



Phenotype analyses of IL-10-producing Foxp3[−] CD4⁺ T cells increased by subcutaneous immunotherapy in allergic airway inflammation

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ABSTRACT

Introduction: The mechanisms of allergen immunotherapy are not fully elucidated. Here, we sought to develop a murine model to demonstrate the effectiveness of subcutaneous immunotherapy (SCIT) for allergic responses. As excessive antigen dosages may induce immune tolerance in sensitized mice, the effects of SCIT were assessed by varying the antigen dosage. The mechanisms of SCIT were analyzed by focusing on the induction of Foxp3⁺ Treg cells and IL-10-producing Foxp3[−] CD4⁺ T cells, as well as on the phenotype of the latter cells.

Methods: Ovalbumin (OVA) + Al(OH)₃-sensitized mice received subcutaneous dosages of OVA at 0.01, 0.1 or 1 mg/animal for SCIT, followed by intratracheal challenges with OVA at 5, 50 or 500 µg/animal.

Results: The maximum effects of SCIT were observed with 1 mg/animal of OVA for airway inflammation induced by 5 µg/animal of OVA, in which airway eosinophilia and Th2 cytokine production were markedly suppressed. The increase in the OVA-specific IgE level was significantly suppressed by SCIT. The development of bronchial epithelial thickening and mucus accumulation were also suppressed by SCIT. Concomitantly, IL-10-producing Foxp3[−] CD4⁺ T cells were increased in the lungs by SCIT, but Foxp3⁺ Treg cells were not. Most of the induced IL-10-producing Foxp3[−] CD4⁺ T cells were negative for either IL-5 or LAG-3, but positive for CD49b.

Conclusion: We successfully developed an airway allergic model for SCIT. It was suggested that most of IL-10-producing Foxp3[−] CD4⁺ regulatory T cells increased by SCIT in the lungs were CD49b⁺ CD4⁺ regulatory T cells, but neither Th2 cells nor Tr1 cells.

1. Introduction

Allergic diseases, such as allergic asthma, are induced by repetitive exposure to allergens [1]. The production of allergen-specific IgE and expansion of allergen-specific Th2 cells are characteristic features of the pathogenesis of allergic diseases. Th2 cells produce inflammatory cytokines, such as IL-4, IL-5 and IL-13, leading to eosinophilic airway inflammation [2, 3]. Several drugs, including glucocorticoids and adrenergic β₂ receptor agonistic bronchodilators, have been developed to treat asthma; however, these pharmacotherapies are only symptomatic treatments. Allergen-specific immunotherapy (AIT) is the only treatment that is able to reduce the symptoms of allergic diseases [4–6]. The effectiveness of AITs, such as subcutaneous immunotherapy (SCIT) and sublingual immunotherapy (SLIT), has been clinically demonstrated for allergic asthma [7].

SCIT and SLIT treatment induced subsets of regulatory T (Treg) cells in clinical studies. Treg cells are generally divided into two groups, the thymus-derived Treg cells and the peripherally-derived Treg cells, the

former of which express the transcription factors, Foxp3 and CD25 [8], whereas the latter include Foxp3⁺ cells and type 1 regulatory T (Tr1) cells. Tr1 cells highly express CD49b and lymphocyte-activation gene 3 (LAG-3), and highly secrete IL-10, but are negative for Foxp3 [9, 10]. SCIT and SLIT increased not only CD4⁺ CD25⁺ Foxp3⁺ T cells but also IL-10-producing Foxp3[−] CD4⁺ T cells in the peripheral blood of allergic patients [11]. However, it is difficult to analyze the detailed mechanisms of Treg cells using peripheral blood mononuclear cells. Moreover, the phenotype of the increased Treg cells at the local inflammatory site was not fully elucidated. Thus, the use of leukocytes at the local airway inflammatory site is required to elucidate the exact phenotypes of Treg cells. On the other hand, antigen-specific IgG₄ was also induced by SCIT and SLIT treatment in clinical studies [12, 13]. Similar to human IgG₄ [14], murine IgG₁ is unable to activate the complement system by the classical pathway but preferentially binds to inhibitory FcγRIIB [15, 16]. Thus, it has been generally accepted that murine IgG₁ is equivalent to human IgG₄ [17, 18].

In order to clarify the mechanisms of AIT, several murine models

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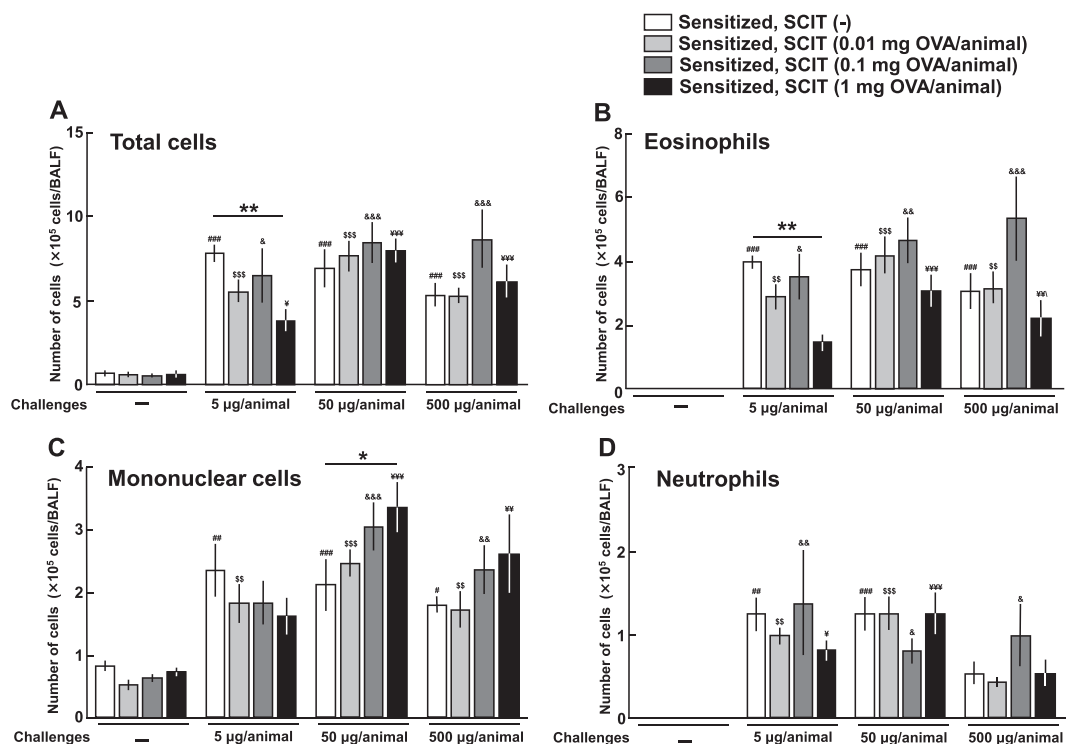


