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IL-33 induces Th17-mediated airway inflammation via mast cells in ovalbumin-challenged mice

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Cho KA, Suh JW, Sohn JH, Park JW, Lee H, Kang JL, Woo SY, Cho YJ. IL-33 induces Th17-mediated airway inflammation via mast cells in ovalbumin-challenged mice. *Am J Physiol Lung Cell Mol Physiol* 302: L429–L440, 2012. First published December 16, 2011; doi:10.1152/ajplung.00252.2011.—Allergic asthma is characterized by infiltration of eosinophils, elevated Th2 cytokine levels, airway hyperresponsiveness, and IgE. In addition to eosinophils, mast cells, and basophils, a variety of cytokines are also involved in the development of allergic asthma. The pivotal role of eosinophils in the progression of the disease has been a subject of controversy. To determine the role of eosinophils in the progression of airway inflammation, we sensitized and challenged BALB/c wild-type (WT) mice and eosinophil-deficient Δ dblGATA mice with ovalbumin (OVA) and analyzed different aspects of inflammation. We observed increased eosinophil levels and a Th2-dominant response in OVA-challenged WT mice. In contrast, eosinophil-deficient Δ dblGATA mice displayed an increased proportion of mast cells and a Th17-biased response following OVA inhalation. Notably, the levels of IL-33, an important cytokine responsible for Th2 immune deviation, were not different between WT and eosinophil-deficient mice. We also demonstrated that mast cells induced Th17-differentiation via IL-33/ST2 stimulation in vitro. These results indicate that eosinophils are not essential for the development of allergic asthma and that mast cells can skew the immune reaction predominantly toward Th17 responses via IL-33 stimulation.

ASTHMA IS RECOGNIZED AS A heterogeneous disorder, involving the activation of both innate and adaptive host defense mechanisms. The activation of local innate host defense pathways in the lungs has been associated with the development, prolongation, and severity of asthma (36, 47). The phenotypic heterogeneity of asthma implies the possible existence of a range of mechanisms that regulate asthma pathogenesis. Airway inflammation in allergic asthma is characterized by increased numbers of eosinophils and mast cells, hypersecretion of mucus, and activation of T cells. Classically, the recruitment of Th2 lymphocytes that secrete IL-4, IL-5, and IL-13, accompanied by the recruitment of eosinophils to the airways, has been considered integral to the pathogenesis of asthma (14, 43).

Despite the strength of the Th2-eosinophil paradigm, some features of human asthma cannot be explained by this mech-

anism alone. For instance, neutrophilic inflammation is commonly observed in bronchial biopsies of asthmatic patients, and the degree of neutrophilia significantly correlates with asthma severity (44). Previous studies have demonstrated that 50% of asthma cases are noneosinophilic; in these cases, the predominant mechanism is CXCL8-mediated neutrophil inflammation in the airway (7). Neutrophil infiltration into the airway can be induced by IL-1, TNF, and IL-17 (8, 18, 20). It has been shown, using ovalbumin (OVA)-challenged OT-II mice, that mast cell-derived TNF promotes Th17 cell-dependent neutrophil recruitment in airway inflammation (27). IL-33 plays a role in immune responses mediated by both mast cells and eosinophils. However, the possible independent effects of IL-33 on mast cells and on eosinophils in airway inflammation have yet to be clearly elucidated.

IL-33 is a recently described member of the IL-1 family, and induces signaling via its heterodimeric receptor, ST2, and the IL-1R accessory protein (IL-1RAP) (3, 6). ST2 is abundantly expressed on the surfaces of mast cells and Th2 cells, and, like IL-33, is critical for the Th2 response (37). ST2 expression has also been demonstrated on basophils and dendritic cells (30, 32, 39). IL-33 is host-protective against helminth infection and reduces atherosclerosis by promoting Th2-type immune responses. However, IL-33 can also promote the pathogenesis of asthma by inducing the production of Th2 cells, as is evident from studies in which blocking IL-33 signaling resulted in the suppression of the asthmatic response (12, 21).

In the present study, we investigated eosinophil-independent mechanisms of the development of airway inflammation in response to OVA challenge, using eosinophil-deficient and wild-type (WT) BALB/c mice (41). Our results provide information on the possible effects of IL-33 on noneosinophilic asthma and on the relationship between mast cells and Th17 cells in airway inflammation.

MATERIALS AND METHODS

Mice. WT BALB/c mice were purchased from Daehan Biolink (Eumsung, Korea), and Δ dblGATA mice (C.Cg-*Gata1*^{tm6Sho}/J) were purchased from Jackson Laboratory (Bar Harbor, ME). All procedures were approved by the Animal Care and Use Committee of the Ewha Womans University, School of Medicine (ESM 09-0116). WT and mutant Δ dblGATA alleles were distinguished via PCR, and *NotI* digestion of the PCR product yielded fragments of 300 and 200 bp (48).

Allergic asthma model. Mice were immunized via intraperitoneal injection on days 0 and 5 with 10 μ g OVA (Grade II; Sigma, St.

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Louis, MO) complexed with 1 mg aluminum hydroxide (Sigma) at a volume of 0.2 ml per mouse. On days 12 to 15 postimmunization, mice were exposed for 30 min per day to an aerosol of either 1% OVA in phosphate-buffered saline (PBS), or PBS only, via a nebulizer (Medel, Vlaeberg, South Africa). The mice were euthanized 24 h after the final aerosol challenge for the collection of bronchoalveolar lavage (BAL) fluid and lung tissues.

Determination of AHR. Airway responsiveness (AHR) was determined from peak pulmonary resistance ($\text{cmH}_2\text{O}\cdot\text{ml}^{-1}\cdot\text{s}^{-1}$) and compliance by use of the FlexiVent system (10 ml/kg tidal volume at 160 breaths/min, SCIREQ, Montreal, Quebec, Canada) after delivery of aerosolized methacholine (Sigma). Methacholine aerosol (3.1–50 mg/ml) was administered for 10 s, after which airway resistance was continuously monitored and recorded.

BAL. After the mice were euthanized with a mixture of Zoletil (30 mg/kg, Virbac Laboratories, Carros, France) and Rompun (10 mg/kg, Bayer, Leverkusen, Germany), the trachea was cannulated with a venipuncture needle. BAL was subsequently conducted via the instillation of 0.5×4 ml PBS into the lungs, along with gentle massaging to maximize cell recovery. BAL cells were pelleted via centrifugation and counted by use of a hemocytometer. Differential leukocyte counts were obtained from a cytospin slide preparation stained with Diff-Quick or Giemsa. For eosinophil-specific staining, an improved version of the Randolph staining method was used (4).

Histopathology. Lung tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Fixed tissues were cut into sections of thickness 5 μm , placed on glass slides, and deparaffinized. Sections were stained with hematoxylin and eosin to detect cellular infiltration, with periodic acid-Schiff staining to detect mucus deposition, and with Sirius Red stain to observe collagen accumulation. To analyze the deposition of mucus and collagen, tissue sections of each mouse were analyzed using Image Analysis Software (analysis FIVE, Olympus, Tokyo, Japan).

Western blot analysis. Lung tissues were homogenized with lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris, pH 7.5) containing protease inhibitors. Appropriate volumes of the homogenates, each containing 15 μg proteins, were separated by 12% SDS-PAGE and then transferred to polyvinylidene fluoride membranes. The blots were probed with primary antibodies against IL-33 (ALX-210-449, Alexis, Lausen, Switzerland), ST2 (ab25877, rabbit polyclonal; Abcam, Cambridge, MA), and β -actin (H11, mouse monoclonal, Applied Biological Materials, Richmond, BC, Canada). Following incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG secondary antibodies, the signals were visualized via enhanced chemiluminescence by using a Thermo ECL kit (Thermo Fisher Scientific, Waltham, MA) in accordance with the manufacturer's instructions.

ELISA. Levels of IFN- γ , IL-4, IL-5 (BD OptEIA, BD Biosciences Pharmingen, San Diego, CA), IL-13 (R&D Systems, Minneapolis, MN), thymic stromal lymphopoietin (TSLP), and IL-17 (BioLegend, San Diego, CA) in BAL fluid were measured by the ELISA kits indicated above in accordance with the manufacturers' instructions.

