

Pathogenic changes in group 2 innate lymphoid cells (ILC2s) in a steroid-insensitive asthma model of mice

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ABSTRACT

A certain population of asthma patients is resistant to steroid therapy, whereas the mechanisms remain unclear. One of characteristic features of steroid-resistant asthma patients is severe airway eosinophilia based on type-2 inflammation. Aims of this study were: 1) to develop a murine model of steroid-resistant asthma, 2) to elucidate that predominant cellular source of a type-2 cytokine, IL-5 was group 2 innate lymphoid cells (ILC2s), 3) to analyze pathogenic alteration of ILC2s in the severe asthma, and 4) to evaluate therapeutic potential of anti-IL-5 monoclonal antibody (mAb) on the steroid-resistant asthma. Ovalbumin (OVA)-sensitized BALB/c mice were intratracheally challenged with OVA at 5 or 500 µg/animal 4 times. Development of airway eosinophilia and remodeling in 5-µg OVA model were significantly suppressed by 1 mg/kg dexamethasone, whereas those in 500-µg OVA model were relatively insensitive to the dose of dexamethasone. ILC2s isolated from the lung of the steroid-insensitive model (500-µg OVA) produced significantly larger amounts of IL-5 in response to IL-33/TSLP than ILC2s from the steroid-sensitive model (5-µg OVA). Interestingly, TSLP receptor expression on ILC2s was up-regulated in the steroid-insensitive model. Treatment with anti-IL-5 mAb in combination with dexamethasone significantly suppressed the airway remodeling of the steroid-insensitive model. In conclusion, multiple intratracheal administration of a high dose of antigen induced steroid-insensitive asthma in sensitized mice. IL-5 was mainly produced from ILC2s, phenotype of which had been pathogenically altered probably through the up-regulation of TSLP receptors. IL-5 blockage could be a useful therapeutic strategy for steroid-resistant asthma.

1. Introduction

Asthma is a chronic inflammatory airway disease characterized by airway eosinophilia, hyperresponsiveness and remodeling (Wenzel, 2012). Long-term treatment with inhaled corticosteroids and a long-acting β_2 agonist is the standard therapy for asthma (Akdiss, 2012). In particular, corticosteroid therapy is indispensable to control airway inflammation, but that in a certain population of patients is not well controlled by inhaled corticosteroids or even by oral corticosteroids (Peters and Wenzel, 2020). It has been suggested that 5–10% of asthma patients receiving medical care are steroid-resistant (Sorkness et al., 2008; Mercado et al., 2012). In general, eosinophilic airway inflammation is well controlled by corticosteroid therapy. However, a certain

patient population with severe asthma exhibits persistent airway eosinophilia based on type-2 inflammation despite long-term corticosteroid therapy (Wenzel, 2012; Chambers et al., 2015; Peters et al., 2019).

Type-2 inflammation is induced by the following mechanisms (Wenzel, 2012; Akdiss et al., 2020): 1) Th2 cells are capable of producing interleukin (IL)-5, a strong chemoattractant of eosinophils, and eosinophils infiltrating into the lung may be involved in the induction or exacerbation of airway inflammation, resulting in airway remodeling and hyperresponsiveness. 2) Group 2 innate lymphoid cells (ILC2s) are also involved in eosinophilic airway inflammation because they produce large amounts of type-2 cytokines, IL-5 and IL-13 in response to airway epithelial cell-derived cytokines such as IL-33 and thymic stromal

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lymphopoietin (TSLP). It was clinically demonstrated that anti-IL-5 monoclonal antibody (mAb), mepolizumab and anti-IL-5 receptor α mAb, benralizumab exerted glucocorticoid-sparing effects in eosinophilic asthma patients (Bel et al., 2014; Nair et al., 2017). These studies suggest that IL-5 or subsequently activated eosinophils make it difficult for airway tissues to react to glucocorticoids. However, the molecular mechanisms associated with the acquisition of steroid resistance have not been clarified in clinical or experimental studies in vivo. One of the reasons is the lack of appropriate animal models for steroid-resistant asthma.

In this study, almost consistent with previous studies to date (De Bie et al., 1996; Blyth et al., 2000; Dorscheid et al., 2003; Ulrich et al., 2008; Alves et al., 2013), it was confirmed that when sensitized BALB/c mice were repeatedly challenged with a relatively low dose (5 μ g/animal/time/day) of antigen, ovalbumin (OVA), asthmatic responses were well suppressed by treatment with a corticosteroid, dexamethasone. In contrast, when a high dose (500 μ g/animal/time/day) of OVA was used for the multiple challenges, the asthmatic responses became relatively insensitive to the glucocorticoid. Thus, this asthma model may reflect a certain pathogenesis of steroid-resistant asthma. Using these steroid-sensitive and-insensitive asthma models, it was elucidated whether major cellular source of IL-5 was ILC2s, and whether ILC2s were phenotypically altered to be pathogenic in the severe asthma. Finally, therapeutic potential of anti-IL-5 mAb on the steroid-insensitive asthma was assessed.

2. Materials and methods

2.1. Sensitization and challenge

As reported previously (Nabe et al., 2005, 2011a; 2011b, 2013, 2015), 5-week-old BALB/c mice (Japan SLC, Hamamatsu, Japan) were sensitized by i.p. injections with OVA (purity of $\geq 98\%$, Grade V, Sigma Chem., St. Louis, MO, USA) adsorbed to Al(OH)₃ at a dose of 50 μ g of OVA/2 mg Al(OH)₃/0.5 ml of saline/animal/time on days 0, 14 and 28. Then, the sensitized mice were intratracheally challenged on days 35, 36, 37 and 40 under inhalation anesthesia with isoflurane with OVA at a dose of 5 or 500 μ g/animal/time/day in a volume of 25 μ l.

Dexamethasone 21-phosphate (Sigma Chem.) was i.p. administered during the intratracheal OVA challenges on days 35, 36, 37, 38, 39 and 40 at a dose of 1 mg/kg/time/day as dexamethasone. When administered on the days of OVA challenges, injection of dexamethasone was conducted 3 h before the respective OVA challenges. In several asthmatic models to date (De Bie et al., 1996; Blyth et al., 2000; Dorscheid et al., 2003; Ulrich et al., 2008; Alves et al., 2013), the development of airway remodeling and airway hyperresponsiveness were markedly suppressed by dexamethasone at 1 mg/kg or less than 1 mg/kg.

As previously reported (Nabe et al., 2011a), anti-IL-5 mAb, TRFK-5 (kindly donated by Prof. Kohtaro Fujihashi, Tokyo University, Tokyo, Japan) was i.p. injected on days 35 and 37 at a dose of 50 μ g/animal/time/day 3 h before OVA challenges.

This animal study was approved by the Experimental Animal Research Committee at Setsunan University. The approval numbers of the animal studies were K17-1, K18-1, K19-1 and K20-1.