Fig. 1. Effects of subcutaneous immunotherapy (SCIT) on multiple antigen challenge-induced increases in total cells (A), eosinophils (B), mononuclear cells (C) and neutrophils (D) in the BALF in sensitized mice. Mice were i.p. treated with OVA + Al (OH)₃ on days 0 and 14. For SCIT treatment, the sensitized mice received subcutaneous injections of OVA solution at 0.01, 0.1 or 1 mg/animal/time/day on days 21, 23 and 25. Mice were challenged with 0.02%, 0.2% or 2% OVA solution at a volume of 25 µl (5, 50 or 500 µg/animal) intratracheally on days 35, 36, 37 and 40 under inhalation anesthesia with isoflurane. BALF was obtained at 4 h after the 4th challenge. Each column represents the mean $\pm$ SEM of 4–12 animals. * and **: P < 0.05 and 0.01, respectively. #, ## and ###: P < 0.05, 0.01 and 0.001 versus non-challenged, non-SCIT-treated mice. \$, \$\$ and \$\$\$: P < 0.01 and 0.001 versus non-challenged, 0.01 mg/animal SCIT-treated mice. &, && and &&&: P < 0.05, 0.01 and 0.001 versus non-challenged, 0.1 mg/animal SCIT-treated mice. ¥, ¥¥ and ¥¥¥: P < 0.05, 0.01 and 0.001 versus non-challenged, 1 mg/animal SCIT-treated mice.

have been used [18–21]. However, the effectiveness of AIT in mice may vary with the methods for sensitization/challenge and therapeutic treatment with antigens because excessive antigen dosages induce immune tolerance to the relevant antigens [22]. Fox et al. [23] compared 3 models of AIT by intraperitoneal administration of OVA using rapid, intermediate and gradual schedules in OVA + Al(OH)₃-sensitized mice, and demonstrated that the effects of AIT on different parameters depended on the duration of AIT treatment. However, intraperitoneal AIT was conducted without antigen challenges [23]; therefore, AIT was assessed without an organ-specific allergic response. A protocol for the development of murine models for SCIT for allergic asthma regarding the clinical effectiveness of SCIT, including effects on IgE production, IgG₁ production, allergic responses and Treg cell induction, has not been reported.

The first goal of the present study was to develop a murine SCIT model demonstrating the effectiveness of SCIT on allergic airway responses, including antigen-specific IgE and IgG₁ production, allergic leukocyte infiltration into the airway tissues, airway remodeling and Treg cell induction. In order to develop the SCIT model, several doses of OVA for intratracheal antigen challenges and SCIT treatment were evaluated in OVA + Al(OH)₃-sensitized mice. The second goal of this study was to elucidate the phenotype of a subset of Treg cells induced by the appropriate SCIT model.

2. Materials and methods

2.1. Sensitization/challenge, and subcutaneous immunotherapy (SCIT)

As reported previously [24–30], 5-week-old BALB/c mice (Japan SLC, Hamamatsu) were sensitized by i.p. injections with OVA (Grade V;

Sigma Chem., St. Louis, MO, USA) adsorbed to Al (OH)₃ at a dose of 50 µg OVA/2 mg Al (OH)₃/0.5 ml saline/animal on days 0 and 14. Then, the sensitized mice were challenged with 0.02%, 0.2% or 2% OVA solution at a volume of 25 µl (5, 50 or 500 µg/animal) intratracheally on days 35, 36, 37 and 40 under inhalation anesthesia with isoflurane. For SCIT treatment, the sensitized mice received subcutaneous injections of OVA solution at 0.01, 0.1 or 1 mg/animal/time/day on days 21, 23 and 25.

This animal study was approved by the Experimental Animal Research Committee at Setsunan University.

2.2. Analyses of cells recovered by bronchoalveolar lavage (BAL)

As we previously reported [24–27, 30], mice were sacrificed 4 h after the fourth airway antigen challenge by lethal i.p. injection of pentobarbital and xylazine. Peripheral blood was drawn from the abdominal aorta, and bronchoalveolar lavage fluid (BALF) was obtained from the right lung lobe for measurement of cytokines and antibodies in the BALF and serum, respectively. To determine differential cell counts, cells were centrifuged onto a glass slide using a cell settling chamber followed by staining with Diff-Quik solution (Sysmex, Kobe, Japan) after treatment with ACK lysis buffer. The left lung lobe was isolated for histological studies.

2.3. Measurement of OVA-specific IgE and OVA-specific IgG₁ in the serum

Amounts of OVA-specific IgE were measured by ELISA using anti-IgE (clone R35-72, BD Biosciences) as a capture antibody, horseradish peroxidase (HRP)-labeled OVA (Abcam, Cambridge, UK) as a detection reagent and OVA-specific IgE (clone E-C1, Chondrex, Redmond, WA,

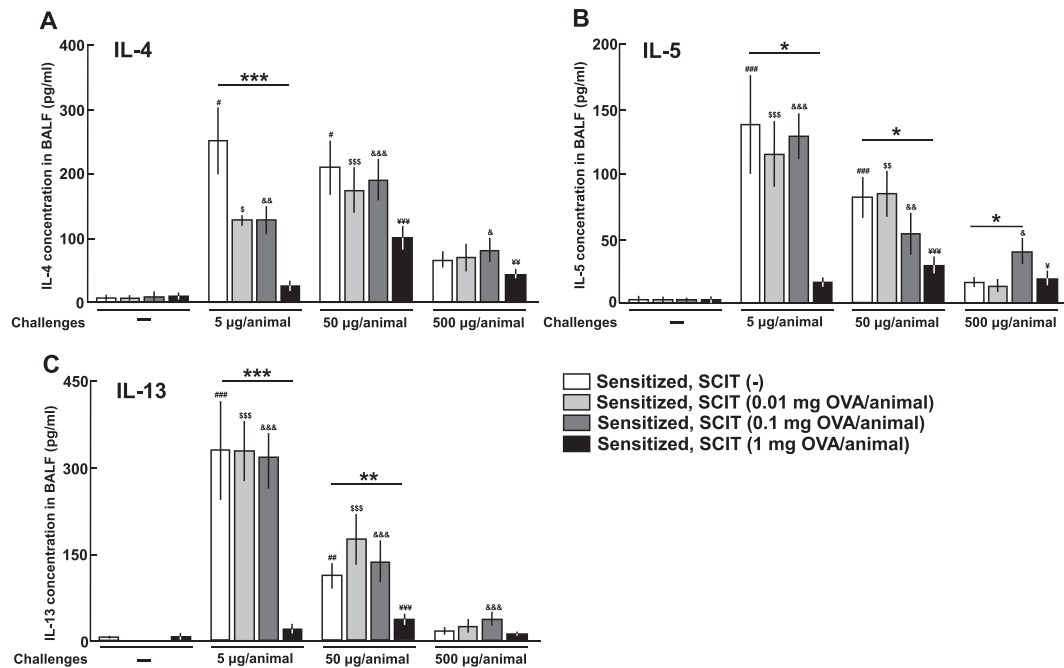


Fig. 2. Effects of subcutaneous immunotherapy (SCIT) on multiple antigen challenge-induced increases in amounts of IL-4 (A), IL-5 (B) and IL-13 (C) in BALF of sensitized mice. Mice were i.p. treated with OVA + Al (OH)₃ on days 0 and 14. For SCIT treatment, the sensitized mice received subcutaneous injections of OVA solution at 0.01, 0.1 or 1 mg/animal/time/day on days 21, 23 and 25. Mice were challenged with 0.02%, 0.2% or 2% OVA solution at a volume of 25 µl (5, 50 or 500 µg/animal) intratracheally on days 35, 36, 37 and 40 under inhalation anesthesia with isoflurane. BALF was obtained at 4 h after the 4th challenge. Each column represents the mean $\pm$ SEM of 4–12 animals. *, ** and ***: $P < 0.05$, 0.01 and 0.001 , respectively. #, ## and ###: $P < 0.05$, 0.01 and 0.001 versus non-challenged, non-SCIT-treated mice. \$, \$\$ and \$\$\$: $P < 0.05$, 0.01 and 0.001 versus non-challenged, 0.01 mg/animal SCIT-treated mice. && and &&&: $P < 0.01$ and 0.001 versus non-challenged, 0.1 mg/animal SCIT-treated mice. ¥, ¥¥ and ¥¥¥: $P < 0.05$, 0.01 and 0.001 versus non-challenged, 1 mg/animal SCIT-treated mice.

