

ST2 Requires Th2-, but Not Th17-, Type Airway Inflammation in Epicutaneously Antigen-Sensitized Mice

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ABSTRACT

Background: IL-33 is known to induce Th2-type cytokine production by various types of cells through its receptors, ST2 and IL-1RAcP. Polymorphism in the ST2 and/or IL-33 genes was found in patients with atopic dermatitis and asthma, implying that the IL-33/ST2 pathway is closely associated with susceptibility to these diseases. Exposure to allergens through damaged skin is suspected to be a trigger for allergen sensitization, resulting in development of such allergic disorders as asthma and atopic dermatitis.

Methods: To elucidate the role(s) of the IL-33/ST2 pathway in asthma in individuals who had been epicutaneously sensitized to an antigen, wild-type and ST2^{-/-} mice were epicutaneously sensitized with ovalbumin (OVA) and then were intranasally challenged with OVA. The degree of airway inflammation, the number of leukocytes and the activities of myeloperoxidase (MPO) and eosinophil peroxidase (EPO) in bronchoalveolar lavage fluids (BALFs), The levels of cytokines and chemokines in lungs and OVA-specific IgE levels in sera were determined by histological analysis, a hemocytometer, colorimetric assay, quantitative PCR or ELISA, respectively.

Results: The number of eosinophils in BALFs, the levels of Th2 cytokines and chemoattractants in the lungs and OVA-specific IgE in sera from ST2^{-/-} mice were significantly reduced compared with wild-type mice. Although the number of neutrophils in BALFs and the pulmonary levels of IL-17 were comparable in both mice, the levels of MPO activity in BALFs and neutrophil chemoattractants in the lung were reduced in ST2^{-/-} mice.

Conclusions: The IL-33/ST2 pathway is crucial for Th2-cytokine-mediated eosinophilic, rather than Th17-cytokine-mediated neutrophilic, airway inflammation in mice that had been epicutaneously sensitized with antigens and then challenged with antigen.

KEY WORDS

asthma, eosinophils, epicutaneous sensitization, IL-33, ST2

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INTRODUCTION

Several longitudinal epidemiological studies proved that eczema is the first clinical manifestation of allergic diseases during infancy, followed by the development of atopic asthma and rhinitis.¹⁻⁴ The underlying mechanisms of allergic disease development remain unclear, but exposure to allergens through skin damaged by disruption of the epidermal barriers is suspected to be a trigger for sensitization in allergic disorders. In support of this notion, genetic deficiency for *filaggrin*, which is crucial for formation of epidermal barriers, is considered to be a predisposing factor for certain allergic diseases such as atopic dermatitis, asthma and rhinitis.⁵⁻⁸ Notably, *filaggrin*-deficient mice develop dermatitis after epicutaneous exposure to protein antigens.⁹ In addition, mice that had been epicutaneously sensitized with an allergen develop allergic airway inflammation after inhalation of the same allergen.¹⁰⁻¹³ These findings suggest that epicutaneous exposure to antigens due to dysfunctional epidermal barriers contributes strongly to induction of allergic diseases.

IL-33, a member of the IL-1 family of cytokines, is able to induce production of Th2-type cytokines by various types of cells such as Th2 cells and mast cells through its ST2 and IL-1RAcP receptors.¹⁴ IL-33 is localized in cell nuclei¹⁵ and released by necrotic cells after tissue injury,¹⁶⁻¹⁸ suggesting that it may be released by damaged skin after scratching, thereby contributing to the development of allergic disorders. In support of this, IL-33 mRNA/protein levels are elevated in skin lesions of patients with atopic dermatitis.¹⁹ Polymorphism of the ST2 and/or IL-33 genes was found in patients with atopic dermatitis, asthma and rhinitis,²⁰⁻²⁶ implying an association with disease severity. However, the role(s) of the IL-33/ST2 pathway in allergic airway inflammation in individuals who had been epicutaneously sensitized to an antigen remains unclear. We attempted to elucidate its role(s) by studies in ST2-deficient mice.