Flow cytometry. To compare cellular profiles after the induction of airway inflammation, BAL cells from WT or $\Delta\text{dblGATA}$ mice were stained and analyzed on a FACSCalibur system (Becton Dickinson, Franklin Lakes, NJ). The antibodies used for flow cytometry are as follows: FITC anti-mouse Ly-6G/Ly-6C (BioLegend), FITC anti-mouse CD49b (BioLegend), PE anti-mouse Fc ϵ RI α (BioLegend), APC anti-mouse IFN- γ (BioLegend), PE anti-mouse CD4 (BioLegend), PerCP anti-mouse CD4 (BioLegend), PE anti-mouse IL-4 (BioLegend), PE anti-mouse IL-9 (BioLegend), PE anti-mouse IL-17A (BioLegend), Alexa Fluor 488 anti-mouse FoxP3 (BioLegend), PE anti-mouse CD117 (BioLegend), FITC anti-mouse IL-1 β (eBioscience, San Diego, CA), FITC anti-mouse IL-6 (eBioscience), FITC anti-mouse ST2 (MD Bioscience, St. Paul, MN), PE anti-mouse CD19 (BioLegend), PE anti-mouse Siglec-F (BD Biosciences Pharmingen), FITC anti-mouse NKp46 (BioLegend).

Mast cell isolation and IL-33 stimulation. Peritoneal mast cells were isolated by peritoneal lavage in euthanized mice (11, 17). In brief, after the mice were anesthetized, 10 ml Tyrode buffer B (137 mM NaCl, 5.3 mM glucose, 12 mM NaHCO_3 , 2.7 mM KCl, 0.3 mM NaH_2PO_4 , pH 7.4) containing 0.1% gelatin (Sigma) was injected into the peritoneal cavity and the abdomen was gently massaged for ~ 90 s. The peritoneal cavity was carefully opened and the fluid containing peritoneal cells was aspirated with a Pasteur pipette. Peritoneal cells were then sedimented at 150 g for 10 min at room temperature and resuspended in Tyrode buffer B. One milliliter of resuspended peritoneal cells was layered over 2 ml of 22.5% wt/vol metrizamide (density 1.120 g/ml, Sigma) and centrifuged at room temperature for 15 min at 400 g. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl_2 , 1 mM MgCl_2 , 5.6 mM glucose, pH 7.4) containing 0.1% BSA. Mast cell preparations were $\sim 95\%$ pure as assessed by toluidine blue staining. For IL-33 stimulation, collected and pooled mast cells (10^5) were cultured in the presence or absence of IL-33 (50 ng/ml, R&D Systems). Additionally, mast cells were pretreated with ST2 blocking Ab (10 $\mu\text{g}/\text{ml}$, R&D Systems) for 1 h to block ST2 receptor-IL-33 ligation.

Th17 cell differentiation. Naive CD4^+ T cells were purified from mouse spleens via magnetic isolation (Miltenyi Biotec, Bergisch Gladbach, Germany). For the preparation of spleen cell suspensions, spleens from 8-wk-old female BALB/c mice were removed and minced with a nylon mesh (70- μm pore size). After the cells were pelleted, erythrocytes were lysed with hypotonic buffer (0.15 M NH_4Cl , 10 mM KHCO_3 , 0.1 mM Na_2EDTA). Cells were washed in PBS and incubated with anti-CD4 antibody for 15 min at 4°C . Cells were then conducted onto a magnetic separator to isolate CD4^+ cells and were collected with positive selection. Cells were seeded at a density of 3×10^5 per well in 96-well plates and cultured in DMEM containing 10% FBS. For Th17 differentiation, cells were stimulated with TGF- β 2 (2 ng/ml, R&D Systems), IL-6 (50 ng/ml, BioLegend), and anti-CD3 (5 $\mu\text{g}/\text{ml}$, BioLegend). To observe Th17 differentiation enhancements via mast cell activation, the cells were cocultured with mast cells (10^5) in the presence or absence of IL-33 (50 ng/ml, R&D Systems). Cells pretreated for 1 h with ST2 blocking antibody (10 $\mu\text{g}/\text{ml}$, R&D Systems, no. 1004-MR) were used in coculture to study the effect of blocking ST2 receptor-IL-33 ligation in mast cell interactions with helper T cells. In addition, during the coculturing of CD4^+ T cells and mast cells, cells were pretreated for 1 h with blocking antibodies against either IL-1 β (1 $\mu\text{g}/\text{ml}$, R&D Systems, no. AB401-NA) or IL-6 (1 $\mu\text{g}/\text{ml}$, R&D Systems, no. AB-406-NA) to ascertain whether IL-33-activated mast cells affect Th17 cell differentiation via the secretion of IL-1 β or IL-6. Isotype antibodies used for blocking were as follows: normal goat IgG for IL-1 β and IL-6 (1 $\mu\text{g}/\text{ml}$, R&D Systems, no. AB-108-C), normal human IgG for ST2 (10 $\mu\text{g}/\text{ml}$, R&D Systems, no. 1-001-A).

Statistical analysis. Data are expressed as means $\pm$ SE. We used SPSS (v11.5) to examine the kurtosis and skewness of our collected data (by choosing "Descriptive Statistics") and analyzed whether our data were normally distributed or not. Skewness refers to the "lean" of a distribution; a positive skew indicates a longer tail to the right than to the left, and a negative skew indicates a longer tail to the left than to the right. Kurtosis refers to how "flat" a distribution is. In general, if kurtosis and skewness are not between -2 and $+2$, the data are too far away from a normal distribution and need to be corrected before tests that have assumptions of normality are applied. For normal distribution data, we used unpaired *t*-test to analyze the results for significant differences (at $P < 0.05$). Otherwise, nonparametric Mann-Whitney tests were applied to analyze the results for significant differences (at $P < 0.05$) with use of GraphPad Prism software (GraphPad Software, San Diego, CA).

RESULTS

OVA-induced airway inflammation in WT and eosinophil-deficient mice. We used OVA in a standard sensitization protocol in WT and Δ dblGATA mice to induce allergic airway inflammation. Prior to inducing airway inflammation, we confirmed the targeted depletion of the palindromic GATA-site as previously described (48). Twenty-four hours after the last challenge via the inhalation of 1% OVA aerosol solution, mice were subjected to BAL for airway inflammation analysis. OVA-challenged WT and Δ dblGATA mice revealed elevated airway hyperreactivities (Fig. 1A) and histological structure changes (Fig. 1B, *top*). These structural changes were accompanied by mucus production, (Fig. 1B, *middle*) and collagen accumulation in both WT and Δ dblGATA mice (Fig. 1B, *bottom*). However, the degrees of AHR or inflammation were more intense in OVA-challenged WT mice than in Δ dblGATA mice (Fig. 1C).

Next, we compared the cellular profiles of infiltrating cells collected from the BAL fluid of OVA-challenged WT and Δ dblGATA mice to determine whether eosinophils might account for the observed airway inflammation and mucus secretion. To visually assess cell morphology, BAL cells were collected and stained by the Giemsa or Diff-Quick methods. As shown in Fig. 2, BAL fluid from OVA-challenged WT BALB/c mice showed higher total cell numbers, particularly in the proportion of eosinophils, which are visible as cells containing red cytoplasmic granules (as previously demonstrated in Fig. 1D). Notably, in a comparison of the numbers of infiltrating cells in BAL collected from OVA-challenged BALB/c and Δ dblGATA mice, Δ dblGATA mice exhibited lower cell numbers in the BAL fluid than did WT mice. This might be attributable to the absence of eosinophils in Δ dblGATA mice, resulting in a compensatory increase in the proportions of other cells.

In particular, in OVA-challenged WT mice, eosinophils were found to accumulate in the parenchyma and bronchial wall (Fig. 1E). To differentiate eosinophils from other polymorphonuclear cells, we conducted an improved version of Randolph staining (4), which is specific to eosinophilic granules and is visible as red dots (Fig. 1F). In Δ dblGATA mice, other cells, particularly neutrophils, were observed in increased numbers in BAL, rather than eosinophils. These data show that eosinophils are dispensable in the induction of lung inflammation, mucus production, and BAL cell increases associated with OVA-induced airway inflammation. In addition, we also observed an increase in airway resistance with increasing numbers of infiltrating cells in the sensitized lungs (compare Figs. 1A and 2A).