USA) as a standard. Color development was performed by incubation with tetramethylbenzidine substrate solution (Thermo Fisher Scientific, Waltham, MA, USA). After the addition of H₃PO₄ solution, the OD at 450 nm was measured. Amounts of OVA-specific IgG₁ were measured using Mouse OVA-IgG₁ ELISA KIT (Shibayagi, Shibukawa, Japan).

2.4. Assays of levels of cytokines in the BALF

IL-4, IL-5 and IL-13 were measured using Ready Set Go! ELISA kits (Thermo Fisher Scientific).

2.5. Histological studies

The left lobe of the lung was fixed with 10% buffered formalin. Tissues were embedded in paraffin, and 4-µm sections were stained with periodic acid–Schiff (PAS) for light microscopic observation. Quantitative analyses of histological changes were conducted as reported previously [25, 30]. Briefly, the degree of epithelial thickening was quantified as follows: the bronchus, the diameter of which was approximately 200–400 µm, was photographed, and then the epithelial area (µm²) and the length of the basement membrane (µm) were measured using Adobe Photoshop CS2 (version 9.0; Adobe Systems, San Jose, CA, USA) and Image J (version 1.49; NIH, Bethesda, MD, USA). The degree of epithelial thickening was expressed as the epithelial area (µm²) divided by the length of the basement membrane (µm). Mucus accumulation in the epithelial layer was also quantified by dividing the PAS⁺ area in the epithelium (µm²) by the length of the basement membrane (µm).

2.6. Detection of IL-10-producing Foxp3⁺ CD4⁺ T cells by FACS

The left lobe of the lung was isolated 3 days after the 3rd challenge. The lung tissue was digested by incubation with 870 units/ml of

collagenase (Thermo Fisher Scientific) at 37 °C for 1 h. The obtained single cell suspension was suspended in RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at a concentration of 2×10^6 cells/ml, and then distributed into the wells of a 96-well plate at 250 µl/well. The cells were incubated with PBS, OVA (10^{-3} g/ml) or a mixture of PMA (50 ng/ml) and ionomycin calcium salt (500 ng/ml) (Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 6 h in the presence of a protein transport inhibitor, monensin sodium salt (2 µM, Calbiochem, San Diego, CA, USA). Under OVA stimulation conditions, monensin sodium salt was added 2 h after the start of incubation.

After incubation, the cells were washed by PBS, and then stained with eFluor® 780-conjugated Fixable Viability Dye (Thermo Fisher Scientific) to prevent the non-specific binding of subsequent antibodies to the dead cells. After washing with 2% FBS-containing PBS, the cells were treated with an anti-CD16/32 (FcγRII/III) antibody (2.4G2) to prevent the non-specific binding of subsequent antibodies to the cells. After the Fc blocking, the cells were stained with PE/Dazzle™594-conjugated anti-mouse CD3 (17A2), PerCP/Cy5.5-conjugated anti-mouse LAG-3 (C9B7W), PE/Cy7-conjugated anti-mouse CD3 (17A2), PE/Cy7-conjugated anti-mouse KLRG1 (2F1/KLRG1) and/or APC-conjugated anti-mouse CD49b (HMA2) (all from BioLegend, San Diego, CA, USA), and/or FITC-conjugated anti-mouse CD4 (H129.19) and/or PE-conjugated anti-mouse CD25 (3C7) (both from BD Biosciences, San Jose, CA, USA) at 4 °C for 20 min. After washing, the cells were incubated with Fixation/Permeabilization Concentrate (Thermo Fisher Scientific) for 12–18 h, and then washed with Permeabilization Buffer (Thermo Fisher Scientific), followed by incubation with PE-conjugated anti-mouse IL-10 (JES5-16E3), and/or eFluor® 660-conjugated anti-mouse/rat Foxp3 (FJK-16s) (all from Thermo Fisher Scientific), and/or APC-conjugated anti-mouse/human IL-5 (TRFK5) (BioLegend) for 40 min. After washing with 2% FBS-containing PBS, cells were analyzed using FACSARIA™ Fusion (Becton Dickinson, San Jose, CA, USA).

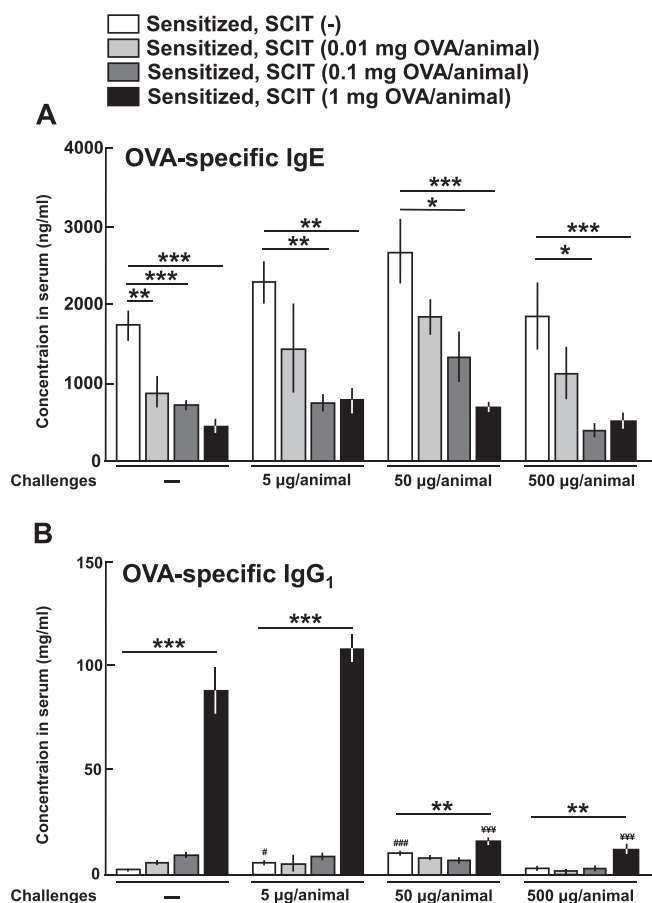


Fig. 3. Effects of subcutaneous immunotherapy (SCIT) on production of OVA-specific IgE (A) and OVA-specific IgG₁ (B) in the sera of sensitized-challenged mice. Mice were i.p. treated with OVA + Al (OH)₃ on days 0 and 14. For SCIT treatment, the sensitized mice received subcutaneous injections of OVA solution at 0.01, 0.1 or 1 mg/animal/time/day on days 21, 23 and 25. Mice were challenged with 0.02%, 0.2% or 2% OVA solution at a volume of 25 µl (5, 50 or 500 µg/animal) intratracheally on days 35, 36, 37 and 40 under inhalation anesthesia with isoflurane. Blood was drawn at 4 h after the 4th challenge. Each column represents the mean ± SEM of 4–12 animals. *, ** and ***: $P < 0.05$, 0.01 and 0.001, respectively. # and ###: $P < 0.05$ and 0.001 versus non-challenged, non-SCIT-treated mice. \$: $P < 0.05$ versus non-challenged, 0.01 mg/animal SCIT-treated mice. ¥¥¥: $P < 0.001$ versus non-challenged, 1 mg/animal SCIT-treated mice.

