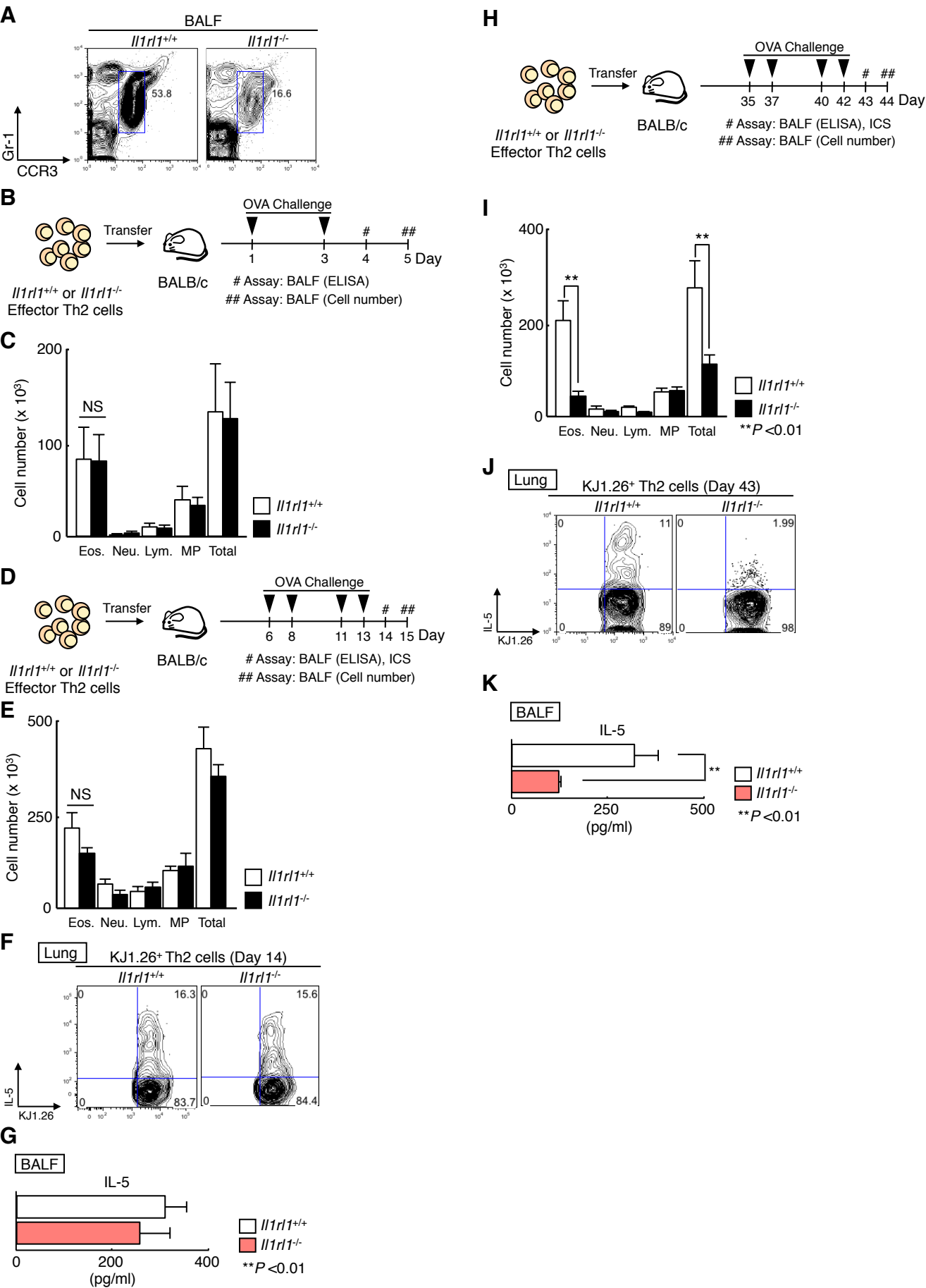


Figure S4 (continued) related to main Figure 4

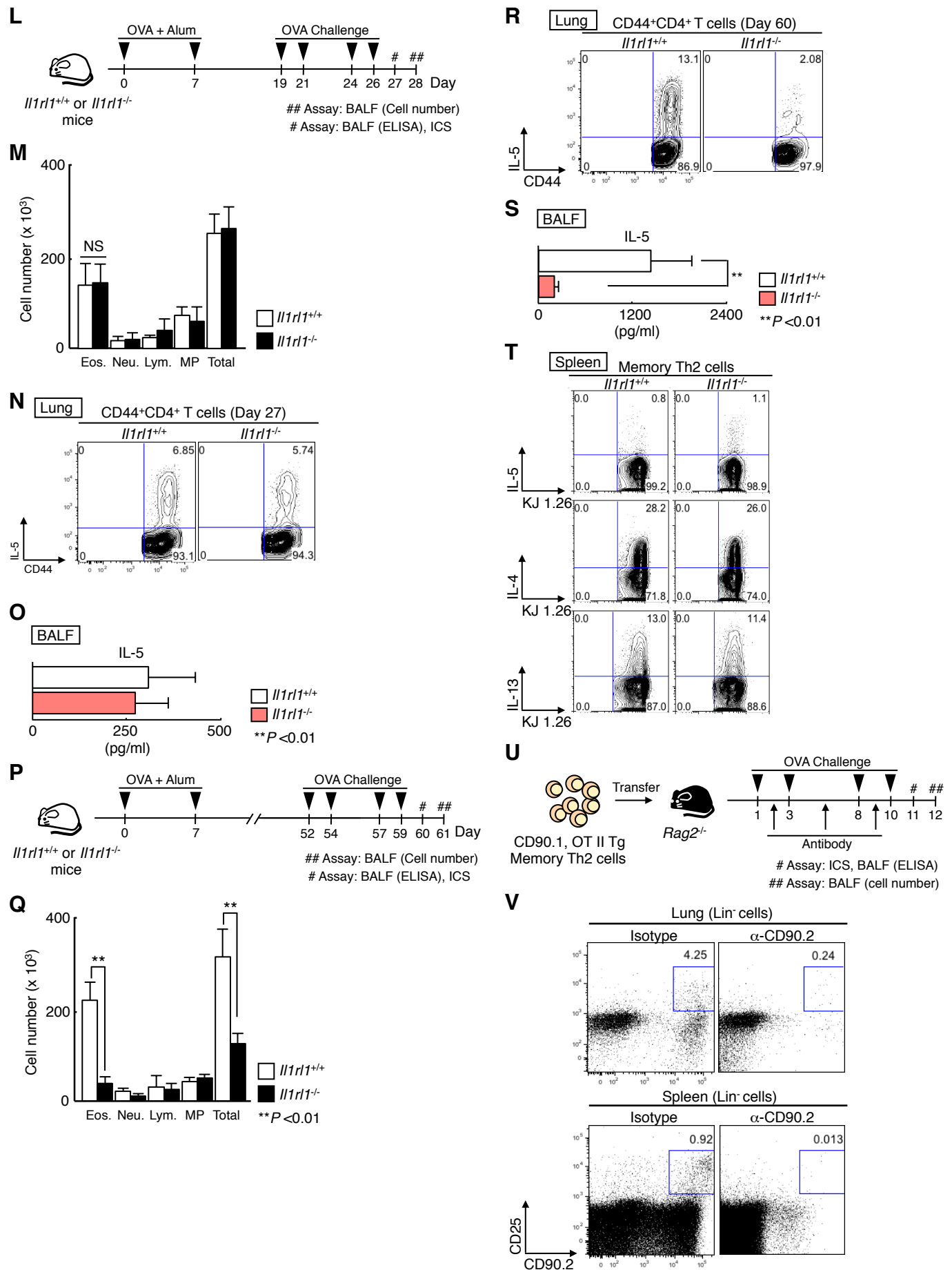


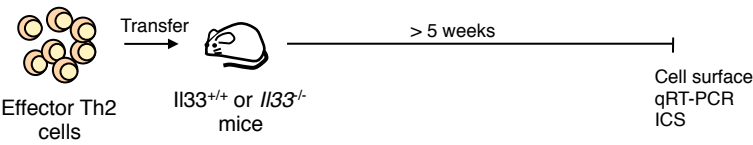
Figure S4. Airway inflammation model induced by OVA and Alum immunization of *Il1rl1*^{+/+} or *Il1rl1*^{-/-} mice (related to Figure 4).

(A) Cell surface expression profiles of Gr-1 and CCR3 on leukocytes collected from the BAL fluid of *Il1rl1*^{+/+} or *Il1rl1*^{-/-} groups. Experimental protocols are shown in **Figure 4A**. The eosinophil population is CCR3⁺Gr-1^{int}. (B) Experimental protocols for effector Th2 cell-dependent allergic inflammation mouse models using *Il1rl1*^{+/+} or *Il1rl1*^{-/-} donor cells. (C) The absolute cell numbers of eosinophils (Eos.), neutrophils (Neu.), lymphocytes (Lym.), and macrophages (Mac.) in the BAL fluid are shown as described in **Figure 4D**. Mean values (5 mice per group) are shown with standard deviations. Two independent experiments were performed with similar results. MS: not significant (D) Experimental protocols for Th2 cell-dependent allergic inflammation mouse models (6 days after cell transfer) using *Il1rl1*^{+/+} or *Il1rl1*^{-/-} donor cells. (E) The absolute cell numbers of eosinophils (Eos.), neutrophils (Neu.), lymphocytes (Lym.), and macrophages (Mac.) in the BAL fluid are shown. Mean values (5 mice per group) are shown with standard deviations. (F) Intracellular staining profiles of IL-5 and KJ1.26 in the lung KJ1.26⁺ Th2 cells (on day 14). (G) ELISA analysis of IL-5 in the BAL fluid from the experimental groups with *Il1rl1*^{+/+} or *Il1rl1*^{-/-} Th2 cell transfer shown in panel D. Two independent experiments were performed with similar results. (H) Experimental protocols for Th2 cell-dependent allergic inflammation mouse models (35 days after cell transfer) using *Il1rl1*^{+/+} or *Il1rl1*^{-/-} donor cells. (I) The absolute cell numbers of eosinophils (Eos.), neutrophils (Neu.), lymphocytes (Lym.), and macrophages (Mac.) in the BAL fluid are shown. Mean values (5 mice per group) are shown with standard deviations (**: $P < 0.01$). (J) Intracellular staining profiles of IL-5 and KJ1.26 in the lung KJ1.26⁺ Th2 cells (on day 43). (K) ELISA analysis of IL-5 in the BAL fluid from the experimental groups