Differential cellular profiles with eosinophil deficiency in AHR. To determine whether the profile of airway-infiltrating cells in Δ dblGATA mice with AHR was eosinophil deficient but otherwise similar that of WT, we analyzed cell surface expression molecules to characterize BAL cells. As shown in Fig. 3, WT mice exposed to OVA exhibited an increased proportion of eosinophils (Siglec F⁺ cells), which correlated well with the results shown in Fig. 1, C–E. In Δ dblGATA mice, as described previously, few eosinophils exist in BAL even after OVA challenge to the lungs. Instead the proportions of mast cells (Fc ϵ RI⁺ and c-kit⁺), neutrophils (Gr-1⁺), and

Th17 (CD4⁺ and IL-17⁺) cells were higher in Δ dblGATA mice with AHR than in WT mice (Fig. 3).

Cytokine secretion upon OVA sensitization and immunization. To analyze and compare the type of immune response elicited by OVA challenge, we used intracellular cytokine staining of BAL cells, as well as ELISA, to measure the concentration of the Th1 cytokine IFN- γ , Th2 cytokines IL-4, IL-5, IL-13, and TSLP, and the Th17 cytokine IL-17 in BAL fluid (Fig. 4). On comparing intracellular cytokine expression levels between CD4⁺ cells and BAL cells by flow cytometric analysis, we found that the proportion of IFN- γ ⁺ cells was lower, and that of Foxp3⁺ cells was higher, in both groups of mice after OVA challenge. The proportion of IL-4⁺ cells was higher only in OVA-challenged WT BALB/c mice. Notably, the proportion of IL-17⁺ cells was highly increased in OVA-challenged Δ dblGATA mice (Fig. 4A). We also determined, using ELISA, that OVA-challenged WT BALB/c mice exhibited increased levels of Th2 cytokines, but OVA-challenged Δ dblGATA mice showed an increase in IL-17 levels, as well as a significant increase in the level of IL-4 and IL-5 (Fig. 4B). Our results indicate that airway inflammation induced by OVA aerosol challenge results in prominent Th2 responses but not Th1 responses (IFN- γ) in WT mice, whereas eosinophil-deficient mice display Th17-dominant immune responses with a profound increase in IL-5 secretion in the lung.

IL-33 expression in lung tissues from OVA-induced AHR. Based on our observations, we attempted to locate the pathway responsible for switching to Th17 responses in eosinophil-deficient mice. IL-33 is expressed at higher levels in asthmatic patients (31) and in a mouse model of asthma (16) relative to healthy individuals or WT mice. In OVA-induced experimental asthma models, IL-33 induces eosinophilic inflammation and airway hyperresponsiveness (19). Furthermore, there is compelling evidence to suggest that IL-33 exacerbates inflammatory responses via the activation of mast cells in rheumatoid arthritis (46). It has been noted that IL-33 from bronchial epithelial cells induces the release of the proinflammatory cytokines IL-1 β and IL-6 from mast cells (26). Since IL-1 β and IL-6 are part of the cytokine milieu required for Th17 cell differentiation, we postulated that IL-33 might operate in part through the amplification of Th17 responses via mast cells to promote inflammation. Accordingly, we carried out immunoblotting to detect IL-33 and ST2 (the IL-33 receptor) in the lung tissues of WT and Δ dblGATA mice. We observed that the expression levels of IL-33 and ST2 did not differ significantly between OVA-challenged WT and Δ dblGATA mice (Fig. 5A and B). In contrast to the rest of the IL-1 cytokine family members, which primarily drive the Th1 immune response, IL-33 has been recognized as a promoter of Th2 immunity. This is particularly true in cases of allergic asthma, where its action on eosinophils contributes to a marked acceleration in pathogenesis (12, 21, 34). The results shown in Fig. 5 indicate that IL-33 expression is not regulated principally by eosinophils only, but by many other target cells, and also possibly by many upstream inflammatory mediators.

Δ dblGATA mice exhibit increased mast cell activation by OVA challenge. We previously demonstrated, by studying profiles of cell recruitment to the lungs upon OVA challenge, that the increase in the proportion of mast cells was characteristic of OVA-challenged Δ dblGATA mice (Fig. 3). Upon activation, mast cells secrete proinflammatory cytokines (26),