2.2. Analysis of eosinophils and neutrophils recovered by bronchoalveolar lavage (BAL)

At 24 h after the 4th intratracheal challenge, the mice were anesthetized by i.p. injection of pentobarbital (70 mg/kg) and xylazine (12 mg/kg). The right lung was lavaged via a tracheal catheter using two aliquots of 0.6 ml of phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS). Total leukocyte numbers were counted by a particle counter and size analyzer (Z2, Beckman Coulter, Brea, CA, USA) after treatment with ammonium-chloride-potassium (ACK) lysis buffer to remove contaminating erythrocytes. For the differential cell counts, cells

were transferred onto a glass slide by centrifugation in a cell settling chamber (Neuro Probe, Gaithersburg, MD, USA). Subsequently, cells were then stained with Diff-Quik solution (Sysmex International Reagent, Kobe, Japan), and then eosinophils and neutrophils were detected under observation by light microscope.

2.3. Analysis of mast cells, basophils, ILC2s in the lung, and expressions of TSLP receptors and IL-33 receptors (ST2) on ILC2s

The left or right lung lobes were isolated 24 h after the 4th challenge under the general anesthetization, and then minced in PBS. The minced lung was digested by incubation with 870 units/ml of collagenase type I (Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C for 1 h. Cells were dispersed with a syringe and then filtered through 70- μ m nylon mesh. After centrifugation, cells were treated with ACK lysis buffer to remove contaminating erythrocytes. The total number of leukocytes was counted using a hemocytometer under staining with trypan blue.

According to previous reports (Nabe et al., 2013; Halim et al., 2012), mast cells, basophils, and ILC2s were detected as IgE⁺ c-kit⁺ cells, IgE⁺ c-kit⁺ CD49b⁺ cells, and lineage[−] CD45⁺ CD278⁺ CD90.2⁺ ST2⁺ cells, respectively. First, lung cells were treated with an anti-CD16/32 (Fc γ RII/III) antibody to prevent non-specific binding of the subsequently used antibodies to the cells. After washing with 2%FBS-containing PBS, the cells were stained with fluorescein isothiocyanate-conjugated anti-mouse IgE (clone R35-72) and phycoerythrin-conjugated anti-mouse c-kit (clone 2B8) (all from BD Biosciences, San Jose, CA, USA), allophycocyanin-conjugated anti-mouse CD49b (clone DX5), fluorescein isothiocyanate-conjugated anti-mouse lineage cocktail including anti-mouse CD3 ϵ (clone 145-2C11), anti-mouse Ly-6G/Ly-6C (clone RB6-8C5), anti-mouse CD11b (clone M1/70), anti-mouse CD45R/B220 (clone RA3-6B2), anti-mouse TER-119/Erythroid cells (clone Ter-119), pacific blue-conjugated anti-mouse CD45 antibody (clone 30-F11), phycoerythrin-conjugated anti-mouse CD278 antibody (clone 15F9), brilliant violet 510™-conjugated anti-mouse CD90.2 (Thy-1.2) antibody (clone 53-2.1), brilliant violet 421™-conjugated anti-mouse ST2 antibody (clone DIH9), brilliant violet 421™-conjugated anti-mouse TSLP receptor antibody (clone 22H9), brilliant violet 421™-conjugated rat IgG2a, κ isotype control antibody (clone RTK2758) (all from BioLegend, San Diego, CA, USA), and/or allophycocyanin-conjugated anti-mouse ST2 antibody (clone RMST2-2, Thermo Fisher Scientific) for 20 min at 4 °C. After washing with 2% FBS-containing PBS, cells were analyzed using FACSARIA™ Fusion (Becton Dickinson, San Jose, CA, USA).

2.4. Measurement of cytokines and chemokines in the lung tissue

The left lung lobes were isolated at 24 h after the 4th challenge with 5 or 500 μ g/animal of OVA. As reported by us (Nabe et al., 2015, 2018), the left lobes were homogenized in 1 ml of protease inhibitor cocktail tablet (Complete, Mini®, Roche, Mannheim, Germany)-containing tissue protein extraction reagent (T-PER, Thermo Fisher Scientific) with the polytron homogenizer. After centrifugation, supernatants were stored at −80 °C until measurement of cytokines by enzyme-linked immunosorbent assay (ELISA). ELISA kits for IL-4, IL-5, IL-13, IL-17A, IL-33 and eotaxin-1 (Thermo Fisher Scientific), IL-10 and TSLP (Biolegend), and KC (R&D Systems, Minneapolis, MN, USA) were used.

2.5. Histological examination

Twenty 4 h after the 4th OVA challenge, the left lobes of the lung were isolated under anesthetization with pentobarbital and xylazine, and then fixed with 10% buffered formalin. Tissues were embedded in paraffin, and 4- μ m sections were stained with Masson trichrome and periodic acid–Schiff (PAS) for light microscopic observation. The lung tissues that had not undergone BAL were used in this examination.

Similar to our previous studies (Nabe et al., 2011a; Matsuda et al., 2018), histological changes were semi-quantified. The degree of epithelial thickening was quantified using PAS-stained sections as follows: the bronchus, the diameter of which was over 150 μm , was photographed, and then the epithelial area (μm^2) and the length of the basement membrane (μm) were measured using Adobe Photoshop CS2 (version 9.0.2; Adobe Systems, San Jose, CA, USA) and Image J (version 1.42; NIH, Bethesda, MD, USA). The degree of epithelial thickening was expressed by dividing the epithelial area by the length of the basement membrane. Mucus accumulation in the epithelial layer was also quantified using PAS-stained sections by dividing the PAS⁺ area in the epithelium (μm^2) by the length of the basement membrane (μm).

Masson trichrome-stained sections were used for assessment of subepithelial fibrosis by scoring the extension of the blue fibrotic area by a 7-point graded scale from 0 to 3: 0, no blue area existed underneath the basement membrane; 0.5, 1, 1.5, 2, 2.5 and 3, blue fibrotic area existed underneath the basement membrane by approximately less than 15 μm , 15–30 μm , 30–45 μm , 45–60 μm , 60–75 μm or more than 75 μm . All specimens were analyzed in a blinded manner.

2.6. Production of IL-5, IL-4, and IL-13 from ILC2s and CD4⁺ T cells in response to IL-33 and TSLP

The lung cells were collected at 24 h after the 4th challenge with 5 or 500 μg /animal of OVA. ILC2s were purified from the lung cells by magnetic beads and flow cytometry. Anti-human/mouse CD278 antibody (clone C398.4A, Biolegend) was labelled by the DSB-X biotin protein labeling kit (Thermo Fisher Scientific). CD278⁺ cells were purified using the DSB-X biotinylated antibody and Dynabeads® Flow-Comp™ Flexi, part A (Thermo Fisher Scientific) according to the protocol provided by the manufacturer. After the isolation of CD278⁺ cells, lineage[−] CD45⁺ CD90.2⁺ ST2⁺ cells were sorted by flow cytometry.

