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Original article

Effects of sublingual immunotherapy in a murine asthma model sensitized by intranasal administration of house dust mite extracts

Kenjiro Shima^a, Toshiyuki Koya^{a,*}, Keisuke Tsukioka^a, Takuro Sakagami^a,
Takashi Hasegawa^b, Chiharu Fukano^c, Katsuyo Ohashi-Doi^c, Satoshi Watanabe^a,
Eiichi Suzuki^b, Toshiaki Kikuchi^a

^a Department of Respiratory Medicine and Infectious Diseases, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan^b Department of General Medicine, Niigata University Medical and Dental Hospital, Niigata, Japan^c Research Laboratory, Torii Pharmaceutical Co., Ltd., Tokyo, Japan

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Abbreviations:

AHR, airway hyperresponsiveness;

AIT, allergen immunotherapy;

BALF, bronchoalveolar lavage fluid

Der f, *Dermatophagoides farinae*;

ELISA, enzyme-linked immunosorbent

assay; HDM, house dust mite; ICS, inhaled

corticosteroid; IL, interleukin; IFN-

γ, interferon-γ; MNC, mononuclear cell;

PAS, periodic acid-Schiff;

SCIT, subcutaneous immunotherapy;

SLIT, sublingual immunotherapy;

TGFβ, transforming growth factor β;

Treg, regulatory T cell

ABSTRACT

Background: Sublingual immunotherapy (SLIT) has received attention as a method for allergen immunotherapy. However, the mechanism of SLIT has not yet been fully investigated. Therefore, we evaluated the effects of SLIT in a murine asthma model, sensitized by intranasal administration of house dust mite (HDM) extracts.

Methods: Female BALB/c mice were intranasally exposed to HDM for either 3 or 5 weeks (5 consecutive days per week). Mice were administered either low-dose (0.5 mg/day) or high-dose (5 mg/day) sublingual HDM extracts for 2 weeks, followed by an additional week of intranasal exposure. Airway hyperresponsiveness (AHR), bronchoalveolar lavage fluid (BALF) cell count, cytokine levels in the BALF and lymph node cell culture supernatants, and allergen-specific antibodies were measured. Lung histology was also investigated.

Results: In mice sensitized for 5 weeks, high-dose SLIT ameliorated AHR, airway eosinophilia and goblet cell metaplasia. In mice sensitized for 3 weeks, even low dose SLIT ameliorated AHR and airway eosinophilia. Th2 cytokine levels in culture supernatants of submandibular lymph node cells in high-dose SLIT mice decreased, whereas IL-10 levels increased. Total IgA in BALF increased in mice sensitized for 3 or 5 weeks, and high-dose SLIT also increased allergen-specific IgG2a in mice sensitized for 5 weeks.

Conclusions: These data suggest that earlier induction of SLIT in HDM-sensitized mice provides superior suppression of AHR and goblet cell metaplasia. The modulation of allergen specific IgG2a and local IgA might play a role in the amelioration of AHR and airway inflammation.

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Introduction

Bronchial asthma is characterized by chronic allergic airway inflammation, variable airway obstruction, and airway

hyperresponsiveness (AHR) to various stimuli. In addition, prolonged airway inflammation induces structural changes in the airway that are difficult to treat.¹ To date, the initial therapy for bronchial asthma is inhaled corticosteroids (ICS), which are powerful enough to control the inflammation, and can alleviate symptoms and restore respiratory functions. However, ICS only treats the symptoms of inflammation and cannot modify the natural history of disease.^{2,3}

Allergen immunotherapy (AIT) involves the administration of specific allergens to patients with IgE-mediated conditions. In this

* Corresponding author. Department of Respiratory Medicine and Infectious Diseases, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Niigata-shi, Niigata 951-8510, Japan.

E-mail address: tkoya@med.niigata-u.ac.jp (T. Koya).

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context, the major objectives of AIT are to reduce responses to allergic triggers, decrease the inflammatory response and prevent the development of persistent disease. Furthermore, AIT is the only curative treatment for allergic diseases, and subcutaneous immunotherapy (SCIT) has been demonstrated to be clinically effective in treating asthma and rhinitis caused by allergic sensitization⁴; however, SCIT requires inconvenient injections and is associated with potentially severe systemic reactions. Thus, sublingual immunotherapy (SLIT) has been introduced to avoid systemic reactions, and has recently received increased attention regarding its potential clinical application in AIT.⁵

House dust mite (HDM) is the major allergen source in cases of human asthma and perennial allergic rhinitis. The randomized controlled trials have demonstrated that SLIT with HDM (using SLIT-droplets or tablets) is effective in treating allergic rhinitis and allergic bronchial asthma.^{6,7} Another large clinical trial of HDM SLIT tablets in both adult and adolescent patients with bronchial asthma is under way. Interestingly, although a large number of animal models of HDM-induced allergic asthma have been developed,^{8,9} the efficacy and mechanism(s) of action for SLIT with HDM are not fully understood. Therefore, this study aimed to develop an animal model of allergic asthma induced by HDM, and to investigate the effects of SLIT in mice intranasally sensitized with HDM.

Methods

Animals

Eight-week-old female BALB/c mice, free of murine-specific pathogens, were used in this study (CLEA Japan, Tokyo, Japan). Animals were housed under specific pathogen-free conditions and a 12:12 h light:dark cycle. All experiments were conducted under a

protocol approved by the Niigata University ethics committee for animal experiments.