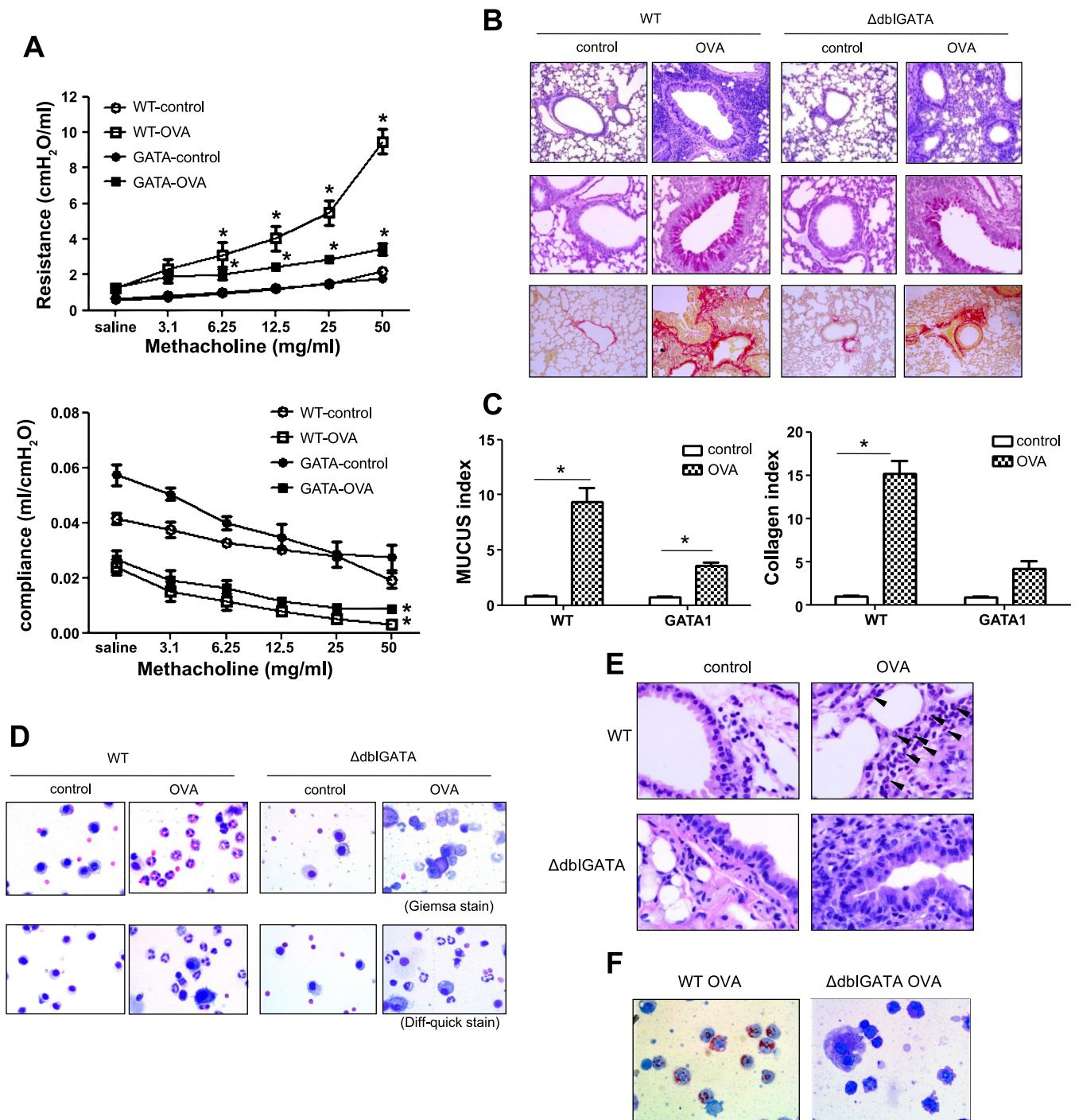


Fig. 1. Ovalbumin (OVA)-induced airway inflammation in BALB/c-wild-type (WT) and Δ dblGATA (GATA) mice. BALB/c-WT and Δ dblGATA mice were systemically sensitized to OVA, and then challenged with 1% OVA or PBS intranasally for 30 min daily. **A**: airway hyperreactivity with increasing concentrations of methacholine was assessed by mechanical ventilation. The data are expressed as means $\pm$ SE. Unpaired *t*-test to analyze the results for significant differences (at $P < 0.05$) between OVA-challenged vs. control groups. **B**: paraffin-embedded lung sections were prepared 24 h after the last OVA challenge and stained with hematoxylin and eosin to observe inflammation (*top*; original magnification $\times 400$). Periodic acid-Schiff stain was used for detection of mucus secretion (*middle*). Tissue sections were also stained for collagen (red) with Sirius red solution (*bottom*, original magnification $\times 200$). **C**: to compare the amount of mucus secretion and collagen deposition between treatment groups of mice, tissue sections of each mouse were analyzed via Image Analysis Software (analysis FIVE, Olympus). Nonparametric Mann-Whitney tests were applied to analyze the results for significant differences (at $P < 0.05$). **D**: cytospin preparations of bronchoalveolar lavage (BAL) cells stained with Giemsa (*top*) or Diff-Quick (*bottom*), demonstrating prominent eosinophilic recruitment in WT-OVA mice, presented at original magnification $\times 1,000$. **E**: paraffin-embedded lung sections were stained with hematoxylin and eosin. Arrowheads indicate eosinophils (red cytoplasm). Images are presented at original magnification $\times 400$. **F**: cytospin preparations of BAL cells stained with an improved Randolph staining method. Red-stained cytoplasmic granules are noted in OVA-challenged WT BALB/c mice. Images are presented at original magnification $\times 1,000$.

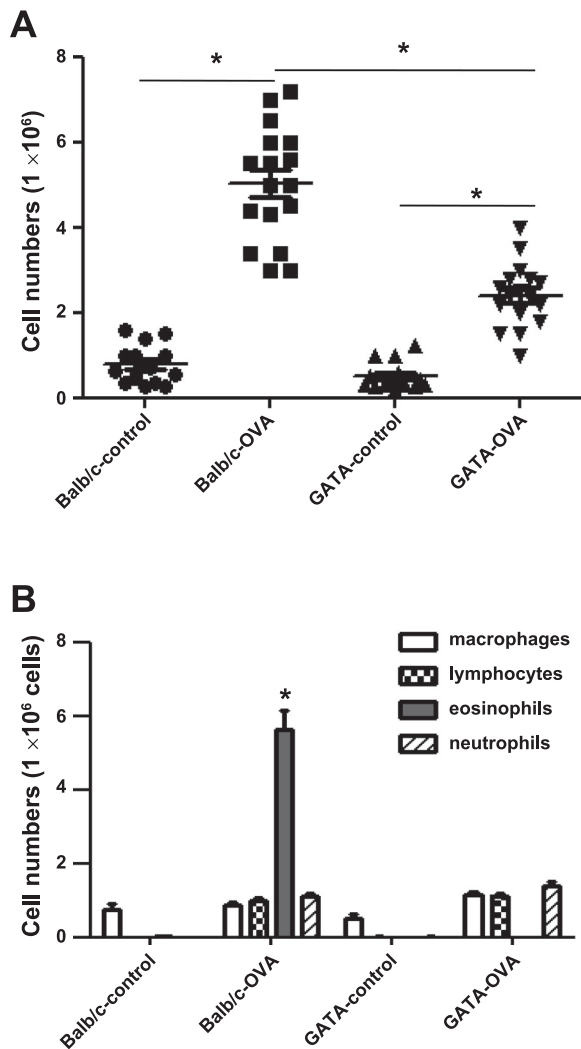


Fig. 2. OVA-induced airway inflammation in BALB/c-WT and Δ dblGATA mice. A: BAL fluid was collected 24 h after the final OVA or PBS challenge and the number of cells in the BAL fluid was determined as described in MATERIALS AND METHODS. The data are expressed as means $\pm$ SE and unpaired *t*-test to analyze the results for significant differences ($*P < 0.05$). B: differential cell count was calculated as (total cell number of BAL cells) $\times$ (percentage of macrophages, lymphocytes, eosinophils, or neutrophils counted in the Giemsa-stained cytospin cell preparation in Fig. 1B). The data are expressed as means $\pm$ SE, and nonparametric Mann-Whitney tests were applied to analyze the results for significant differences ($*P < 0.05$).

which can serve as activation markers; we therefore compared the levels of these cytokines among the different experimental groups. Intracellular staining of mast cells (c-kit⁺ and FcεRI⁺ cells) from Δ dblGATA mice revealed strong expression of IL-1 β and IL-6 after OVA sensitization and challenge (Fig. 6A). These results indicate that mast cells proliferate and secrete proinflammatory cytokines during airway inflammation, particularly in cases of pathogenesis with eosinophil deficiency. Mast cell increases in Δ dblGATA mice were also observed in lung tissue (Fig. 6B).

IL-33 directly drives expression of IL-1 β and IL-6 from mast cells via ST2. Next, we hypothesized that mast cells could be influenced by IL-33 secreted primarily from bronchial epithelial cells, and furthermore, that mast cells might induce Th17

differentiation via the production of IL-1 β and IL-6. To evaluate the contribution of IL-33-activated mast cells to the development of Th17 cells, we first investigated whether IL-33 directly drives mast cells to generate the Th17-skewing cytokines IL-1 β and IL-6. For in vitro stimulation assays, mast cells were purified and identified via toluidine blue staining (data not shown). Since IL-33 is known to exert its effects via binding to cell surface ST2 receptors, we first investigated whether incubation with the anti-ST2 antibody could interfere with the stimulatory effects of IL-33. Indeed, the preincubation of mast cells with anti-ST2 antibody inhibited IL-33-induced IL-1 β and IL-6 production (Fig. 7A). These data indicate that IL-33 signals directly drive the expression of the proinflammatory cytokines, IL-1 β and IL-6, which contribute to the polarization of naive CD4⁺ T cells to Th17 cells.

Mast cells activated by IL-33 enhance Th17 cell differentiation. Finally, we evaluated the effect of the IL-33/mast cell axis on Th17 cell differentiation. IL-33 is capable of triggering mast cell activation (2, 15); however, the effect of IL-33 on Th17 cells via their interactions with mast cells has not yet been elucidated. To reveal the relationship between mast cells and Th17 differentiation via the IL-33 pathway, we stimulated purified naive CD4⁺ T cells from Δ dblGATA mice to differentiate into Th17 cells using either TGF- β and IL-6 or with anti-CD3 antibody only. We subsequently compared the effects of IL-33, mast cells, or a combination of both on Th17 differentiation in vitro.

As shown in Fig. 7B, IL-33 alone did not induce naive CD4⁺ T cells to develop into Th17 cells; however, IL-33 increased the ability of mast cells to induce Th17 differentiation without skewing the immune response of Th17, via the direct stimulation of ST2 receptors on mast cells. Moreover, IL-33-activated mast cells caused the complete differentiation of naive T cells into Th17 cells, under Th17-skewing conditions. Besides, IL-33 stimulation in the presence of anti-CD3 antibody failed to trigger Th17 polarization even under Th17-skewing conditions in the absence of mast cells. However, under mast cell coculture conditions, we noted elevated percentages of IL-17-secreting Th17 cells. This interaction between mast cells and CD4⁺ cells may result from IL-33/ST2 ligation-induced production of IL-1 β and IL-6 from mast cells, as proved by neutralizing ST2, IL-1 β , and IL-6 (Fig. 7, B and C).

