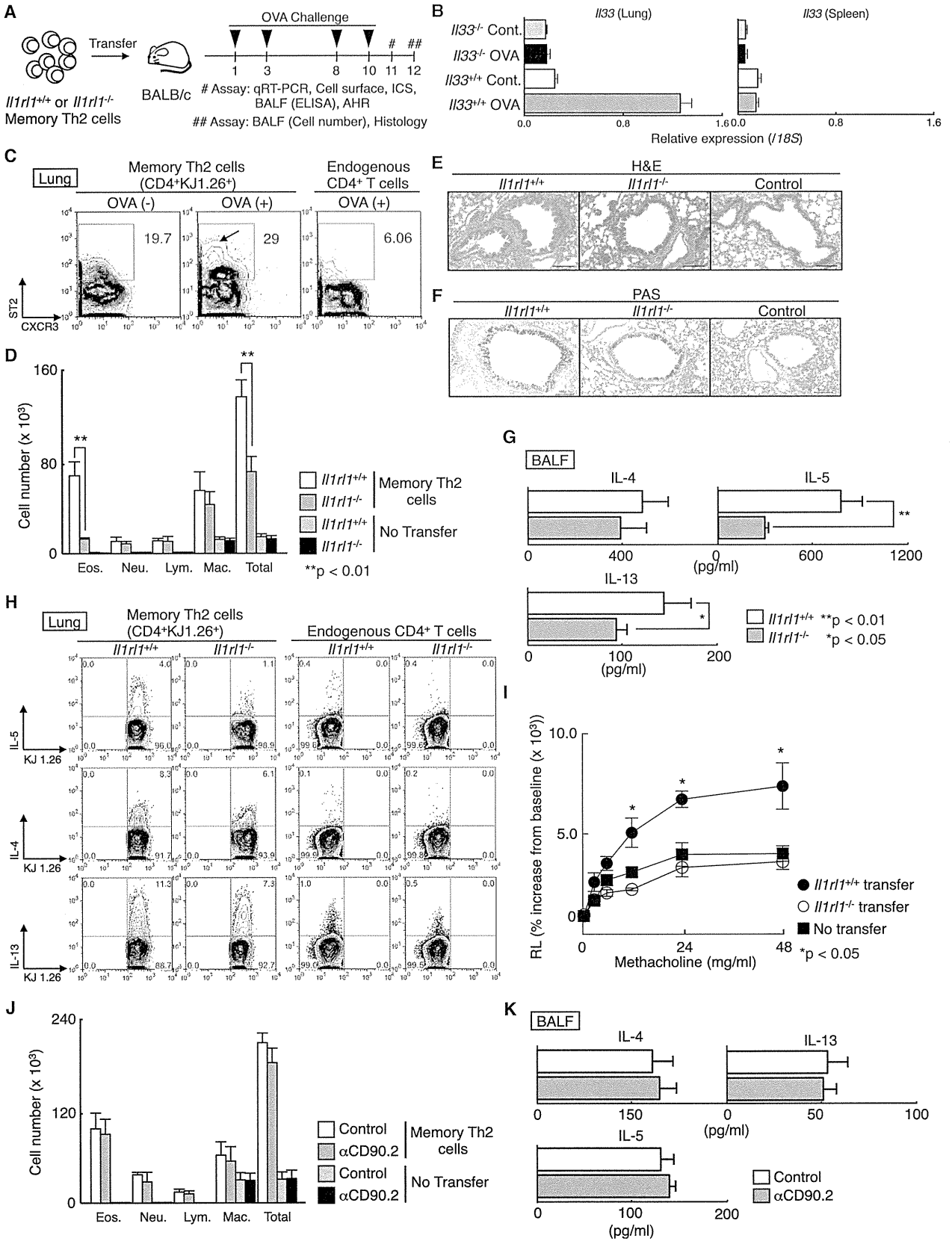

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an arrow, Figure 4C, middle). Only a small number of ST2-expressing cells were detected in endogenous lung CD4$^+$ T cells even after OVA challenge (Figure 4C, right).

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 μm. (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.

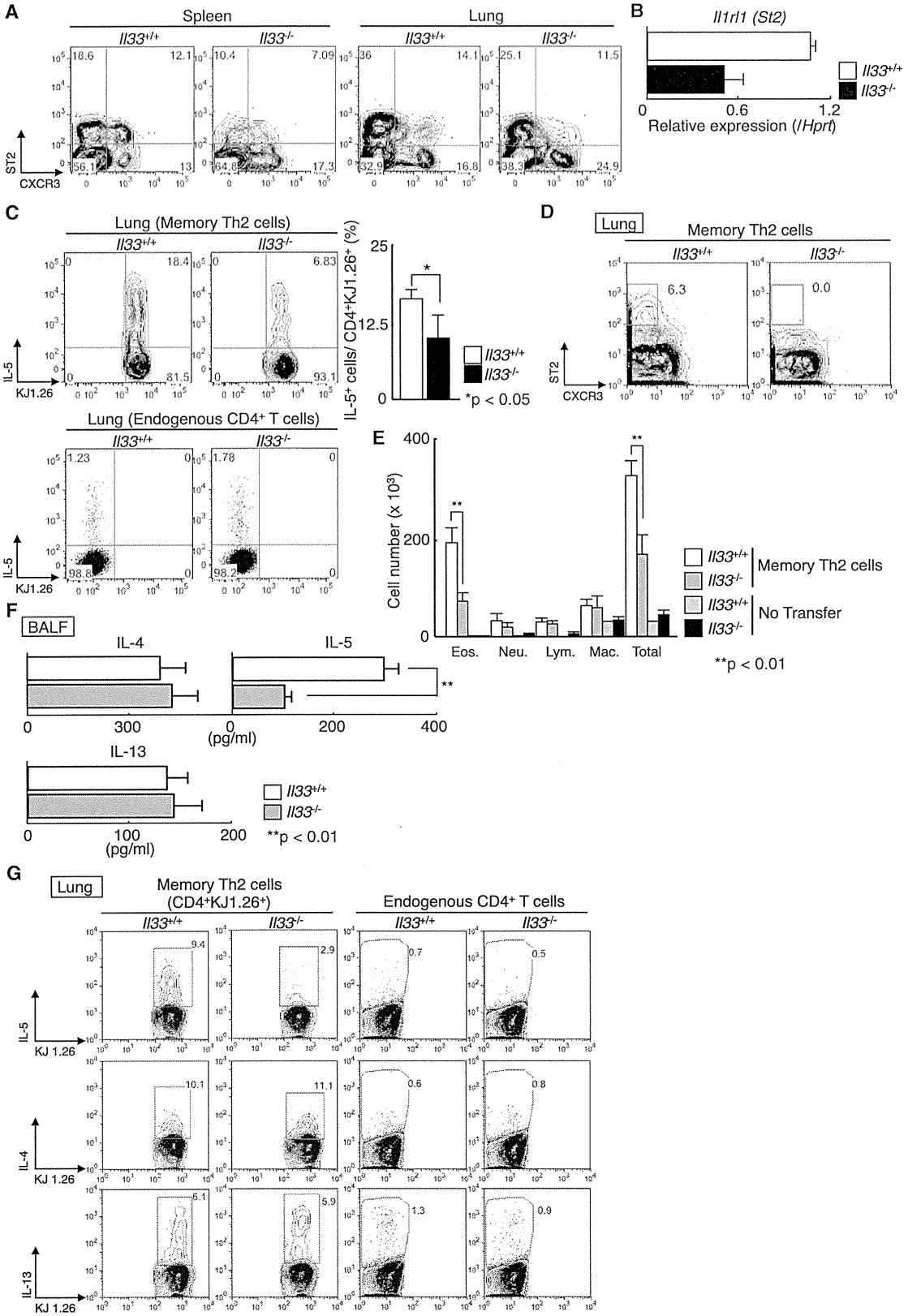

Figure 5. Reduced Memory-Th2-Cell-Dependent Airway Inflammation in *Il33*$^{-/-}$ Mice

(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-κB 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.

