IL-1 Family Cytokines Drive Th2 and Th17 Cells to Innocuous Airborne Antigens

Takao Kobayashi[1], Koji Iijima[1], James L. Checkel[1], and Hirohito Kita[1]

[1]Department of Medicine and Department of Immunology, Mayo Clinic Rochester, Rochester, Minnesota

Allergic asthma is commonly thought to result from dysregulated airway inflammatory responses to ubiquitous environmental antigens mediated by $CD4^+$ T cells polarized to a Th2 or Th17 cell. However, the mechanisms involved in the development of these T-cell responses remain unknown. This study examines the effects of IL-1 family cytokines, such as IL-33 and IL-1β, on the development of antigen-specific Th2 and Th17 cells in the airway. We administered IL-1 family cytokines and model antigens, such as ovalbumin, into the airways of naive BALB/c mice, and examined the cellular and humoral immune responses. To investigate the immunologic mechanisms, we used IL-4 green fluorescent protein reporter mice and mice deficient in the *Il4* gene. Innocuous antigens, such as endotoxin-free ovalbumin and short ragweed extract, did not sensitize naive mice when administered through the airways. However, when mice were exposed to the same antigens with IL-1β or IL-33, they developed IgE antibodies. In particular, IL-33 induced robust and long-lasting Th2 cells that produced a large quantity of IL-5 and IL-13 and asthma-like airway pathology. IL-1β induced Th17 cells. In naive, nonsensitized animals, IL-33 stimulated endogenous IL-4 expression by $CD4^+$ T cells, which was critical for the polarization of $CD4^+$ T cells to the Th2 type. In the absence of IL-4, mice developed Th17 cells and neutrophilic airway inflammation. In conclusion, IL-1 family cytokines possess a potent adjuvant activity to promote both Th2 and Th17 cells to innocuous airborne antigens, and they may play fundamental roles in the immunopathology of asthma.

Keywords: asthma; IL-1β; IL-33; antigen

Allergic asthma is commonly thought to result from dysregulated airway inflammatory responses to ubiquitous environmental antigens mediated by $CD4^+$ T cells polarized to a Th2 cell phenotype (1, 2). In addition, in patients with asthma, IL-17 expression is increased in the airways, and the severity of airway hyperreactivity (AHR) correlates with the increase in IL-17 expression levels (3). A novel subset of memory $CD4^+$ cells that produces both Th2-type cytokines and IL-17 has also been identified in patients with asthma (4). However, little is known about how such pathologic Th2 and Th17 immune responses develop. Generally, respiratory exposure to innocuous antigens is considered to be a tolerogenic event (5).

Innate immune responses can shape adaptive immune responses. Recently, important roles for the IL-1 family molecules in both the innate and adaptive immune responses have been reported (6, 7). IL-33 is a new member of the IL-1 family cytokines (8). In contrast to other IL-1 family members, IL-33 is not typically expressed by hematopoietic cells, but is abundantly expressed by epithelial cells, keratinocytes, and endothelial cells (8, 9), suggesting its involvement in mucosal immunity. IL-33 has been considered an "alarm" or endogenous "danger signal" released by damaged or necrotic cells (10). Several cell types express the IL-33 receptor (i.e., ST2/IL-1 receptor-like 1), including mast cells, basophils, eosinophils, natural killer T cells, natural killer cells, dendritic cells, and fully differentiated Th2 cells (11). An important new development in the field involves the characterization of innate lymphoid cells (ILCs) that produce large quantities of Type 2 cytokines in response to IL-33 or IL-25 (12, 13). These so-called Type 2 ILCs may play important roles in Type 2 airway inflammation and tissue homeostasis in mucosal organs, even in the absence of adaptive immunity (14, 15). However, the effects of IL-33 and other IL-1 family cytokines on adaptive immune responses in the airways, and in particular the development and polarization of antigen-specific $CD4^+$ T cells, are not well understood.

This study demonstrates that IL-1 family cytokines, including IL-1β and IL-33, potently induce the Th2-type and Th17-type differentiation of naive $CD4^+$ T cells in response to innocuous airborne antigens. In particular, IL-33 mediated antigen-specific Th2 cells that were found to be long-lasting and to cause pathologic changes in the airways. In naive animals, IL-33, administered together with airborne antigens, drove $CD4^+$ T-cell endogenous IL-4 production, which was in turn indispensable for the successful polarization of naive $CD4^+$ T cells to the Th2 type. Therefore, IL-1 family cytokines demonstrate potent adjuvant activities in the airways to drive Th2-type and Th17-type $CD4^+$ T cells to innocuous airborne antigens, and hence may play fundamental roles in the pathogenesis of asthma.

CLINICAL RELEVANCE

Asthma is commonly thought to result from dysregulated airway inflammatory responses to ubiquitous environmental antigens mediated by $CD4^+$ T cells polarized to Th2 or Th17 cell phenotypes. However, little is known about how such pathologic Th2 and Th17 immune responses develop. Our research addresses the scientific questions of whether and how IL-1 family cytokines are involved in the development of Th2 and Th17 cells to airborne antigens. The observations in this study suggest that IL-1β and IL-33 induce the development of Th2-type and Th17-type $CD4^+$ T cells to innocuous airborne antigens, to which animals are normally tolerant. IL-1 family cytokines may hence play pivotal roles in dysregulated airway immune responses in asthma and allergic diseases.

MATERIALS AND METHODS

Mice

BALB/cJ, BALB/c-*Il4*^{tm2nNt}/J (*Il4*$^{-/-}$), C.129-*Il4*^{tm1Lky}/J (4 get), and C.129S7(B6)-*Rag1*^{tm1Mom}/J (*Rag1*$^{-/-}$) mice were obtained from Jackson

(*Received in original form November 3, 2012 and in final form June 19, 2013*)

This work was supported by National Institutes of Health grants R01 AI49235 and R01 AI71106, and by the Mayo Foundation.

Author Contributions: T.K., K.I., J.L.C., and H.K. designed the study and analyzed and interpreted the data. T.K., K.I., and J.L.C. acquired the data. T.K. and H.K. drafted and revised the manuscript. T.K., K.I., J.L.C., and H.K. approved the submitted version of the manuscript.

Correspondence and requests for reprints should be addressed to Hirohito Kita, M.D., Department of Medicine and Department of Immunology, Mayo Clinic Rochester, 200 First Street SW, Guggenheim Building, Room 401A, Rochester, MN 55905. E-mail: kita.hirohito@mayo.edu

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 49, Iss. 6, pp 989–998, Dec 2013

Originally Published in Press as DOI: 10.1165/rcmb.2012-0444OC on July 9, 2013
Internet address: www.atsjournals.org

Laboratory (Bar Harbor, ME). The 4 get mice and *Rag1*$^{-/-}$ mice were then bred under specific pathogen–free conditions. Female mice were used at 7–9 weeks of age. The procedures and handling of the mice were reviewed and approved by the Mayo Institutional Animal Care and Use Committee (Mayo Clinic, Rochester, MN).

Antigens and Cytokines

Endotoxin-free ovalbumin (OVA), that is less than 0.5 EU/mg by limulus lysate assay (Wako USA, Richmond, VA), was prepared from specific pathogen–free chicken eggs (Charles River Laboratories, Wilmington, MA) under sterile conditions (16) (additional information on methods is available in the online supplement).

Airway Exposure of Mice to Antigens and Cytokines

On Days 0 and 7, mice were lightly anesthetized with tribromoethanol and intranasally administered with 100 μg OVA in the presence or absence of 100 ng of IL-33 or IL-1β in 50 μl of PBS (Sigma-Aldrich, St. Louis, MO) (17) (Figure 1A). On Day 14, the plasma and spleen were collected for analysis of the specific antibodies and *in vitro* cytokine production, respectively. On Days 21, 22, and 23, mice were challenged intranasally with 100 μg OVA, and on Day 24, mice were killed with an overdose of pentobarbital (additional information on methods is available in the online supplement).

OVA-Specific IgE, IgG1, and IgG2a

The OVA-specific IgE, IgG1, and IgG2a levels in the plasma specimens were measured by ELISA (17) (additional information on methods is available in the online supplement).

Analyses of Cytokine Production by Splenocytes and Draining Lymph Node Cells

Single-cell suspensions of the spleen or mediastinal lymph node (MLN) were harvested and resuspended in RPMI-1640 medium (Gibco, Foster City, CA). One hundred microliters of the cell suspension were dispensed into each round-bottom well of 96-well culture plates, and stimulated by adding 100 μl of 200 μg/ml OVA for 96 hours. The concentrations of IL-4, IL-5, IL-13, IFN-γ, and IL-17A in the supernatant were measured by ELISA (additional information on methods is available in the online supplement).