2.7. Analysis of IL-10 production from lung cells in culture supernatant

As shown in 2.6, obtained lung cells were stimulated by OVA or PMA + ionomycin in the absence of monensin for 6 h. After the incubation, cell culture supernatant was collected. IL-10 concentration in cell culture supernatant was determined using an ELISA kit (R&D systems).

2.8. Statistical analyses

Two groups were compared with the unpaired *t*-test. To compare multiple groups, one-way ANOVA was performed, followed by Dunnett's multiple comparison test. A *P*-value < 0.05 was considered significant. Statistical analyses were performed with JMP® Pro (Version 11.2.0, SAS Institute Japan, Tokyo, Japan).

3. Results

3.1. Effects of SCIT on antigen-induced cellular inflammation

As shown in Fig. 1, compared with the non-challenged mice, the numbers of leukocytes were markedly increased in the BALF of mice intratracheally challenged with OVA at 5, 50 or 500 µg/animal (Fig. 1A). More than 50% and approximately 30% of the infiltrated leukocytes consisted of eosinophils (Fig. 1B) and mononuclear cells (Fig. 1C), respectively. Neutrophils were also significantly increased in the BALF at 5 and 50 µg/animal of OVA, whereas they tended to be reduced by the increasing the dose of intratracheal OVA challenge to 500 µg/animal (Fig. 1D). In our preliminary study, at a lower dose of OVA (0.5 µg/animal), only slight increases in the numbers of leukocytes in the BALF were observed (data not shown).

SCIT treatment consisted of 3 subcutaneous injections of 0.01, 0.1 or 1 mg/animal of OVA before the intratracheal OVA challenges. SCIT treatment with 1 mg/animal of OVA significantly suppressed the eosinophil infiltration induced by intratracheal challenges with 5 µg/animal of OVA (Fig. 1B). The increases in neutrophil and mononuclear cell numbers by 5 µg/animal of OVA also tended to be suppressed by SCIT at 1 mg/animal (Fig. 1C and D). However, in the groups challenged by 50 or 500 µg/animal of OVA, significant suppressive effects on the cell infiltration into BALF were not observed by SCIT at any dose of OVA (Fig. 1A–D).

On the other hand, the number of mononuclear cells was significantly increased by SCIT (1 mg/animal) in the group challenged by 50 µg OVA/animal (Fig. 1C). This increase in mononuclear cells was similarly observed in the group challenged by 500 µg OVA/animal, but it was not significant (Fig. 1C).

3.2. Effects of SCIT on Th2 cytokine production

In comparison with the non-challenged group, IL-4, IL-5 and IL-13 were increased in the BALF by the OVA challenges at 5, 50 or 500 µg/animal (Fig. 2A–C), whereas the degree of Th2 cytokine production tended to be reduced by increasing the dose of OVA for challenge (Fig. 2A–C). In our preliminary study, with a lower dose of OVA (0.5 µg/animal), only slight increases in the amounts of IL-4, IL-5 and IL-13 were observed (data not shown).

In the 5 µg/animal OVA-challenged group, IL-4, IL-5 and IL-13 were significantly decreased by SCIT at 1 mg OVA/animal (Fig. 2A–C). In the group challenged by OVA at 50 µg/animal, IL-5 and IL-13 were significantly reduced by SCIT at 1 mg OVA/animal (Fig. 2B and C). However, when sensitized mice were challenged with OVA at 500 µg/animal, the production of IL-4, IL-5 and IL-13 was not significantly reduced by SCIT at any dose (Fig. 2A–C).

3.3. Effects of SCIT on antigen-specific IgE and antigen-specific IgG₁ production

As shown in Fig. 3A, there was a large amount of OVA-specific IgE in the serum of non-challenged mice, indicating that intraperitoneal sensitization with OVA + Al(OH)₃ produced IgE even without the intratracheal OVA challenges. The intratracheal OVA challenges at 5, 50 or 500 µg/animal further elevated the amount of IgE. The OVA-specific IgE production was significantly reduced by SCIT in each challenged group (Fig. 3A).

In contrast, the amount of OVA-specific IgG₁ (murine equivalent to human IgG₄) in the serum was low before the intratracheal OVA challenges in the sensitized mice (Fig. 3B). The IgG₁ level was weakly but significantly up-regulated by the intratracheal OVA challenges at 5 or 50 µg/animal (Fig. 3B). SCIT treatment significantly enhanced the increase in serum antigen-specific IgG₁. In particular, the increases by SCIT (1 mg OVA/animal) in the non-challenged and the relatively low dose (5 µg/animal) antigen-challenged groups were markedly large