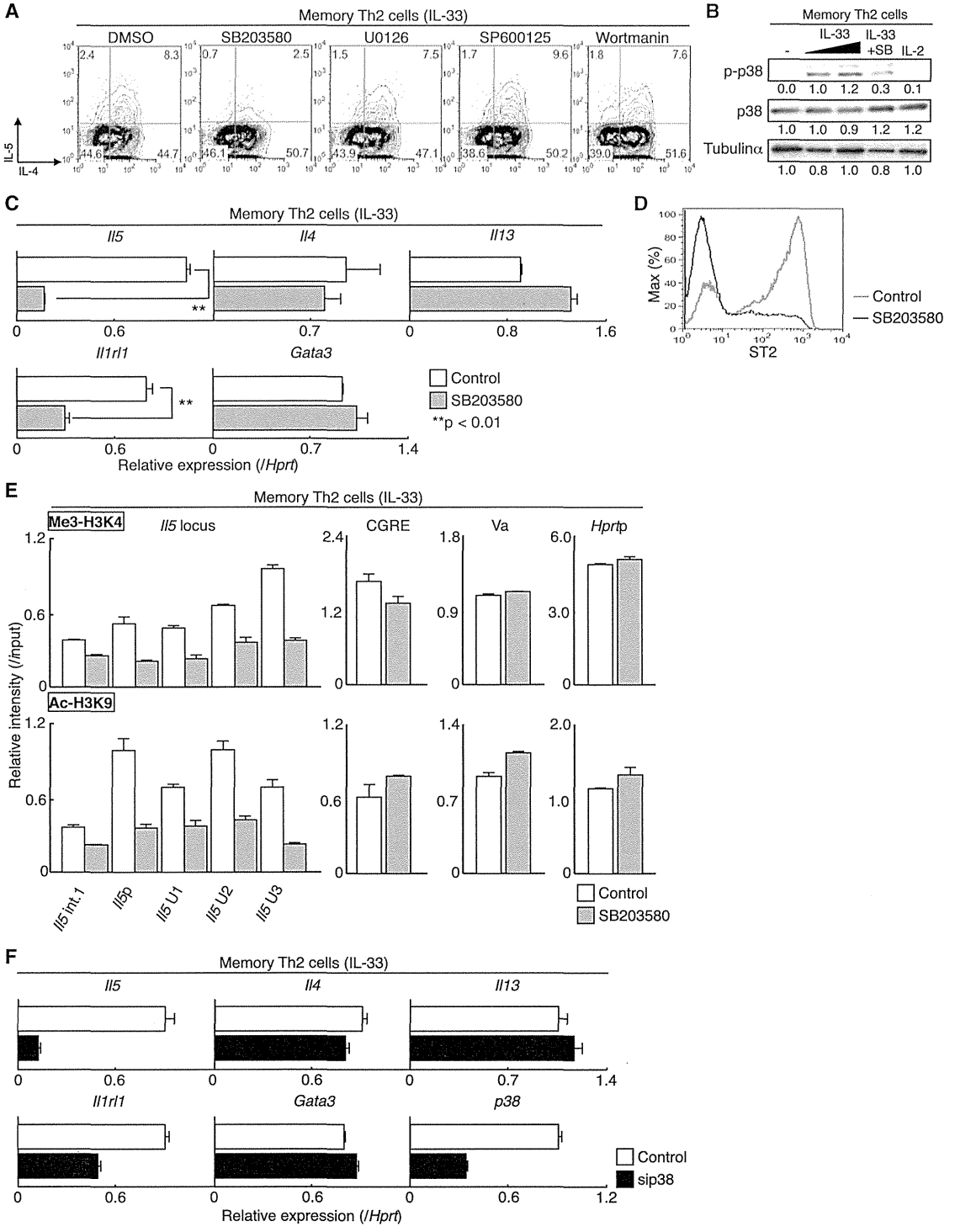

Figure 6. p38 Signaling Is Crucial for IL-33-Dependent IL-5 Induction by Memory Th2 Cells

(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-κB, 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-TCRβ is shown.

At least three (A–D) or two (E and F) independent experiments were performed and showed similar results. See also Figure S6.