AHR and Lung Histology

On Day 24 before the mice were killed, AHR to methacholine was assessed in conscious mice by whole-body plethysmography (Buxco Electronics, Wilmington, NC) (17, 18) or in anesthetized mice by the FlexiVent forced oscillation technique (Scireq, Montreal, PQ, Canada) (19) (additional information on methods is available in the online supplement).

Analysis of IL-4 Expression *In Vivo* by Reporter Mice

Nonsensitized naive 4 get mice were administered intranasally with OVA, with or without 100 ng of IL-33 or IL-1β. Forty-eight or 96 hours later, MLN cells were harvested and stained with anti-CD3ε and anti-CD4. After washing, cells were resuspended, fixed, and analyzed with a FACScan flow cytometer (BD Biosciences, San Jose, CA) by gating on a lymphocyte population or entire leukocytes, using scattergrams (additional information on methods is available in the online supplement).

Statistical Analysis

Data are presented as the means ± standard errors of the mean for the mice or experiments indicated. The statistical significance of the differences between various treatment groups was assessed with the Student *t* test. $P < 0.05$ was considered significant.

RESULTS

IL-33 Promotes Th2-Type Sensitization to an Innocuous Antigen

To examine the effects of IL-1 family cytokines on the development and differentiation of antigen-specific CD4$^+$ T cells in the airways, we intranasally exposed naive mice to endotoxin-free

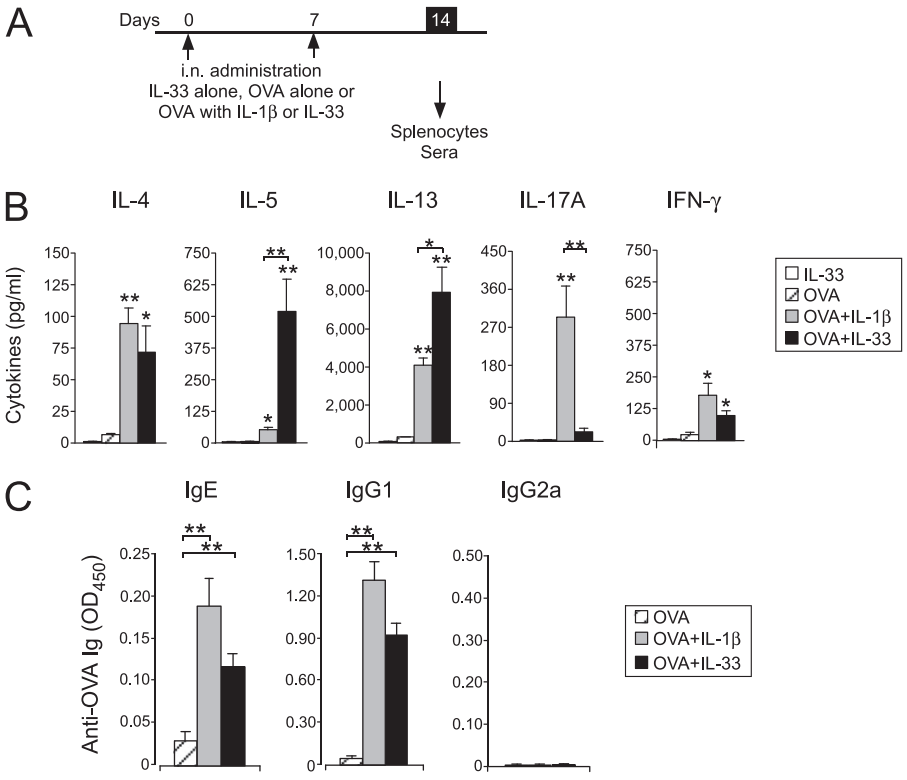

Figure 1. IL-33 promotes Th2-type airway sensitization. (*A*) A schematic representation of a mouse airway sensitization model. On Days 0 and 7, naive BALB/c mice were intranasally (i.n.) exposed to 100 μg ovalbumin (OVA), with or without 100 ng IL-1β or IL-33. On Day 14, spleens and plasma were collected for *in vitro* culture and analyses of the anti-OVA antibodies levels, respectively. (*B*) Day 14 splenocytes were cultured with OVA for 4 days, and the levels of cytokines in the supernatants were measured according to ELISA. (*C*) Plasma antibody levels were measured according to ELISA. Results are presented as means ± SEMs ($n = 4$–5 in each group). *$P < 0.05$ and **$P < 0.01$, compared with mice previously exposed to OVA alone or between the groups indicated by *horizontal lines*. OD_{450} = optical density at 450 nm.

OVA, with or without cytokines (Figure 1A). No adjuvants, such as aluminum hydroxides (alum), were used in these experiments. As previously reported (5), exposure to endotoxin-free OVA alone did not sensitize the mice, and the splenocytes from these animals produced either no or minimal cytokines when they were restimulated with OVA *in vitro* (Figure 1B). In contrast, splenocytes from mice that had been exposed to OVA + IL-33 produced significant amounts of IL-4, IL-5, and IL-13, upon restimulation with OVA. Splenocytes from mice previously exposed to OVA + IL-1β produced amounts of IL-4 roughly comparable to those from mice exposed to OVA + IL-33. On the other hand, mice exposed to OVA + IL-1β produced significantly less IL-5 and IL-13, but more IL-17A, compared with mice exposed to OVA + IL-33 ($P < 0.05$ and $P < 0.01$, respectively).

Airway exposure to OVA alone induced a minimal antibody response (Figure 1C). In contrast, significant increases in the levels of anti-OVA IgE and IgG1 antibodies were observed in mice exposed to OVA along with IL-33 or IL-1β. No or little production of anti-OVA IgG2a antibody was observed. The antibody responses were abolished in *Rag1*$^{-/-}$ mice (Figure E1A in the online supplement). These findings suggest that the IL-1 family of cytokines, specifically IL-33 and IL-1β, induces systemic T-cell and B-cell immune responses to OVA antigen present in the airways. IL-33 likely promotes a robust Th2-type sensitization, and IL-1β promotes a mixed Th2-type and Th17-type sensitization.

IL-33–Driven Antigen-Specific Th2-Type Immunity Mediates Pathological Changes in the Lungs

To examine the physiological importance of these results, we challenged the animals intranasally with OVA alone 2 weeks after the final exposure to OVA, together with IL-33 or IL-1β (Figure 2A). This time point was selected because IL-33–mediated, as well as IL-1β–mediated, airway inflammation resolves completely in 2 weeks (Figure 2B). Mice previously exposed to IL-33 alone or OVA alone displayed a minimal inflammatory response to OVA challenge (Figure 2B). In contrast, when mice previously exposed to OVA + IL-33 were challenged with OVA, they exhibited a marked increase in eosinophils. When mice previously exposed to OVA + IL-1β were challenged with OVA, they developed modest airway inflammation, with a roughly equal composition of lymphocytes, neutrophils, and eosinophils. Furthermore, significant increases in IL-4, IL-5, and IL-13, but not IFN-γ or IL-17, were observed in the airways of mice that had previously been exposed to OVA + IL-33 and were challenged with OVA (Figure 2C). In contrast, mice previously exposed to OVA + IL-1β showed a modest increase in Th2 cytokines and a marked increase in IL-17. To verify that IL-33 mediates antigen-specific adaptive immune responses to OVA, wild-type (WT) or *Rag1*$^{-/-}$ mice were exposed to OVA + IL-33. Unlike WT mice, *Rag1*$^{-/-}$ mice failed to develop airway eosinophilia, and showed no increase in Th2 cytokines in bronchoalveolar lavage (BAL) fluid when they were challenged with OVA (Figures E1B and E1C). A modest increase in neutrophils was observed in the BAL fluid of OVA-challenged *Rag1*$^{-/-}$ mice.

Histologic changes in the lungs were analyzed by periodic acid-Schiff staining. Mucus production in the airway epithelium was observed in mice previously exposed to OVA + IL-33, and then challenged with OVA (Figure 3A). In contrast, no or only minimal mucus production was observed in mice previously exposed to IL-33 alone, OVA alone, or OVA + IL-1β, and subsequently challenged with OVA (Figure 3A and E2). In addition, mice previously exposed to OVA + IL-33 and then challenged with OVA developed AHR to inhaled methacholine, as shown by whole-body plethysmography (Figure 3B, *left*) and direct assessment in intubated and ventilated animals (Figure 3B, *right*). Mice previously exposed to OVA alone did not develop AHR, compared with mice previously exposed to PBS (Figure E2). Collectively, these findings indicate that the airway exposure of naive animals to IL-33 induces a Th2-type sensitization and memory response to an inhaled innocuous antigen (i.e., OVA), resulting in asthma-like airway pathology upon re-exposure to the same antigen.