CD4⁺ T cells were purified from the lung isolated at 24 h after the 4th challenge in both models by flow cytometry. CD4⁺ T cells were stained with fluorescein isothiocyanate-conjugated anti-mouse CD3 ϵ (clone 145-2C11, BD Biosciences) and phycoerythrin-conjugated anti-mouse CD4 (clone H129.19, BD Biosciences). After the fluorescent immunostaining, CD4⁺ T cells were sorted by flow cytometry.

ILC2s (5×10^4 cells/ml) and CD4⁺ T cells (5×10^4 cells/ml) were incubated with IL-33 (Biolegend, 1–100 ng/ml), TSLP (R&D systems, 1–100 ng/ml), or their combination (10 ng/ml IL-33 and 10 ng/ml TSLP) for 72 h at 37 °C. A mixed solution of phorbol myristate acetate (PMA) and ionomycin (10^{-8} M PMA and 10^{-6} g/ml ionomycin) was also used as a stimulant of ILC2s and CD4⁺ T cells. Dynabeads™ Mouse T-Activator CD3/CD28 (Thermo Fisher Scientific) was used to stimulate CD4⁺ T cells.

After centrifugation, the concentrations of IL-5, IL-4, and IL-13 in the supernatant were measured by respective ELISA kits (Thermo Fisher Scientific).

2.7. Total RNA sequencing and bioinformatics

Total RNA of the lung and purified ILC2s derived from non-challenged (N=5), 5- μg OVA-challenged (N=5), and 500- μg OVA-challenged mice (N=5) were extracted using miRNeasy Mini kit (Qiagen, Venlo, Netherlands). The quality and quantity of total RNA were assessed by NanoDrop One (Thermo Fisher Scientific) and Agilent 2100 Bioanalyzer System (Agilent Technologies, Santa Clara, CA, USA), respectively. One hundred ng of total RNA was treated with NEBNext rRNA Depletion kit (New England BioLabs, Ipswich, MA, USA) according to the manufacturer's protocol, and then RNA-seq libraries were generated with TruSeq Stranded Total RNA Library Prep Kit (Illumina, San Diego, CA, USA). The cDNA libraries were sequenced with 50 base paired-end sequencing on an NovaSeq 6000 System (Illumina). The sequencing run and the base call analysis were performed according to

NovaSeq 6000 Sequencing System Guide with NovaSeq 6000 SP Reagent Kit (100 cycles). After the sequencing, sequence raw data was generated with processing by bcl2fastq-v2.20.0.422.

Differential gene expression analysis was performed by the Cufflinks suite of tools v2.0.2 (Trapnell et al., 2012). Differences were considered significant when q-value was <0.05.

2.8. Statistical analyses

A one-way analysis of variance was performed. If significant differences were detected, individual group differences were determined by Dunnett's tests or Tukey's tests. The unpaired *t*-test was used to compare between two groups. A probability value (P) below 0.05 was considered significant. These statistical analyses were conducted using JMP Pro 11.2.0 (SAS Institute Japan, Tokyo, Japan). A statistical analysis of RNA-sequencing was described above.

3. Results

3.1. Steroid insensitivity in infiltration of eosinophils and neutrophils into the lung

As shown in Fig. 1A, infiltration of eosinophils into the lung was comparably induced in both 5- and 500- μg OVA-induced models. Multiple treatments with dexamethasone (1 mg/kg/time/day) potentially suppressed the airway eosinophilia in the 5- μg OVA model. This effect of dexamethasone was almost similar to other murine models, which had been reported to date (De Bie et al., 1996; Blyth et al., 2000; Dorscheid et al., 2003; Ulrich et al., 2008; Alves et al., 2013). In contrast, the eosinophil infiltration induced by 500- μg OVA was not significantly inhibited by dexamethasone at the same dosage. On the other hand, regarding infiltration of neutrophils into the lung, the degree in the 500- μg OVA model was significantly higher than that in the 5- μg OVA model (Fig. 1B). As in the case of eosinophils, although the neutrophilia in the 5- μg OVA model was almost completely suppressed by dexamethasone, that in the 500- μg OVA model was not significantly affected (Fig. 1B).

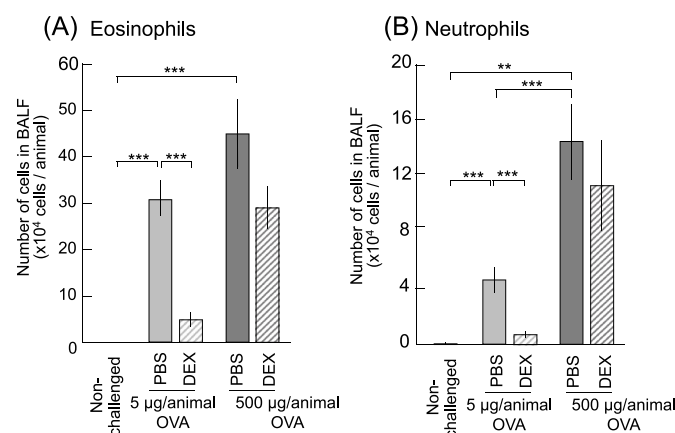


Fig. 1. Effects of dexamethasone on 5- or 500- μg OVA-induced increases in the numbers of eosinophils (A) and neutrophils (B) in the BALF of sensitized mice. BALB/c mice were i.p. sensitized with OVA+Al(OH)₃ on days 0, 14, and 28, followed by intratracheal OVA challenges on days 35, 36, 37, and 40 at a dose of 5 or 500 μg /animal. Dexamethasone (DEX, 1 mg/kg/time/day) or PBS was i. p. administered 3 h before the respective 4 challenges, and on days 38 and 39. BALF was collected 24 h after the 4th challenge, followed by differential cell count. Each column represents the mean $\pm$ S.E. of 9–14 animals. ** and ***: significant at $p < 0.01$ and 0.001 , respectively.

3.2. Steroid insensitivity in the development of airway remodeling

In both 5- and 500- μ g OVA models, bronchial epithelial thickening and mucus accumulation in the epithelium were significantly developed (Fig. 2A, C and 2D). However, subepithelial fibrosis was detected only in the 500- μ g OVA model (Fig. 2B and E). The multiple treatments with dexamethasone almost completely suppressed the development of epithelial thickening and mucus accumulation induced by 5- μ g OVA (Fig. 2A, C and 2D). In contrast, such epithelial changes were not significantly inhibited by dexamethasone (1 mg/kg/time/day) in the 500- μ g OVA model (Fig. 2A, C and 2D). In addition, subepithelial fibrosis induced only in the 500- μ g OVA model was hardly affected by dexamethasone (Fig. 2B and E).