METHODS

MICE

BALB/cA wild-type mice were purchased from Sankyo Lab (Tsukuba, Japan). ST2^{-/-} mice (on the BALB/c background) were generated as described elsewhere.²⁷ Six- to 9-week-old female mice were used in all experiments. Mice were housed under specific-pathogen-free conditions at the National Research Institute for Child Health and Development, and the animal protocols were approved by the Institutional Review Boards of the National Research Institute for Child Health and Development and The Institute of Medical Science, The University of Tokyo.

EPICUTANEOUS SENSITIZATION

Mice were epicutaneously sensitized with OVA as de-

scribed elsewhere,¹³ with minor modifications. In brief, the dorsal skin of mice was shaved with hair clippers and then stripped 6 times with adhesive cellophane tape (Nichiban, Tokyo, Japan). A patch (Finn chamber disk; ϕ 8 mm; Smart Practice, Phoenix, AZ, USA) containing an antigen solution (400 μ g of OVA [grade V; Sigma-Aldrich, St. Louis, MO, USA] in 40 μ l of PBS) or PBS alone (control) was placed on the tape-stripped skin for 3 days and then removed. One week later, a fresh patch having the same content was applied to the same skin site. This cycle was repeated three times, resulting in a total of 9 days' exposure to the patch.

INDUCTION OF ALLERGIC AIRWAY INFLAMMATION

One week after removal of the last patch, each mouse was intranasally challenged with OVA (200 μ g in 20 μ l of PBS) or PBS alone for 3 days (one challenge per day). Twenty-four hours after the last challenge, bronchoalveolar lavage fluid (BALF) was collected from each mouse, as described elsewhere.²⁸ The total cell count and leukocyte profile were determined with a hemocytometer (Sysmex XT-1800i; Sysmex Corporation, Hyogo, Japan), as described previously.²⁹

MEASUREMENT OF MYELOPEROXIDASE (MPO) AND EOSINOPHIL PEROXIDASE (EPO) ACTIVITIES

MPO and EPO activities were measured as described elsewhere.²⁸ Recombinant human MPO and EPO (Calbiochem) were used as standard reagents.

QUANTITATIVE REAL-TIME PCR

Twenty-four hours after the last challenge, the lungs were harvested. Total RNA in the lung homogenates was isolated and Quantitative real-time PCR was performed as described elsewhere.²⁹ The mRNA expression levels were normalized to the GAPDH level in each sample. PCR primers were designed as shown in Table 1.

HISTOLOGY

Twenty-four hours after the last challenge, the lungs were harvested and fixed in Carnoy's solution. The fixed tissue was embedded in paraffin and sliced into 5- μ m sections, followed by hematoxylin-eosin or periodic acid-Schiff (PAS) staining.

MEASUREMENT OF OVA-SPECIFIC IMMUNOGLOBULINS IN SERA

Sera were collected twenty-four hours after the last challenge. The level of OVA-specific IgE in each serum was determined by ELISA, as described elsewhere.³⁰ Anti-OVA mouse IgE (TOS-2), as a standard OVA-specific IgE, was kindly provided by Dr. Mamoru Kiniwa (Taiho Pharmaceutical, Saitama, Ja-

Table 1 Primer designs

Gene	Forward (5'-3')	Reverse (5'-3')
GAPDH	CCCACTCTTCCACCTTCGATG	AGGTCCACCACCCTGTTGCT
ST2 short	TCAACCGCCTAGTGAACACACC	CAAAGCCCCAAGTCCCATTCTC
IL-33	CAGGCCTTCTTCGTCCTTCAC	TCTCCTCCACTAGAGCCAGCTG
IL-4	TCCAAGGTGCTTCGCATATTTT	CAGCTTATCGATGAATCCAGGC
IL-5	CCCTCATCCTCTTCGTTGCAT	ATGTGATCCTCCTGCGTCCAT
IL-13	GGCAGCAGCTTGAGCACATT	GGCATAGGCAGCAAACCATG
IL-17A	CCGCAATGAAGACCCTGATAGAT	AGAATTCATGTGGTGGTCCAGC
CCL11	GAATCACCAACAACAGATGCAC	ATCCTGGACCCACTTCTTCTT
CCL22	ATCCTGGACCCACTTCTTCTT	CGGCAGGATTTTGAGGTCCA
CXCL1	CGGCAGGATTTTGAGGTCCA	TGAACGTCTCTGTCCCGAGC
CXCL2	AACTGACCTGGAAGGAGGAGC	ACTCTCAGACAGCGAGGCACAT