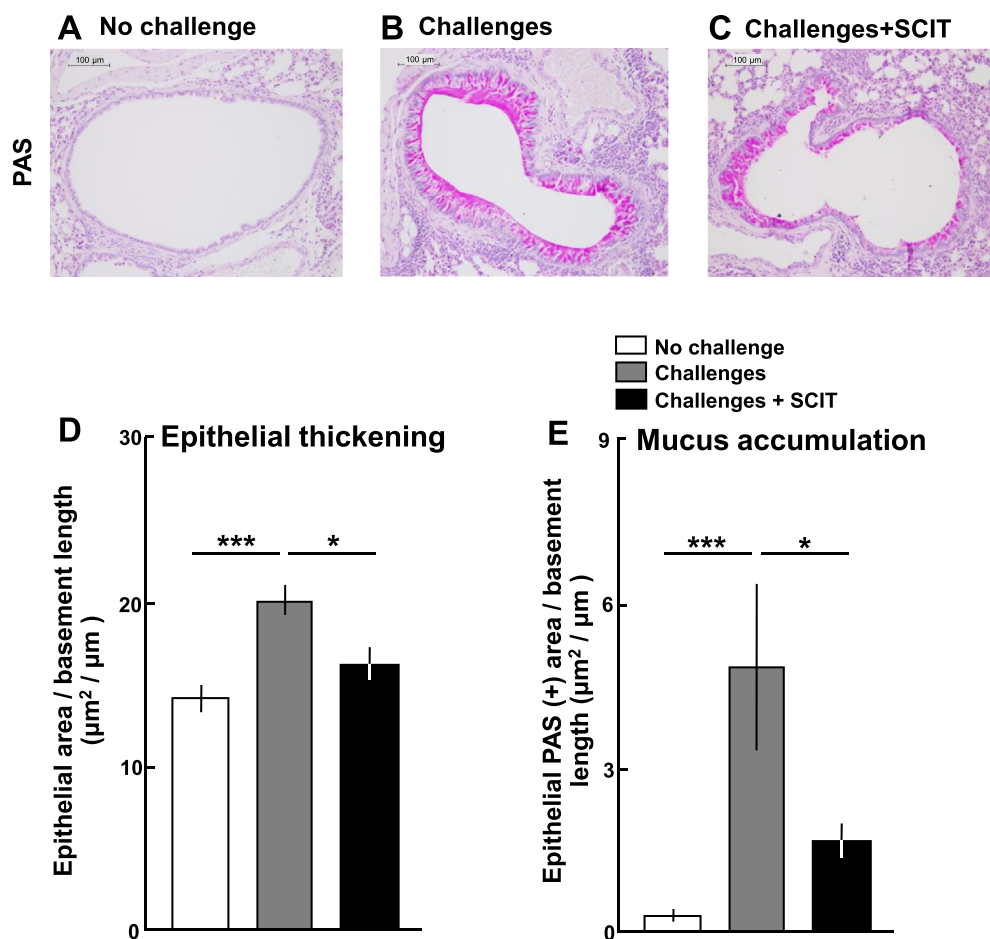


Fig. 4. Effects of subcutaneous immunotherapy (SCIT) on multiple antigen challenge-induced development of airway remodeling in sensitized mice. Mice were i.p. treated with OVA + Al (OH)₃ on days 0 and 14. For SCIT treatment, the sensitized mice received subcutaneous injections of OVA solution at 1 mg/animal/time/day on days 21, 23 and 25. Mice were challenged with 0.02% OVA solution at a volume of 25 μl (5 μg /animal) intratracheally on days 35, 36, 37 and 40 under inhalation anesthesia with isoflurane. Lung tissue was isolated at 4 h after the 4th challenge, followed by staining with periodic acid-Schiff (PAS, A, B and C). The degree of epithelial thickening was quantified using PAS-stained sections by dividing the epithelial area (μm^2) in a bronchus by the length of the basement membrane (μm) (D). The degree of mucus deposition was calculated by dividing the PAS⁺ area (μm^2) in a bronchus by the length of the basement membrane (μm) (E). Each column represents the mean $\pm$ SEM of 6 or 12 animals. * and ***: P < 0.05 and 0.001, respectively.

(Fig. 3B).

3.4. Effects of SCIT on the development of airway remodeling

As shown in Figs. 1 and 2, both allergic eosinophilic inflammation and Th2 cytokine production were suppressed by SCIT at 1 mg OVA/animal in the group challenged with 5 μg OVA/animal. Thus, it was histologically examined whether airway remodeling was affected. Compared with the histology of the lung tissue isolated from non-challenged mice (Fig. 4A), epithelial changes in the bronchus exhibiting epithelial thickening and mucus accumulation were evidently induced in the tissue of challenged mice (Fig. 4B). Both epithelial thickening and mucus accumulation were significantly suppressed by SCIT (Fig. 4A–E).

3.5. Effects of SCIT on numbers of Foxp3⁺ Treg cells and IL-10-producing Foxp3⁺ CD4⁺ T cells in the lungs

We next evaluated whether SCIT treatment increased the number of Foxp3⁺ Treg, CD4⁺ CD25⁺ Foxp3⁺ T cells in the lungs. As described above, this examination was also conducted in the 5- μg OVA/animal-challenged and 1-mg OVA/animal-SCIT-treated group. Compared with the challenged and non-SCIT-treated mice, the number of Foxp3⁺ Treg cells in the lungs was not increased by SCIT treatment (Fig. 5B).

We also examined whether the number of IL-10-producing Foxp3⁺ CD4⁺ T cells was increased by SCIT. In order to detect the IL-10-producing cells, the lung cells obtained by enzymatic digestion were stimulated with OVA or PMA + ionomycin in vitro. Interestingly, more than 95% of OVA-induced IL-10-producing CD4⁺ T cells were negative for Foxp3 (Fig. 5C). The number of IL-10-producing Foxp3⁺ CD4⁺ T cells was significantly increased by SCIT in both stimulation conditions

(Fig. 5D).

When the lung cells were stimulated with OVA or PMA + ionomycin in vitro in the absence of the protein transport inhibitor, IL-10 concentration in culture supernatant was increased compared with non-challenged. However, the level of IL-10 in the supernatant was not further increased in the SCIT-treated group (data not shown). Reasons of the difference in effects of SCIT between IL-10-producing Foxp3⁺ CD4⁺ T cells and IL-10 level in culture supernatant are unclear. It may be due to the fact that lung cells contain a variety of IL-10-producing leukocytes such as dendritic cells [31].

3.6. Phenotypes of the induced IL-10-producing Foxp3⁺ CD4⁺ T cells

It has been reported that IL-10-producing CD4⁺ T cells may include Th2 cells [32], Tr1 cells [10] and CD4⁺ CD49b⁺ regulatory T cells [33]. Therefore, we focused on marker molecules in the OVA-induced IL-10-producing Foxp3⁺ CD4⁺ T cells. As shown in Fig. 5C, most of the IL-10⁺ CD4⁺ T cells were not Foxp3⁺ Treg. More than 70% of the IL-10⁺ CD4⁺ T cells were negative for IL-5, a Th2 cytokine (Fig. 6B). As shown in Fig. 6C, only 2.6% of IL-10⁺ CD4⁺ T cells were double-positive for LAG-3 and CD49b, Tr1 markers. In contrast, 61.6% of IL-10⁺ CD4⁺ T cells were CD49b⁺ LAG-3⁺ (Fig. 6C). Vicente et al. [33] reported that CD4⁺ CD49b⁺ regulatory T cells highly expressed killer cell lectin-like receptor G1 (KLRG1). Of the IL-10-producing CD4⁺ T cells, 19.3% were double-positive for CD49b and KLRG1 (Fig. 6D).

4. Discussion

It is well-known that repeated exposure to high antigen doses induces immune tolerance to said antigen in mice [19]. Under such