3.3. IL-5-producing cells, ILC2s and Th2 cells

Both Th2 cells and ILC2s are major sources of IL-5 in allergic airway inflammation (Akdis et al., 2020). Thus, we investigated which cells were the main producer of IL-5 in the lung of the asthma models. According to a previous report (Halim et al., 2012), ILC2s in the lung were detected and sorted by flow cytometry as lineage[−] CD45⁺ CD278⁺ ST2⁺ CD90.2⁺ cells. CD4⁺ T cells in the lung were also sorted by flow cytometry. Compared with CD4⁺ T cells (Fig. 3B), ILC2s produced large amounts of IL-5 in response to combination treatment with IL-33 and TSLP, which are airway epithelial cell-derived cytokines (Mitchell and

O'Byrne, 2017), or PMA/ionomycin (Fig. 3A). Interestingly, the amounts of IL-5 produced from ILC2s of the 500- μ g OVA model were significantly larger than those from the 5- μ g OVA model (Fig. 3A). In contrast, CD4⁺ T cells produced relatively small amounts of IL-5 even after stimulation with anti-CD3/CD28 antibodies (Fig. 3B). The response of CD4⁺ T cells collected from the 500- μ g OVA model to anti-CD3/CD28 antibodies was rather significantly lower than that of the 5- μ g OVA model (Fig. 3B).

Capabilities of other type-2 cytokine production from ILC2s were also compared between the steroid-sensitive and insensitive models. Consistent with the results of IL-5, production of IL-13 and IL-4 from the ILC2s of the steroid-insensitive model was also up-regulated, although the amount of IL-4 was approximately 100-fold lower than those of IL-5 and IL-13 (Fig. 4A, Fig. 4B).

3.4. TSLP receptors and IL-33 receptors (ST2) on the ILC2s

In order to clarify the molecular mechanisms underlying the IL-5 hyper-producing activity of ILC2s of the steroid-insensitive model, expression of TSLP receptors and ST2 on ILC2s were analyzed by flow cytometry. As shown in Fig. 5A and B, the median fluorescence intensity (MFI) of TSLP receptors on ILC2s from the 500- μ g OVA model was significantly potentiated compared with that of those from the 5 μ -OVA model. In contrast, the MFI of ST2 on ILC2s from the 500 μ -OVA model was not different from that of those from the 5 μ -OVA model, although

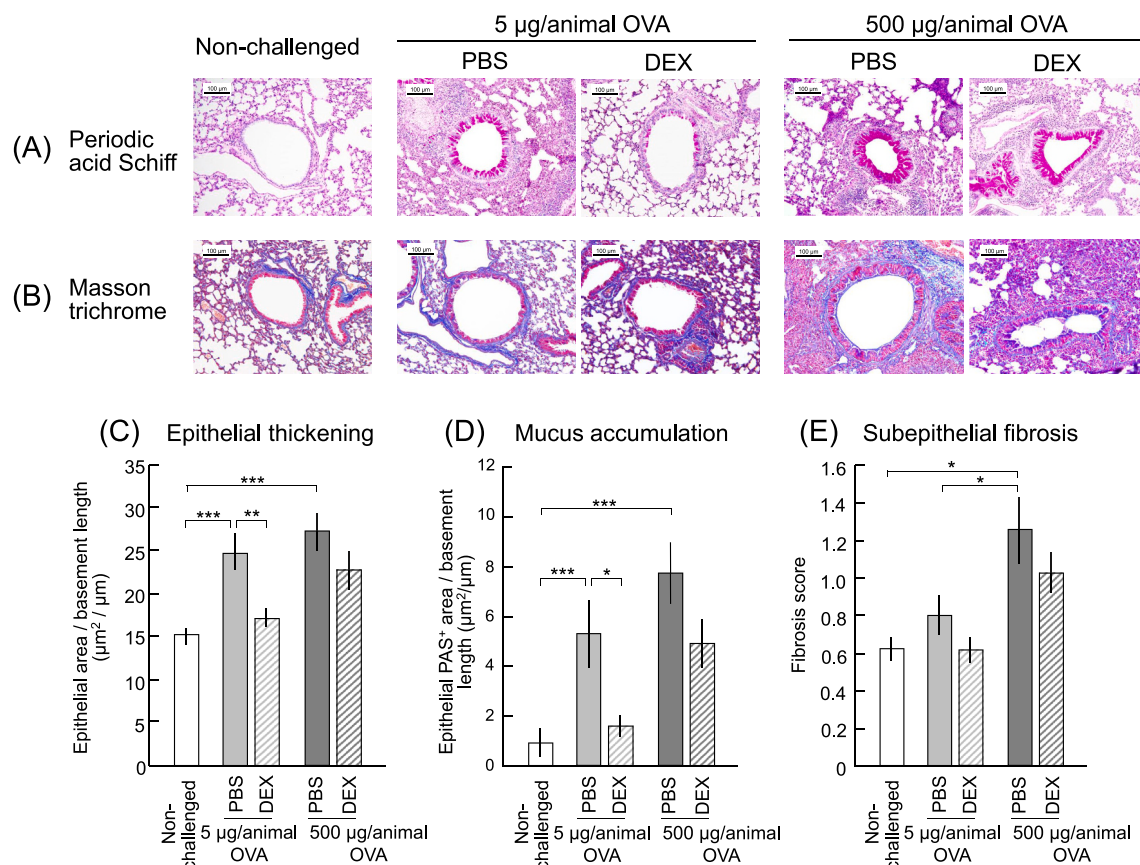


Fig. 2. Effects of dexamethasone on 5- or 500- μ g OVA-induced development of airway remodeling in sensitized mice. BALB/c mice were i.p. sensitized with OVA+Al (OH)₃ on days 0, 14, and 28, followed by intratracheal OVA challenges on days 35, 36, 37, and 40 at a dose of 5 or 500 μ g/animal. Dexamethasone (DEX, 1 mg/kg/time/day) or PBS was i.p. administered 3 h before the respective 4 challenges, and on days 38 and 39. The lung was isolated 24 h after the 4th OVA challenge or without OVA challenges. Isolated lung tissue was fixed with 10% formalin, embedded in paraffin, and cut into 4- μ m sections, followed by staining with PAS (A) or Masson trichrome (B). The degree of epithelial thickening was quantified using PAS-stained sections by dividing the epithelial area in a bronchus (diameter: >150 μ m) by the length of the basement membrane (C). The degree of mucus accumulation was calculated by dividing the PAS⁺ area in a bronchus by the length of the basement membrane (D). Subepithelial fibrosis was assessed using Masson trichrome-stained sections by scoring the extension of the blue fibrotic area by a 4-point graded scale from 0 to 3. Each column represents the mean $\pm$ S.E. of 10–17 animals. *, ** and ***: significant at $p < 0.05$, 0.01, and 0.001, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