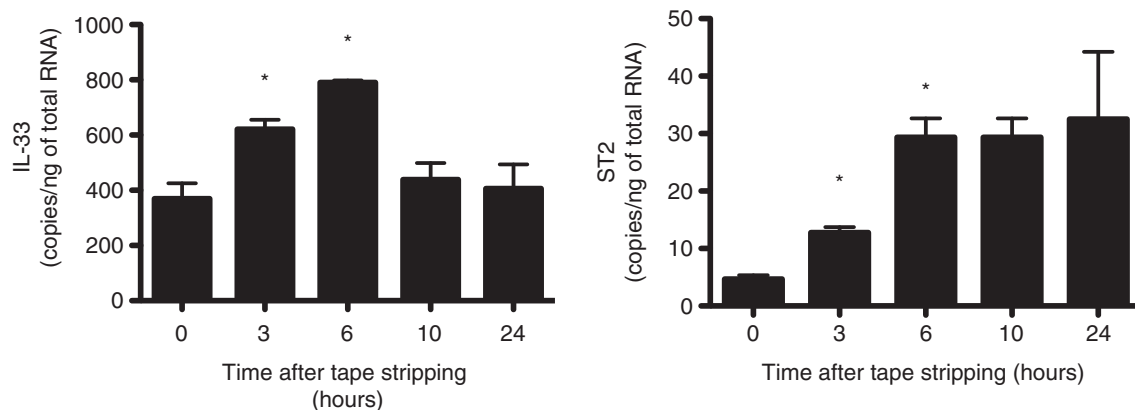


Fig. 1 Expression of IL-33 and ST2 mRNA was upregulated in the skin after tape stripping. mRNA was isolated from the dorsal skin of wild-type mice at the indicated time points after tape stripping. The expression of IL-33 and ST2 mRNA was determined by quantitative PCR. Data show the mean + SE ($n = 4$). * $P < 0.05$ vs. 0 (hours).

pan).

STATISTICS

Unless otherwise specified, the unpaired Student's *t*-test, two-tailed, was used for statistical evaluation of the results. All results are shown as the mean + SEM.

RESULTS

EXPRESSION OF IL-33 AND ST2 mRNA WAS UPREGULATED IN THE SKIN AFTER TAPE STRIPPING

An active form of IL-33 that is localized in the cell nucleus¹⁵ is considered to be released by necrotic cells during tissue injury.¹⁶⁻¹⁸ Patients with atopic dermatitis scratch inflamed skin lesions, resulting in exacerbation of the symptoms due to mechanical skin injury.³¹ Since increased expression of IL-33 was observed in the lesions of patients with atopic dermatitis,¹⁹ mechanical skin injury by scratching may result in release of IL-33, contributing to the disease development. Indeed, we found that expression of IL-33 and ST2 mRNA was significantly increased in the

skin of wild-type mice at 3 and 6 hours after tape stripping, which was used to mimic mechanical skin injury due to scratching (Fig. 1). These observations suggest that scratching-induced IL-33 and ST2 production may contribute to the pathogenesis of allergic diseases.

ALLERGIC AIRWAY INFLAMMATION WAS ATTENUATED IN ST2^{-/-} MICE SENSITIZED EPICUTANEOUSLY WITH OVA

Allergic airway inflammation was induced by OVA inhalation in mice that had been epicutaneously sensitized with OVA by tape stripping.^{10,13} To elucidate the role of the IL-33/ST2 pathway in that setting, we epicutaneously sensitized BALB/c-wild-type (WT) and ST2^{-/-} mice with OVA and then induced airway inflammation by intranasal challenge with OVA, as shown in Figure 2. Pulmonary inflammation accompanied by eosinophil and neutrophil infiltration, goblet cell hyperplasia and mucus secretion, but not epithelial and smooth muscle cell hyperplasia, was observed in epicutaneously OVA-sensitized WT mice af-