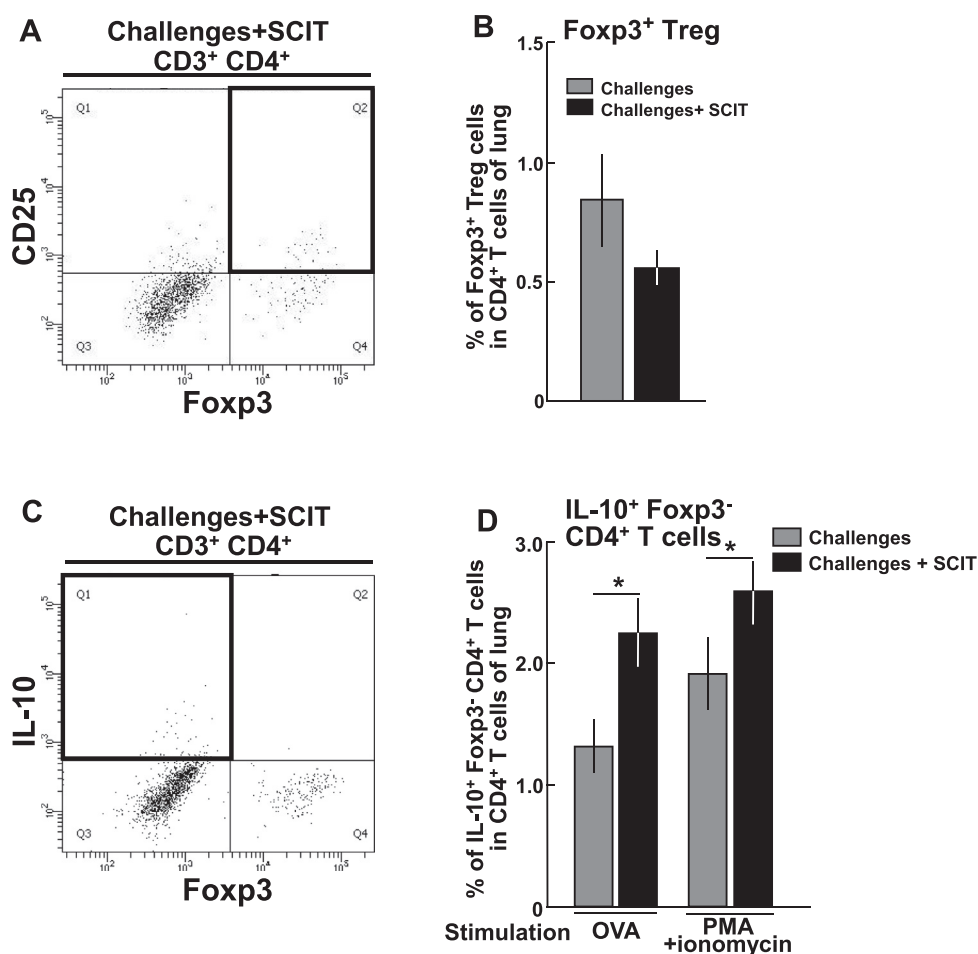


Fig. 5. Effects of subcutaneous immunotherapy (SCIT) on numbers of Foxp3⁺ Treg cells (A and B) and IL-10-producing Foxp3⁻ CD4⁺ T cells (C and D) in the lungs. Each fluorescence minus one (FMO) control is shown in Supplementary Fig. 1. Mice were i.p. treated with OVA + Al (OH)₃ on days 0 and 14. For SCIT treatment, the sensitized mice received subcutaneous injections of OVA solution at 1 mg/animal/time/day on days 21, 23 and 25. Mice were challenged with 0.02% OVA solution at a volume of 25 μ l (5 μ g/animal) intratracheally on days 35, 36 and 37 under inhalation anesthesia with isoflurane. Lungs were isolated 3 days after the 3rd challenge, followed by enzymatic digestion to obtain a single cell suspension. For detection of IL-10-producing cells, the lung cells were stimulated with 10⁻³ g/ml OVA or PMA + ionomycin for 6 h. Numbers in respective panels (A and C) are the means $\pm$ SEM of 11 or 12 animals. Each column (B and D) represents the mean $\pm$ SEM of 11 or 12 animals. *: $P < 0.05$.

immune tolerance, the effects of AIT may be reduced. Indeed, in the present protocol for antigen challenges in the sensitized mice, more than 5 μ g of OVA/animal induced allergic Th2 and eosinophilic airway inflammation, whereas at 50 or 500 μ g of OVA/animal, allergic inflammation tended to be weakened, as demonstrated by Th2 cytokine production in BALF. This may be related with immune tolerance. However, the number of eosinophils in BALF was not dramatically decreased. Although the reason of the difference is unclear, it may be due to that of each half-life period in the lung. Significant inhibition of Th2 and eosinophilic airway inflammation by SCIT was observed only with 1 mg of OVA/animal. The effects of SCIT were predictably reduced when the immune tolerance was induced at 500 μ g of OVA/animal for challenge. Therefore, we selected the doses of 5 μ g of OVA/animal and 1 mg of OVA/animal for antigen challenge and SCIT, respectively. Although we chose intratracheal administration for the airway antigen challenge, when other challenge routes, such as intranasal or inhalation, are used for antigen challenge, the doses should be reassessed.

In order to elucidate mechanisms of AIT, we first focused on Foxp3⁺ Treg (CD4⁺ CD25⁺ Foxp3⁺ T) cells. In this study, it was found that the Foxp3⁺ Treg cells were decreased instead of increased in the lungs by SCIT treatment. Similar with the present finding, the proportion of Foxp3⁺ Treg cells in PBMCs of patients treated with wasp venom immunotherapy (VIT) was significantly decreased one month after initiating VIT [34]. Böhm et al. [18] also demonstrated that pulmonary Foxp3⁺ Treg cells were not significantly increased by AIT in a mouse model based on OVA-driven experimental asthma. In contrast, Foxp3⁺ Treg cells in mediastinal lymph nodes were significantly increased [18]. Moreover, Maazi et al. [35] reported that the number of Foxp3⁺ Treg cells in the spleen and blood was significantly increased by AIT in a mouse model of OVA-induced asthma. In our preliminary study,

Foxp3⁺ Treg cells were not increased in the mediastinal lymph nodes by SCIT (data not shown). Whether Foxp3⁺ Treg cells increase may be dependent on experimental protocols, including sensitization/challenge, SCIT and organ examined. In our present model, it was suggested that the mechanisms of SCIT are independent of the pathways causing the increase in Foxp3⁺ Treg cells.

On the other hand, IL-10-producing Foxp3⁻ CD4⁺ T cells are a subset of Treg cells involved in the effects of allergen immunotherapy [36, 37]. In our model, the IL-10-producing Foxp3⁻ CD4⁺ T cells were significantly up-regulated in the lungs. It has been reported that AIT effectively increased IL-10-producing Foxp3⁻ CD4⁺ T cells in murine models of asthma [38]. In this study, we demonstrated that most of the IL-10-producing Foxp3⁻ CD4⁺ T cells were negative for IL-5, suggesting that the cells were not Th2 cells. However, there are few reports that analyzed the phenotypes of the IL-10-producing Foxp3⁻ CD4⁺ T cells induced by AIT. Recently, Gagliani et al. [10] found that murine IL-10-producing Foxp3⁻ CD4⁺ T cells highly expressed CD49b and LAG-3, and termed these cells as Tr1 cells. We induced IL-10-producing Foxp3⁻ CD4⁺ T cells in vitro by 7-day culture of spleen cells isolated from sensitized mice with an antigen and several cytokines, and found that the antigen-induced IL-10-producing Foxp3⁻ CD4⁺ T cells included the Tr1 cells [33]. However, in the present in vivo SCIT model, most of the IL-10-producing Foxp3⁻ CD4⁺ T cells induced by SCIT were negative for LAG-3, suggesting that the cells did not correspond to Tr1 cells. On the other hand, most of the increased IL-10-producing Foxp3⁻ CD4⁺ T cells by SCIT were positive for CD49b. Vicente et al. [33] demonstrated remarkable heterogeneity in IL-10-producing Foxp3⁻ CD4⁺ T cell populations, and revealed that IL-10-producing Foxp3⁻ CD49b⁺ Treg cells have an effect or memory phenotype. Similarly, Benoist et al. [39] reported that CD4⁺ CD49b⁺ Treg cells of