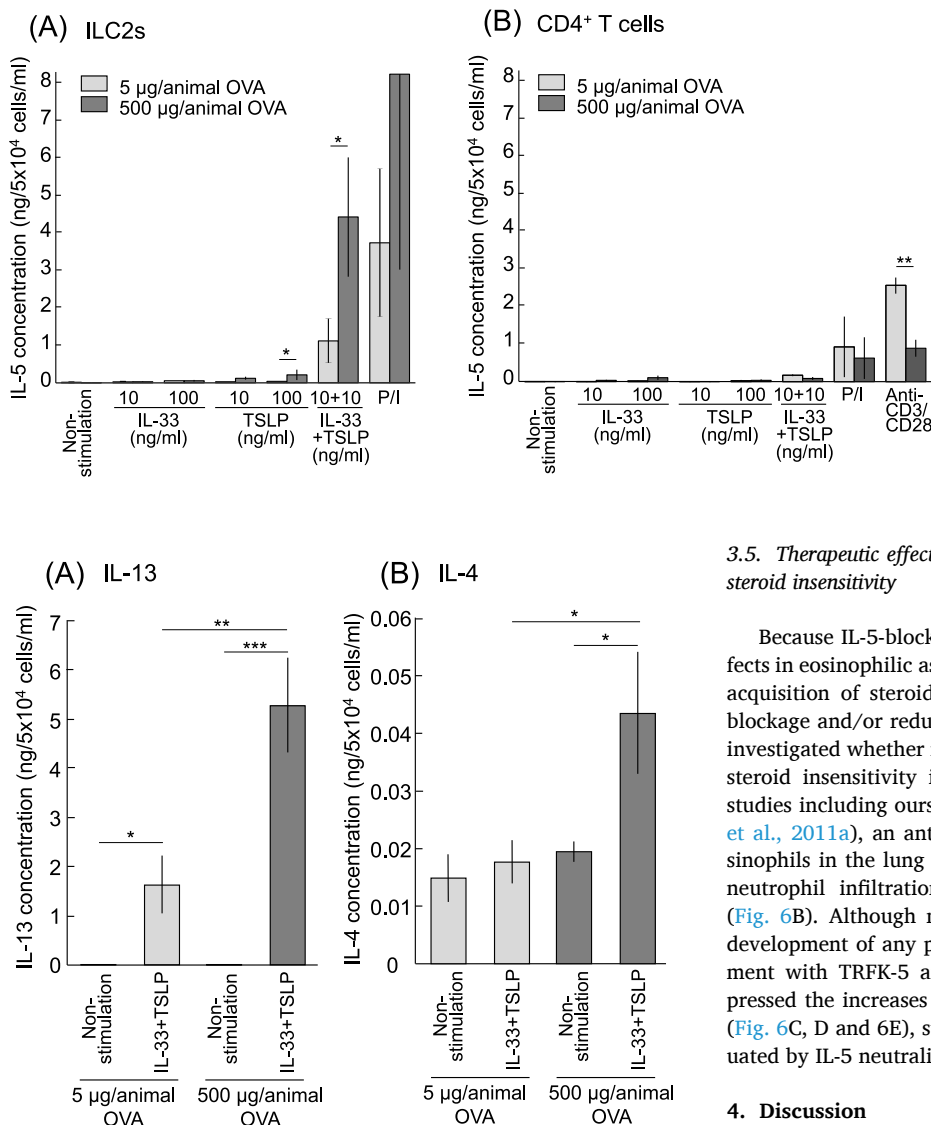


Fig. 4. Hyper-production of IL-13 (A) and IL-4 (B) from ILC2s of the steroid-resistant model *in vitro*. BALB/c mice were i.p. sensitized with OVA+Al(OH)₃ on days 0, 14, and 28, followed by intratracheal OVA challenges on days 35, 36, 37, and 40 at a dose of 5 or 500 µg/animal. The left lung lobes were collected 24 h after the 4th challenge. ILC2s in the dispersed lung tissue were detected by flow cytometry. Purified ILC2s were incubated with TSLP and IL-33 (both 10 ng/ml) or vehicle (no stimulation) for 72 h. IL-4 and IL-13 in the supernatant were measured by ELISA. Each column represents the mean $\pm$ S.E. of 5 experiments. *, ** and ***: significant at $p < 0.05$, 0.01, and 0.001, respectively.

it significantly increased even in the 5-µg OVA model (Fig. 5C and D).

RNA-seq was performed on ILC2s collected from the lung of non-challenged, and 5- and 500-µg OVA models. As shown in Table 1, gene expression of *Crlf2* (i.e. TSLP receptors) and *Il1rl1* (i.e. ST2) in ILC2s were up-regulated in both 5- and 500-µg OVA models in comparison with those from non-challenged. Consistent with the results of protein expression (Fig. 5), the expression of *Crlf2* but not *Il1rl1* in ILC2s derived from 500-µg OVA model was significantly higher than that from 5-µg OVA model. Gene expressions of Janus kinase (JAK) and signal transducer and activator of transcription (STAT) other than *Stat4* and *Stat5b* were significantly up- or down-regulated in ILC2s derived from both 5- and 500-µg OVA models. Among of the genes of JAKs and STATs, expressions of *Jak3* and *Stat5a* in ILC2s derived from 500-µg OVA model were markedly and significantly higher than those from 5-µg OVA model.

Fig. 3. Capability of IL-5 production *in vitro* by ILC2s (A) and CD4⁺ T cells (B) derived from lung cells of sensitized mice challenged with 5 or 500 µg of OVA. BALB/c mice were i.p. sensitized with OVA+Al(OH)₃ on days 0, 14, and 28, followed by intratracheal OVA challenges on days 35, 36, 37, and 40 at a dose of 5 or 500 µg/animal. Twenty-four hours after the 4th challenge, the lung was isolated and then dispersed by collagenase, followed by sorting of ILC2s and CD4⁺ T cells by flow cytometry. Purified ILC2s and CD4⁺ T cells were incubated with IL-33 and/or TSLP, PMA/ionomycin (P/I), vehicle, or anti-CD3/CD28 antibodies for 72 h. IL-5 in the supernatant was measured by ELISA. Each column represents the mean $\pm$ S.E. of 5 experiments. * and **: significant at $p < 0.05$ and 0.01, respectively.

3.5. Therapeutic effectiveness of anti-IL-5 mAb on the acquisition of steroid insensitivity

Because IL-5-blocking antibodies exerted glucocorticoid-sparing effects in eosinophilic asthma patients (Bel et al., 2014; Nair et al., 2017), acquisition of steroid resistance in asthma may be relieved by IL-5 blockage and/or reduction of airway eosinophilia. Thus, it was finally investigated whether neutralization of IL-5 can affect the acquisition of steroid insensitivity in the 500 µg-OVA model. Similar to previous studies including ours (Mathur et al., 1999; Tomaki et al., 2000; Nabe et al., 2011a), an anti-IL-5 mAb, TRFK-5, reduced the number of eosinophils in the lung of the 500-µg OVA model (Fig. 6A). In contrast, neutrophil infiltration into the lung was not affected by TRFK-5 (Fig. 6B). Although neither TRFK-5 nor dexamethasone affected the development of any parameter of airway remodeling, combined treatment with TRFK-5 and dexamethasone partly but significantly suppressed the increases in all assessed parameters of airway remodeling (Fig. 6C, D and 6E), suggesting that the steroid insensitivity was attenuated by IL-5 neutralization.