Fig. 2 Experimental protocol.

ter the last OVA, but not PBS, challenge (Fig. 3A, B). On the other hand, epicutaneously OVA-sensitized ST2^{-/-} mice showed suppressed eosinophil and neutrophil infiltration, but not goblet cell hyperplasia and mucus secretion, compared with epicutaneously OVA-sensitized WT mice after the last OVA challenge (Fig. 3A, B). Consistent with this, the numbers of cells such as eosinophils, neutrophils and lymphocytes in BALFs were significantly increased in both epicutaneously OVA-sensitized WT and ST2^{-/-} mice after intranasal challenge with OVA in comparison with after PBS inhalation (Fig. 3C). However, the total-cell, eosinophil and macrophage, but not lymphocyte, counts in BALFs from ST2^{-/-} mice were significantly reduced compared with WT mice (Fig. 3C). The levels of EPO activity in BALFs from ST2^{-/-} mice were also decreased in that setting (Fig. 4A). Despite a slight, but not statistically significant, decrease in neutrophils in BALFs from ST2^{-/-} mice (Fig. 3C), the level of MPO activity was markedly decreased in those BALFs compared with BALFs from WT mice (Fig. 4A). In addition, after the challenge with OVA, the level of OVA-specific IgE in sera from epicutaneously OVA-sensitized ST2^{-/-} mice was also significantly reduced compared with epicutaneously OVA-sensitized WT mice (Fig. 4B).

IL-33-ST2 PATHWAY IS REQUIRED FOR Th2-TYPE, BUT NOT Th17-TYPE, IMMUNE RESPONSES IN MICE AFTER EPICUTANEOUS Ag SENSITIZATION

Others demonstrated that airway eosinophilia is mediated by Th2 cytokines, while airway neutrophilia is mediated by Th17 cytokines, in mice epicutaneously sensitized with OVA and then subjected to intranasal OVA challenge.^{11,12} Consistent with previous reports, we observed that in addition to expression of mRNA for Th2 cytokines (i.e., IL-4, IL-5 and IL-13) and Th2-associated chemokines (i.e., CCL11 and CCL22) the expression of mRNA for IL-17A and neutrophil chemoattractants (i.e., CXCL1 and CXCL2) in the lungs from epicutaneously OVA-sensitized WT mice after the last OVA challenge was significantly increased in comparison with after PBS inhalation (Fig. 5). Consistent with the number of eosinophils and the level of EPO activity in BALFs, mRNA expression for IL-4, IL-5, IL-13, CCL11 and CCL22 in the lungs from epicutaneously OVA-sensitized ST2^{-/-} mice was significantly decreased compared with epicutaneously OVA-sensitized WT mice after the last OVA challenge (Fig. 5). Likewise, expression of mRNA was de-

creased for CXCL1 and CXCL2 but normal for IL-17A in the lungs from epicutaneously OVA-sensitized ST2^{-/-} mice compared with epicutaneously OVA-sensitized WT mice after the last OVA challenge (Fig. 5). These observations suggest that the IL-33/ST2 pathway is crucial for development of Th2-type immune responses and contributes to, but not is essential for, the development of Th17-type immune responses during antigen sensitization due to mechanical skin injury.