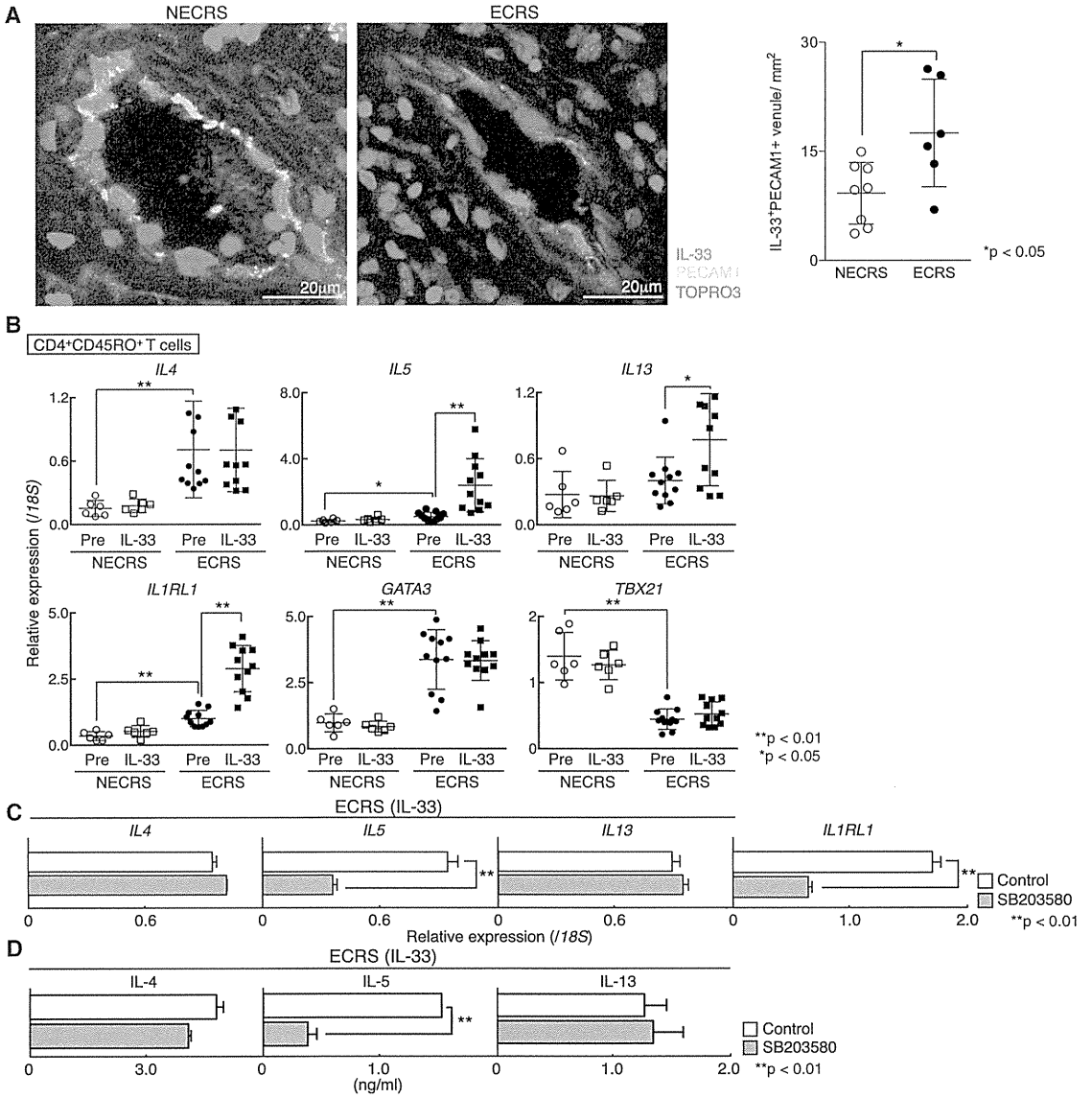

Figure 7. Human IL-33 Enhances IL-5 Production in Memory CD4+ T Cells Prepared from Nasal Polyps of ECRS Patients

(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 ± 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-αβ (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 μM) 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 μg 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.

ACKNOWLEDGMENTS

We thank Kaoru Sugaya, Miki Kato, and Toshihiro Ito for their excellent technical assistance. This work was supported by the Global COE Program (Global Center for Education and Research in Immune System Regulation and Treatment) and by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (Grants-in-Aid for Scientific Research [S] 26221305, (B) 21390147, and (C) 24592083; Grants-in-Aid for Young Scientists (B) 24790461 and 25860352; Research Activity start-up 25893032), the Ministry of Health, Labor, and Welfare, the Astellas Foundation for Research on Metabolic Disorders, the Uehara Memorial Foundation, the Kanae Foundation for the Promotion of Medical Science, the Princes Takamatsu Cancer Research Fund, and the Takeda Science Foundation. D.J.T. was supported by a Japanese Society for the Promotion of Science postdoctoral fellowship (2109747).

Received: December 27, 2013
Revised: September 10, 2014
Accepted: January 26, 2015
Published: February 17, 2015

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Clinical & Experimental Immunology
The Journal of Translational Immunology
British Society for immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12399

Activation of invariant natural killer T cells in regional lymph nodes as new antigen-specific immunotherapy via induction of interleukin-21 and interferon-γ

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Accepted for publication 12 June 2014
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Summary

Invariant natural killer T (iNKT) cells play important immunoregulatory functions in allergen-induced airway hyperresponsiveness and inflammation. To clarify the role of iNKT cells in allergic rhinitis (AR), we generated bone marrow-derived dendritic cells (BMDCs), which were pulsed by ovalbumin (OVA) and α-galactosylceramide (OVA/α-GalCer-BMDCs) and administered into the oral submucosa of OVA-sensitized mice before nasal challenge. Nasal symptoms, level of OVA-specific immunoglobulin (IgE), and T helper type 2 (Th2) cytokine production in cervical lymph nodes (CLNs) were significantly ameliorated in wild-type (WT) mice treated with OVA/α-GalCer-BMDCs, but not in WT mice treated with OVA-BMDCs. These anti-allergic effects were not observed in Jα18$^{-/-}$ recipients that lack iNKT cells, even after similar treatment with OVA/α-GalCer-BMDCs in an adoptive transfer study with CD4$^+$ T cells and B cells from OVA-sensitized WT mice. In WT recipients of OVA/α-GalCer-BMDCs, the number of interleukin (IL)-21-producing iNKT cells increased significantly and the Th1/Th2 balance shifted towards the Th1 dominant state. Treatment with anti-IL-21 and anti-interferon (IFN)-γ antibodies abrogated these anti-allergic effects in mice treated with α-GalCer/OVA-BMDCs. These results suggest that activation of iNKT cells in regional lymph nodes induces anti-allergic effects through production of IL-21 or IFN-γ, and that these effects are enhanced by simultaneous stimulation with antigen. Thus, iNKT cells might be a useful target in development of new treatment strategies for AR.

Keywords: α-galactosylceramide, bone marrow-derived dendritic cells, invariant natural killer T cell, IFN-γ, IL-21

Introduction

During the past several decades, the prevalence of allergic rhinitis (AR) has increased globally [1,2] and AR now affects 400 million people worldwide as a common allergic inflammatory disease that causes medical and socioeconomic problems [3]. Significant improvement of AR symptoms can be achieved using readily available drugs such as H1-anti-histamines and topical corticosteroids, but these drugs do not treat the underlying disease [4]. Antigen-specific immunotherapy may potentially alter the natural course of AR [4–6]; however, conventional immunotherapies, including subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT), are not convenient, because several years are required to establish a stable and adequate response. In addition, some patients do not show significant improvement of symptoms even with long-term therapy [7–9]. These burdens on patients would be reduced by a new method with enhanced therapeutic efficacy and a shortened duration of treatment without serious adverse events.

Invariant natural killer T (iNKT) cells, a major subset of NK T cells, express a unique semi-invariant T cell receptor (TCR) with a Vα14-Jα18 chain in mice and a Vα24-Jα18 chain in humans [10,11]. These cells produce many T helper type 1 (Th1)- and Th2-type proinflammatory cytokines, including interferon (IFN)-γ and interleukin (IL)-4, resulting in immune modulation of autoimmune diseases and responses to tumour and infectious agents [12–16]. TCRs of iNKT cells recognize monomorphic

major histocompatibility complex (MHC) class I-like CD1d molecules on antigen-presenting cells (APCs), and glycolipid antigens such as α-galactosylceramide (α-GalCer) presented on CD1d preferentially activate iNKT cells [17]. iNKT cells have been suggested to be tolerogenic in allergic airway inflammation [18–20], but it is unclear whether iNKT cells regulate development of AR.

In this study, we administered ovalbumin (OVA)- and α-GalCer-pulsed bone marrow-derived dendritic cells (BMDCs) into the oral submucosa of OVA-sensitized mice and examined the role of activated iNKT cells in an AR mouse model. Administration of OVA/α-GalCer-pulsed BMDCs suppressed antigen-specific responses, whereas OVA-pulsed BMDCs did not do so. These results show that activation of iNKT cells in draining lymph nodes ameliorated nasal allergic responses in an AR mouse model, and that this anti-allergic effect is associated with IL-21 and IFN-γ production through activated iNKT cells.

Materials and methods

Mice

Female BALB/c mice (8 weeks old) were purchased from SLC Inc. (Hamamatsu, Japan). Jα18-deficient (Jα18$^{-/-}$) mice were established by specific deletion of the Jα18 gene segment [15] and back-crossing 10 times to the BALB/c background. These mice were also used at 8 weeks of age. Mice were maintained under specific pathogen-free conditions. Use of the mice was approved by the Chiba University Institutional Animal Care and Use Committee and the experiments were conducted in conformity with the guidelines of the committee.