4. Discussion

In general, asthmatic responses in murine models were markedly suppressed by dexamethasone at 1 mg/kg or less than 1 mg/kg (De Bie et al., 1996; Blyth et al., 2000; Dorscheid et al., 2003; Ulrich et al., 2008; Alves et al., 2013). Asthmatic responses induced by 5-µg OVA in this study were also almost completely suppressed by 1 mg/kg dexamethasone. However, when the challenging dose of OVA was increased to 500 µg, the responses became relatively insensitive to the same dose of dexamethasone. In our previous studies using the 500-µg OVA model, when dexamethasone was administered at 3 mg/kg, the airway eosinophilia and remodeling were suppressed, whereas the suppression was only partial (Nabe et al., 2011a; Matsuda et al., 2018).

Molecular mechanisms underlying steroid-resistant asthma have not been clarified *in vivo*. This should be due to lack of appropriate models. Similar to the present study, multiple intratracheal challenges with 3 allergens, OVA, cockroach allergen and house dust mite, produced steroid-resistant asthma in mice (Duechs et al., 2014), in which steroid resistance developed after 69 days. The present model required only 43 days after the initial sensitization. These models can be utilized for analyses of mechanisms of steroid-resistant asthma in humans.

It is unclear why the high-dose OVA induced the steroid-insensitive asthma. Sharpy et al. (2014) reported that OVA (Grade V, Sigma Chem.) that we used contained lipopolysaccharide (LPS) at 400 ng/10 mg OVA. It has been reported that LPS contributes to asthma exacerbation and development of corticosteroid insensitivity (Goleva et al.,

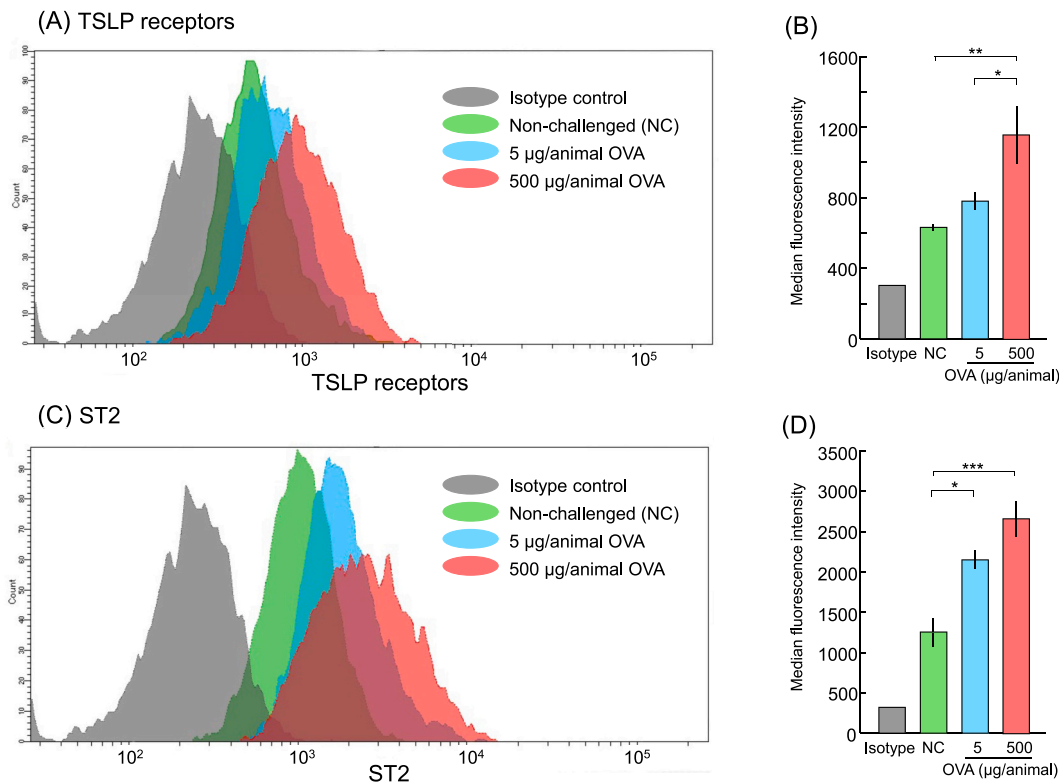


Fig. 5. Expression of TSLP receptors (A and B) and ST2 (C and D) on ILC2s derived from lung cells of sensitized mice challenged with 5 or 500 µg of OVA. BALB/c mice were i.p. sensitized with OVA+Al(OH)₃ on days 0, 14, and 28, followed by intratracheal OVA challenges on days 35, 36, 37, and 40 at a dose of 5 or 500 µg/animal. Twenty-four hours after the 4th challenge, the lung was isolated and then dispersed by collagenase. The median fluorescence intensity of TSLP receptors and ST2 on ILC2s was calculated by flow cytometry. Each column of non-challenged, 5 µg of OVA and 500 µg of OVA represents the mean ± S.E. of 4 experiments. The column of isotype control represents 1 experiment. NC: Non-challenged.

Table 1

Relative gene expression of TSLP receptor (*Crlf2*), ST2 (*Il1rl1*) and JAK-STAT signaling molecules in ILC2s derived from non-challenged, and 5- and 500-µg OVA models.

Gene	5-µg OVA/Non-challenged			500-µg OVA/Non-challenged			500-µg OVA/5-µg OVA		
	Log ₂ FC	q value	Significance	Log ₂ FC	q value	Significance	Log ₂ FC	q value	Significance
<i>Crlf2</i>	0.817	0.0003	Yes	1.351	0.0003	Yes	0.534	0.0003	Yes
<i>Il1rl1</i>	0.337	0.033	Yes	0.388	0.013	Yes	0.051	0.816	No
<i>Jak1</i>	-0.304	0.015	Yes	-0.304	0.014	Yes	0.0007	0.996	No
<i>Jak2</i>	0.242	0.048	Yes	0.257	0.034	Yes	0.015	0.933	No
<i>Jak3</i>	1.445	0.0003	Yes	1.766	0.0003	Yes	0.321	0.013	Yes
<i>Tyk2</i>	0.387	0.001	Yes	0.439	0.0003	Yes	0.052	0.737	No
<i>Stat1</i>	0.443	0.0003	Yes	0.337	0.004	Yes	-0.106	0.457	No
<i>Stat2</i>	0.546	0.0003	Yes	0.544	0.0003	Yes	0.001	0.994	No
<i>Stat3</i>	0.395	0.001	Yes	0.528	0.0003	Yes	0.132	0.368	No
<i>Stat4</i>	-0.147	0.3132	No	-0.216	0.120	No	-0.069	0.681	No
<i>Stat5a</i>	0.357	0.002	Yes	0.745	0.0003	Yes	0.388	0.0003	Yes
<i>Stat5b</i>	-0.071	0.635	No	-0.018	0.919	No	0.053	0.723	No
<i>Stat6</i>	-0.388	0.004	Yes	-0.396	0.006	Yes	-0.008	0.971	No

RNA-seq analyses were conducted using ILC2s collected from 5 animals of each group. FC: fold change.