DISCUSSION

IL-33 is known to induce secretion of Th2-type cytokines by various types of cells such as Th2 cells, mast cells, basophils, eosinophils and innate-type lymphoid cells (i.e., natural helper cells, nuocytes, MPP2 cells and ih2 cells) through its ST2 and IL-1RAcP receptors,^{14,32-36} suggesting involvement of IL-33 in the pathogenesis of Th2-type allergic disorders. Indeed, the levels of soluble ST2 proteins and IL-33 mRNA/protein are increased in sera and/or tissue specimens from patients with asthma³⁷⁻³⁹ and in the lungs of mice that developed allergic airway inflammation.^{40,41} Moreover, it is suspected that single-nucleotide polymorphisms in the regions of the *IL-33* and *ST2* genes may influence susceptibility to allergic disorders.²⁰⁻²⁶ However, the role of the IL-33/ST2 pathway in the development of OVA-induced allergic airway inflammation in mice remains controversial, as reviewed elsewhere.¹⁴ Following two sensitizations with OVA emulsified with alum (OVA/Alum) and then challenge by OVA inhalation, respiratory function and/or eosinophilic airway inflammation were observed to be normal in ST2^{-/-} mice⁴²⁻⁴⁴ but attenuated in anti-ST2 mAb-treated wild-type mice.^{45,46} On the other hand, airway inflammation was attenuated in ST2^{-/-} mice that had been sensitized only once with OVA/alum and then challenged by OVA inhalation.⁴³ The reason for the discrepancy remains unclear. However, it might be partially due to an immunomodulatory effect of alum, which was used as an adjuvant, on the antigen sensitization. For example, mast cells, B cells, IgE, IL-1, TNF and CCR8 are not required for development of OVA-induced airway inflammation in mice that had been sensitized with OVA/alum, whereas they are essential for that event in mice that had been sensitized with OVA in the absence of alum, as reviewed elsewhere.⁴⁷ In the present study, we used another asthma-like mouse model in which mice were sensitized with OVA in the absence of alum via an epicutaneous route after tape stripping of the skin. It is known that airway inflammation observed in epicutaneously antigen-sensitized mice is associated with Th2-type cytokine-mediated eosinophilia and Th17-type cytokine-mediated neutrophilia.¹¹ Using that model, we clearly demonstrated that development of Th2-type allergic airway inflammation was significantly attenuated in epicutaneously