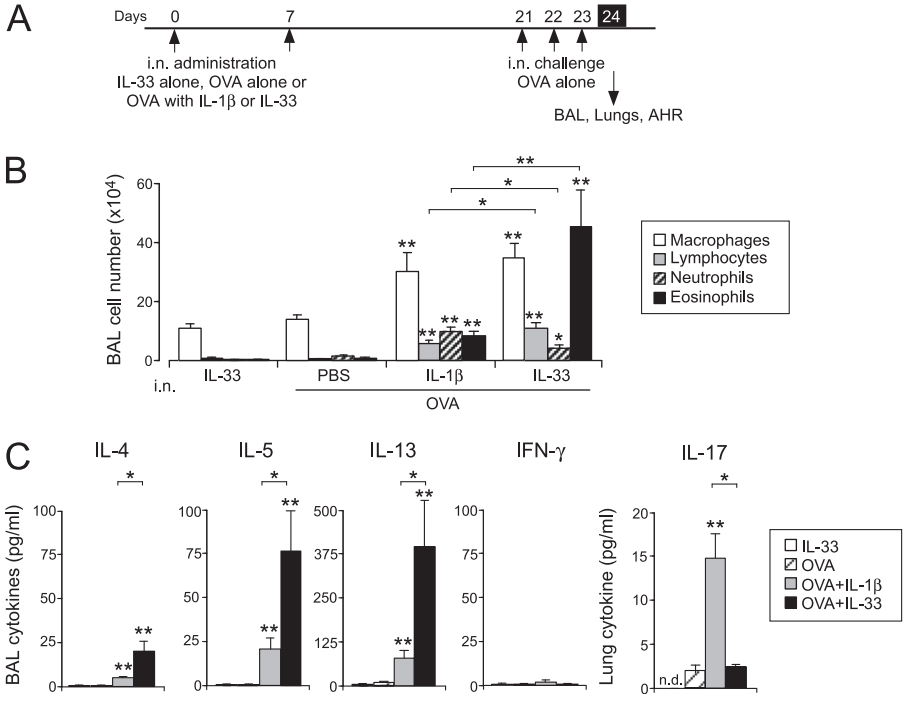

Figure 2. Airway inflammatory responses in mice challenged with ovalbumin (OVA). (*A*) A schematic representation of the challenge protocol. Mice were intranasally exposed to OVA with or without IL-1β or IL-33, as described in Figure 1A, and then intranasally challenged with 100 μg OVA alone on Days 21, 22, and 23. On Day 24, airway hyperresponsiveness (AHR) was assessed, and bronchoalveolar lavage (BAL) and lung specimens were collected. (*B*) BAL fluids were analyzed for numbers of inflammatory cells. (*C*) The levels of cytokines in the supernatants of BAL fluids and lung homogenates were measured according to ELISA. Results are presented as means ± SEMs (n = 4–5 in each group). *$P < 0.05$ and **$P < 0.01$, compared with mice previously exposed to OVA alone or between the groups indicated by *horizontal lines*.

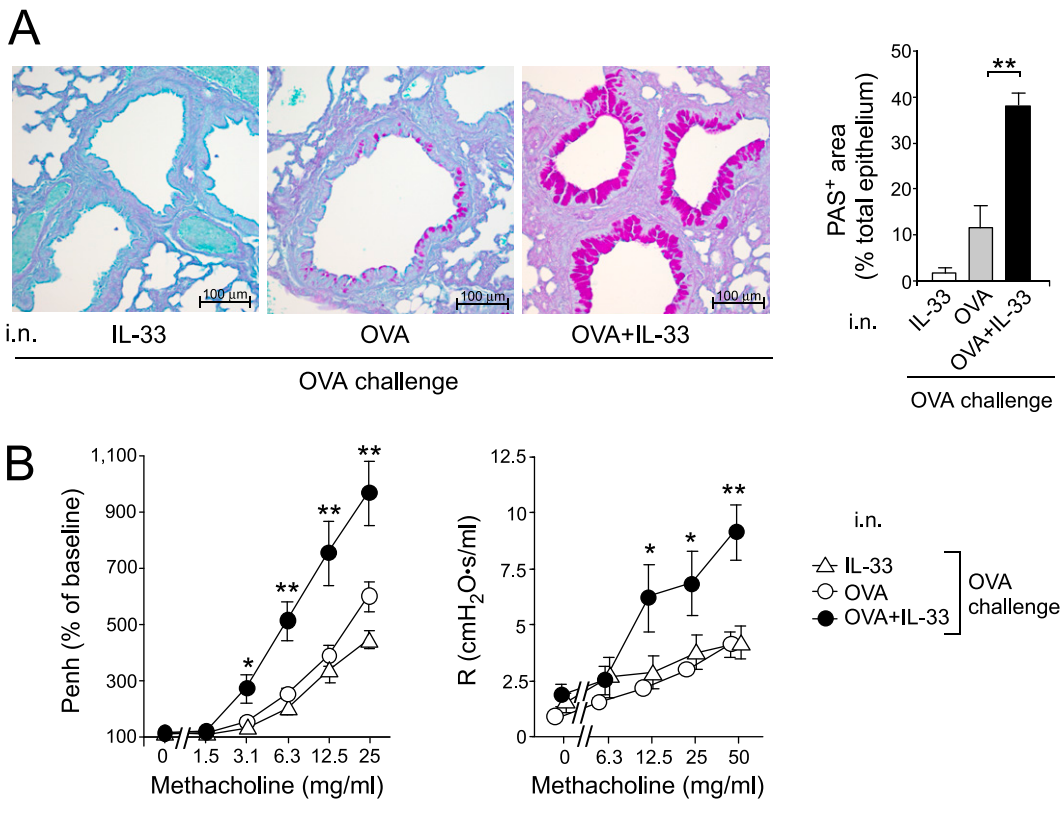

Figure 3. Airway mucus production and airway reactivity in mice challenged with ovalbumin (OVA). Mice were exposed to OVA with or without IL-33, as shown in Figure 2A. On Day 24, mucus production and AHR were analyzed. (*A*) *Left*: Photomicrographs of periodic acid-Schiff (PAS)-stained lung-tissue specimens. Original magnification, ×160. *Right*: PAS-positive areas were determined in at least 10 randomly selected airway sections per lung section, and are expressed as percentages of epithelium. Results are presented as means ± SEMs (n = 5–6 mice in each group). $^{**}P < 0.01$, compared with mice exposed to OVA alone. (*B*) AHR to methacholine was examined by whole-body plethysmography (*left*) and a forced oscillation technique (*right*). Results are presented as a percentage of the baseline value (before PBS or methacholine challenge) and as means ± SEMs (n = 5–6 mice in each group). $^{*}P < 0.05$ and $^{**}P < 0.01$, compared with mice exposed to OVA alone. Penh = enhanced pause; R = resistance.

IL-33 Induces Long-Term Memory Responses

Differentiated $CD4^+$ T-cell populations exhibit some plasticity, and can alter the range of cytokines they produce under different conditions (20). Therefore, to examine whether IL-33 induces a long-term memory response, mice were exposed intranasally to OVA + IL-33, and the kinetics of antibody responses were examined for up to 70 days, without any booster treatment during the waiting period. The serum levels of anti-OVA IgE antibodies continued to increase for 70 days in mice exposed to OVA + IL-33. No significant increase in anti-OVA IgE was observed in mice exposed to OVA alone (Figure 4B). Similarly, the levels of the IgG1 and IgG2a subclasses of anti-OVA antibodies tended to increase for up to 70 days in mice exposed to OVA + IL-33. The serum levels of anti-OVA IgE antibodies slightly increased after nasal antigen challenge, whereas IgG1 and IgG2a antibody levels decreased (Figure 4B). When these mice were subsequently challenged intranasally with OVA alone on Days 71 through 73, they developed a marked increase in airway eosinophilia, as well as a significant increase in lymphocytes and neutrophils (Figure 4C). BAL fluid and lung analyses revealed increased levels of IL-5 and IL-13, but not IFN-γ or IL-17A, in mice previously exposed to OVA + IL-33 and challenged with OVA (Figure 4D).