Allergen

The species of HDM used was *Dermatophagoides farinae* (Der f), which was collected for preparing the dried extract (Torii Pharmaceutical, Tokyo, Japan). To characterize the extract, the major allergens (Der f 1; 27 kDa and Der f 2; 15 kDa) were detected by SDS-PAGE. The BCA assay was used to determine the protein concentration by absorption spectrophotometry with bovine serum albumin as the internal standard ($0.31 \text{ mg}^{\text{BSA}}/\text{mg}^{\text{extract}}$). The major allergens' concentrations were measured using enzyme-linked immunosorbent assays (ELISA) (Der f 1; $4.8 \text{ } \mu\text{g}/\text{mg}^{\text{extract}}$, Der f 2; $4.0 \text{ } \mu\text{g}/\text{mg}^{\text{extract}}$).

HDM-induced asthma model and treatment protocol

To induce AHR with HDM, mice were intranasally exposed to the HDM extract ($25 \text{ } \mu\text{g}$ in $10 \text{ } \mu\text{L}$ of saline) for 3, 5 or 7 weeks (5 consecutive days/week), without immunization (Fig. 1A). SLIT was performed for 2 weeks (5 consecutive days/week) after intranasal exposure, followed by an additional week of intranasal exposure (Fig. 1B, C). Saline was sublingually administered to mice as the control for SLIT. Twenty-four hours after the last HDM challenge, AHR was assessed, and bronchoalveolar lavage fluid (BALF), serum and lungs were obtained for further analyses.

Sublingual administration of allergen extract

SLIT was performed by grasping the mouse by the scruff of the neck, and carefully applying $10 \text{ } \mu\text{L}$ of the allergen extract under the tongue. The mouse was maintained in this position for 20–30 s after administration, to prevent the animal from immediately

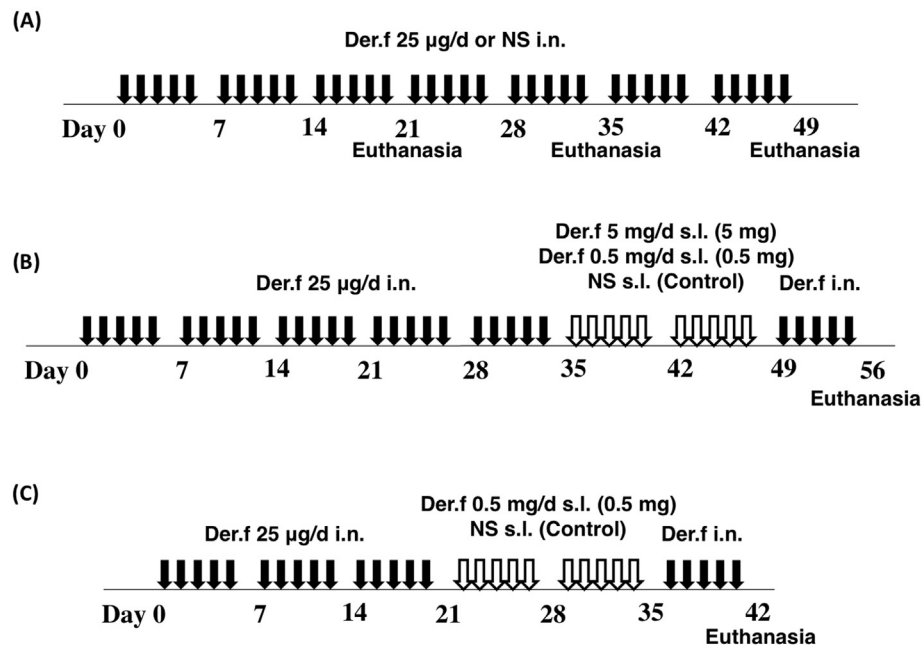


Fig. 1. Experimental protocols. (A) Mice were intranasally exposed to purified house dust mite (HDM) extract (black arrows, $25 \text{ } \mu\text{g}$ Der. f in $10 \text{ } \mu\text{L}$ saline) for 5 consecutive days/week for 3–7 weeks without immunization. Sublingual immunotherapy (white arrows) was administered for 5 consecutive days/week for 2 weeks after intranasal exposure for 5 weeks (B) or 3 weeks (C), as either high dose (5 mg/day) or low dose (0.5 mg/day), followed by an additional week of intranasal exposure. Instead of HDM extract, control mice received saline (NS) for SLIT. At 24 h after the last HDM challenge, airway hyperresponsiveness was assessed, and bronchoalveolar lavage fluid (BALF), serum and lungs were obtained for further analyses. i.n., intranasal; s.l., sublingual.

swallowing the allergen extract. To ensure the mice did not swallow the SLIT solution, they were sacrificed after our preliminary experiments, and the stomach examined for the presence of Trypan blue. Doses of 0.5 or 5 mg/mouse were used in this experiment, and to achieve the higher dose, the allergen solution was administered twice over a 10 min interval.

Determination of AHR

AHR was assessed by measuring the changes in respiratory resistance in response to increasing doses of inhaled methacholine, using the flexiVent system (SCIREQ, Montreal, Quebec, Canada), as reported previously.¹⁰ Briefly, anesthetized and tracheostomized mice were mechanically ventilated. Increasing concentrations (0–12.5 mg/ml) of methacholine aerosol were administered through an inline nebulizer. After delivery of aerosolized methacholine, the single-compartment model was used to assess respiratory resistance. Data were expressed as the percent change from baseline resistance and compliance values.