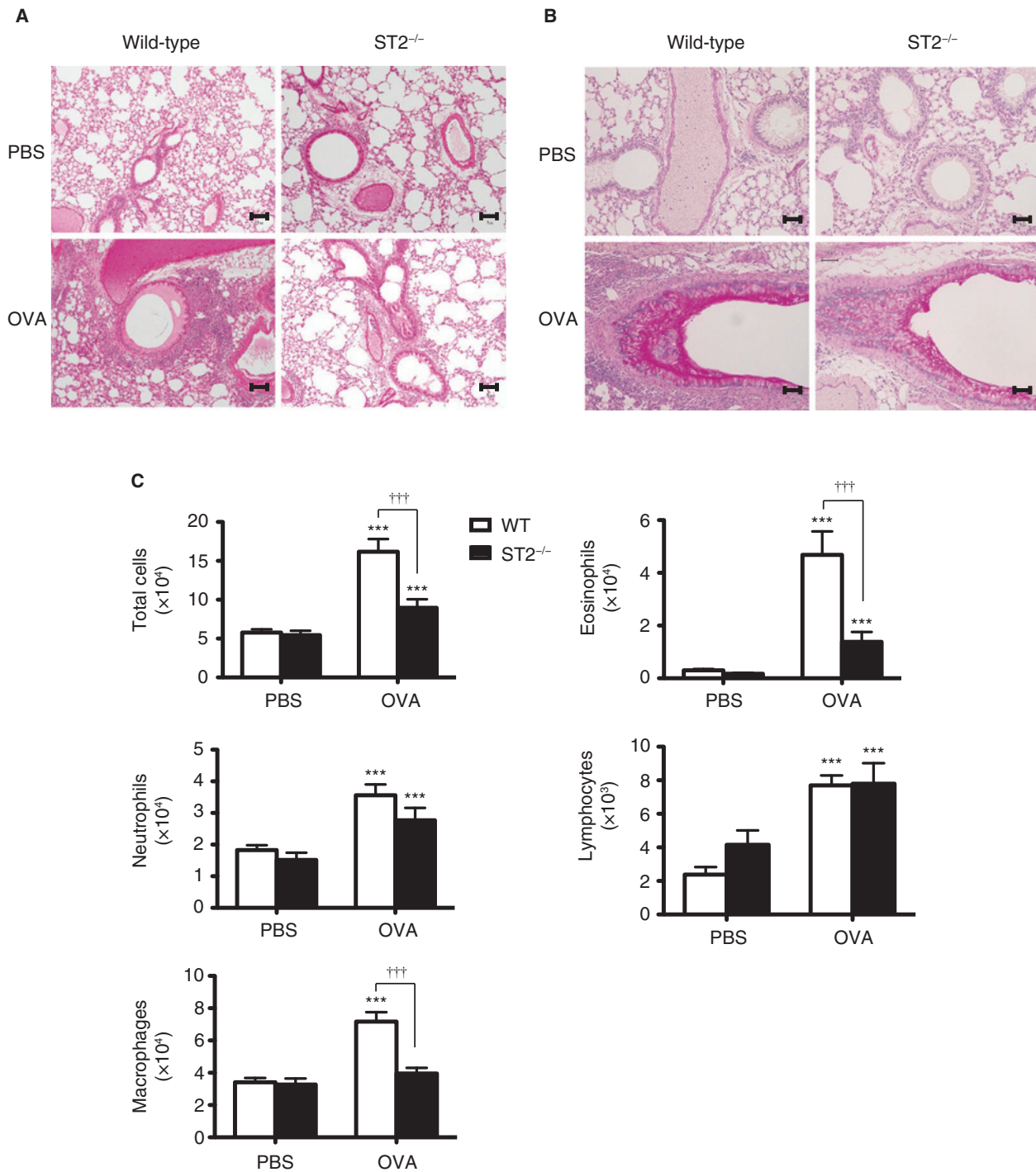


Fig. 3 Pulmonary inflammation was attenuated in epicutaneous OVA-sensitized ST2^{-/-} mice after OVA challenge. Mice were epicutaneously sensitized with OVA, followed by intranasal challenge with OVA or PBS, as shown in Figure 2. Twenty-four hours after the last OVA or PBS inhalation, the lungs, BAL cells and fluids, and sera were collected. (**A**, **B**) Lung histology. H&E (**A**) and PAS (**B**) staining. Bar = 100 μ m. The data show representative results from 8-12 mice in each experimental group, as indicated. (**C**) The number of BAL cells. The data show the mean $\pm$ SEM (wild-type [WT] mice: PBS, $n = 16$, and OVA, $n = 20$; ST2^{-/-} mice: PBS, $n = 14$, and OVA, $n = 20$). *** $P < 0.001$ vs. the corresponding values for PBS-treated mice, and ††† $P < 0.001$ vs. the indicated group.