with *Il1rl1*^{+/+} or *Il1rl1*^{-/-} Th2 cell transfer shown in panel H. Two independent experiments were performed with similar results (**: $P < 0.01$). (L) Experimental protocols for an OVA and Alum immunization-induced allergic inflammation mouse model (12 days after the last immunization) using *Il1rl1*^{+/+} or *Il1rl1*^{-/-} mice. (M) The absolute cell numbers of eosinophils (Eos.), neutrophils (Neu.), lymphocytes (Lym.), and macrophages (Mac.) in the BAL fluid are shown. Mean values (5 mice per group) are shown with standard deviations. NS: not significant (N) Intracellular staining profiles of IL-5 and CD44 in the lung CD44⁺CD4⁺ T cells (on day 27). (O) ELISA analysis of IL-5 in the BAL fluid from the experimental groups with *Il1rl1*^{+/+} or *Il1rl1*^{-/-} mice in panel M. (P) Experimental protocols for OVA and Alum immunization-induced allergic inflammation mouse model (45 days after immunization) using *Il1rl1*^{+/+} or *Il1rl1*^{-/-} mice. (Q) The absolute cell number of eosinophils (Eos.), neutrophils (Neu.), lymphocytes (Lym.), and macrophages (Mac.) in the BAL fluid. Mean values (5 mice per group) are shown with standard deviations (**: $P < 0.01$). (R) Intracellular staining profiles of IL-5 and CD44 in the lung CD44⁺CD4⁺ T cells (on day 60). (S) ELISA analysis of IL-5 in the BAL fluid from the experimental groups with *Il1rl1*^{+/+} or *Il1rl1*^{-/-} mice in panel Q. Two independent experiments were performed with similar results (**: $P < 0.01$). (T) Intracellular staining profiles of IL-4, IL-5, and IL-13 in the *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory Th2 cells in the spleen after challenge with OVA 4 times. Three independent experiments were performed with similar results. (U) Experimental protocols for the depletion of ILC2 cells in memory Th2 cell-dependent allergic inflammation mouse models using CD90.2 antibody. (V) Cell surface expression profiles of CD90.2 and CD25 on Lin⁻ cells (Monticelli et al., 2011; Roediger et al., 2013) from the lung and the spleen of isotype control or anti-CD90.2 groups. Two independent experiments were performed with similar results.

Figure S5 related to main Figure 5

A



B

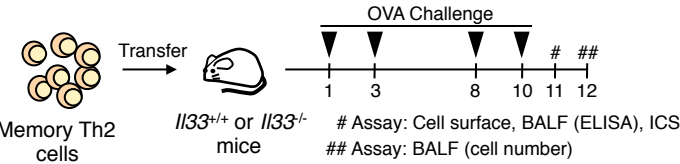


Figure S5. Experimental protocols for the generation and in vivo function of memory Th2 cells using $IL33^{-/-}$ mice (related to Figure 5).

(A) Experimental protocols for the generation of memory Th2 cells using $IL33^{+/+}$ or $IL33^{-/-}$ mice as recipients. (B) Experimental protocols for memory Th2 cell-dependent allergic inflammation models using $IL33^{+/+}$ or $IL33^{-/-}$ recipient mice.

Figure S6 related to main Figure 6

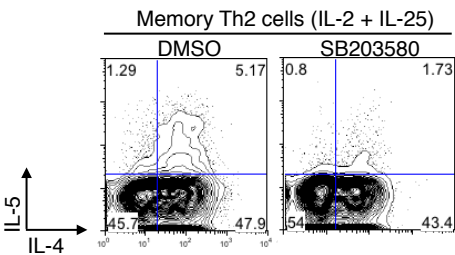


Figure S6. The p38 signaling is required for IL-2 and IL-25-dependent IL-5 induction by memory Th2 cells (related to Figure 6).

Intracellular staining profiles of IL-5 and IL-4 in IL-2- and IL-25-treated memory Th2 cells with DMSO and p38 inhibitor (SB203580; 10 μ M). Two independent experiments were performed with similar results.