Measurement of cytokine levels in BALF and culture supernatants of cervical lymph node cells and lung cells

Immediately after AHR was assessed, BAL was performed using a tracheal tube, as described previously.¹¹ Submandibular lymph nodes were removed, and mononuclear cells (MNCs) were

prepared by using mechanical dissociation. Lung cells were isolated as previously described using collagenase digestion.¹² MNCs and lung cells (2.0×10^5 cells/well) were cultured on BD Biocoat™ T-cell activation assay plates (BD Biosciences, San Jose, CA, USA) for 72 h, at which time supernatants were recovered for the cytokine assays. In some experiments, cells were cultured without serum because of TGF- β measurement. The supernatants from cell cultures or BAL fluid were used for the measurement of cytokines and growth factors using commercially available enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturers' instructions. ELISA kits for the detection of IL-4, IL-5 and IFN- γ were obtained from eBioscience (San Diego, CA). The IL-10, IL-13 and TGF- β 1 ELISA kits were purchased from R&D Systems.

Detection of total IgE and IgA, and Der f specific IgE and IgG antibodies

To measure total IgE and IgA levels, we used ELISA kits specific for mouse IgE (Shibayagi, Gunma, Japan) and mouse IgA (Bethyl Laboratories, TX, USA).

Der f specific IgE antibodies were measured using ELISA. Briefly, 96-well plates were coated with 10 μ g/mL a monoclonal anti-mouse IgE antibody (23G3; Southern Biotech, AL, USA) in 0.1 M carbonate buffer (pH 9.5) overnight at 4 °C. The blocking buffer was 10% foetal bovine serum in phosphate-buffered saline, and blocking performed for 1 h. Binding was detected using biotin-conjugated

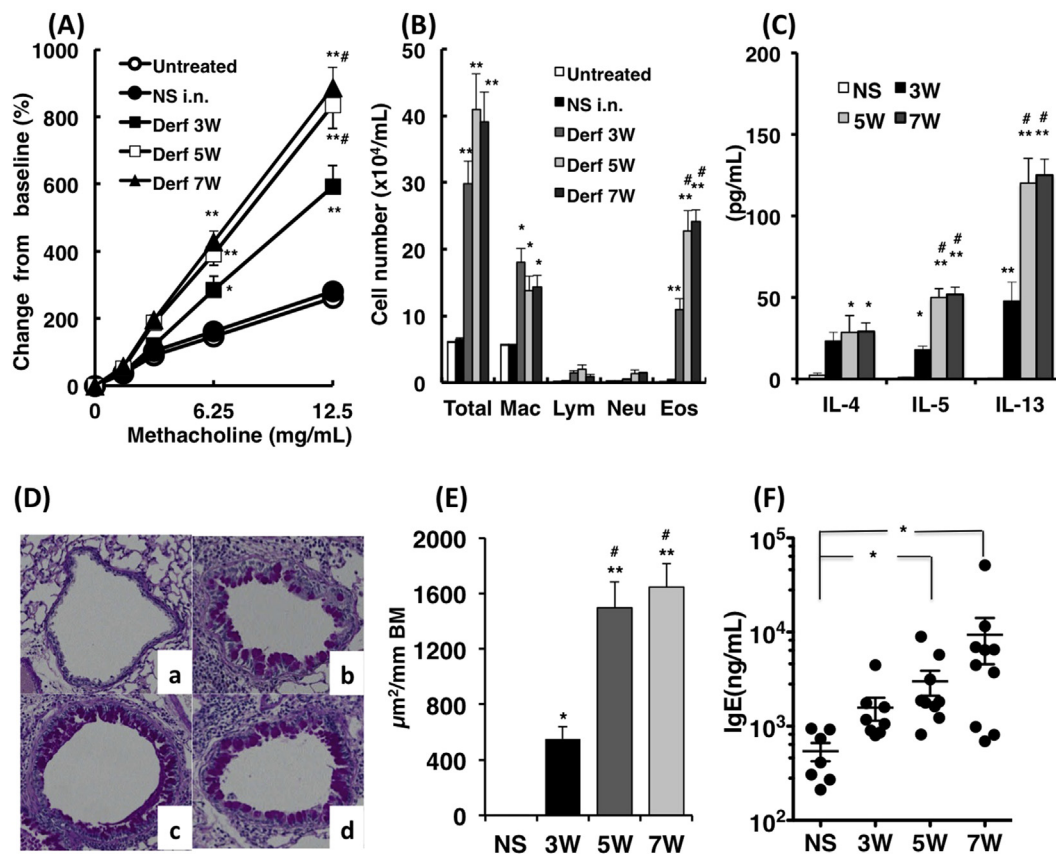


Fig. 2. Comparison of the development of airway hyperresponsiveness, eosinophilia and lung histology in the intranasal sensitization model. Mice were intranasally exposed to HDM extract for 3–7 weeks. The detailed procedures are described in the [Methods](#). (A) Changes in airway resistance with increasing concentrations of nebulized methacholine. (B) Changes in the cellular composition of BALF. (C) Levels of the indicated cytokines in BALF. (D) Representative periodic acid-Schiff (PAS) staining (bottom), performed in lung sections 24 h after the last challenge (100 $\times$ magnification). Saline group (NS or NS i.n.) (a), Der. f. for 3 weeks (b), Der. f. for 5 weeks (c), Der. f. for 7 weeks (d). (E) Quantitative analysis of PAS-positive cells in bronchial tissue. (F) The levels of total IgE in serum. Data presented as mean $\pm$ standard error of the mean (SEM). * $P < 0.05$ or ** $P < 0.01$ compared to the saline group or as indicated. # $P < 0.05$ compared to the Der. f. exposure for 3 weeks. BM, basement membrane; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils.