Reagents

α-GalCer (KRN7000) was obtained from Kirin Brewery (Gunma, Japan). OVA (grade 5) was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in endotoxin-free D-phosphate-buffered saline (PBS) (Wako Pure Chemical Industries, Osaka, Japan). RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS), l-glutamine (2 μM), penicillin (100 U/ml), streptomycin (100 μg/ml), HEPES (10 mM), 2-mercaptoethanol (55 μM), 1% non-essential amino acids and 1 mM sodium pyruvate (all from GIBCO BRL, Grand Island, NY, USA) was used in cell culture experiments. Anti-FcγRII/III monoclonal antibodies (mAbs) (2·4G2) (BD Biosciences, San Jose, CA, USA) were used for Fc blocking. Allophycocyanin-conjugated α-GalCer-loaded CD1d tetramer was purchased from Proimmune (Oxford, UK). Fluorescein isothiocyanate (FITC)-anti-CD11c (N418) (eBiosciences, San Diego, CA, USA), phycoerythrin (PE)-conjugated mAbs including anti-CD4 (GK1·5), anti-CD19 (6D5), anti-CD40 (3/23), anti-CD86 (GL-1) (BioLegend, San Diego, CA, USA), anti-MHC class II IA + IE (M5/114·15) or anti-CD80 (16-10A-1) (eBiosciences) were used for fluorescence activated cell sorter (FACS) analysis. Anitbodies including anti-IL-4 (11B11), anti-IL-5 (TRFK5; Mabtech Ab, Nacka, Sweden), anti-IFN-γ (AN18; BioLegend) or anti-immunoglobulin (Ig)E (RME-1; BD Pharmingen, San Jose, CA, USA), and biotin-conjugated antibodies including anti-IL-4 mAb (BVD6-24G2), anti-IL-5 (TRFK4) (Mabtech antibody), anti-IFN-γ (R4-6A2; BioLegend) or anti-IgE (R35-72; BD Pharmingen) were used in enzyme-linked immunosorbent assays (ELISAs) [21]. A mouse IL-13 ELISA Ready-SET-Go! Kit (eBiosciences) and a mouse anti-OVA IgE antibody assay kit (Chondrex, Redmond, WA, USA) were also used in the study.

Generation of BMDCs

Bone marrow cells obtained from the femurs of naive BALB/c mice were cultured with 20 ng/ml murine granulocyte–macrophage colony-stimulating factor (GM-CSF) (PeproTech, Rocky Hill, NJ, USA) for 8 days. Non-adherent cells were harvested and pulsed with or without 100 ng/ml α-GalCer for 3 h, followed by a 6-h incubation with or without 1 mg/ml OVA in 24-well plates at 1×10^6 cells/well. These cells were stimulated with 10 μg/ml lipopolysaccharide (LPS) (O111:B4; Sigma-Aldrich) for 3 h and then washed three times with PBS. DCs were analysed based on surface markers using FACS analysis (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ, USA).

Administration of BMDCs in OVA-sensitized mice

BALB/c mice were sensitized intraperitoneally with 100 μg of OVA and 2 mg of alum (Pierce, Rockford, IL, USA) once a week for 3 weeks. One week after the last sensitization, the mice ($n = 5$–6 in each group) received 5×10^6 cells of BMDCs in 100 μl of PBS to the sublingual submucosa by injection and were treated intranasally with 1 mg of OVA for 7 consecutive days (challenge group) or with PBS for 6 consecutive days followed by OVA on the seventh day (control group). After the last treatment, the behaviour of the mice was documented for 5 min using a videorecorder. Sneezing and nasal-rubbing events were counted by an investigator who was blinded to the treatment. The mice were then killed and the serum, cervical lymph nodes (CLNs) and spleens were collected.

Adoptive transfer of CD4$^+$ T cells and B cells

CD4$^+$ T cells and B cells were sorted from OVA-sensitized wild-type (WT) mice by negative selection using a magnetic affinity cell sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany). A single-cell suspension was prepared from spleens [22]. After Fc blocking, splenic cells

Table 1. Polymerase chain reaction (PCR) primers used in the study.

Primer set	Sense primer, 5′–3′	Anti-sense primer, 5′–3′
Vα14	CACTGCCACCTACATCTGTGT	AGTCCCAGCTCCAAAATGCA
IL-21	GCCAGATCGCCTCCTGATTA	CATGCTCACAGTGCCCCTTT
Bcl6	CCGGCTCAATAATCTCGTGAA	GGTGCATGTAGATGTGTGAGTGA
IL-17RA	AGTGTTTCCTCTACCCAGCAC	GAAAACCGCCACCGCTTAC
RORγt	CTTTCAATACCTCATTGTAT	AGGTCCTTCTGGGGGCTTGC
Beta-actin	CCAGCCTTCCTTCTTGGGTAT	TGGCATAGAGGTCTTTACGGATGT

IL = interleukin; RORγt = RAR-related orphan receptor gamma t.

were incubated with a mixture of biotinylated antibodies, including anti-IgM (MA-69), anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-CD11c (N418), anti-TER-119 (TER-119), anti-Gr-1 (RB6-MC5) (BioLegend) or allophycocyanin-conjugated α-GalCer-loaded CD1d tetramer to collect $CD4^+$ T cells, or with a mixture of biotinylated antibodies including anti-CD3 (145-2C11; BioLegend), anti-CD11b, anti-CD11c, anti-TER-119, anti-Gr-1 or allophycocyanin-conjugated α-GalCer-loaded CD1d tetramer to collect B cells. After washing, these cells were incubated with anti-biotin beads and anti-allophycocyanin-beads (Miltenyi Biotec) and then subjected to MACS analysis. The purity of the cells was analysed using FACSCalibur (BD Biosciences) and CellQuest software (Becton Dickinson). Data were analysed with FlowJo software (TreeStar, Ashland, OR, USA). The isolated $CD4^+$ T cells (1×10^7 cells) and B cells ($1{\cdot}5 \times 10^7$ cells) were then transferred intravenously to WT or $J\alpha18^{-/-}$ mice. One day later, injection of BMDCs and nasal challenge were performed as described above.

Neutralization assay

Anti-mouse IL-21 antibody (TY25), rat IgG2a antibody (54447) (R&D Systems), anti-mouse IFN-γ antibody (R4-6A2) or rat IgG2a antibody (RTK2758) (BioLegend) (250 μg) was injected intravenously in OVA-sensitized mice 1 day before BMDC administration and on day 3 of nasal challenge.

Proliferation assay

$CD4^+$ T cells isolated from CLNs were cultured with OVA and irradiated splenic feeder cells for 48 h, with tritium-labelled thymidine (37 kBq/well) added for the last 8 h. The cells were then harvested with a cell harvester (Perkin Elmer, Waltham, MA, USA) onto a β plate and the radioactivity was measured using a liquid scintillation counter (Perkin Elmer).

Restimulation of $CD4^+$ T cells

Single-cell suspensions were prepared from CLNs and $CD4^+$ T cells were sorted by the MACS technique using a biotinylated anti-CD4 antibody (GK1·5; BioLegend) and anti-biotin beads (Miltenyi Biotec). The cells were cultured for 48 h at a density of $1{\cdot}5 \times 10^5$ cells/well in round-bottomed 96-well microculture plates in the presence of 1 mg/ml OVA with $CD4^+$ T cell-depleted and irradiated splenic feeder cells (5×10^5 cells) obtained from naive mice. The concentration of cytokines in the supernatant was measured by ELISA.