DISCUSSION

In this study, we have demonstrated that IL-33 acts directly on mast cells to generate IL-1 β and IL-6, thereby enhancing Th17 differentiation, which may eventually induce neutrophil-dominant airway inflammation in response to OVA challenge. IL-33 was upregulated in OVA-induced airway inflammation in both WT and eosinophil-deficient mice, and this was accompanied by an increase in ST2 in lung tissue. In particular, Δ dblGATA mice developed neutrophilic airway inflammation on OVA challenge and exhibited increased percentages of mast cells relative to WT mice. Our results demonstrate that the IL-33/mast cell axis polarizes the Th17-mediated neutrophilic response under conditions of eosinophil deficiency.

Eosinophils are crucially important effector cells in asthma and allergic disease; however, their role in disease progression,

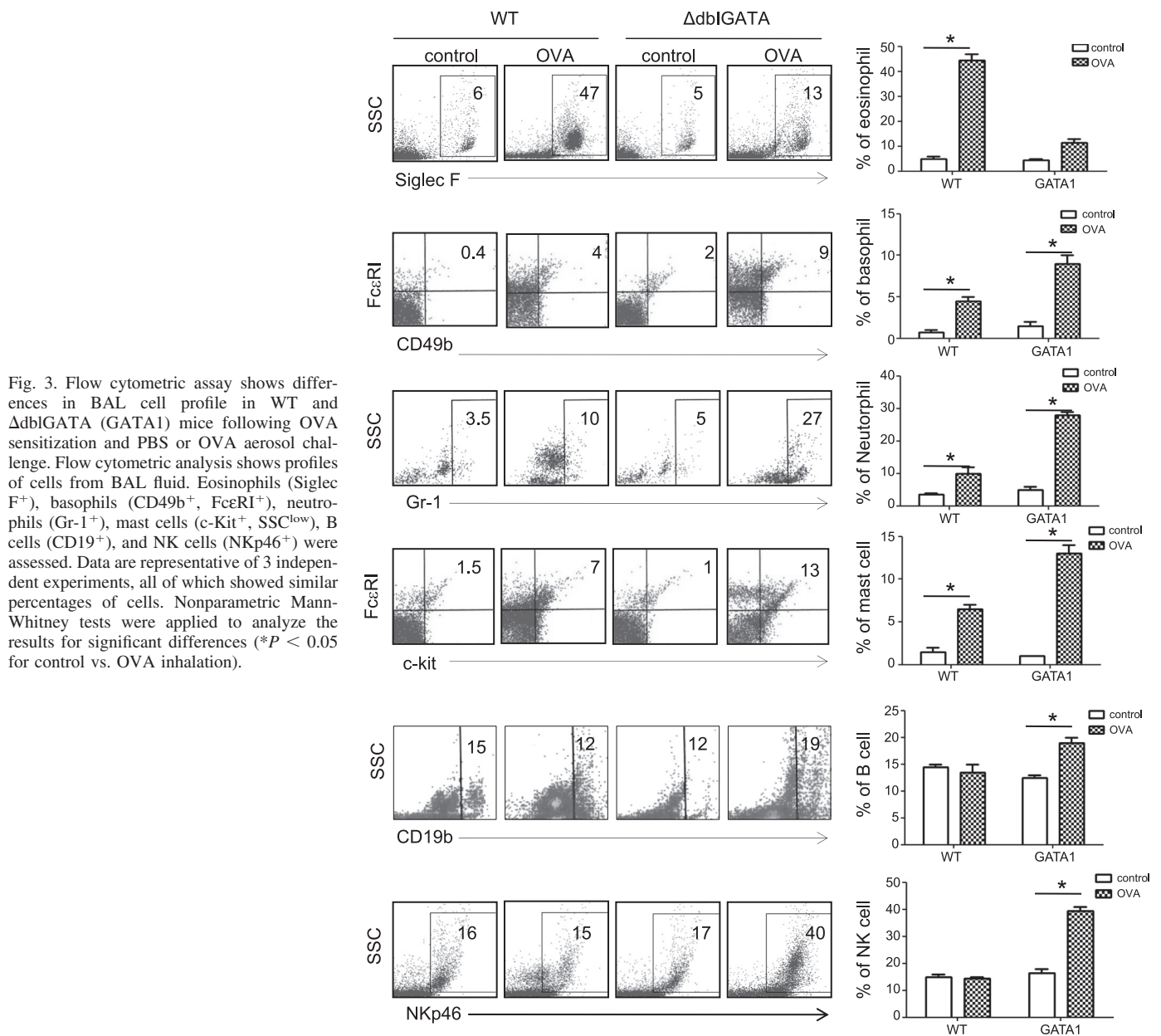


Fig. 3. Flow cytometric assay shows differences in BAL cell profile in WT and Δ dblGATA (GATA1) mice following OVA sensitization and PBS or OVA aerosol challenge. Flow cytometric analysis shows profiles of cells from BAL fluid. Eosinophils (Siglec F⁺), basophils (CD49b⁺, FcεRI⁺), neutrophils (Gr-1⁺), mast cells (c-Kit⁺, SSC^{low}), B cells (CD19⁺), and NK cells (NKp46⁺) were assessed. Data are representative of 3 independent experiments, all of which showed similar percentages of cells. Nonparametric Mann-Whitney tests were applied to analyze the results for significant differences (* $P < 0.05$ for control vs. OVA inhalation).

as well as mechanisms that regulate the pathogenesis of asthma, remain open questions because of the heterogeneity of the disease. In light of the association between increased airway reactivity and the rapid recruitment and accumulation of eosinophils in the tissue, we evaluated the function of eosinophils in the development of airway inflammation in a mouse model. To model eosinophil deficiency, we used mice that possessed a deletion of the double GATA-binding site in the GATA-1 promoter region, an element presumed to mediate the positive autoregulation of GATA-1 expression. The transcription factor GATA-1 reprograms the differentiation of immature myeloid cells into three different hematopoietic lineages (erythroid cells, megakaryocytes, and eosinophils), and the double GATA deletion leads to a selective loss of the eosinophil lineage (48). Δ dblGATA mice therefore exhibit selective eosinophil deficiency with no other significant phenotypic abnormalities and

are useful in ascertaining the functions of eosinophils in a variety of inflammatory or allergic disorders.

Our data indicate that OVA sensitization and challenge induce AHR accompanied by pulmonary eosinophilia and increases in the levels of IL-4, IL-5, IL-13 and TSLP, in WT BALB/c mice. Interestingly, Δ dblGATA mice also exhibit the AHR phenotype, although to a lesser degree than do WT mice. IL-4 and IL-5 produced by Th2 cells have been postulated to play central roles in the initiation and maintenance of allergic asthma (28). The development of airway eosinophilia and allergen-induced AHR is also associated with increased IL-4, IL-5, and IL-13 levels in BAL fluid, consistent with the development of a Th2-mediated allergic response. In addition to Th2 cells, basophils and mast cells, which are activated during an allergic response, are also potent sources of IL-4 and IL-5 (40). IL-13 production is impaired in IL-5/eotaxin double-