Mite Extract Dfb (Biostir, Japan) and Streptavidin- β -galactosidase conjugate (Roche, Basel, Switzerland). The substrate buffer was 10 mmol/L 4-methyl umbelliferyl- β -D-galactosidase/DMF.

For detection of HDM-specific IgG, horseradish peroxidase-conjugated anti-mouse IgG antibodies (Cat No. 074-1802, KPL, MD, USA) were used. For measurement of HDM-specific IgG subclass antibodies, either biotinylated IgG1 (Cat. No. 1070-08, Southern Biotech), biotinylated IgG2a (R19-15, BD Biosciences), or biotinylated IgG2b (R12-3, BD Biosciences) was used. Levels of Der f specific IgE and IgG were expressed as potency (%), which was calculated using the value of fluorescence intensity. The potency (%) = (sample – negative control)/(positive control – negative control) $\times$ 100. The pooled serum of Der f-sensitized mice was used as a positive control.

Lung histology

Left lungs were fixed in 10% formalin and immersed in paraffin. After deparaffinization, samples were stained with haematoxylin and eosin and periodic acid-Schiff (PAS) stain for histological analysis. The histological analyses were performed as described previously.¹²

Statistical analyses

Mann–Whitney *U* tests were used to evaluate the differences between all groups. The data from 3 independent experiments (4 mice/group in each experiment [$n = 12$]) were pooled. Comparisons of all paired values were performed using the Kruskal–Wallis test. Significance was assumed at P values < 0.05 for all tests. The values for all measurements were expressed as mean $\pm$ standard error of the mean.

Results

Comparison with intranasal exposure duration on AHR, airway inflammation, goblet cell metaplasia and total IgE levels in serum

Mice that were exposed to HDM for 3 weeks showed obvious AHR to methacholine, airway eosinophilia, elevation of Th2 cytokines in BALF, and goblet cell metaplasia compared with animals that were exposed to saline. AHR and allergic airway inflammation were significantly augmented in mice exposed to HDM for 5 weeks and these parameters were sustained in mice exposed for 7 weeks (Fig. 2A–E). Total IgE levels in serum were significantly increased in mice exposed to HDM for 5 and 7 weeks compared to mice exposed to saline (Fig. 2F). There were no significant changes between mice exposed for 5 weeks and 7 weeks. From these data, we used mice exposed for 3 or 5 weeks in this SLIT study.