IL-33 Induces the Endogenous Expression of IL-4 in $CD4^+$ T Cells in Naive Mice

Traditionally, IL-4 has been viewed as the keystone for the differentiation of Th2 cells *in vitro* (21, 22). Therefore, to investigate immunological mechanisms, we hypothesized that IL-4 may be expressed early during the sensitization process induced by IL-33. To examine the real-time *in vivo* expression of IL-4, we used mice with a bicistronic IL-4/internal ribosome entry site/enhanced green fluorescent protein (eGFP) gene knocked into the IL-4 locus (4 get) (23). In these mice, the cells that activate the *Il4* locus accumulate eGFP in their cytoplasm (23). When naive 4 get mice were exposed to OVA alone or IL-33 alone, no expression of IL-4eGFP in the $CD3^+CD4^+$ or $CD3^+CD4^-$ population in MLN cells was evident (Figure 5A). In contrast, when mice were exposed to OVA + IL-33, a proportion of $CD4^+$ T cells expressed IL-4eGFP (Figure 5A). No expression of IL-4eGFP was observed in the $CD4^-$ population. An increased expression of IL-4eGFP in $CD4^+$ T cells was also observed in mice exposed to OVA with IL-1β. Quantitatively, mice exposed to OVA + IL-33 or OVA + IL-1β showed roughly comparable numbers of $CD4^+$ T cells expressing IL-4 (i.e., $eGFP^+CD3^+CD4^+$) (Figure 5B). The kinetics analysis showed that the increase in $eGFP^+$ cells is observed mainly in the $CD3^+CD4^+$ cell population (Figure 5C). Furthermore, in this population, robust IL-4eGFP expression occurred between 48 and 96 hours after exposure to OVA + IL-33. Our analysis of IL-4eGFP expression was extended to the $CD3^+CD4^-$ lymphocyte population and $CD3^-$ leukocyte population. However, no significant increase in IL-4eGFP expression was observed for up to 96 hours in these populations (Figure 5C, *middle* and *right*).

To examine whether IL-33 can sensitize animals to various other innocuous airborne antigens, we exposed naive 4 get mice intranasally to the experimental antigen keyhole limpet hemocyanin (KLH) (*Megathura crenulata*) or the natural allergen short

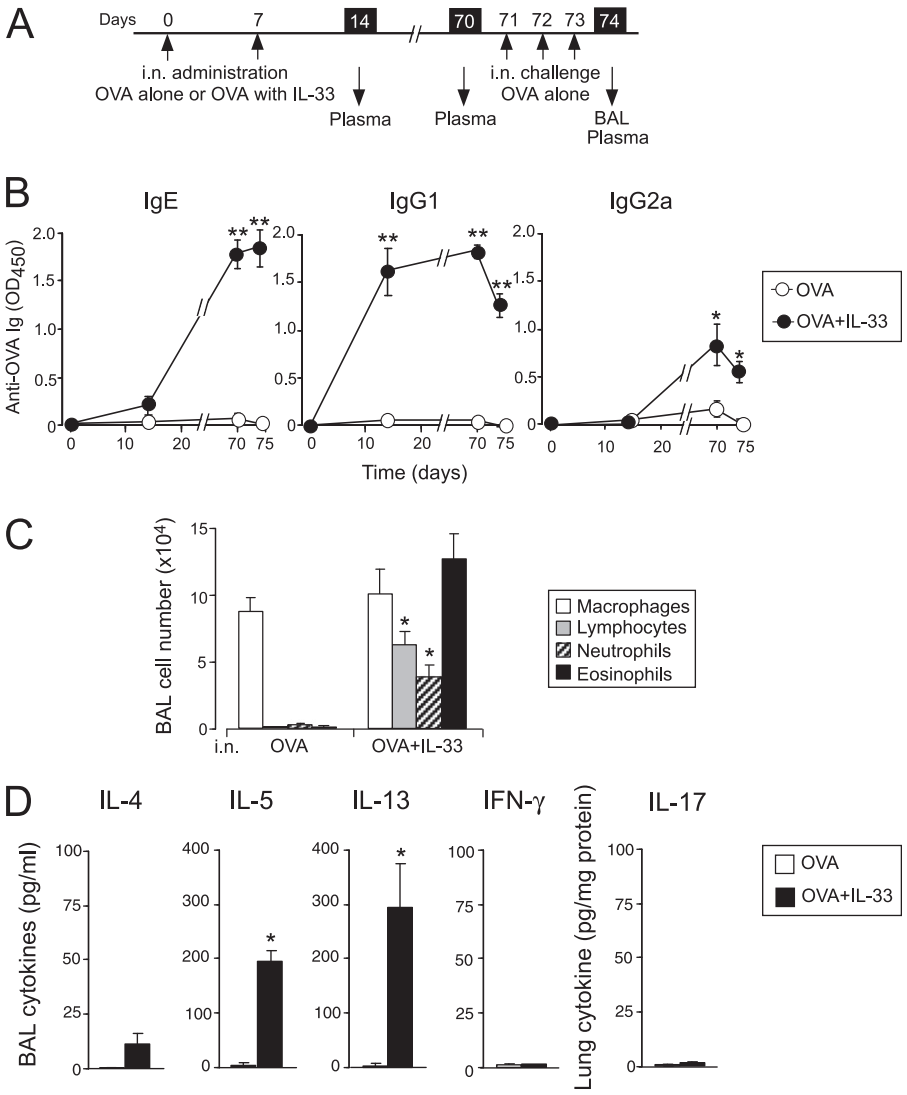

Figure 4. IL-33 promotes long-term sensitization to ovalbumin (OVA). (*A*) A schematic representation of the mouse airway sensitization model. On Days 0 and 7, naive BALB/c mice were intranasally exposed to 100 μg OVA, with or without 100 ng IL-33. Mice were intranasally challenged with 100 μg OVA alone on Days 71, 72, and 73. On Day 74, BAL and lung specimens were collected. Plasma specimens were collected on Days 14, 70, and 74 for analyses of antibody levels. (*B*) The kinetics of the production of the anti-OVA IgE, IgG1, and IgG2a antibodies was analyzed. (*C*) BAL fluids were analyzed for the number of inflammatory cells. (*D*) The levels of cytokines in the supernatant of BAL fluids and lung homogenates were measured according to ELISA. Results are presented as means ± SEMs ($n = 4$–5 in each group). *$P <$ 0.05 and **$P < 0.01$, compared with mice exposed to OVA alone.

ragweed (SRW) extract, with or without IL-33. Exposure to KLH alone or SRW extract alone without IL-33 did not induce IL-4eGFP expression (Figure 6A). In contrast, when these antigens were administered together with IL-33, a proportion of the $CD4^+$ T cells clearly expressed IL-4eGFP (Figures 6A and 6B). Furthermore, when mice previously exposed intranasally to KLH with IL-33 were subsequently challenged with KLH alone, they developed marked airway eosinophilia (Figure 6C) and increased BAL levels of IL-4, IL-5, and IL-13 (Figure 6D). Similar findings were observed in mice previously exposed to SRW extract with IL-33 and then challenged with SRW extract. Altogether, these findings suggest that IL-33 induces IL-4 expression by $CD4^+$ T cells in naive mice within 4 days of exposure to innocuous airborne antigens.

Endogenous IL-4 Plays a Pivotal Role in the Differentiation of the Antigen-Specific $CD4^+$ T Cells Induced by Airway IL-33

The roles for IL-4 in Th2-associated disease models are controversial (24–26). To investigate the role of endogenous IL-4 in the IL-33–mediated differentiation of Th2 cells *in vivo*, we exposed WT or *Il4*$^{-/-}$ mice intranasally to OVA + IL-33. When stimulated *ex vivo* with OVA antigen, MLN cells from WT mice produced a large quantity of Th2 cytokines, and in particular IL-5 and IL-13 (Figure 7A). MLN cells from *Il4*$^{-/-}$ mice previously exposed to OVA + IL-33 produced no IL-4 and partly decreased amounts of IL-5 and IL-13 (~ 30% decrease), compared with WT mice. Importantly, a significant increase in IL-17A was observed in *Il4*$^{-/-}$ mice. At the single-cell level, a proportion of $CD4^+$ T cells in the MLNs from WT mice previously exposed to OVA + IL-33 expressed IL-5 when they were restimulated *ex vivo* with OVA. No IL-17–producing $CD4^+$ T cells were detectable. In *Il4*$^{-/-}$ mice, an increased proportion of $CD4^+$ T cells produced IL-17A.