Detection of IL-21-producing iNKT cells

IL-21-producing iNKT cells were detected by an enzyme-linked immunospot (ELISPOT) assay [21] with anti-mouse IL-21 antibody and biotinylated anti-mouse IL-21 antibody (mouse IL-21 DuoSet; R&D Systems, Minneapolis, MN, USA). A single-cell suspension was prepared from CLNs and spleens, as described above. Splenocytes were incubated with anti-FcγRII/III mAbs and depleted with biotinylated antibodies, including anti-IgM, anti-B220, anti-CD11b, anti-CD11c, anti-TER-119, anti-Gr-1 and anti-biotin beads, using the MACS technique. The enriched spleen cells were incubated with allophycocyanin-conjugated α-GalCer-loaded CD1d tetramer and splenic iNKT cells were sorted using FACS ARIA II (BD Biosciences). CLN cells (2×10^5 cells/well) were cultured with α-GalCer (100 ng/well) and splenic iNKT cells (5×10^4 cells/well) were co-cultured with BMDCs (5×10^4 cells per well) in 96-well filtration plates (Multiscreen; Millipore Corp., Bedford, MA, USA) for 3 days.

Real-time reverse transcription–polymerase chain reaction (RT–PCR)

Total RNA was extracted from $CD4^+$ cells in CLNs using TRIzol reagent (Life Technologies, Gaithersburg, MD, USA) and reverse transcribed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Relative gene expression was calculated by a sequence detection system (StepOnePlus™; Applied Biosystems) and the amount of cDNA was normalized using the *beta-actin* housekeeping gene. Primer sets (Table 1) were purchased from Eurofins Operon MWG (Ebersberg, Germany).

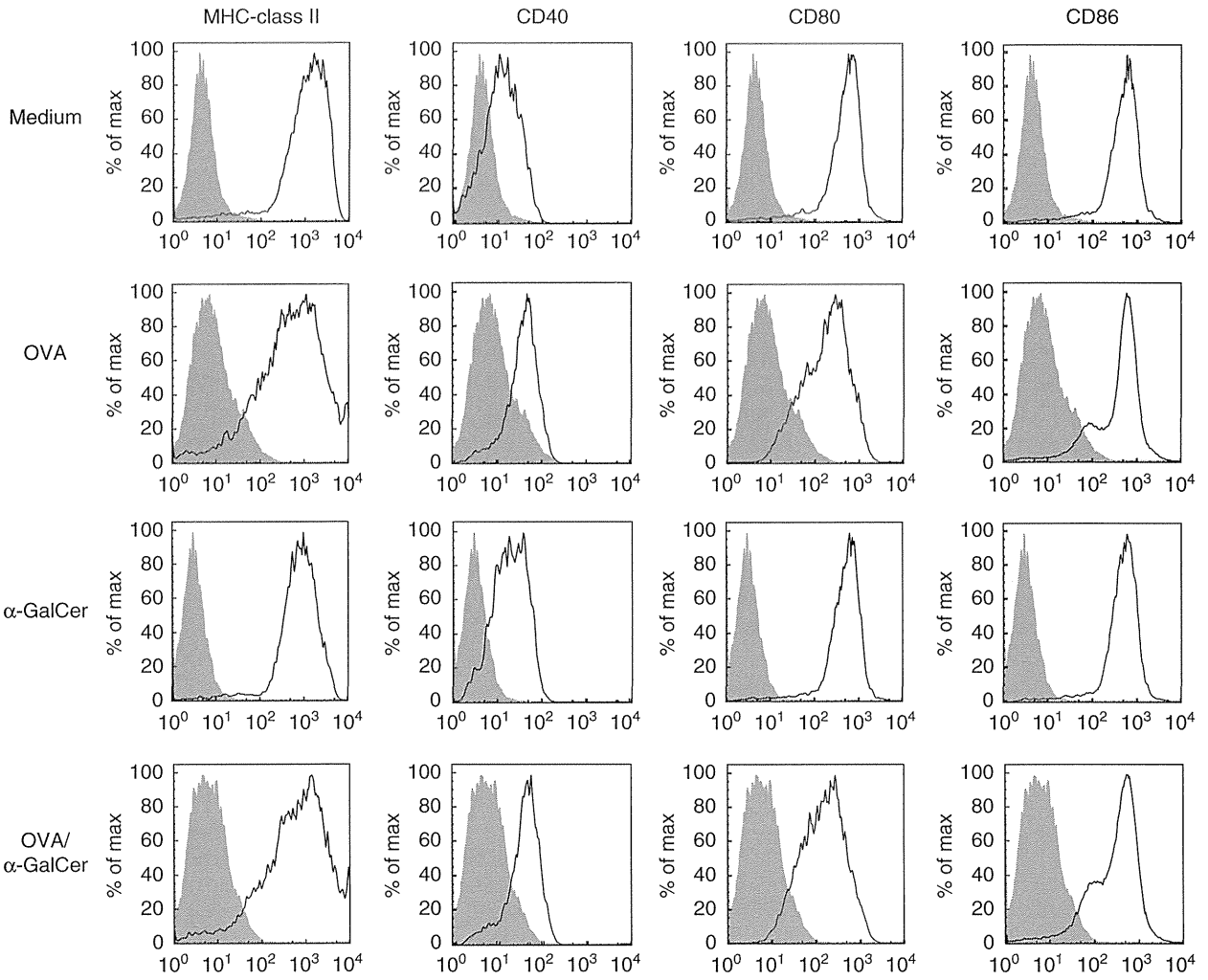

Fig. 1. Similar expression levels of surface markers in each group of bone marrow-derived dendritic cells (BMDCs). BMDCs were incubated with medium only, ovalbumin (OVA), α-galactosylceramide (α-GalCer) or OVA/α-GalCer, followed by addition of lipopolysaccharide (LPS), and surface markers were analysed by fluorescence activated cell sorter (FACS). BMDCs were gated on $CD11c^+$. Shaded profiles in the histograms show background staining with rat immunoglobulin (Ig)G2a. Data are representative of three independent experiments.

Statistical analysis

Statistical analysis was performed using a two-tailed Student's *t*-test with $P < 0{\cdot}05$ considered to be significant. Data are shown as the mean ± standard deviation.

Results

Characteristics of BMDCs

Surface marker expression levels on stimulated BMDCs were evaluated by FACS analysis prior to administration into AR mice. The percentage of $CD11c^+$ cells in the generated BMDCs was approximately 95%. Based on the MHC class II levels, there were few differences among BMDCs cultured with medium, OVA, α-GalCer and OVA plus α-GalCer. Similar patterns were observed for the expression levels of CD40, CD80 and CD86 (Fig. 1).

Oral submucosal administration of BMDCs in OVA-sensitized mice

On the 7th day of nasal challenge with OVA, mice administered OVA/α-GalCer-BMDCs showed significant decreases in the number of sneezing and nasal rubbing attacks, and in the levels of both OVA-specific and total IgE, compared with mice administered BMDCs. There were no significant differences in nasal symptoms and IgE levels among mice that received BMDCs, OVA-BMDCs or α-GalCer-BMDCs (Fig. 2a,b).