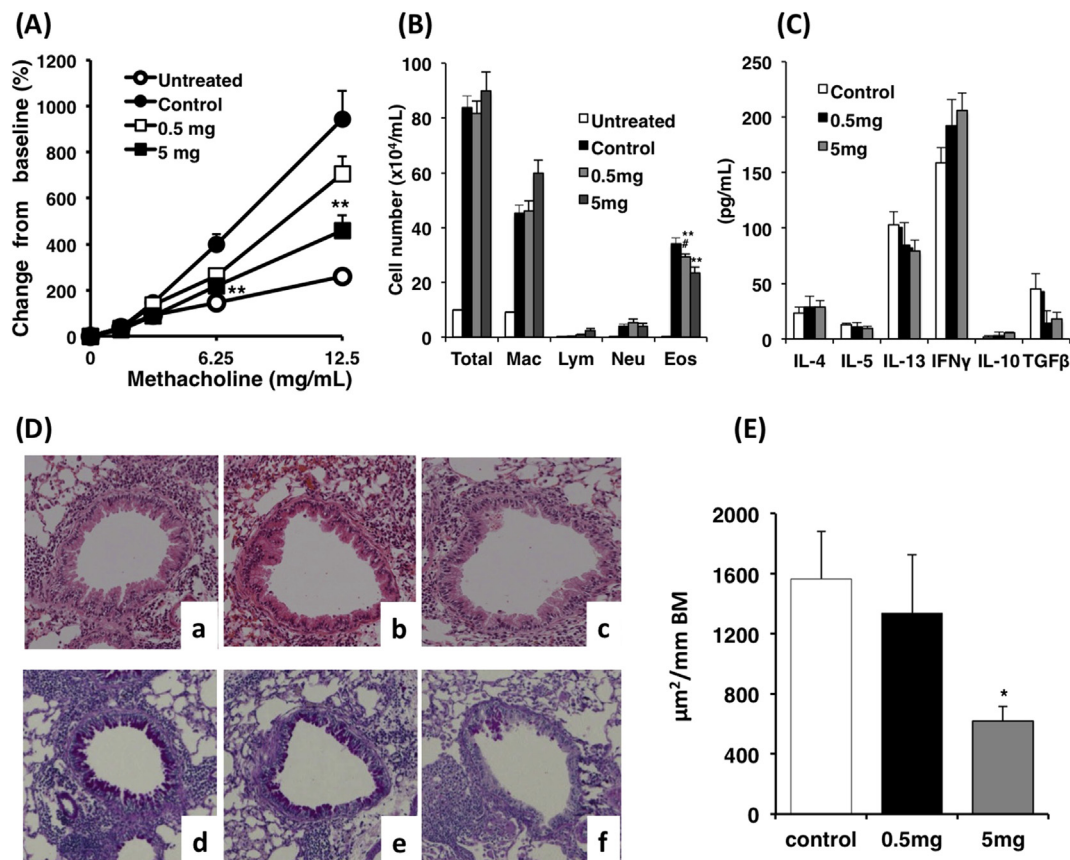


Fig. 3. Comparison of the effects of sublingual immunotherapy (SLIT) on the development of airway hyperresponsiveness, eosinophilia and lung histology in the 5-week intranasal sensitization model. Mice were intranasally exposed to HDM extract for 5 weeks, and received SLIT at high dose (5 mg), low dose (0.5 mg) or saline (control). The detailed procedures are described in the Methods. **(A)** Changes in airway resistance with increasing concentrations of nebulized methacholine. **(B)** Changes in the cellular composition of BALF. **(C)** Levels of the indicated cytokines in BALF. **(D)** Representative haematoxylin and eosin staining (top) and periodic acid-Schiff (PAS) staining (bottom), performed in lung sections 24 h after the last challenge (100 $\times$ magnification). Control (**a, d**), 0.5 mg SLIT (**b, e**), 5 mg SLIT (**c, f**). **(E)** Quantitative analysis of PAS-positive cells in bronchial tissue. Data presented as mean $\pm$ SEM from 3 independent experiments ($n = 12$). * $P < 0.05$ or ** $P < 0.01$ compared to the control group. # $P < 0.05$ compared to 0.5 mg SLIT group. BM, basement membrane; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils.

Effect of SLIT on AHR, airway inflammation and histological change in the treatment model

For the 5-week period of sensitization, mice that received high dose SLIT (5 mg/day) showed an obvious decrease in AHR compared with control animals. In mice that received low dose SLIT (0.5 mg/day), AHR was not significantly decreased compared to control animals (Fig. 3A). In cell composition in BALF, the number of eosinophil in both mice received high dose and low dose SLIT was decreased compared to control animals (Fig. 3B). The other cell types were not significantly different between SLIT mice and control mice (Fig. 3B). The cytokine levels in BALF were not significantly different between control mice and SLIT mice (Fig. 3C). Regarding histological features, inflammatory cell infiltrates, including eosinophils, around the bronchus were ameliorated in high dose SLIT (5 mg/day), compared to control animals. The presence of goblet cell metaplasia, a feature of airway remodelling, was easily identified using PAS staining of the mucus glycoproteins. Mice that received high dose SLIT, but not those that received the low dose, showed decreased goblet cell metaplasia (Fig. 3D, E).

For the 3-week period of sensitization, low dose SLIT (0.5 mg/day) induced a significant reduction of AHR. The number of eosinophils in the BALF was also decreased in mice that received SLIT, compared to mice that received saline (Fig. 4A, B). The cytokine levels in BALF were not also significantly different between control mice and SLIT mice (Fig. 4C). The inflammatory cell infiltrates

around the bronchus were apparently reduced in mice that received SLIT (Fig. 4D). PAS-positive goblet cells in the bronchus were significantly reduced in mice that received SLIT, compared to mice that received saline (Fig. 4D, E).

To investigate cytokine production capacity, MNCs were harvested from submandibular lymph nodes and stimulated against anti-CD3 coated plates. MNC-derived supernatant levels of IL-5 and IL-13 significantly decreased in high-dose SLIT mice compared to levels in control mice. Moreover, IL-13 levels in low-dose SLIT mice also significantly decreased relative to levels in control mice. Levels of the suppressive cytokine IL-10 significantly increased in high-dose SLIT mice compared to control mice, whereas the levels of TGF- β 1 did not differ significantly among groups (Fig. 5). Lung cell culture supernatants did not differ significantly between SLIT mice and control mice (data not shown).

Effect of SLIT on immunoglobulin in the treatment model

The IgE levels in SLIT mice sensitized for both 3 and 5 weeks were not significantly different compared to control mice, whereas IgG1 and IgG2a levels in high-dose SLIT mice significantly increased compared to levels in low-dose SLIT mice and control mice (Fig. 6A–D). The IgA levels in serum were not significantly different between groups (data not shown). However, BALF IgA levels in SLIT mice sensitized for 3 weeks were significantly increased compared to the levels in the control mice (Fig. 6E). The BALF IgA levels after 5-week sensitization and high dose SLIT (5 mg/day) mice were also