Figure S7 related to main Figure 7

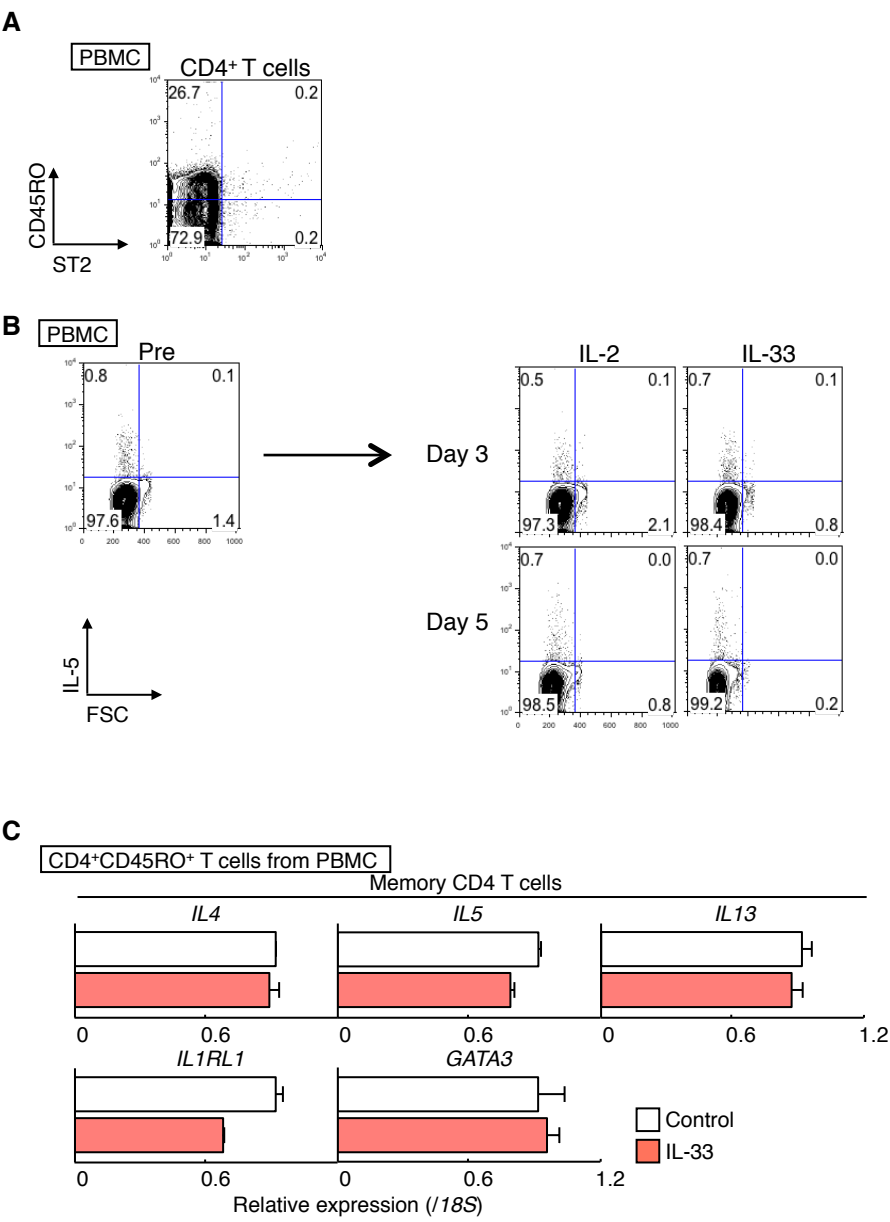


Figure S7. IL-33 showed no obvious effect on the induction of ST2 and Th2 cytokines in CD45RO⁺ memory CD4⁺ T cells collected from PBMC of healthy donors (related to Figure 7).

(A) Cell surface expression profiles of CD45RO and ST2 on CD4⁺ T cells from PBMC of human healthy donors. (B) Intracellular staining profiles of IL-5 in IL-2 or IL-33 cultured CD45RO⁺ memory CD4⁺ T cells from PBMC of human healthy donors are shown. (C) Quantitative RT-PCR analysis of *IL4*, *IL5*, *IL13*, *IL1RL1*, or *GATA3* in CD45RO⁺ memory CD4⁺ T cells before and after cultured with IL-33 for 5 days are shown. Three independent experiments were performed with similar results.

Supplemental Experimental Procedures

Reagents.