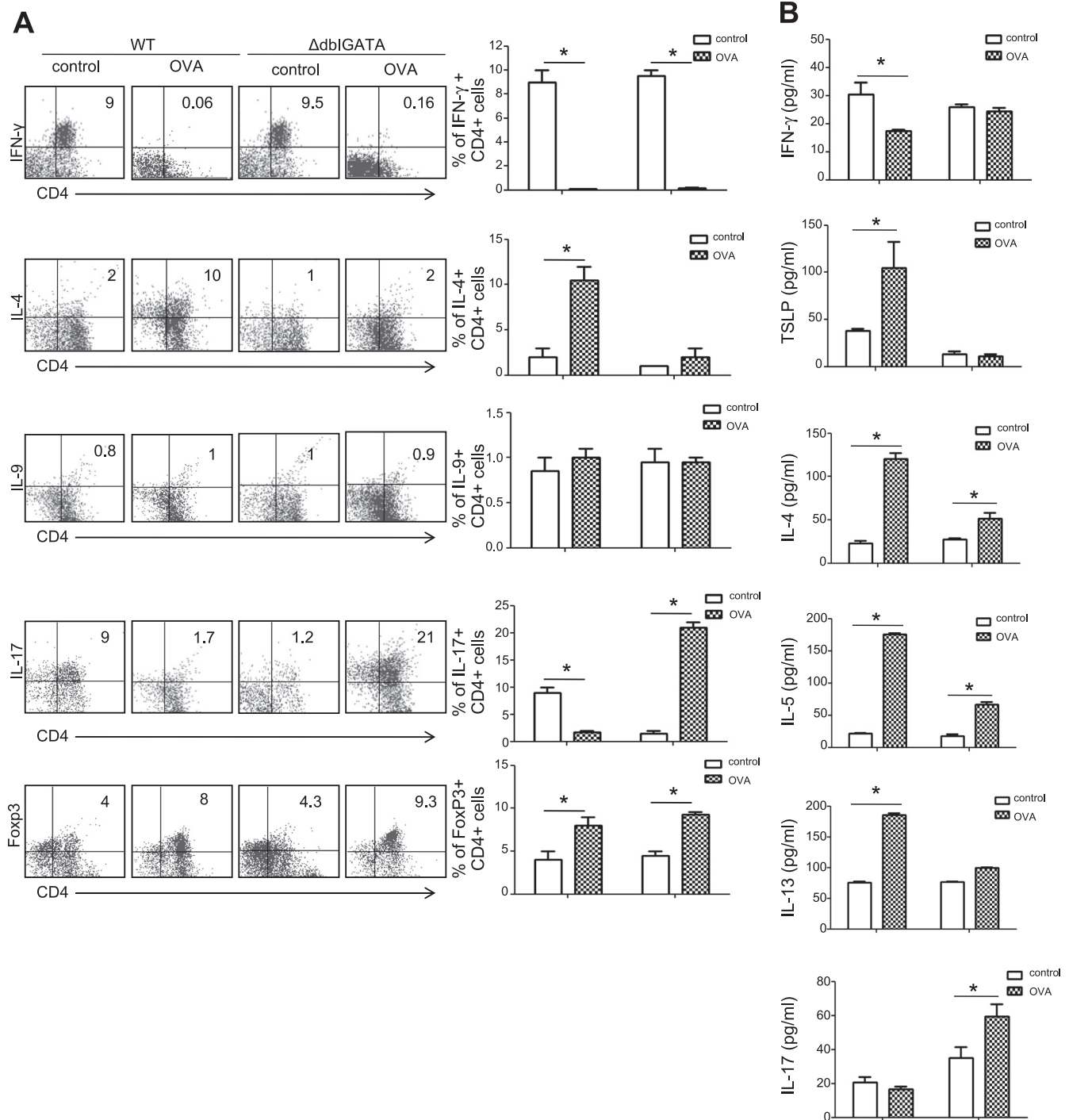


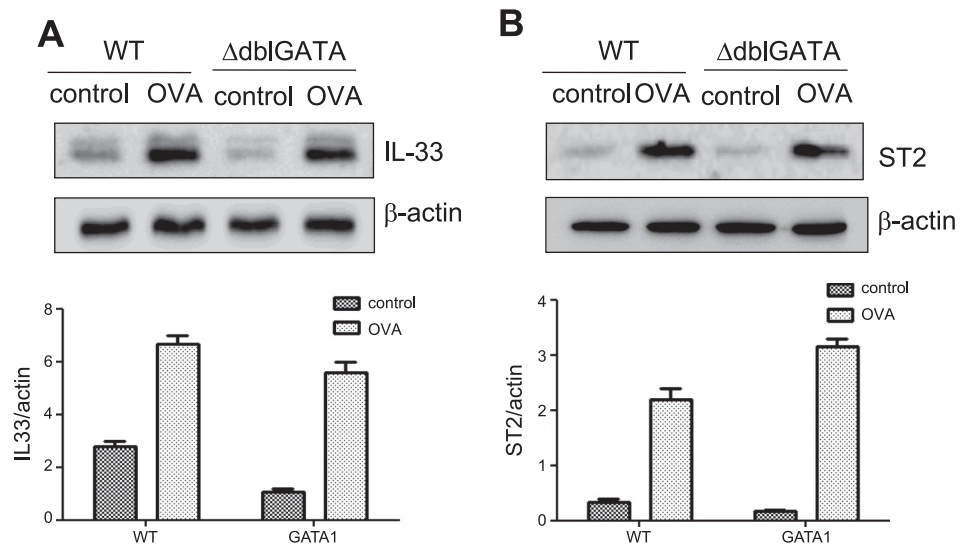
Fig. 4. Comparison of Th1, Th2, and Th17 responses after OVA challenge. **A**: proportions of CD3⁺ T cells were determined according to the intracellular levels of cytokines, for IFN- γ -secreting T cells (IFN- γ ⁺), IL-4-secreting T cells (IL-4⁺), IL-9-secreting T cells (IL-9⁺), Th17 cells (IL-17⁺ and IFN- γ ⁻), and T_{reg} (Foxp3⁺). Data are representative of 3 independent experiments, all of which showed similar percentages of cells. **B**: concentrations of IFN- γ , TSLP, IL-4, IL-5, IL-13, and IL-17 in BAL fluid from WT and Δ dblGATA mice after OVA or PBS challenge. Cytokine concentrations in BAL fluid from OVA- or PBS-challenged (control) mice were measured by ELISA. Data are expressed as the means $\pm$ SE (n = 5). Nonparametric Mann-Whitney tests were applied to analyze the results for significant differences (* P < 0.05 for control vs. OVA inhalation).

knockout mice (23), and human eosinophils produce functional IL-13 in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) or IL-5 (42).

We observe that eosinophil deficiency also leads to a significant increase in cytokine levels upon OVA challenge; this might be due to the intact populations of mast cells or baso-

phils. Recently, TSLP, the Th2-polarizing cytokine, has been well defined as an important player in Th2-mediated inflammation. TSLP is an epithelial cell-derived cytokine expressed in the skin, lungs, and thymus that exerts a profound influence on the polarization of dendritic cells, thus driving the production of Th2 cytokines (43, 49). Allergic diseases such as asthma and

Fig. 5. IL-33 and ST2 are expressed in both OVA-challenged WT and Δ dblGATA mice. The expression levels of IL-33 (A) and ST2 (B) were evaluated in lung homogenates from each group via immunoblotting. Similar results were obtained from 3 independent experiments. Band pixel densities of IL-33 and ST2 were acquired, normalized to β -actin band densities, and expressed as means $\pm$ SE ($*P < 0.05$) in bar graphs (bottom).



atopic dermatitis (AD) are characterized by their Th2 inflammatory profile, with the release of Th2 cytokines and chemokines, and the infiltration of their corresponding leukocyte targets (9). Epidemiological analyses demonstrate that AD is frequently the initial step in the so-called “atopic march”; over 50% of patients with moderate to severe AD develop asthma later in life, and the severity of AD affects the course of respiratory allergy. TSLP might be instrumental in the onset of the atopic march (25).

In the present study, the observed increase in IL-17-secreting $CD3^+$ T cells in OVA-challenged airway inflammation was consistent with the increased IL-17 protein expression measured by ELISA. This is a matter of particular interest to us, because the neutrophil increase in the BAL cell profile may contribute to airway inflammation. OVA inhalation induces the development of Th17 cells, which are responsible for the infiltration of neutrophils in the airways. According to previous research, IL-17-secreting Th17 cells are one of the major pathogenic immune components in neutrophilic AHR (10, 24); we confirmed that the Th17-neutrophilic response can be exclusively developed under airway inflammation conditions in the absence of eosinophils. IL-17 has been mainly reported to be a T cell-secreted cytokine, but recently the role of “innate” IL-17-producing cells has emerged (45). Furthermore, we also observe that the proportions of $CD4^+$ IL-17 $^+$ cells were higher both in OVA-challenged Δ dblGATA mice and in vitro (Fig. 7); these might be $CD3^+$ $\gamma\delta$ T cells (22, 38). Therefore, other possible mechanisms relevant to IL-17-dominant airway inflammation need to be investigated, including innate IL-17 producing cells.