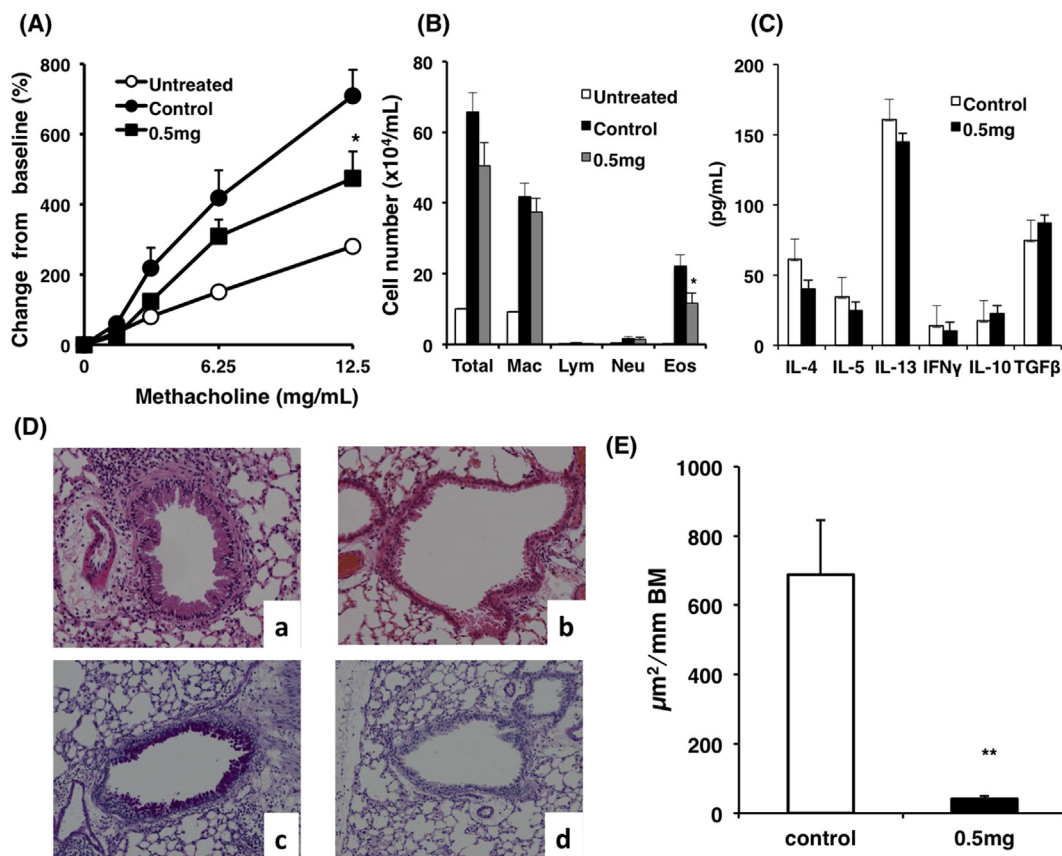


Fig. 4. Comparison of the effects of sublingual immunotherapy (SLIT) on the development of airway hyperresponsiveness (AHR), eosinophilia and lung histology in the 3-week intranasal sensitization model. Mice were intranasally exposed to HDM, and received SLIT with HDM (0.5 mg) or saline (control). (A) Changes in airway resistance with increasing concentrations of nebulized methacholine. (B) Changes in the cellular composition of BALF. (C) Levels of the indicated cytokines in BALF. (D) Representative haematoxylin and eosin staining (top) and periodic acid-Schiff (PAS) staining (bottom), performed in lung sections 24 h after the last challenge (100 $\times$ magnification). Control (a, c) and SLIT (b, d). (E) Quantitative analysis of PAS-positive cells in bronchial tissue. Data presented as mean $\pm$ SEM from 3 independent experiments ($n = 12$). * $P < 0.05$ or ** $P < 0.01$ compared to the control group. BM, basement membrane; Mac, macrophages; Lym, lymphocytes; Neu, neutrophils; Eos, eosinophils.

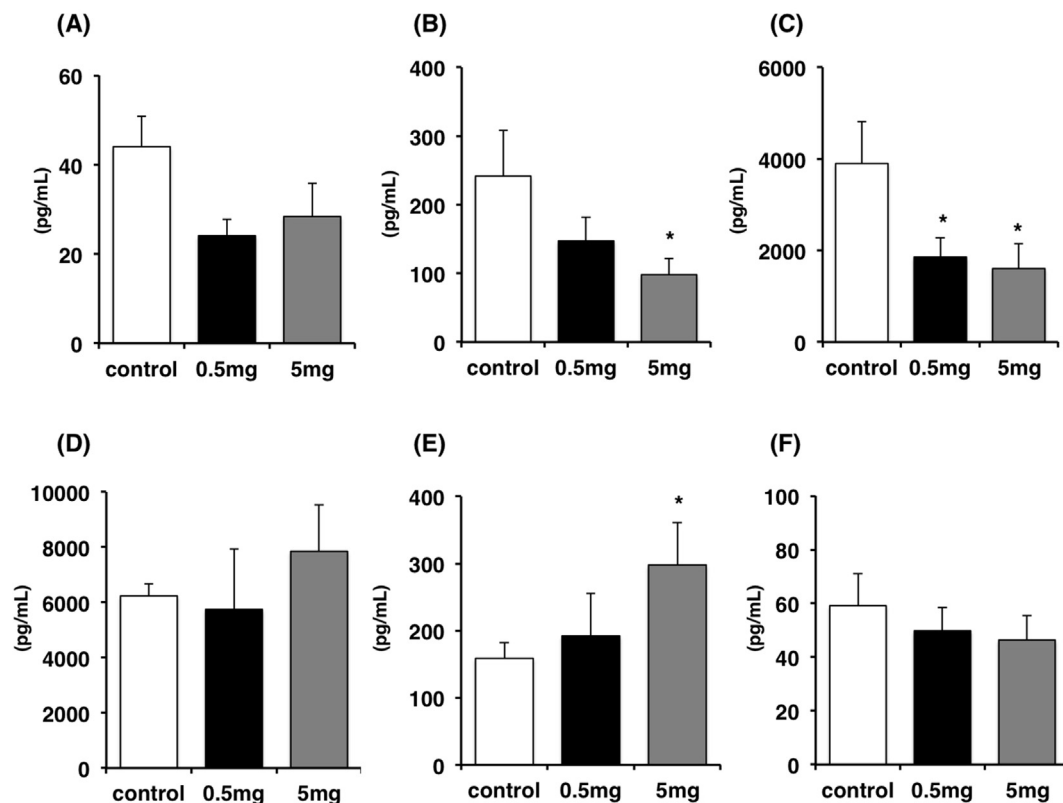


Fig. 5. In vitro cytokine levels. Levels of interleukin (IL-4) (A), IL-5 (B), IL-13 (C), interferon γ (D), IL-10 (E), and transforming growth factor β 1 (F) were determined in supernatants from submandibular lymph node mononuclear cells (MNCs) isolated from mice that received saline (control) or either high-dose (5 mg/day house dust mite extract) or low-dose (0.5 mg/day house dust mite extract) sublingual immunotherapy. MNCs were cultured on BD Biocoat T-cell activation assay plates for 72 h. Data are presented as mean $\pm$ standard error of the mean from 3 independent experiments carried out in triplicate. * $P < 0.05$ compared to the control group.