The reagents used in this study are as follows: FITC- or APC-conjugated anti-CD4, FITC-, PE-, APC-conjugated KJ1-26 (anti-clonotypic for DO11.10 TCR), PE-, APC-conjugated anti-CD62L, PE/Cy7-conjugated anti-CXCR3, and PE-conjugated Gr-1 were purchased from BD-PharMingen, San Diego, CA. PE-, APC-conjugated anti-CXCR3, APC-conjugated anti-CCR3, and APC-conjugated anti-ST2 were purchased from R&D Biotech. Anti-FcR γ II and III (2.4G2) and unconjugated anti-IL-4 (11B11), anti-IL-12 (C17.8) and anti-IFN γ (R4-6A2) were used as culture supernatants. Recombinant mouse IL-4 was from TOYOBO, Osaka, Japan. Recombinant mouse and human IL-33 and mouse IL-25 were purchased from R&D Biotech. The OVA peptide (residues #323-339; ISQAVHAAHAEINEAGR) was synthesized by BEX Corporation, Tokyo, Japan. For inhibition of cytokine-mediated signals, SB203580 (Merck), U0126 (Merck), SP600125 (Merck), and Wortmannin (Merck) were used.

Detection of apoptotic cells.

For the detection of apoptotic cells, Annexin V^{FITC} apoptosis detection kit II (BD Biosciences) was used according to the manufacturer's protocol.

Proliferation assay.

For the analysis of memory Th2-cell proliferation in response to cytokines, [³H]-thymidine (37 kBq per well) was added to the culture for the last 16 hours, and the incorporated radioactivity was measured on a β plate.

ELISA for the measurement of cytokine concentration.

Cells were stimulated with immobilized anti-TCR β (3 μ g/ml) in 48-well flat bottom plates (1.0 x 10⁵ cells/well) for 16 hours at 37°C. The production of IL-4, IL-5 and IFN γ was assessed by ELISA as described previously (Yamashita et al., 2006). The production of IL-13 was evaluated with a mouse IL-13 ELISA kit (R&D systems) according to the manufacturer's protocol.

Chromatin Immunoprecipitation (ChIP) assay.

ChIP assay was performed as described previously (Yamashita et al., 2002). The antibodies used in ChIP assay as follows; anti-trimethyl histone H3-K4 (LP Bio), anti-acethyl histone H3-K9 (Millipore), anti-trimethyl histone H3K27 (Millipore), anti-RNA pol II (Abcam), anti-p300 (Santa Cruz), and normal rabbit IgG (Santa Cruz). The specific primers used in ChIP assay as follows;

//4p FW: 5'-AAGGCAGACTTTCTTGATATT-3'

//4p RV: 5'-GGCCTTTCAGACTAATCTT-3'

//5 int.1 FW: 5'-TGGCCTTGAACCTCACAGAGA-3'

//5 int.1 RV: 5'-TTGGCCTTTAATCCCAGAAC-3'

//5p FW: 5'-AAGTCTAGCTACCGCCAATA-3'

//5p RV: 5'-AGCAAAGGTGAGTTCAATCT-3'

//5 U1 FW: 5'-CGGCAGATTTGGGGAGATA-3'

//5 U1 RV: 5'-TTGTGAGTTTCACATCATGCAC-3'

//5 U2 FW: 5'-CATGGTGTTGAATCACTCTGAAA-3'

//5 U2 RV: 5'-TCAGGGGAATTGCCAAGTAA-3'

Il5 U3 FW: 5'-TGCACATAACCACACACATACATATAA-3'

Il5 U3 RV: 5'-GTAATAAATCTTAAAGAAGGCGGAGA-3'

Il13p FW: 5'-GGTCAGCGGGTGAATTAC-3'

Il13p RV: 5'-AAAATCCAGTGGCCGCTAAA-3'

CGRE FW: 5'-GGCTGGACCGCTGGATCAGT-3'

CGRE RV: 5'-CCTCTCCTGGTGGCGTGTTG-3'

V_α FW: 5'-TCCATACAGTTCCTGCAGTAGCTG-3'

V_α RV: 5'-CATCTCCCCAACCCCAAGATATA-3'