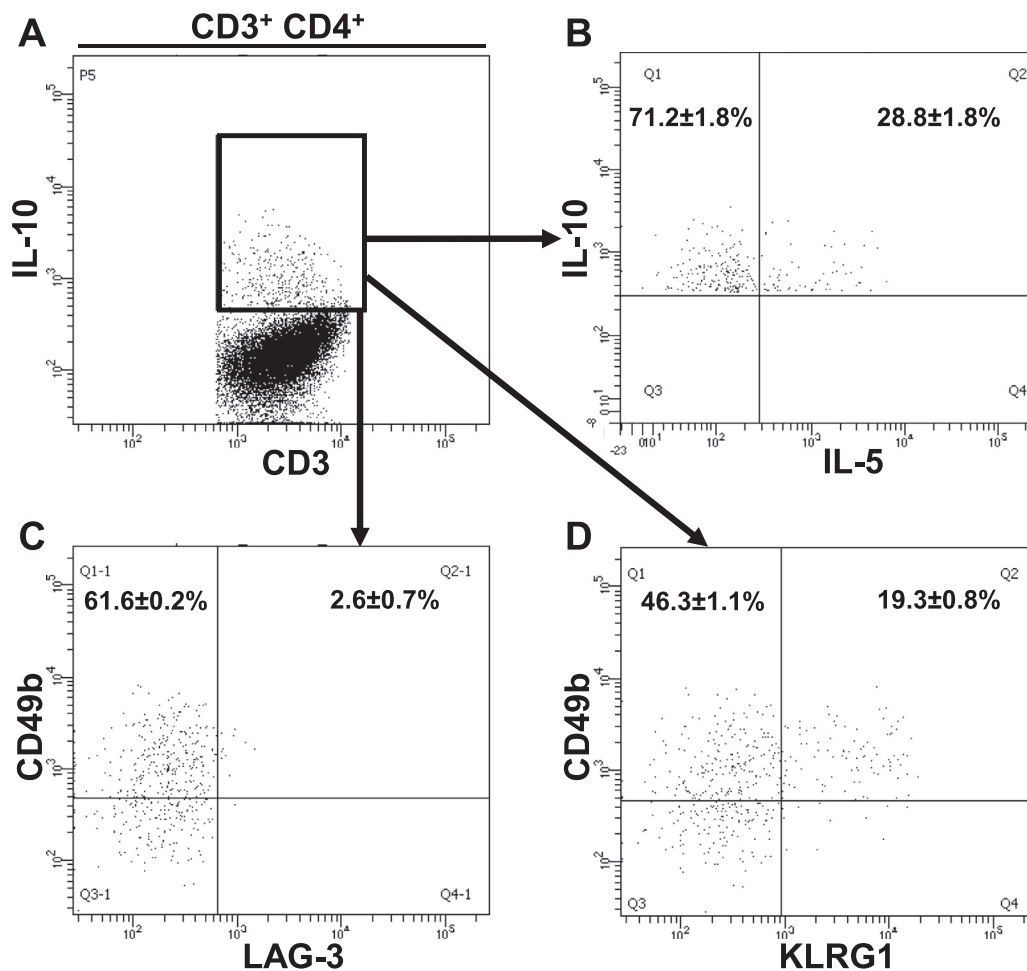


Fig. 6. Expression of intracellular IL-10 (A), IL-5 (B), cell surface CD49b and LAG-3 (C), cell surface CD49b and KLRG1 (D) in IL-10-producing CD4⁺ T cells in the lung tissue of sensitized-challenged mice. Each fluorescence minus one (FMO) control is shown in Supplementary Fig. 2. Mice were i.p. treated with OVA + Al(OH)₃ on days 0 and 14. For SCIT treatment, the sensitized mice received subcutaneous injections of OVA solution at 1 mg/animal/time/day on days 21, 23 and 25. Mice were challenged with 0.02% OVA solution at a volume of 25 μ l (5 μ g/animal) intratracheally on days 35, 36 and 37 under inhalation anesthesia with isoflurane. Lungs were isolated 3 days after the 3rd challenge, followed by enzymatic digestion to obtain a single cell suspension. The lung cells were stimulated with 10⁻³ g/ml OVA for 6 h. Numbers in respective panels (A, B, C and D) are the means $\pm$ SEM of 3 animals.

naive mice were more efficient in suppressing the onset of diabetes than CD4⁺ CD25⁺ Treg cells. Thus, it was suggested that the effectiveness of SCIT in the present study may be related with the Foxp3⁻ CD49b⁺ CD4⁺ T cells, which responded to the relevant antigen to produce IL-10.

Previous studies have demonstrated that depletion of IL-10 signaling in a mouse model of AIT leads to abrogation of AIT-mediated suppression [19, 40]. Furthermore, the ability of CD4⁺ CD25⁺ Treg cells of thymic origin to attenuate airway inflammation was found to be dependent on IL-10 [41]. Additionally, *in vivo* neutralization of IL-10 function by an anti-IL-10 receptor antibody augmented allergic airway inflammation, indicating that IL-10 functioned as a negative regulator in asthma pathogenesis [27]. As suggested elsewhere [42], the IL-10 produced from regulatory T cells can affect antigen-presenting cells, followed by dampening activation of Th2 cells [42]. IL-10 also inhibits activities of mast cells and eosinophils [42]. In addition, it was reported that IL-10 stimulates IgG₄ production from human B cells [43]. Therefore, the increased IL-10 by SCIT in our study may have affected IgE-producing B cells to class switch to IgG₁-producing B cells. Taken together, the increased IL-10 has pivotal roles in the mechanisms of SCIT. The effects of SCIT observed in the present study are likely associated with IL-10 secreted from the Foxp3⁻ CD49b⁺ CD4⁺ T cells.

On the other hand, in this study, serum OVA-specific IgG₁ (murine equivalent to human IgG₄) was markedly increased by SCIT, whereas serum OVA-specific IgE was predictably decreased by SCIT. The increased IgG₁ is thought to compete with IgE as a blocking antibody in a similar way to IgG₄ in the human system [44, 45]. It is speculated that antigen-specific IgG₁ was consumed when large amounts (50 and 500 μ g/animal) of antigen were used for multiple challenges in this

study. Although allergen-specific IgG₁ has been proposed to contribute to the effects of AIT, the relationship between the efficacy of AIT and induction of the allergen-specific IgG₁ remains controversial. Shirinbak et al. [21] demonstrated that allergen-specific IgG₁ induced by AIT in sensitized mice is not involved in the suppression: Even B cell-deficient mice are affected by AIT [21], suggesting that there is a suppressive pathway independent of the antibody hypothesis. In contrast, Burton et al. [46] reported that administration of allergen-specific IgG₁ before sensitization suppressed the production of IgE and induced regulatory T cells in mice with food allergies. Furthermore, IgE-mediated mast cell activation was suppressed by signaling through the low affinity IgG receptor (Fc γ RIIB) [47]. In addition, IgE production was inhibited by cross-linking allergen-specific IgG₄ with Fc γ RIIB receptors on memory B cells [48, 49]. In the mouse, IL-10 production from macrophages was also induced by allergen-IgG complexes [50, 51]. In short, it seems that there are both antigen-specific IgG-dependent and independent pathways for the effects of AIT.

We established a murine model that can assess effectiveness of SCIT for allergic airway inflammation by clarifying appropriate doses of antigen for SCIT and challenges, and suggested that increased IL-10-producing Foxp3⁻ CD4⁺ regulatory T cells by SCIT in the lungs were CD49b⁺ CD4⁺ regulatory T cells, but neither Th2 cells nor Tr1 cells.