significantly increased, compared to the levels in the control mice (Fig. 6E). However, the BALF IgA levels after 5-week sensitization and low dose SLIT (0.5 mg/day) were similar to those in the control mice.

Discussion

In the present study, we demonstrated that sublingual administration of HDM extract was able to reduce the development of AHR, airway eosinophilia and goblet cell metaplasia in an intranasal HDM sensitization model. SLIT is currently established as a safe and efficacious treatment for human allergic disease,⁶ and murine models of SLIT have found that it is effective in reducing AHR and airway inflammation.¹³ However, the optimal protocol for evaluating the efficacy of SLIT in animal models remains unclear. In this study, we performed SLIT in two different protocols, using a model with mice intranasally sensitized to HDM. Our results indicate that earlier induction of SLIT was associated with suppression of AHR and goblet cell metaplasia.

Another priority of this study was to examine the use of intranasal HDM as a sensitization allergen. In most rodent model, ovalbumin is used as the allergen, whereas HDM is a more clinically relevant asthma allergen, and can also be used as an alternative allergen for animal model.⁸ Moreover, intranasal sensitization is a closer method in clinical situation than the intraperitoneal sensitization used in rodent asthma models, as many allergens enter the respiratory system through breathing. In clinical data, chronic exposure of indoor allergens is thought to be one of the causes for the development of asthma.^{14–16} However, compared to intraperitoneal sensitization, intranasal sensitization tends to show lower AHR and airway inflammation, such as airway eosinophilia and Th2

cytokine production. Therefore, changes in cytokine levels in BALF in response to SLIT might be minimal at best. Moreover, intranasal sensitization may stimulate innate lymphoid cells type 2 (ILC2) as well as Th2 type cells involved with eosinophilic inflammation. These ILC2 might be insensitivity to antigen-specific immunotherapy.

In our study, daily intranasal HDM administration induced AHR, airway allergic inflammation and airway remodelling, starting at 3 weeks, and subsequently increased at 5 weeks. In this study, early induction of SLIT showed greater effects in AHR, airway eosinophilia and goblet cell metaplasia. For example, goblet cell metaplasia in 3-week model was greatly decreased in low dose SLIT. However, the absolute areas of PAS-positive cells were much lower in control mice in the 3-week model compared to the control mice in the 5-week model. In addition, the positive cells in the 3-week model stained more faintly than those in the 5-week model. In the 5-week model, tenfold doses of HDM were required to induce tolerance comparable to the 3-week model. In the prophylactic model using immunotherapy prior to sensitization, a shorter period of SLIT was sufficient to induce tolerance comparable to the therapeutic model.¹³ Furthermore, it has been reported that the longer period of SLIT was necessary to suppress AHR in a murine model of birch pollen asthma.¹⁷ Therefore, a robust immune response can be established after a relatively short period of sensitization, and it becomes difficult to modulate the response with immunotherapy. In our current model, substantial doses of allergen (0.5 mg or 5 mg) were required to induce SLIT. One previous study reported that 50 μ g of HDM extracts (25 μ g each of *Dermatophagoides pteronyssinus* and *D. farinae* extracts) effectively inhibited AHR and airway inflammation.¹⁸ The difference between their dosage and