When WT mice previously exposed to OVA + IL-33 were challenged with OVA, marked airway eosinophilia was observed (Figure 8A). When *Il4*$^{-/-}$ mice were challenged with OVA, the number of airway eosinophils was significantly decreased by approximately 70%, and a marked increase in neutrophils was observed. Analyses of BAL and lung cytokines revealed a significant decrease in Th2 cytokines and a marked increase in IL-17A in *Il4*$^{-/-}$ mice, compared with WT mice (Figure 8B). Furthermore, the BAL levels of CXCL1 and CCL3 significantly increased and decreased, respectively, in *Il4*$^{-/-}$ mice compared with WT mice ($P < 0.05$). CXCL1 levels positively correlated with the neutrophils in the BAL ($r = 0.860$, $P < 0.001$) and the IL-17 level in the lung homogenate ($r = 0.845$, $P < 0.001$). *Il4*$^{-/-}$ mice previously exposed to OVA + IL-33 developed a slight increase in AHR, compared with mice previously exposed to OVA (*see* Figure E3). These findings suggest that IL-33 drives the Th2-type or

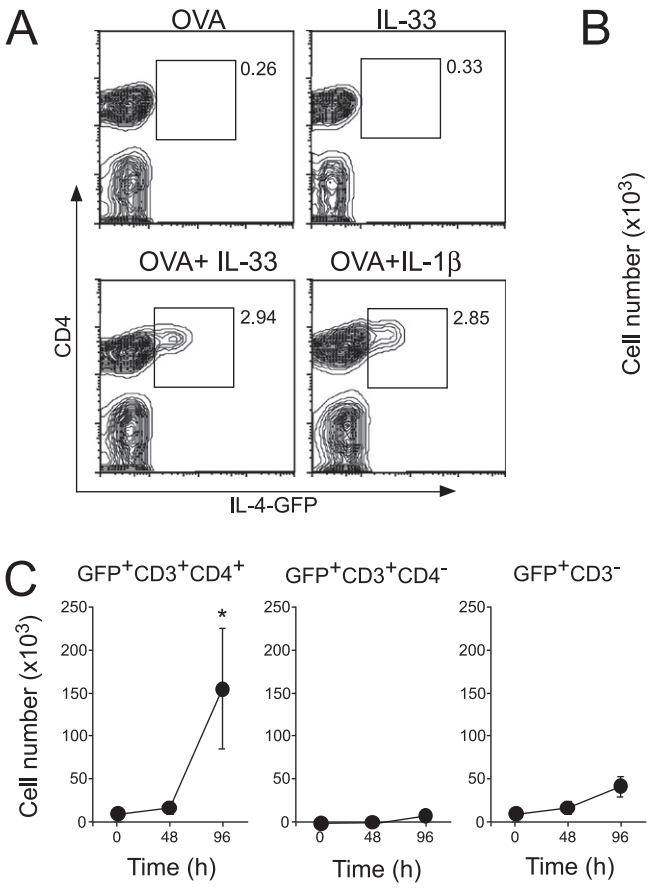

Figure 5. In naive mice, airway exposure to ovalbumin (OVA) antigen with IL-33 induces the expression of IL-4. Naive C.129-*Il4*^{tm1Lky}/J (4 get) mice were intranasally exposed to OVA, with or without IL-33 or IL-1β. Two or 4 days later, mediastinal lymph node (MLN) cells were collected, and the expression of IL-4/enhanced green fluorescent protein (eGFP) was analyzed by FACS with gating on a lymphocytic cell population (*A*) or entire leukocyte population (*B*). (*A*) Representative scattergrams show the expression of CD4 and IL-4eGFP in CD3$^+$ lymphocytic cell populations on Day 4. (*B*) Numbers of IL-4eGFP$^+$CD3$^+$CD4$^+$ cells on Day 4 were calculated, and are presented as means ± SEMs ($n = 4$ in each group). *$P < 0.05$ and **$P < 0.01$, compared with mice exposed to OVA alone. (*C*) Kinetic changes in the numbers of IL-4eGFP$^+$ cells in mice exposed to OVA + IL-33 were analyzed by gating on CD3$^+$CD4$^+$ cells, CD3$^+$CD4$^-$ cells, and CD3$^-$ cells. Data are presented as means ± SEMs ($n = 4$ in each group). *$P < 0.05$, compared with Time 0.

Th17-type polarization of antigen-specific T cells, depending on the availability of endogenous IL-4.

DISCUSSION

The key finding in this study involves the potent capacity of IL-33 to drive antigen-specific Th2-type and perhaps Th17-type CD4$^+$ T cells to react to a variety of innocuous airborne antigens. IL-33 has been considered an "alarm" or endogenous "danger signal" released by tissue cells upon damage (10). Consistent with the tolerogenic environment in the lungs (5), airway exposure to innocuous antigens alone, such as OVA, SRW extract, and KLH, does not induce memory responses (Figures 1 and 6). In contrast, airway exposure to IL-33 facilitates a sensitization to these airborne antigens, and produces asthma-like lung pathological and physiological changes upon re-exposure to the same antigens. The observation that *Rag1*$^{-/-}$ mice failed to develop such antigen-specific Th2 responses (Figure E1) further supports the involvement of the adaptive immune system. The lack of regulatory T cells in *Rag1*$^{-/-}$ mice may explain the modest increase in neutrophils among OVA-challenged *Rag1*$^{-/-}$ mice, because these cell types likely play important roles in the resolution of airway inflammation (27). Thus, IL-33 may be a key factor in the dysregulated response to ubiquitous environmental antigens (1) in patients with allergic airway diseases.

Our findings are consistent with past *in vitro* observations, in which IL-33 induced polarized cytokine production by naive CD4$^+$ T cells. For example, IL-33, with or without IL-2, induced Th2-type cytokine production by naive CD4$^+$ T cells when they were cultured with T-cell receptor–crosslinking antibodies or antigen-presenting cells (APCs) (16, 28). On the other hand, the roles for IL-33 have been controversial in OVA-induced airway inflammation models *in vivo*, in which mice were sensitized by intraperitoneal injections of OVA with alum adjuvant. In several studies, eosinophilic inflammation or AHR was not affected or even exacerbated in mice deficient in the IL-33 receptor, ST2 (29, 30). The BAL levels of Th2-type cytokines and serum OVA-specific IgE production were not affected in *Il33*$^{-/-}$ mice (31). In contrast, in a short-term priming model in which mice were injected intraperitoneally with OVA plus alum once and challenged as early as 8 days after immunization, airway eosinophilia was partly attenuated in *ST2*$^{-/-}$ mice (32). The reasons for the differences in these observations may be attributable to the dominant role of innate immune cells, such as Type 2 ILCs, in the short-term model of airway inflammation (33). In our study, IL-33, when administered intranasally with antigens, effectively sensitized naive animals to OVA and other antigens, without the need for any other adjuvants. Furthermore, IL-33–driven Th2-type T-cell and B-cell memory responses persisted for at least several months. Perhaps the timing/frequency (acute versus chronic) and route (peritoneal cavity versus airway) of antigen exposure comprise key factors in determining the effects of IL-33 on Th2-type adaptive immune responses.

The molecular mechanisms for Th2 cell differentiation have been studied extensively *in vitro* (34). When naive CD4$^+$ T cells are stimulated with cognate antigens, T cells rapidly produce GATA3 and activate signal transducer and activator of transcription (STAT)–5, resulting in T-cell receptor–dependent/IL-4–independent early IL-4 transcription (induction) (35). These T cells then complete their differentiation into Th2 cells by responding to the endogenously produced IL-4 and continued STAT5 activation (polarization). Our experiments suggest that this two-step model may also apply to the *in vivo* airway setting, as summarized in the online supplement (Figure E4). In naive animals, CD4$^+$ T cells in MLN cells began to express IL-4 within 4 days of airway exposure to antigens in the presence of IL-33 (Figures 5 and 6). The airway exposure to IL-33 alone or antigens alone