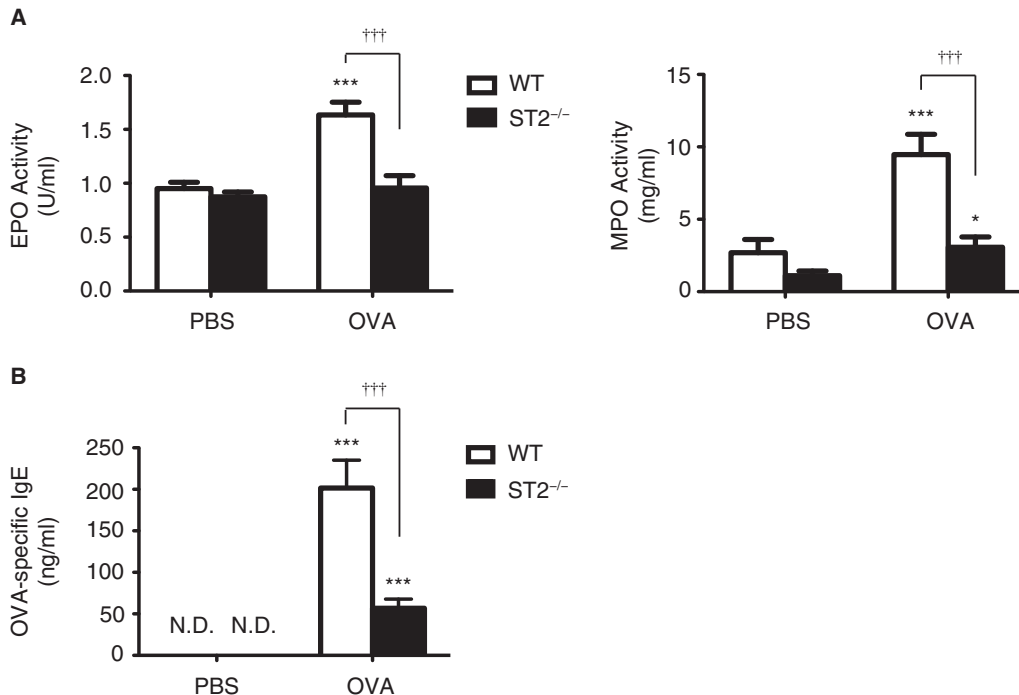


Fig. 4 The levels of EPO and MPO activities in BALFs and serum IgE were reduced in epicutaneous OVA-sensitized ST2^{-/-} mice after OVA challenge. **(A)** The levels of EPO and MPO activities in BALFs and **(B)** the levels of OVA-specific IgE in sera of the wild-type (WT) and ST2^{-/-} mice used in Figure 3B. * $P < 0.05$ and *** $P < 0.001$ vs. the corresponding values for PBS-treated mice, and ††† $P < 0.001$ vs. the indicated groups.

OVA-sensitized ST2^{-/-} mice after intranasal OVA challenge (Fig. 3B, 4A, 5), suggesting that the IL-33/ST2 pathway is crucial for induction of Th2-type allergic airway inflammation, especially under adjuvant-free experimental conditions.

It was recently reported that Th2-type eosinophilic airway inflammation after OVA inhalation was dramatically suppressed in IL-4^{-/-} IL-13^{-/-} mutant mice that had been epicutaneously sensitized with OVA, whereas Th17-type neutrophilic airway inflammation was exaggerated.¹² IL-17 production by T cells can be inhibited by both IL-4 and IFN- γ ,⁴⁸ suggesting that IL-4 deficiency resulted in increased IL-17 production and led to the enhanced IL-17-mediated immune responses observed in IL-4^{-/-} IL-13^{-/-} mice. In contrast to IL-4^{-/-} IL-13^{-/-} mice,¹² we found normal levels of pulmonary IL-17 mRNA expression and airway neutrophilia in epicutaneously OVA-sensitized ST2^{-/-} mice after the last OVA challenge (Fig. 3B, 5). It may be explained that IL-4 mRNA expression was significantly decreased, but not completely abrogated, in ST2^{-/-} mice (Fig. 5).

Despite the normal airway neutrophilia and IL-17 levels described above, the levels of CXCL1 and CXCL2 mRNA expression and MPO activity were markedly decreased in lungs and BALFs, respectively, from epicutaneously OVA-sensitized ST2^{-/-}

mice after the last OVA challenge (Fig. 4A, 5). This suggests that the IL-33/ST2 pathway may be partially involved in neutrophil recruitment, independently of IL-17. Indeed, we previously reported that IL-33 can directly induce production of neutrophil chemoattractants (i.e., IL-8) by both endothelial and epithelial cells.⁴⁹ However, since the effect of the IL-33/ST2 pathway on neutrophil recruitment was less than that of IL-17, ST2 deficiency may not exert a significant effect on airway neutrophilia in our experimental system.

In summary, we demonstrated that the IL-33/ST2 pathway is crucial for Th2-cytokine-mediated eosinophilic, rather than Th17-cytokine-mediated neutrophilic, airway inflammation in mice that had been epicutaneously sensitized with antigen. Our findings may provide a clue for development of novel therapeutics for asthma.