Recently, IL-33, a member of the IL-1 family, has also been implicated in the promotion of the Th2 cytokine response in vivo. IL-33 signaling through the IL-1R-like molecule T1/ST2, which is expressed on Th2 cells, eosinophils, basophils, and mast cells, has been shown to function as an important effector of Th2 responses in a variety of experimental settings, including mouse asthma models. Like other members of the IL-1 family, IL-33 is produced in its precursor form by airway epithelial cells, smooth muscle cells, and fibroblasts and can be cleaved by caspase-1. However, the involvement of caspase-1 in IL-33 maturation in vivo has not been established, and other reports have shown that

IL-33 is inactivated after maturation by caspase-1 (5). Intraperitoneal administration of IL-33 to mice results in an allergic phenotype including blood eosinophilia and increases in the serum concentrations of IL-5, IL-13, IgA, and IgE. The administration of IL-33 also induces pathological alterations in the lung and digestive tract, including eosinophilic and mononuclear infiltrates, elevated mucus production, and epithelial cell hyperplasia and hypertrophy (34). Although IL-33 has been demonstrated to drive immune reactions toward the Th2 response, we noted no differences in IL-33 and ST2 expression under OVA-challenged conditions between WT mice (with increased Th2 populations) and Δ dblGATA mice (with increased Th17 populations), thereby suggesting that IL-33 has diverse functions in the modulation of airway inflammation. The increased proportions of mast cells, neutrophils, and Th17 cells in the BAL fluid of eosinophil-deficient mice suggest that mast cells might function as direct effector cells to mediate airway inflammation in response to IL-33.

Mast cells express cell-surface ST2 at high density and are reported to respond directly to IL-33 to generate inflammatory cytokines and chemokines in vitro (2, 13, 34). Although IL-33 has been previously described as a general inducer of the Th2 response, the effect of IL-33 on IL-6, IL-1 β , and TNF- α secretion suggest that, in addition, this cytokine may also enhance the proinflammatory activities of mast cells under various conditions, including arthritis. In particular, IL-1 β production by mast cells has been shown to play a central role in the initiation of joint inflammation in a model of autoantibody-induced arthritis (29). We therefore investigated the role of mast cells regulated by IL-33, particularly with regard to the induction of the Th17 response mediated by high concentrations of IL-6 and IL-1 β .

Aside from an increase in their proportions in the BAL fluid, Fc ϵ RI $^+$ and c-kit $^+$ mast cells also promoted inflammatory activity in OVA-challenged Δ dblGATA mice via IL-6 and IL-1 β expression, accompanied by ST2 upregulation. Furthermore, our results demonstrate that IL-33 triggered an increase in both cytokines in mast cells via the ST2 receptor. Collectively, these results show that the proinflammatory activity of mast cells in airway inflammation might be triggered via IL-33/ST2 receptor signaling. Because IL-6 and IL-1 β are

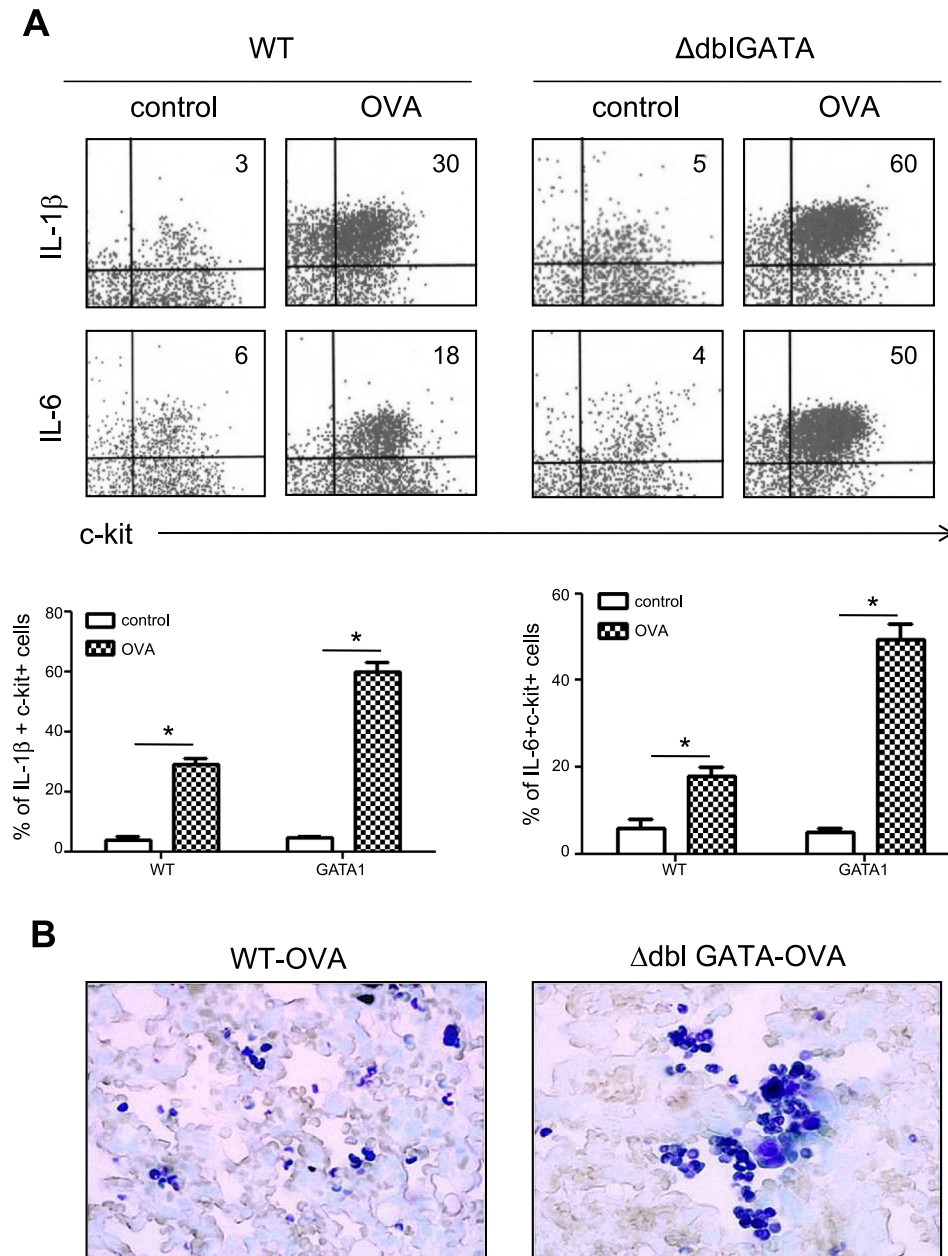


Fig. 6. OVA-challenged Δ dblGATA mice exhibit mast cell increases with IL-1 β and IL-6 production. **A**: mast cells from BAL produce more IL-1 β and IL-6 in OVA-challenged Δ dblGATA mice (Fc ϵ RI $^{+}$ cells were gated for analysis). Intracellular expression of IL-1 β or IL-6 in Fc ϵ RI $^{+}$ and c-kit $^{+}$ mast cells was determined via flow cytometry. Nonparametric Mann-Whitney tests were applied to analyze the results for significant differences (* $P < 0.05$). **B**: mast cells (blue-colored cells) in lung tissue of OVA-challenged WT and Δ dblGATA mice (toluidine blue stain, original magnification $\times 400$).

direct inducers of Th17 differentiation, we subsequently investigated whether IL-33-activated mast cells could influence Th17 differentiation.

Th17 cells secrete the cytokine IL-17, which induces neutrophil-rich inflammation and is a key player in noneosinophilic and neutrophilic asthma (33). In contrast, the Th2 cytokines IL-4 and IL-13 suppress excessive neutrophil recruitment and proinflammatory cytokine production in Th2-driven parasite infection (35). Clinically, many asthma patients fail to respond satisfactorily to standard steroid therapy, and this type of steroid-resistant, severe asthma has been previously linked to the presence of neutrophilic inflammation in the lung (1). Both Th2 and Th17 cells are capable of inducing AHR, but Th17 cell-mediated airway inflammation and AHR are steroid resistant, thereby indicating a potential role of Th17 cells in steroid-resistant asthma (24).