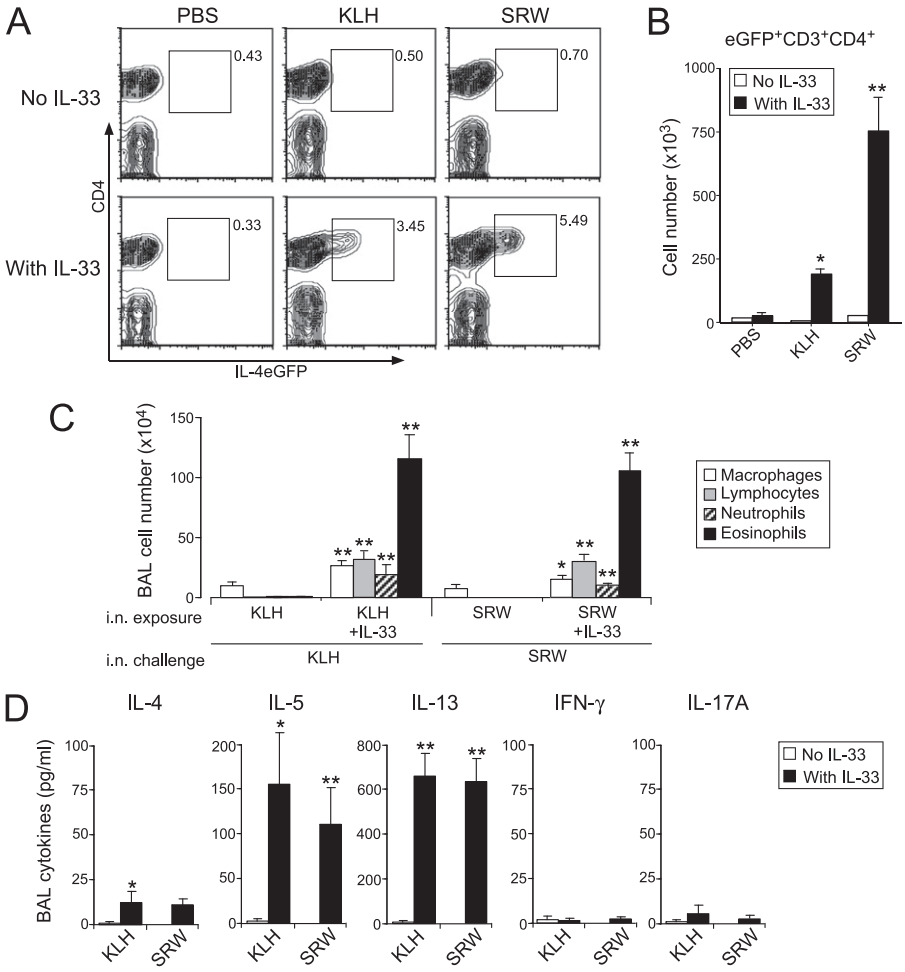

Figure 6. IL-33 induces IL-4 expression by $CD4^+$ T cells in response to various airborne antigens. (*A* and *B*) Naive 4 get mice were intranasally exposed to keyhole limpet hemocyanin (KLH) or short ragweed (SRW) extract, with or without IL-33. Four days later, the expression of IL-4eGFP by $CD3^+$ T cells in MLN cells was analyzed according to FACS. (*A*) Representative scattergrams show the expression of CD4 and IL-4eGFP in $CD3^+$ lymphocytic cell populations. (*B*) The numbers of IL-4eGFP$^+$CD3$^+$CD4$^+$ cells were calculated, and are presented as means ± SEMs ($n = 4$ in each group). *$P < 0.05$ and **$P < 0.01$, compared with mice exposed to antigen alone without IL-33. (*C* and *D*) Naive BALB/c mice were intranasally exposed to KLH or SRW extract with or without IL-33 on Days 0 and 7, and then challenged with the respective antigens on Days 21, 22, and 23. On Day 24, BAL and lung specimens were collected. (*C*) BAL fluids were analyzed for the number of inflammatory cells. (*D*) The levels of cytokines in the supernatants of BAL fluids were measured according to ELISA. Results are presented as means ± SEMs ($n = 4$–5 in each group). *$P <$ 0.05 and **$P < 0.01$, compared with mice previously exposed to antigens alone without IL-33.

failed to induce this early, endogenous IL-4 expression by $CD4^+$ T cells, suggesting that IL-33 facilitates the initial T-cell interaction with APCs. Indeed, IL-33 activates cytokine production and the expression of major histocompatibility complex–II and costimulatory molecules by dendritic cells (16), and IL-33 also directly enhances STAT5 activation in $CD4^+$ T cells (28). We also verified the increased expression of major histocompatibility complex–II by lung dendritic cells when mice were intranasally exposed to IL-33 (Figure E5). Importantly, in the absence of IL-4, the development of antigen-specific Th2 cells decreased, whereas the development of Th17 cells increased (Figures 7 and 8). IL-4 is known to inhibit the development of Th17 cells (36, 37). Therefore,

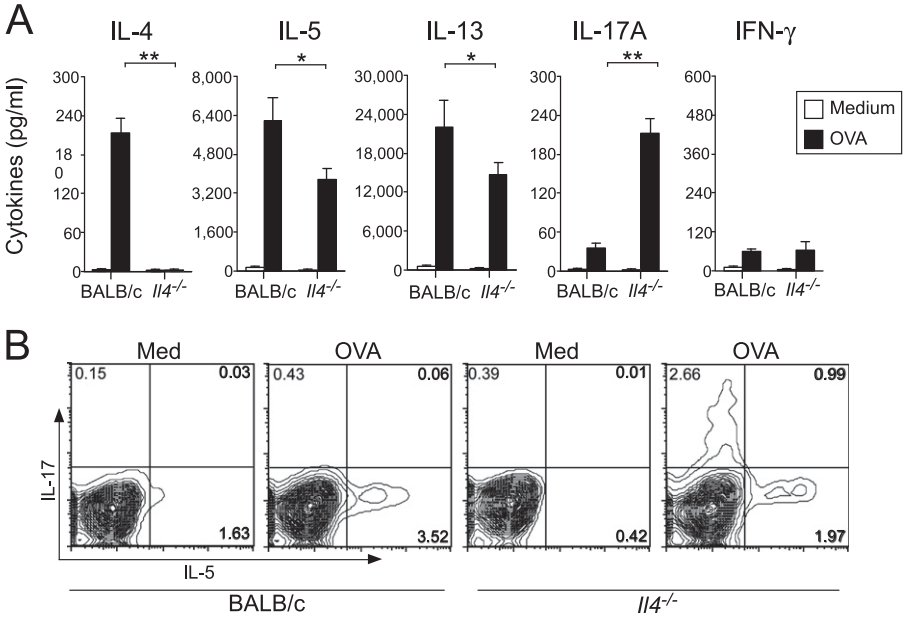

Figure 7. In the absence of IL-4, Th2-type sensitization by IL-33 is partly decreased, and Th17-type sensitization is enhanced. Wild-type BALB/c and *Il4*$^{-/-}$ mice were intranasally exposed to ovalbumin (OVA) + IL-33, as described in Figure 1A. On Day 11, MLN cells were collected and restimulated with or without OVA for 4 days. (*A*) The levels of cytokines in the supernatant were analyzed according to ELISA. Results are presented as means ± SEMs ($n = 4$ in each group). *$P < 0.05$ and **$P < 0.01$, between groups indicated by *horizontal lines*. (*B*) The expression of IL-5 and IL-17A by $CD4^+$ T cells was examined according to flow cytometry. Representative scattergrams are shown from two independent experiments showing similar results. Med, medium.