2008, 2013). Stimulation of toll-like receptor 4 by LPS is followed by p38 mitogen-activated protein kinase (MAPK)-mediated intracellular events, resulting in proinflammatory cytokine production (Ulevitch and Tobias, 1999). Other studies demonstrated that up-regulation of p38 MAPK expression in peripheral blood mononuclear cells from corticosteroid-insensitive asthma patients was negatively correlated with the steroid responsiveness (Bhavsar et al., 2010; Mercado et al., 2011). Therefore, LPS contained in OVA may be involved in the decreased sensitivity to glucocorticoids in immune cells. Regarding roles of LPS in the development of steroid insensitivity, further analyses are required using our models.

Type-2 inflammatory responses manifested by airway eosinophilia

were developed in both the steroid-sensitive and insensitive models. Not only Th2 cells but also ILC2s are important to cause type-2 inflammation (Wenzel, 2012; Akdis et al., 2020). Indeed, the number of ILC2s was markedly increased in the lung of the present models (Supplementary Table 1). Interestingly, ILC2s rather than Th2 cells were the predominant source of IL-5, and the phenotype of ILC2s in the steroid-insensitive model was pathogenically altered to be IL-5 hyper-producing in response to TSLP/IL-33, both of which were increased in the lung (Supplementary Table 2). As TSLP receptors on the ILC2s were up-regulated in the steroid-insensitive model, the pathogenic alteration presumably resulted in the increased ability of ILC2s to produce IL-5 in response to TSLP. Innate type-2 immune responses are also increased in

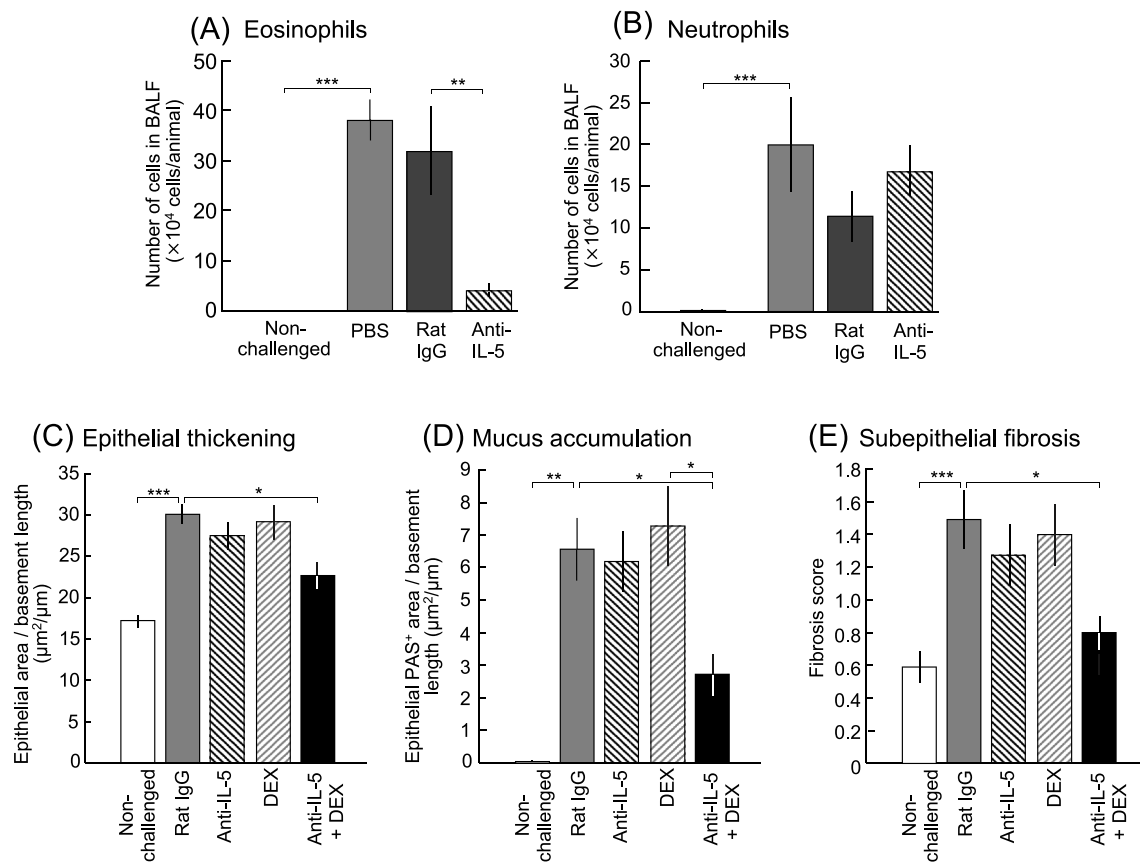


Fig. 6. (A and B) Selective depletion of eosinophils by anti-IL-5 mAb and (C, D and E) effects of anti-IL-5 mAb on the acquisition of steroid resistance in the 500- μg OVA-induced development of airway remodeling in sensitized mice. BALB/c mice were i.p. sensitized with OVA+Al(OH)₃ on days 0, 14, and 28, followed by intratracheal OVA challenges on days 35, 36, 37, and 40 at a dose of 500 μg /animal. Dexamethasone (DEX, 1 mg/kg/time/day) or PBS was i.p. administered 3 h before the respective 4 challenges, and on days 38 and 39. Anti-IL-5 mAb (TRKF-5, 50 μg /animal/time/day) was i.p. administered 3 h before the 1st and 3rd challenges (days 35 and 37). BALF was collected using the right lobes 24 h after the 4th challenge, followed by differential cell count (A and B). The left lung lobes were isolated 24 h after the 4th OVA challenge or without OVA challenges. Isolated lung tissue was fixed with 10% formalin, embedded in paraffin, and cut into 4- μm sections, followed by staining with PAS or Masson trichrome. The degree of epithelial thickening was quantified using PAS-stained sections by dividing the epithelial area in a bronchus (diameter: >150 μm) by the length of the basement membrane (C). The degree of mucus accumulation was calculated by dividing the PAS⁺ area in a bronchus by the length of the basement membrane (D). Subepithelial fibrosis was assessed using Masson trichrome-stained sections by scoring the extension of the blue fibrotic area by a 4-point graded scale from 0 to 3. Each column represents the mean $\pm$ S.E. of 8 or 9 (A and B) and 10 or 11 (C, D and E) animals. *, ** and ***: significant at $p < 0.05$, 0.01, and 0.001, respectively.

asthmatic patients (Bartemes et al., 2014). To our knowledge, this is the first report to demonstrate that the pathogenic change in ILC2s under severe inflammatory conditions was due to up-regulation of TSLP receptors.