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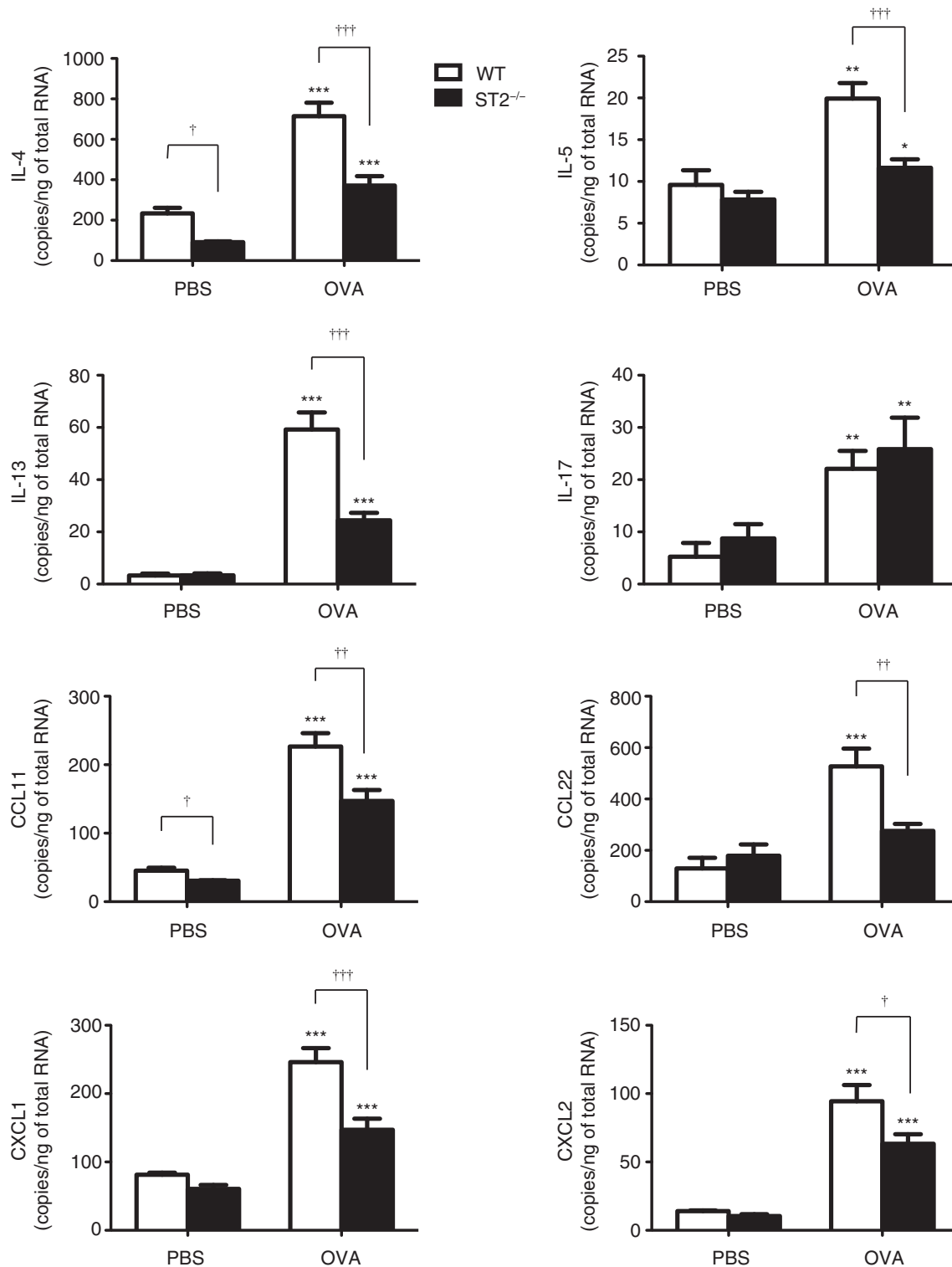


Fig. 5 The expression levels of Th2 cytokines, but not IL-17A, were reduced in lungs from epicutaneous OVA-sensitized ST2^{-/-} mice after OVA challenge. Twenty-four hours after the last OVA challenge, lungs were harvested from the wild-type (WT) and ST2^{-/-} mice used in Figure 3B, and the total mRNA was isolated. The expression of mRNA for cytokines and chemokines was determined by quantitative PCR. Data show the mean + SEM (WT mice: PBS, *n* = 16, and OVA, *n* = 20; ST2^{-/-} mice: PBS, *n* = 14, and OVA, *n* = 20). **P* < 0.05, ***P* < 0.01 and *** *P* < 0.001 vs. the corresponding values for PBS-treated mice, and † *P* < 0.05, †† *P* < 0.01 and ††† *P* < 0.001 vs. the indicated groups.

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