Conflict of interest

The authors have no financial conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2018.06.014>.

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Supplementary Figures:

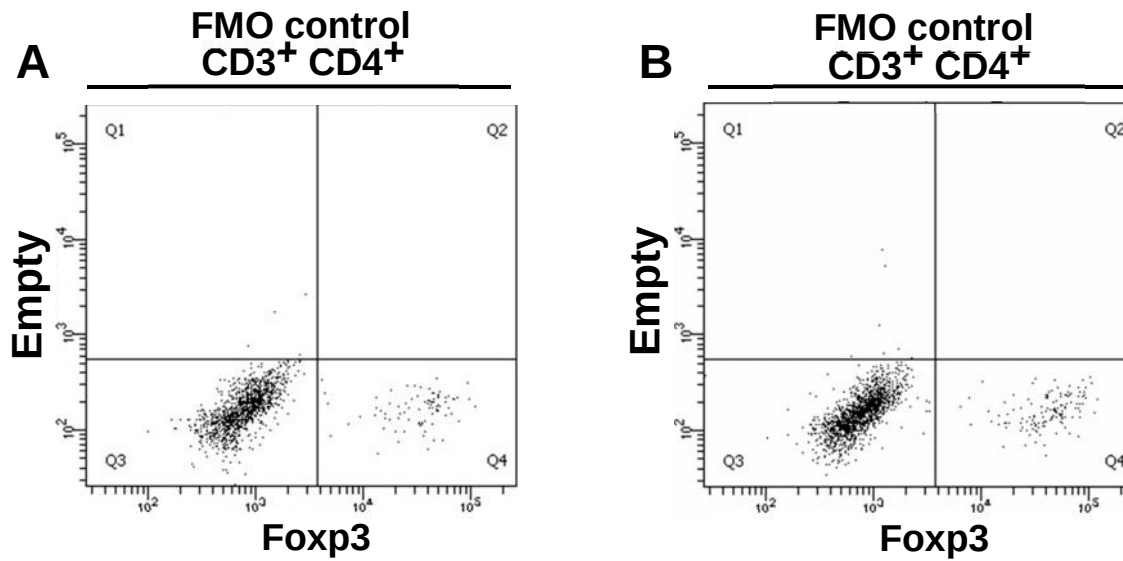


Fig. S1. Fluorescence minus one (FMO) control for Fig. 5A (A) and Fig. 5C (B). Mice were i.p. treated with OVA+Al(OH)₃ on days 0 and 14. For SCIT treatment, the sensitized mice received subcutaneous injections of OVA solution at 1 mg/animal/time/day on days 21, 23 and 25. Mice were challenged with 0.02% OVA solution at a volume of 25 μ l (5 μ g/animal) intratracheally on days 35, 36 and 37 under inhalation anesthesia with isoflurane. Lungs were isolated 3 days after the 3rd challenge, followed by enzymatic digestion to obtain a single cell suspension. The lung cells were stimulated with 10⁻³ g/ml OVA for 6 h.

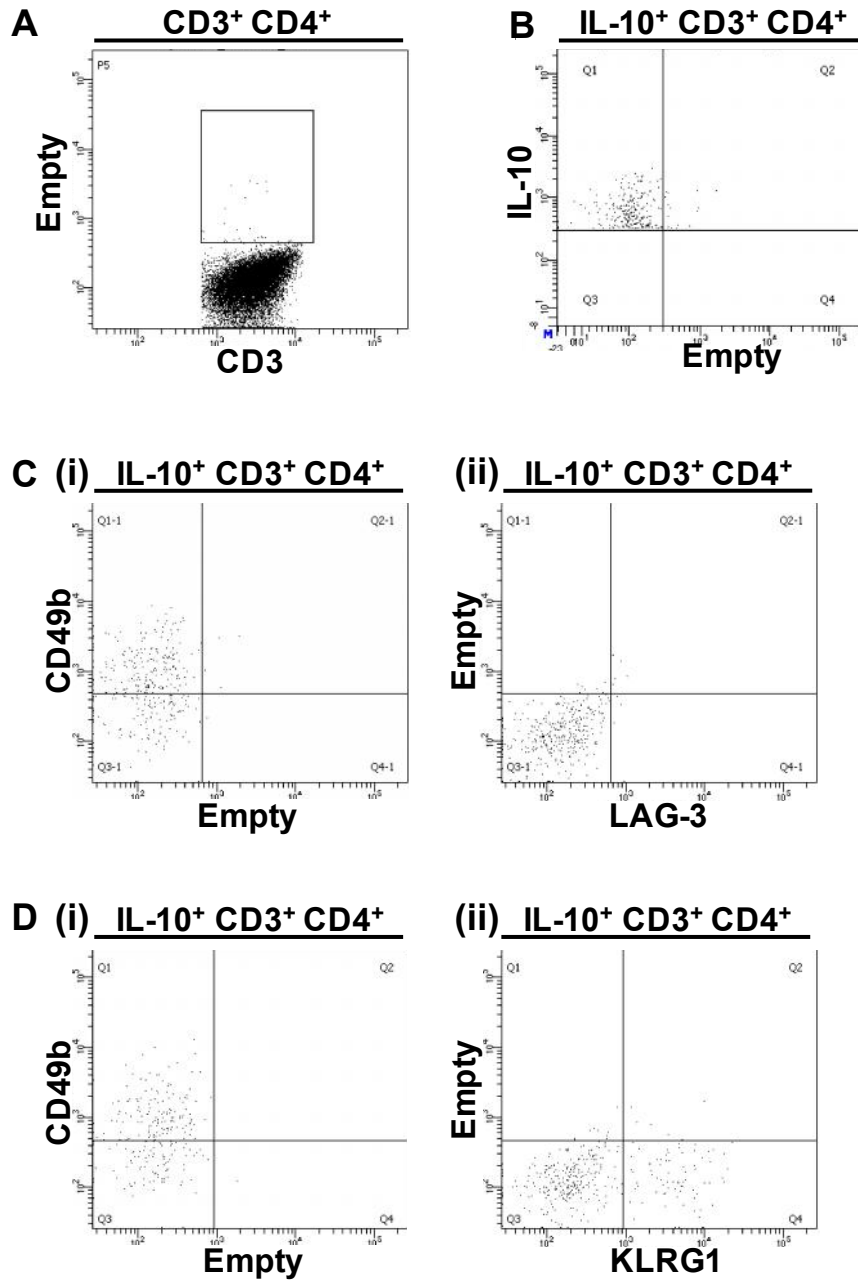


Fig. S2. Fluorescence minus one (FMO) control for Fig. 6A (A), Fig. 6B (B), Fig. 6C (C (i) and (ii)) and Fig. 6D (D (i) and (ii)). Mice were i.p. treated with OVA+Al(OH)₃ on days 0 and 14. For SCIT treatment, the sensitized mice received subcutaneous injections of OVA solution at 1 mg/animal/time/day on days 21, 23 and 25. Mice were challenged with 0.02% OVA solution at a volume of 25 μ l (5 μ g/animal) intratracheally on days 35, 36 and 37 under inhalation anesthesia with isoflurane. Lungs were isolated 3 days after the 3rd challenge, followed by enzymatic digestion to obtain a single cell suspension. The lung cells were stimulated with 10⁻³ g/ml OVA for 6 h.