TSLP was reported to be important in steroid resistance of airway ILC2s in severe asthma patients (Liu et al., 2018). Additionally, an anti-TSLP mAb was a potential therapy for severe uncontrolled asthma (Corren et al., 2017). Binding of TSLP on TSLP receptors forms ternary complex, TSLP/TSLP receptor/IL-7 receptor α , which triggers activation of intracellular signaling pathways, including JAK1/JAK2, leading to phosphorylation of STAT3/STAT5 (Zhong et al., 2014). Kabata et al. (2013) reported that STAT5 signaling was involved in steroid-resistant response of ILC2s. The present RNA-seq study revealed that *Stat5a* gene expression was significantly up-regulated in ILC2s of the steroid-insensitive model compared to that of the steroid-sensitive model. Therefore, the up-regulation of TSLP receptors and STAT5a could be involved in the transformation of ILC2s into IL-5-hyper producing cells. *Jak3* gene expression was also significantly up-regulated in the ILC2s. Studies have shown that γc family cytokines including IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 play critical roles in the function of ILC2s (Moro et al., 2010; Xue et al., 2014) through activation of respective γc -sharing receptors, which interact with JAK3, followed by STAT5a phosphorylation (Leonard et al., 2019). Thus, the up-regulation

of JAK3 may also participate in the ILC2 transformation.

Toki et al. (2020) reported that TSLP and IL-33 signaling reciprocally promoted their receptor expression on lung ILC2s. As shown in Supplementary Table 1, it was demonstrated that the number of ILC2s increased in the lung of the steroid-insensitive model was comparable to that of the steroid-sensitive model. In contrast, the lung IL-33 level of the former model was significantly higher than that in the latter model (Supplementary Table 2). Thus, the up-regulation of TSLP receptors on the ILC2s of the steroid-insensitive model should be due to exposure the cells to the increased IL-33 in vivo.

Consistent with a previous study (Klein Wolterink et al., 2012), IL-4 production from ILC2s derived from the steroid-sensitive model was markedly less than production of IL-5 and IL-13. However, IL-4 production from ILC2s was significantly up-regulated in the steroid-insensitive model. Moro (2019) reported that ILC2s produced IL-4 under certain inflammatory conditions, leading to increased polyclonal IgE levels, followed by survival and expansion of Fc ϵ R⁺ cells. The number of mast cells in our steroid-insensitive model was markedly higher than that in the steroid-sensitive model (Supplementary Table 1) although serum total IgE levels were not different between them (data not shown). The increase in IL-4 production by the pathogenic ILC2s may be associated with the increased severity of the pathogenesis.

Not only anti-IL-5 but also anti-IL-5 receptor α mAb exerted oral

glucocorticoid-sparing effects in eosinophilic asthma patients (Bel et al., 2014; Nair et al., 2017), suggesting that IL-5 and/or infiltrated eosinophils make airway cells insensitive to glucocorticoids. In this study, when the anti-IL-5 was administered alone, the development of airway remodeling in the steroid-insensitive model was not suppressed. This result was similar to that in a previous study (Mathur et al., 1999). In contrast, under combination treatment with the anti-IL-5 and dexamethasone, the development of airway remodeling was significantly suppressed. Thus, the steroid-insensitive model resembles the severe asthma phenotypes in the clinical settings (Bel et al., 2014; Nair et al., 2017). IL-5 blockage should be a useful therapeutic strategy for steroid-resistant severe asthma. Furthermore, the present finding implies that IL-5 and/or infiltrated eosinophils contribute to the acquisition of steroid insensitivity.

We hypothesize that a vicious cycle was formed in the microenvironment of inflamed lung: The expression of TSLP receptors on ILC2s was up-regulated by airway epithelium-derived IL-33 as reported by Toki et al. (2020), thereby increasing intracellular TSLP signaling, resulting in IL-5 hyper-production. Barretto et al. (2020) demonstrated that activation of IL-5 receptors expressed on nasal epithelial cells by IL-5 induced phosphorylation of extracellular signal-regulated kinase (ERK), which contributes to IL-33 production from the epithelial cells (Park et al., 2016). Collectively, the increased IL-5 derived from ILC2s may induce IL-33 production from airway epithelial cells, followed by the upregulation of TSLP receptors on ILC2s. Therefore, anti-IL-5 therapy could block the vicious cycle in the severe asthma.

Under the proposed vicious cycle, various inflammatory cells including eosinophils acquire the steroid resistance. In our preliminary experiments, gene expression of glucocorticoid receptor (GR) β , a dominant negative of GR α , in eosinophils isolated from the lung of the steroid-insensitive model was up-regulated compared to that in the steroid-sensitive model (data not shown). GR β was reported to inhibit mRNA transcription by the glucocorticoid-GR α complex (Patel et al., 2018). We are currently investigating IL-5-associated expression of GR β in inflammatory cells.

IL-5 production of CD4⁺ T cells collected from the steroid-insensitive model in response to anti-CD3/CD28 antibodies was lower than that of the steroid-sensitive model (Fig. 3B). Multiple antigen exposure is thought to be a major cause for the induction of exhausted T cells, which are functionally characterized by reduced capacity in proliferative and cytokine production (Pauken and Wherry, 2015). Hallmarks of exhausted T cells are enhanced expression of immune checkpoints, such as programmed death receptor-1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), lymphocyte activation gene-3 (LAG-3), T-cell immunoglobulin and ITIM domain (TIGIT), and T-cell immunoglobulin-3 (TIM-3) (Blackburn et al., 2009). As shown in Supplementary Table 3, gene expressions of *Lag3*, *Havcr2* (i.e. TIM-3) and *Ctla4* but not *Tigit* and *Pdcd1* (i.e. PD-1) in the lung tissue derived from the steroid-insensitive model were significantly higher than those of the steroid-sensitive model. Therefore, the reduced IL-5 production of CD4⁺ T cells may be associated with T-cell exhaustion.

It was tried to analyze relationship between in vivo cytokine/chemokine production and the development of steroid-insensitive asthma. However, the levels in the steroid-insensitive model were not consistently more than those of the steroid-sensitive model (Supplementary Table 2). These results may be due to 1) diversity of cellular source of respective cytokines/chemokines, 2) influence of regulatory cytokines produced from Treg cells as manifested by increase in IL-10 (Supplementary Tables 2) and 3) the exhaustion of CD4⁺ T cells as described above. Thus, it was difficult to analyze the relationship.

5. Conclusion

Multiple intratracheal administration of a high dose of antigen induced steroid-insensitive asthma in sensitized mice. In the severe asthma model, a representative type-2 cytokine, IL-5 was mainly

produced from ILC2s. Phenotype of ILC2s was pathogenically altered in the severe asthma to be IL-5 hyper-producer probably through up-regulation of TSLP receptors and STAT5a. IL-5 blockage could be a useful therapeutic strategy for steroid-resistant asthma.

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CRediT authorship contribution statement

Masaya Matsuda: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Yoshiyuki Tanaka:** Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Hayato Shimora:** Methodology, Investigation. **Naoki Take-moto:** Methodology, Investigation. **Miku Nomura:** Methodology, Investigation. **Ryogo Terakawa:** Methodology, Investigation. **Kenno-suke Hashimoto:** Methodology, Investigation. **Harumi Sakae:** Methodology, Investigation. **Akira Kanda:** Conceptualization, Writing – review & editing. **Hiroshi Iwai:** Conceptualization, Writing – review & editing. **Kazuyuki Kitatani:** Conceptualization, Writing – review & editing. **Takeshi Nabe:** Conceptualization, Data curation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition, All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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