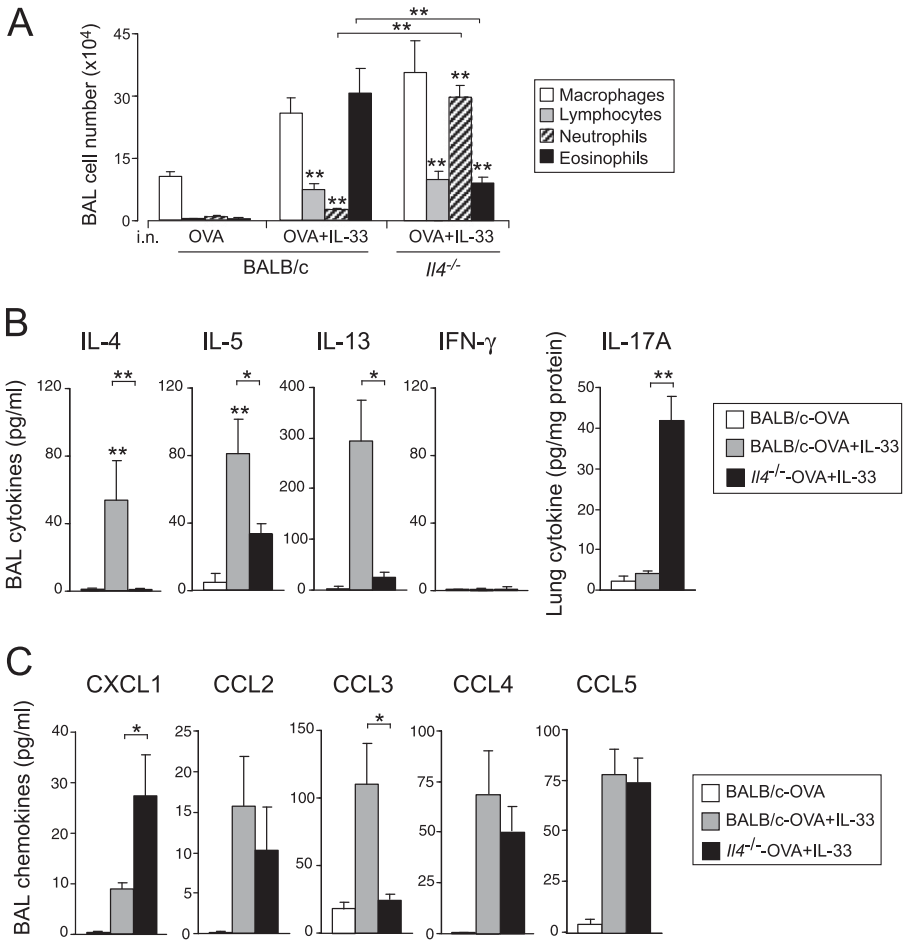

Figure 8. Endogenous IL-4 plays a pivotal role during airway inflammation and cytokine production in mice exposed to ovalbumin (OVA) + IL-33, and then challenged with OVA. Wild-type BALB/c and $Il4^{-/-}$ mice were intranasally exposed to OVA with or without IL-33, as described in Figure 2A, and were then challenged with OVA alone on Days 21, 22, and 23. On Day 24, BAL and lung specimens were collected. (*A*) BAL fluids were analyzed for the number of inflammatory cells. (*B*) The cytokine levels in the supernatant of BAL fluids and lung homogenates were measured according to ELISA. (*C*) The chemokine levels in the BAL fluid supernatant were measured by a Bioplex assay (Bio-Rad, Hercules, CA). Results are presented as means ± SEMs (n = 4–5 in each group). *$P < 0.05$ and **$P < 0.01$, compared with mice previously exposed to OVA alone, or between groups, as indicated by *horizontal lines*.

it is tempting to speculate that endogenous IL-4, which is produced by naive $CD4^+$ T cells when they first encounter antigens in the presence of IL-33, plays a pivotal role in the IL-33–driven development of Th2 cells. Nonetheless, we should not rule out a potential role for other innate immune cells, such as basophils and Type 2 ILCs (11), which may produce cytokines and express accessory molecules in response to IL-33, and thus support the differentiation of Th2 cells (Figure E4).

Interestingly, although both IL-33 and IL-1β induced early IL-4 expression by $CD4^+$ T cells within 4 days of antigen exposure (Figure 5), only IL-33 induced robust Th2 cell differentiation and mediated eosinophilic airway inflammation (Figure 2). In contrast, mice exposed to OVA with IL-1β developed a Th17 response, together with a modest Th2 response (Figure 2). Thus, although IL-1β may mediate the early induction of naive $CD4^+$ T cells toward the Th2 type, it may then redirect them to the Th17 type. Th17 cells express IL-1R and require IL-1 for their differentiation from naive T cells (7). The systemic administration of IL-1β results in enhanced and prolonged T-cell responses of both the Th2 type and Th17 type (38). Furthermore, the receptor for IL-1β, IL-1R1, was expressed early during the differentiation of naive $CD4^+$ T cells to Th17 cells (28). Importantly, in our study, mice exposed to OVA + IL-1β produced levels of anti-OVA IgE and IgG1 antibodies (Figure 1) comparable to those in mice exposed to OVA + IL-33, suggesting that, under certain conditions, antigen-specific IgE antibody can be generated independently of fully committed Th2 cells. These observations may explain the heterogeneity in the immunologic phenotypes of human asthma, which could demonstrate eosinophilic or neutrophilic airway inflammation (39). It is important to determine whether the Th17-like $CD4^+$ T cells induced by an airway administration of OVA + IL-1β derive from the early differentiation of Th2 cells or T follicular helper cells, which are capable of producing IL-4 and promoting the B-cell production of antibodies (40, 41). Accumulating evidence supports a link between IL-33 and human allergic airway diseases. For example, an increased expression of IL-33 in the airway epithelium is observed in patients with allergic rhinitis (42) or severe asthma (43). Single-nucleotide polymorphisms in the IL-33 gene have been associated with asthma, increased eosinophils, and allergic rhinitis (44, 45). In addition, a genome-wide study of more than 10,000 patients with asthma revealed a significant association with both ST2 and IL-33 (46, 47). The demonstration that IL-33 drives the development of antigen-specific Th2 and Th17 cells to innocuous airborne antigens sheds new light on the potential mechanisms for dysregulated immune responses in the airways of patients with asthma and other allergic airway diseases. Because IL-33 (as well as other IL-1 family cytokines) is most likely to be among the earliest molecules that are released during the airway immune response (15), they may provide an important target for both preventing and treating human allergic airway diseases.

Author disclosures are available with the text of this article at www.atsjournals.org.

***Acknowledgments*:** The authors thank Ms. Diane L. Squillace, Ms. Gail M. Kephart, Ms. Kathleen R. Bartemes, and Mr. James E. Tarara for technical assistance, and Ms. LuRaye Eischens for secretarial help.

References

1. Umetsu DT, McIntire JJ, Akbari O, Macaubas C, DeKruyff RH. Asthma: an epidemic of dysregulated immunity. *Nat Immunol* 2002;3: 715–720.