Our results demonstrate that IL-33 did not exert a direct effect on the induction of Th17 cells in vitro, whereas coculture with mast cells slightly enhanced the proportion of differentiated Th17 cells. With only anti-CD3 antibody stimulation, mast cells facilitated the differentiation of Th17 cells from naive CD4 $^{+}$ T cells and strongly induced the differentiation of Th17 cells in the presence of IL-33. These effects were markedly reduced in pretreatment with the soluble ST2 receptor, which blocks the ligation of IL-33 and membrane-bound ST2 on mast cells. In combination with the Th17-skewing cytokines TGF- β and IL-6, the effect of the IL-33/mast cell axis enhanced the differentiation of Th17 cells. Although the role of IL-33-mediated T-cell stimulation has been relatively well documented in Th2-mediated immune responses and disease, this result illustrates the ability of IL-33 to directly stimulate mast cells and enhance the Th17 response. These data sup-

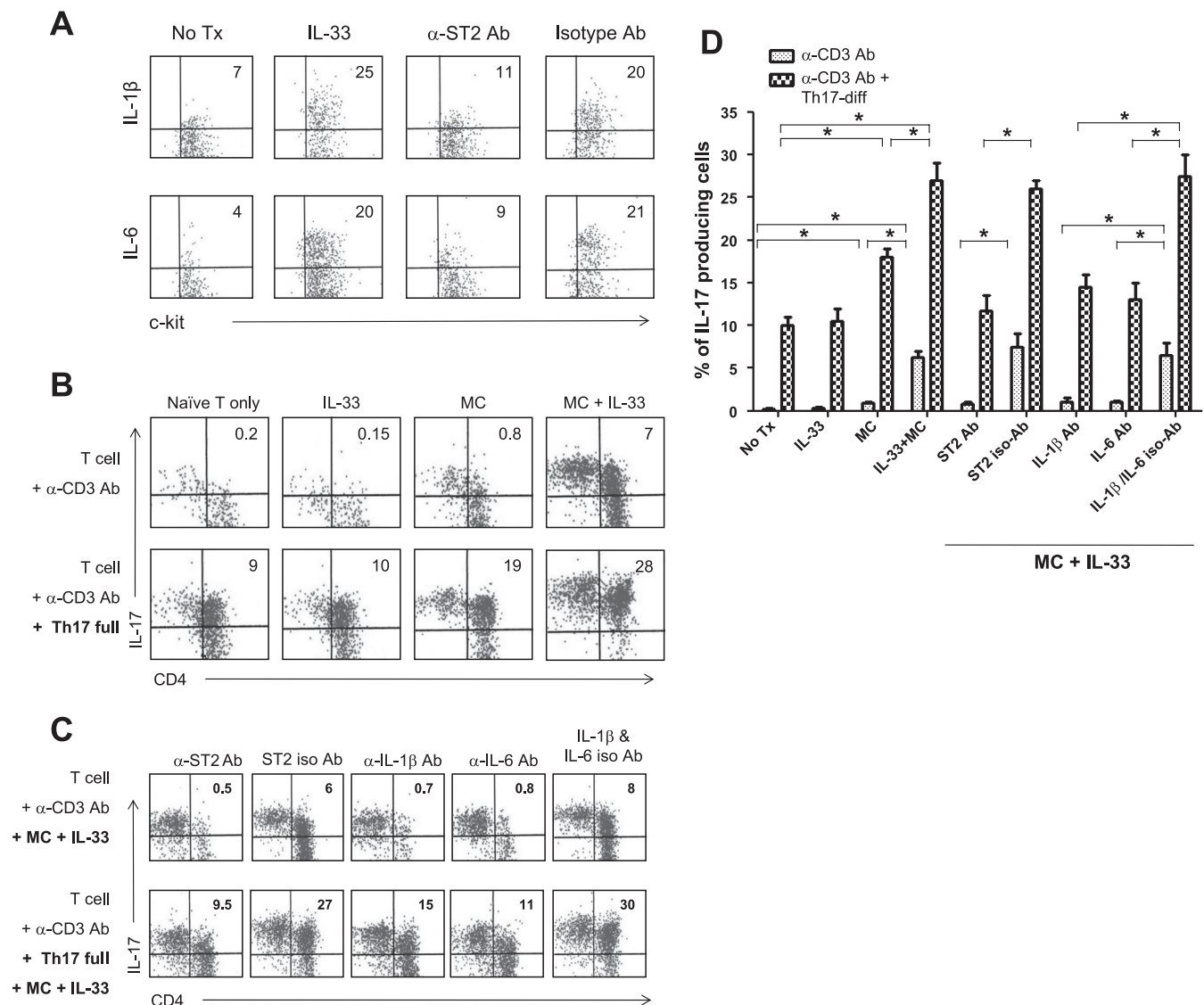


Fig. 7. Differentiation of naive CD3 T cells into Th17 cells is enhanced by IL-33-stimulated mast cells. **A**: mast cells were isolated from peritoneal cavity and cultured (10^5) with or without IL-33 (50 ng/ml). To block stimulation via the ST2/IL-33 pathway, the mast cells were pretreated with ST2 blocking Ab (10 μ g/ml) for 1 h prior to IL-33 treatment. Proportions of Fc ϵ RI $^+$ and c-kit $^+$ mast cells expressing IL-1 β or IL-6 intracellularly were determined by flow cytometry. Data are representative of 3 independent experiments, all of which yielded similar percentages of cells. No Tx, no treatment. **B**: naive CD4 $^+$ T cells were cultured with only anti-CD3 and IL-33, or with mast cells (MC), and compared with those that were stimulated with the Th17-skewing cytokines IL-6 (50 ng/ml) and TGF- β (2 ng/ml) (Th17 full). Flow cytometric analysis data are representative of 3 independent experiments. **C**: naive CD4 $^+$ T cells were cultured in the presence of IL-33 or mast cells with or without Th17-skewing cytokines and the proportions of Th17 cells were compared. To determine whether IL-33-activated mast cells affect Th17 cell differentiation via secretion of IL-1 β or IL-6, cells were neutralized with antibodies against ST2, IL-1 β , or IL-6. **D**: frequency of CD4 $^+$ IL-17 $^+$ cells in **B** and **C**. Mast cells or combination with IL-33 significantly increased the proportions of CD4 $^+$ IL-17 $^+$ cells from naive CD4 $^+$ T cells. Blockage of ST2, IL-1 β , or IL-6 by use of neutralizing antibodies inhibited production of IL-17-secreting T cells compared with those of isotype antibody treatment. The data are expressed as means $\pm$ SE ($n = 4$). Nonparametric Mann-Whitney tests were applied to analyze the results for significant differences (at $P < 0.05$).

port the potential role of the IL-33/mast cell axis in the pathogenesis of asthma, particularly in eosinophil-independent phenotypes.

Eosinophils are major proinflammatory cells that are major contributors to inflammatory processes, and IL-5 is central to their maturation and release from the bone marrow as well as their subsequent accumulation and activation in tissues. Mepolizumab is a humanized monoclonal antibody with potent IL-5 neutralizing effects that represents a potential treatment option for eosinophilic diseases. However, clinical outcomes are not consistent because human asthma is heterogeneous with respect to clinical features and cellular sources of inflamma-

tion; however, a large body of experimental evidence supports the hypothesis that Th2 cytokines orchestrate allergic airway inflammation in animal models. Therefore, in targeting a mediator with the aim of disease modification or prevention, the goal is to find a target that is a central regulator of immunological activity. As such, IL-33 is an attractive candidate for therapeutic intervention using decoy receptors for ST2. Whereas other therapies are limited by the fact that they block various individual components of the downstream immune response in asthma, it is hoped that blockade of IL-33, a central regulator of the inflammatory response, or its receptors, will deliver on the promise of immune modulation.

In summary, our results corroborate the increasing evidence that eosinophils are not indispensable in the development of allergic asthma. Moreover, IL-33-activated mast cells might provide insights into the mechanisms underlying noneosinophilic and Th17-mediated neutrophilic asthma and also may provide a therapeutic target to modulate the relevant mast cell-dependent pathology and beyond, in a broad range of inflammatory diseases.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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