Analysis of $CD4^+$ T cells isolated from CLNs

Cytokine production by $CD4^+$ T cells in CLNs is shown in Fig. 2c. Of the Th2 cytokines examined, IL-4, IL-5 and IL-13 levels were significantly lower in $CD4^+$ T cells from mice that received OVA/α-GalCer-BMDCs compared with

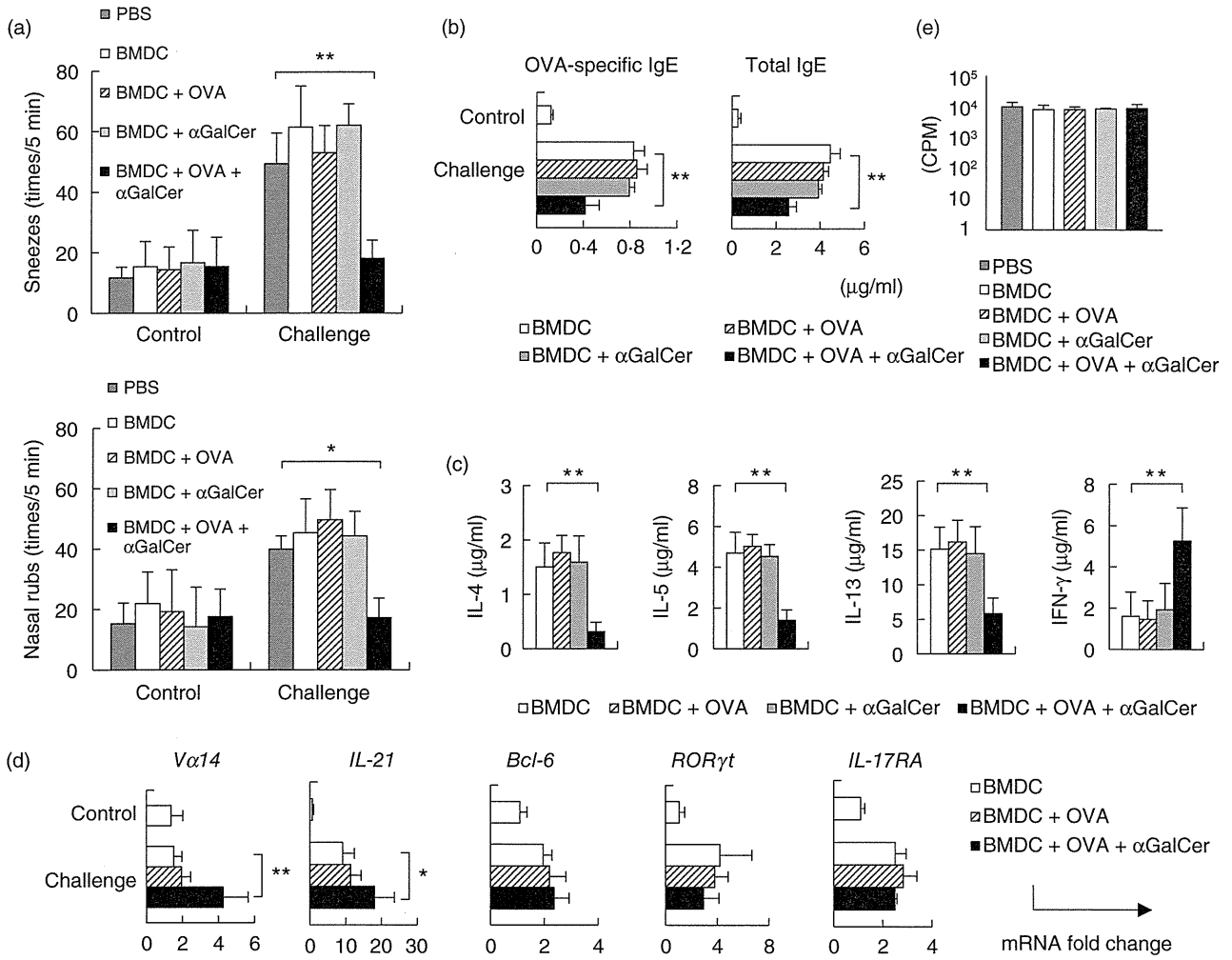

Fig. 2. Prevention of development of nasal allergic symptoms by administration of ovalbumin, α-galactosylceramide-bone marrow-derived dendritic cells (OVA, α-GalCer-BMDCs). (a) Number of sneezes and nasal rubs. (b) OVA-specific and total immunoglobulin (Ig)E levels in serum. (c,e) Cytokine production and proliferation of $CD4^+$ T cells obtained from cervical lymph nodes (CLNs). Counts per minute, cpm. (d) Relative gene expression of $CD4^+$ cells obtained from CLNs. Data are representative of three independent experiments. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

those from mice that received BMDCs or OVA-BMDCs. Enhanced IFN-γ production occurred in mice that received OVA/α-GalCer-BMDCs. Gene expression profiles from quantitative RT–PCR analysis (Fig. 2d) showed higher *Vα14* and *IL-21* expression in the CLNs of OVA/α-GalCer-BMDC-treated mice compared with other groups. However, expression of *Bcl-6*, a Tfh cell-related transcript, and Th17 cell-related transcripts such as *IL-17RA* and *RORγt*, did not differ among the groups. Proliferation of $CD4^+$ T cells also showed no differences among the groups of mice (Fig. 2e).

Adoptive transfer of $CD4^+$ T cells and B cells into $J\alpha18^{-/-}$ mice

Following adoptive cell transfer of $CD4^+$ T cells (excluding iNKT cells) and B cells from spleen of OVA-sensitized WT mice, nasal symptoms after OVA challenge in WT mice were significantly suppressed by oral submucosal administration of OVA/α-GalCer-BMDCs compared with mice administered other BMDCs. However, similar suppression was not observed in $J\alpha18^{-/-}$ [iNKT knock-out (KO)] mice that received OVA/α-GalCer-BMDCs (Fig. 3).

IL-21-producing iNKT cells in CLNs

After stimulation with α-GalCer, IL-21-producing cells increased significantly in CLN cells of mice treated with OVA/α-GalCer BMDCs (Fig. 4a), whereas IL-21 was not detected in culture supernatants of $CD4^+$ T cells (data not shown). To determine whether iNKT cells produce IL-21 in response to α-GalCer presented on BMDCs, splenic iNKT cells of naive mice were co-cultured with BMDCs plus α-GalCer. The results showed that OVA/α-GalCer BMDCs stimulated IL-21 production in iNKT cells (Fig. 4b).