2. Cohn L, Elias JA, Chupp GL. Asthma: mechanisms of disease persistence and progression. *Annu Rev Immunol* 2004;22:789–815.
3. Molet S, Hamid Q, Davoine F, Nutku E, Taha R, Pagé N, Olivenstein R, Elias J, Chakir J. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. *J Allergy Clin Immunol* 2001;108:430–438.
4. Wang YH, Voo KS, Liu B, Chen CY, Uygungil B, Spoede W, Bernstein JA, Huston DP, Liu YJ. A novel subset of CD4(+) T(H)2 memory/effector cells that produce inflammatory IL-17 cytokine and promote the exacerbation of chronic allergic asthma. *J Exp Med* 2010;207:2479–2491.
5. Akbari O, DeKruyff RH, Umetsu DT. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. *Nat Immunol* 2001;2:725–731.
6. Sims JE, Smith DE. The IL-1 family: regulators of immunity. *Nat Rev Immunol* 2010;10:89–102.
7. Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, Kang HS, Ma L, Watowich SS, Jetten AM, Tian Q, *et al*. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* 2009;30:576–587.
8. Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, Zurawski G, Moshrefi M, Qin J, Li X, *et al*. IL-33, an interleukin-1–like cytokine that signals via the IL-1 receptor–related protein ST2 and induces T helper Type 2–associated cytokines. *Immunity* 2005;23:479–490.
9. Moussion C, Ortega N, Girard JP. The IL-1–like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells *in vivo*: a novel "alarmin"? *PLoS ONE* 2008;3:e3331.
10. Lüthi AU, Cullen SP, McNeela EA, Duriez PJ, Afonina IS, Sheridan C, Brumatti G, Taylor RC, Kersse K, Vandenabeele P, *et al*. Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases. *Immunity* 2009;31:84–98.
11. Smith DE. IL-33: a tissue derived cytokine pathway involved in allergic inflammation and asthma. *Clin Exp Allergy* 2010;40:200–208.
12. Saenz SA, Noti M, Artis D. Innate immune cell populations function as initiators and effectors in Th2 cytokine responses. *Trends Immunol* 2010;31:407–413.
13. Barlow JL, McKenzie AN. Nuocytes: expanding the innate cell repertoire in Type-2 immunity. *J Leukoc Biol* 2011;90:867–874.
14. Kim HY, Chang YJ, Subramanian S, Lee HH, Albacker LA, Matangkasombut P, Savage PB, McKenzie AN, Smith DE, Rottman JB, DeKruyff RH, Umetsu DT. Innate lymphoid cells responding to IL-33 mediate airway hyperreactivity independently of adaptive immunity. *J Allergy Clin Immunol* 2012;129:216–227.
15. Bartemes KR, Iijima K, Kobayashi T, Kephart GM, McKenzie AN, Kita H. IL-33–responsive lineage–CD25$^+$ CD44(hi) lymphoid cells mediate innate Type 2 immunity and allergic inflammation in the lungs. *J Immunol* 2012;188:1503–1513.
16. Rank MA, Kobayashi T, Kozaki H, Bartemes KR, Squillace DL, Kita H. IL-33–activated dendritic cells induce an atypical Th2-type response. *J Allergy Clin Immunol* 2009;123:1047–1054.
17. Kobayashi T, Iijima K, Radhakrishnan S, Mehta V, Vassallo R, Lawrence CB, Cyong JC, Pease LR, Oguchi K, Kita H. Asthma-related environmental fungus, *Alternaria*, activates dendritic cells and produces potent Th2 adjuvant activity. *J Immunol* 2009;182:2502–2510.
18. Kobayashi T, Iijima K, Kita H. Marked airway eosinophilia prevents development of airway hyper-responsiveness during an allergic response in IL-5 transgenic mice. *J Immunol* 2003;170:5756–5763.
19. Wagner EM, Jenkins J. Effects of airway distension on leukocyte recruitment in the mouse tracheal microvasculature. *J Appl Physiol* 2007;102:1528–1534.
20. O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4$^+$ T cells. *Science* 2010;327:1098–1102.
21. Le Gros G, Ben-Sasson SZ, Seder R, Finkelman FD, Paul WE. Generation of interleukin 4 (IL-4)–producing cells *in vivo* and *in vitro*: IL-2 and IL-4 are required for *in vitro* generation of IL-4–producing cells. *J Exp Med* 1990;172:921–929.
22. Swain SL, Weinberg AD, English M, Huston G. IL-4 directs the development of Th2-like helper effectors. *J Immunol* 1990;145:3796–3806.
23. Mohrs M, Shinkai K, Mohrs K, Locksley RM. Analysis of Type 2 immunity *in vivo* with a bicistronic IL-4 reporter. *Immunity* 2001;15:303–311.
24. Else KJ, Finkelman FD, Maliszewski CR, Grencis RK. Cytokine-mediated regulation of chronic intestinal helminth infection. *J Exp Med* 1994;179:347–351.
25. Cohn L, Homer RJ, Marinov A, Rankin J, Bottomly K. Induction of airway mucus production by T helper 2 (Th2) cells: a critical role for interleukin 4 in cell recruitment but not mucus production. *J Exp Med* 1997;186:1737–1747.
26. Cohn L, Tepper JS, Bottomly K. IL-4--independent induction of airway hyperresponsiveness by Th2, but not Th1, cells. *J Immunol* 1998;161:3813–3816.
27. D'Alessio FR, Tsushima K, Aggarwal NR, West EE, Willett MH, Britos MF, Pipeling MR, Brower RG, Tuder RM, McDyer JF, *et al.* CD4$^+$CD25$^+$ Foxp3$^+$ Tregs resolve experimental lung injury in mice and are present in humans with acute lung injury. *J Clin Invest* 2009;119:2898–2913.
28. Guo L, Wei G, Zhu J, Liao W, Leonard WJ, Zhao K, Paul W. IL-1 family members and STAT activators induce cytokine production by Th2, Th17, and Th1 cells. *Proc Natl Acad Sci USA* 2009;106:13463–13468.
29. Hoshino K, Kashiwamura S, Kuribayashi K, Kodama T, Tsujimura T, Nakanishi K, Matsuyama T, Takeda K, Akira S. The absence of interleukin 1 receptor–related T1/ST2 does not affect T helper cell Type 2 development and its effector function. *J Exp Med* 1999;190:1541–1548.
30. Mangan NE, Dasvarma A, McKenzie AN, Fallon PG. T1/ST2 expression on Th2 cells negatively regulates allergic pulmonary inflammation. *Eur J Immunol* 2007;37:1302–1312.
31. Oboki K, Ohno T, Kajiwara N, Arae K, Morita H, Ishii A, Nambu A, Abe T, Kiyonari H, Matsumoto K, *et al.* IL-33 is a crucial amplifier of innate rather than acquired immunity. *Proc Natl Acad Sci USA* 2010;107:18581–18586.
32. Kurowska-Stolarska M, Kewin P, Murphy G, Russo RC, Stolarski B, Garcia CC, Komai-Koma M, Pitman N, Li Y, Niedbala W, *et al.* IL-33 induces antigen-specific IL-5$^+$ T cells and promotes allergic-induced airway inflammation independent of IL-4. *J Immunol* 2008;181:4780–4790.
33. Kung TT, Stelts D, Zurcher JA, Jones H, Umland SP, Kreutner W, Egan RW, Chapman RW. Mast cells modulate allergic pulmonary eosinophilia in mice. *Am J Respir Cell Mol Biol* 1995;12:404–409.
34. Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations. *Annu Rev Immunol* 2010;28:445–489.
35. Yamane H, Zhu J, Paul WE. Independent roles for IL-2 and GATA-3 in stimulating naive CD4$^+$ T cells to generate a Th2-inducing cytokine environment. *J Exp Med* 2005;202:793–804.
36. Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q, *et al*. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 2005;6:1133–1141.
37. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT. Interleukin 17–producing CD4$^+$ effector T cells develop via a lineage distinct from the T helper Type 1 and 2 lineages. *Nat Immunol* 2005;6:1123–1132.
38. Ben-Sasson SZ, Hu-Li J, Quiel J, Cauchetaux S, Ratner M, Shapira I, Dinarello CA, Paul WE. IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. *Proc Natl Acad Sci USA* 2009;106:7119–7124.
39. Wenzel SE. Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 2012;18:716–725.
40. King C, Tangye SG, Mackay CR. T follicular helper (TFH) cells in normal and dysregulated immune responses. *Annu Rev Immunol* 2008;26:741–766.
41. Lüthje K, Kallies A, Shimohakamada Y, Belz GT, Light A, Tarlinton DM, Nutt SL. The development and fate of follicular helper T cells defined by an IL-21 reporter mouse. *Nat Immunol* 2012;13:491–498.
42. Haenuki Y, Matsushita K, Futatsugi-Yumikura S, Ishii KJ, Kawagoe T, Imoto Y, Fujieda S, Yasuda M, Hisa Y, Akira S, *et al.* A critical role of IL-33 in experimental allergic rhinitis. *J Allergy Clin Immunol* 2012;130:184.
43. Préfontaine D, Nadigel J, Chouiali F, Audusseau S, Semlali A, Chakir J, Martin JG, Hamid Q. Increased IL-33 expression by epithelial cells in bronchial asthma. *J Allergy Clin Immunol* 2010;125:752–754.
44. Sakashita M, Yoshimoto T, Hirota T, Harada M, Okubo K, Osawa Y, Fujieda S, Nakamura Y, Yasuda K, Nakanishi K, *et al*. Association of serum interleukin-33 level and the interleukin-33 genetic variant with Japanese cedar pollinosis. *Clin Exp Allergy* 2008;38:1875–1881.
45. Gudbjartsson DF, Bjornsdottir US, Halapi E, Helgadottir A, Sulem P, Jonsdottir GM, Thorleifsson G, Helgadottir H, Steinthorsdottir V, Stefansson H, *et al.* Sequence variants affecting eosinophil numbers associate with asthma and myocardial infarction. *Nat Genet* 2009;41:342–347.
46. Moffatt MF, Gut IG, Demenais F, Strachan DP, Bouzigon E, Heath S, von Mutius E, Farrall M, Lathrop M, Cookson WO. GABRIEL

Consortium: a large-scale, consortium-based genomewide association study of asthma. *N Engl J Med* 2010;363:1211–1221.

47. Torgerson DG, Ampleford EJ, Chiu GY, Gauderman WJ, Gignoux CR, Graves PE, Himes BE, Levin AM, Mathias RA, Hancock DB, *et al*. Mexico City Childhood Asthma Study (MCAAS), Children's Health Study (CHS) and HARBORS Study, Genetics of Asthma in Latino Americans (GALA) Study, Study of Genes–Environment and Admixture in Latino Americans (GALA2) and Study of African Americans, Asthma, Genes and Environments (SAGE), Childhood Asthma Research and Education (CARE) Network, Childhood Asthma Management Program (CAMP), Study of Asthma Phenotypes and Pharmacogenomic Interactions by Race–Ethnicity (SAPPHIRE), Genetic Research on Asthma in African Diaspora (GRAAD) Study: meta-analysis of genome-wide association studies of asthma in ethnically diverse North American populations. *Nat Genet* 2011; 43:887–892.