Il1rl1 int.4 FW: 5'-TGGAGAGCTGTGAACAGTGG-3'

Il1rl1 int.4 RV: 5'-TCAATATATCCATGACAAAGACGTG-3'

Il1rl1 int.7 FW: 5'-AATTCCCTTGCTTGGTCCTT-3'

Il1rl1 int.7 RV: 5'-ATCGGATCAGGTTGGTCATC-3'

Il1rl1p FW: 5'-TGGGGGAACAGTCTTGGA-3'

Il1rl1p RV: 5'-CCAGTACTCAVAGATAGGCATCA-3'

Il1rl1 U1 FW: 5'-GTATTTGGCAACTTTCGCTTG-3'

Il1rl1 U1 RV: 5'-TCATTTCAAAGTGAACGAGCA-3'

Hprtp FW: 5'-TCCTCCTCAGACCGCTTTT-3'

Hprtp RV: 5'-TCTGCTGGAGTCCCCTTG-3'

Immunoblotting assay.

Immunoblotting was performed as described previously (Yamashita et al., 2006). Briefly, cytoplasmic extracts and nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagent (Thermo Fisher Scientific). The antibodies used for the

immunoblot analysis were anti-Phospho-p38 (Cell signaling), anti-p38 (Cell signaling), and anti-Tub α (NewMarkers).

The culture conditions of effector and memory Th2 cells.

In some experiments, effector and memory Th2 cells were cultured with cytokines (IL-33, IL-2, IL-7, or IL-25) for a further 5 days. For the administration of inhibitors (SB203580; 10 μ M, U0126; 10 μ M, SP600125; 20 μ M and Wortmannin; 1 μ M), memory Th2 cells were cultured with IL-33 and limited amounts of IL-2 in the presence or absence of inhibitors. For the administration of IL-33, *Il1rl1*^{+/+} or *Il1rl1*^{-/-} memory Th2 mice were injected with recombinant IL-33 at a dose of 1 μ g per mouse on days 0, 2 and 4, then were sacrificed and the organs collected on day 6. For the generation of non-TCR transgenic memory CD4⁺ T cells *in vivo*, BALB/c mice were immunized with OVA+Alum twice. 42 days after the immunization, CD4⁺CD44⁺ T cells prepared from spleen were cultured with IL-33 for 5 days *in vitro*.

The purification of Peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were purified from whole blood. Human CD45RO⁺ memory CD4⁺ T cells were purified from mononuclear cells using a Memory CD4⁺ T cell isolation kit (Miltenyi Biotec.).

Patients

Patients with chronic rhinosinusitis with nasal polyps (CRSwNP) were recruited from Chiba University Hospital, Kimitsu Chuo Hospital and the Chiba Otolaryngology Surgical Center. All patients met the criteria for CRSwNP defined in the European position paper on

rhinosinusitis and nasal polyps (NPs). All patients signed informed consent forms and the study was approved by the Ethics Committee of the Chiba University Graduate School of Medicine and each participating hospital (#1006). NP tissues were obtained during endoscopic sinus surgery. None of the patients received systemic steroids for at least 4 weeks before surgery. CRSwNP was classified as ECRS when tissue eosinophils were >70/ high power field (HPF). The number of tissue eosinophils was counted under a HPF (HPF x 400) in a blinded manner by two observers.

Immunofluorescent staining and confocal microscopy.

For immunofluorescent staining, samples (n=6 for ECRS, n=8 for NECRS) were fixed in 4% paraformaldehyde and equilibrated in 30% sucrose/PBS solution. Cryostat sections of adult femurs were stained and mounted with fluorescent mounting medium (DakoCytomation). Histological analysis was carried out with a confocal laser microscope (LSM710; Carl Zeiss). The antibodies used for the staining were anti-IL-33 (SIGMA), anti-PECAM1 (Santa Cruz), or TO-PRO3 (Invitrogen).

Supplemental References

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