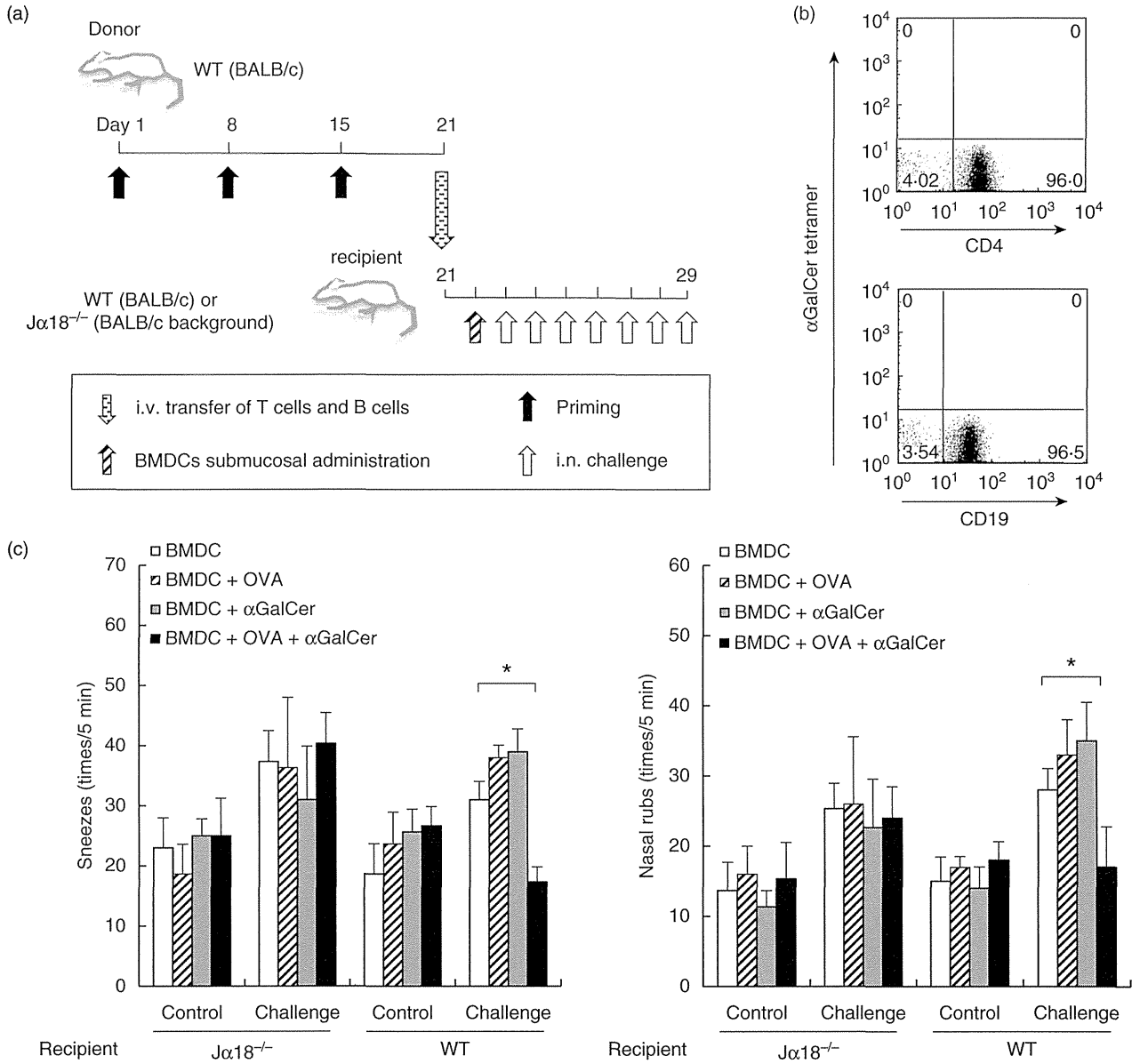

Fig. 3. Suppression of nasal symptoms by ovalbumin, α-galactosylceramide-bone marrow-derived dendritic cells (OVA, α-GalCer-BMDCs) in an invariant natural killer T (iNKT) cell-dependent manner. (a) Protocol of adoptive transfer. (b) Splenic $CD4^+$ T cells and B cells of OVA-sensitized mice were transferred after removal of iNKT cells. (c) Nasal symptoms after the final nasal challenge. Data are representative of three independent experiments. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

Treatment with anti-IL-21 or anti-IFN-γ neutralizing antibody

Treatment with anti-IL-21 mAb or anti-IFN-γ mAb and nasal challenge in OVA/α-GalCer BMDC-treated mice increased the number of sneezes and nasal rubs, compared with control mAb-treated mice (Figs 5a and 6a). OVA-specific and total IgE levels were increased by anti-IL-21 mAb, whereas only OVA-specific IgE was increased by anti-IFN-γ mAb (Figs 5b and 6b).

Discussion

The goal of this study was to assess the anti-allergic effects of activated iNKT cells in CLNs, which are regional draining lymph nodes, in an AR mouse model. Single administration of OVA/α-GalCer-BMDCs into the oral submucosa of OVA-sensitized mice suppressed nasal symptoms and the level of OVA-specific IgE in association with IL-21 and IFN-γ in an iNKT cell-dependent manner. Other BMDCs failed to alleviate the Th2 responses and, therefore, the

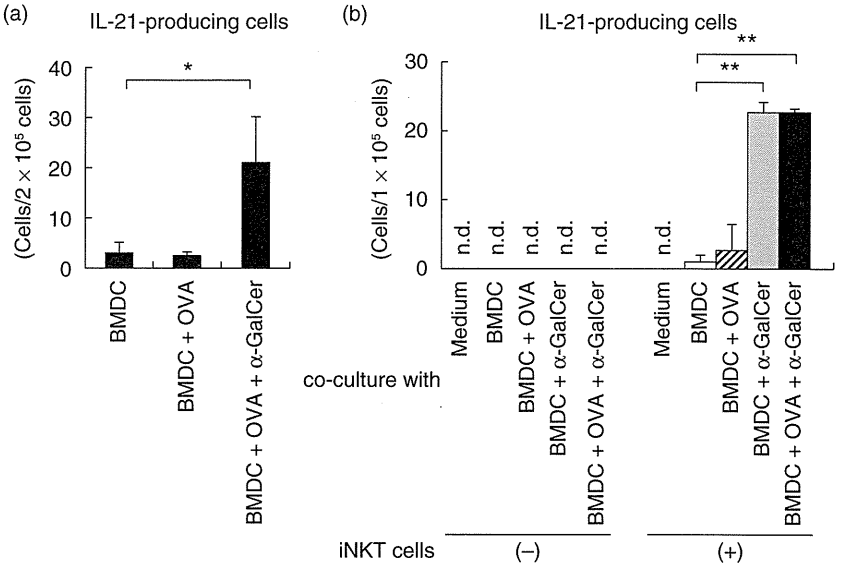

Fig. 4. Increase of invariant natural killer T (iNKT) cells producing interleukin (IL)-21 in cervical lymph nodes (CLNs). (a) CLN cells were cultured with α-galactosylceramide (α-GalCer) and IL-21-secreting cells were detected using an enzyme-linked immunospot (ELISPOT) assay. (b) Splenic iNKT cells and bone marrow-derived dendritic cells (BMDCs) were co-cultured, and IL-21-secreting cells were detected by ELISPOT assay. Data are representative of three independent experiments. n.d. = not detected; $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

production of both OVA-specific and total IgE was up-regulated. These findings indicate that, if antigen stimulation is provided simultaneously, activated iNKT cells in CLNs can suppress a nasal allergic reaction by producing IL-21 and IFN-γ.

IL-21, a type I cytokine, prevents B cell proliferation and correspondingly augments B cell death under certain conditions [23–26]. This cytokine is produced preferentially by activated iNKT cells and $CD4^{+}$ T cells, including Tfh cells and Th17 cells [27–29]. Expression of *Bcl6*, a Tfh cell-related transcript, and Th17 cell-related transcripts such as *IL-17RA* and *RORγt*, did not increase in this study; but *Vα14*, an iNKT cell-related transcript, was markedly up-regulated and IL-21-producing iNKT cells increased significantly in CLNs of mice treated with OVA/α-GalCer-BMDCs in the oral submucosa. In addition, a neutralization assay revealed that IL-21 plays a critical role in suppressing OVA-specific IgE production. These results are congruent with those reported by Hiromura *et al.* showing that intranasal administration of recombinant mouse IL-21 reduces nasal symptoms and the serum level of OVA-specific IgE [30].

The Th1/Th2 balance in CLNs changed towards a Th1-skewed phenotype after administration of OVA/α-GalCer-BMDCs. An IFN-γ neutralization indicated that this Th1 cytokine can play a pivotal role in suppressing the level of OVA-specific IgE. In type I allergic diseases, allergens trigger a Th2-dominant immune response that generates

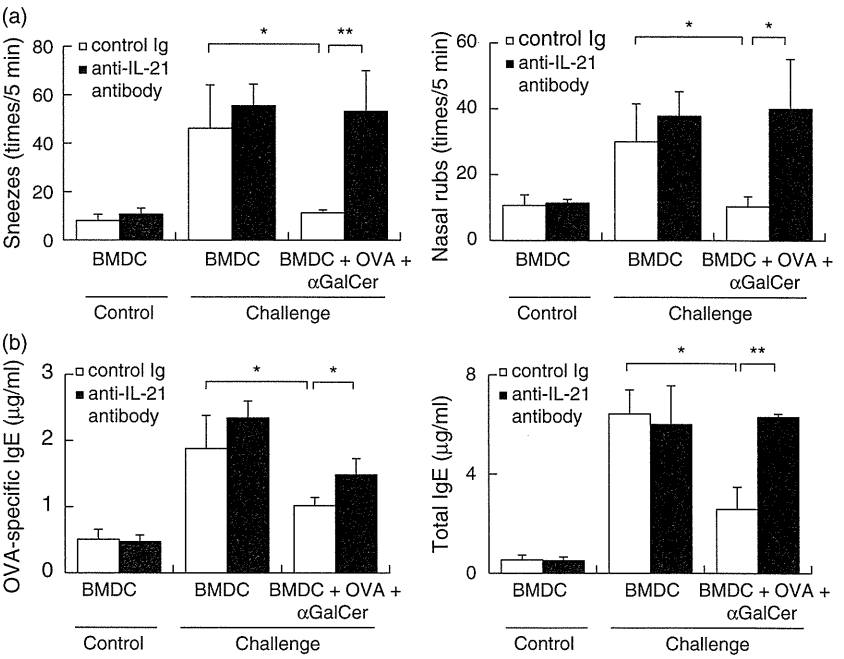

Fig. 5. Failure of ovalbumin, α-galactosylceramide-bone marrow-derived dendritic cells (OVA, α-GalCer-BMDCs) to suppress nasal symptoms by neutralization of interleukin (IL)-21. (a) Nasal symptoms were observed for 5 min after the final nasal challenge. (b) OVA-specific and total immunoglobulin (Ig)E levels in serum. Data are representative of three independent experiments. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

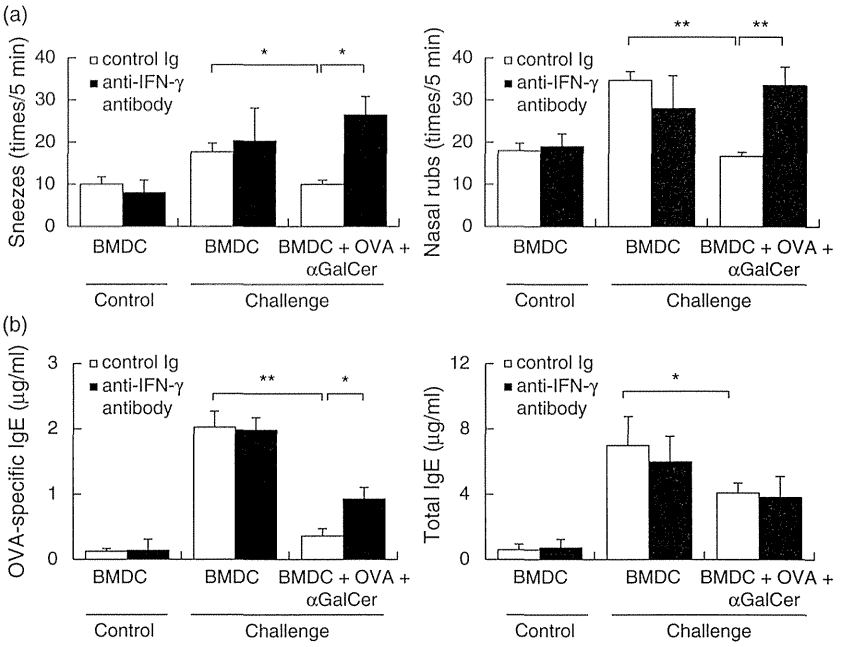

Fig. 6. Failure of ovalbumin, α-galactosylceramide-bone marrow-derived dendritic cells (OVA, α-GalCer-BMDCs) to suppress nasal symptoms by neutralization of interferon (IFN)-γ. (a) Nasal symptoms were observed for 5 min after the final nasal challenge. (b) OVA-specific and total IgE levels in serum. Data are representative of three independent experiments. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

antigen-specific IgE-producing memory B cells, but the role of IFN-γ in IgE production remains unclear [31,32]. Antigen-specific IgE is produced mainly in draining lymph nodes [21,33] and CLNs are regional lymph nodes from the oral cavity, as well as the nasal cavity [33,34]. In the present study, treatment with anti-IFN-γ antibody exacerbated nasal symptoms and the OVA-specific IgE titre in mice treated with OVA/α-GalCer-BMDCs. These results suggest that IFN-γ exerts a potent inhibitory effect on IgE production in AR.

The role of iNKT cells in allergic reactions is unclear. These cells have been suggested to have a suppressive effect on allergic disease [19,20,31,35]; however, other reports show that iNKT cells have an essential role in development of airway hyperreactivity [18,36,37]. This plasticity of iNKT cells may arise partially from differences in systemic *versus* topical administration of α-GalCer and the diversity of APCs. In the present study, OVA/α-GalCer-BMDCs led to suppress OVA-induced nasal allergic symptoms and OVA-specific IgE production. These findings share some features with the previous report demonstrating that mice administered OVA/α-GalCer-BMDCs intratracheally prior to OVA challenge failed to develop airway hyperresponsiveness [38].

Brimnes *et al.* showed that repeated sublingual administration of OVA for 5 days each week for 9 weeks resulted in relief from nasal allergic symptoms in an AR mouse model [39]. Direct administration of OVA and α-GalCer to the oral mucosa failed to have this effect because α-GalCer is not a water-soluble antigen and is not readily phagocytosed by oral dendritic cells. In the present study, α-GalCer-BMDCs did not exacerbate nasal allergic symptoms and simultaneous administration of OVA and α-GalCer using BMDCs led to efficient suppression of OVA-induced allergic reactions.

We have reported previously that DCs isolated from PBMCs of patients with head and neck cancer migrated to CLNs after oral submucosal administration [34], and we showed that this treatment was safe [40]. Simultaneous administration of an antigen with α-GalCer-DCs is thus an accessible way to activate iNKT cells in regional lymph nodes; however, further studies are needed to clarify the role of activated iNKT cells in regional lymph nodes in treatment of AR.

In conclusion, oral submucosal administration of OVA/α-GalCer-pulsed BMDCs activated iNKT cells in CLNs and suppressed Th2 responses in OVA-sensitized mice. In the present study, simultaneous stimulation with antigen and α-GalCer were considered essential to exert anti-allergic effects and led to relief of nasal allergic symptoms. This finding indicates that the activated iNKT cells have the potential to alleviate nasal allergic symptoms in the presence of antigen. Thus, activation of iNKT cells in regional lymph nodes might be an important target in new treatment strategies for AR.

Acknowledgements

We appreciate all the help given by staff of Department of Otolaryngology and immunology of Chiba University. This work was supported by a grant-in-aid for research on allergic disease and immunology from the Ministry of Health, Labor, and Welfare in Japan, and grant-in-aid for the Global Center for Education and Research in Immune System

Regulation and Treatment Program from Ministry of Education, Culture, Sports, Science and Technology (MEXT) in Japan.

Disclosure

The authors have no conflicts of interest to declare.

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