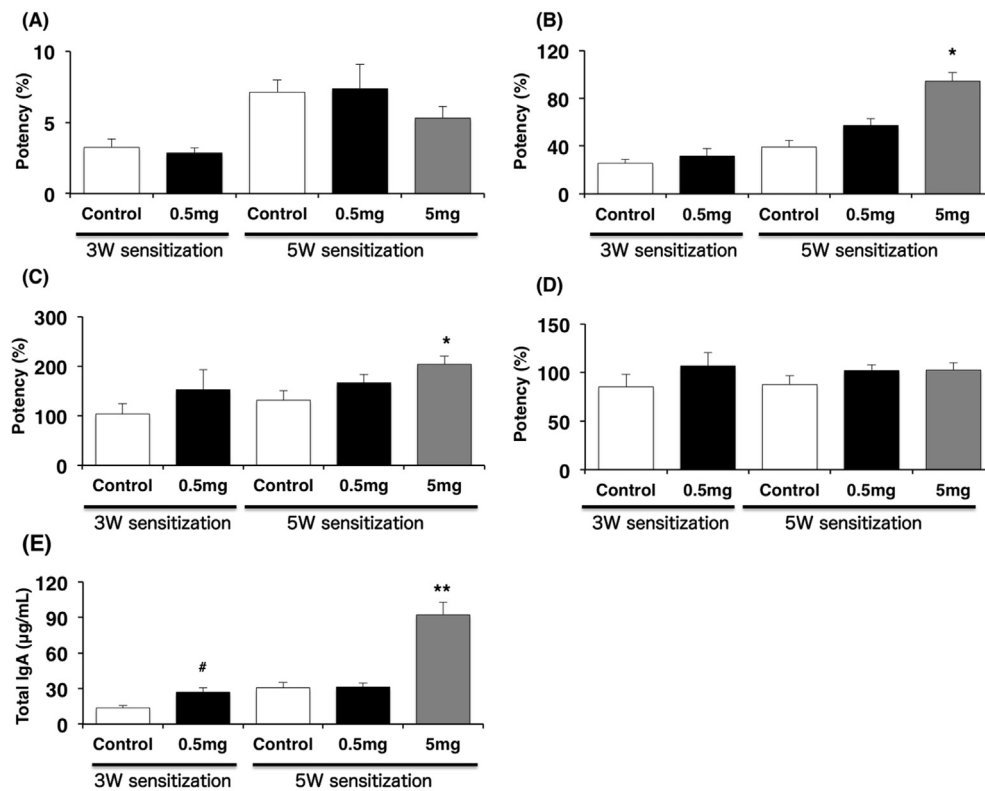


Fig. 6. Serum levels of *Dermatophagoides farina* (Der f)-specific IgE and IgG, and total IgA levels in BALF. The levels of Der f-specific IgE (A), IgG1 (B), IgG2a (C), IgG2b (D) antibodies were measured by ELISA and were expressed as potency (%). Total IgA levels in BALF (E) were measured by commercial ELISA. Control: serum from mice that received saline in place of sublingual immunotherapy (SLIT). 0.5 mg and 5 mg: serum from mice that received low dose SLIT (0.5 mg/day) and high dose SLIT (0.5 mg/day), respectively. 3 W and 5 W sensitization: 3 and 5 week-intranasal sensitization mice. Data presented as mean $\pm$ SEM from three independent experiments carried out in triplicate ($n = 12$). ** $P < 0.01$ compared to the control group (5 W). # $P < 0.05$ compared to the control group (3 W).

ours might be attributed to the context of allergens, the duration of SLIT or the route of allergen administration. Previous studies performed SLIT for longer times than those used in the current study.^{18,19} In preliminary experiments, we found that AHR to methacholine and the cellular composition of BALF were not significantly different in 2-week SLIT versus 4-week SLIT (data not shown). It is possible that longer durations of SLIT maintain the effects of immunotherapy when lower doses of allergen are used.

The mechanisms of allergen specific immunotherapy are likely decreased mast cell and basophil activity and degranulation, induction of regulatory T cells (Tregs) that predominantly produce IL-10, deviation of the Th2 immune response to a Th1 response, or production of blocking antibodies (e.g., IgG4) against IgE or IgG1.^{20–22} In the present study, Th2 cytokines levels from sub-mandibular lymph node culture supernatants decreased in SLIT mice compared to levels in control mice, and IL-10 levels significantly increased in high-dose SLIT mice. Although FoxP3-positive regulatory T cells in lung or lymph node were not apparently different between groups (data not shown), our data might reflect the reinforcement of regulatory T cells in the draining lymph nodes. In this study, the expansion of Tregs in lung or draining lymph node and the significant differences in the number of mast cells stained by toluidine blue, or the levels of mast cell protease-1 in the BALF were not observed in both low dose and high dose SLIT group. However, the levels of Der f specific IgG1 and IgG2a, but not IgE, were increased in high dose SLIT group.

Antigen-specific IgG2a is thought to indicate crucial evidence of successful allergen specific immunotherapy, thus indicating immunotherapeutic efficacy,^{23,24} thus it can be considered to

reflect a modulation of airway allergic inflammation. A previous report found that IgA production at local sites was important in suppressing airway allergic inflammation.¹⁸ IgA is also thought to play a role in tolerance induction,²⁵ and has been associated with an improved clinical course of SLIT in grass pollen allergy patients.²⁶ In the present study, BALF IgA levels in SLIT mice sensitized for 3 weeks were significantly increased compared to control mice (Fig. 5C). The BALF IgA levels after 5-week sensitization and high dose SLIT (5 mg/day) mice were also increased, although that change was not observed for low dose SLIT (0.5 mg/day) mice (Fig. 5C). Although these IgA values were not allergen-specific, our data suggest that local IgA production may play a role in suppressing airway allergic inflammation in this model, especially after early SLIT induction.

In SLIT models, the retention or swallowing of the sublingual allergen is a critical factor. Given the small volume of the allergen (10 μ L), we were able to successfully apply it sublingually, and the allergen was not detected in the stomach (using Trypan blue). If there are some amount of unavoidable allergen influx to stomach, AHR and airway inflammation do not appear be affected, as our preliminary data also showed that 5 mg of orally administered HDM extract did not suppress AHR and airway eosinophilia (data not shown).

In summary, we demonstrated the efficacy of SLIT in HDM sensitized mice, using a model of established airway allergic inflammation with repeated intranasal allergen administration. Our data suggests that early induction of SLIT provides more effective suppression of AHR and goblet cell metaplasia in the therapeutic model. Moreover, the modulation of allergen-specific

IgG and local IgA production may play a role in the amelioration of AHR and airway allergic inflammation. However, the precise mechanism(s) of action remain unknown, and should be addressed in the future.

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Conflict of interest

The authors have no conflict of interest to declare.

Authors' contributions

KS and TKo designed the study and wrote the manuscript. KS, KT and CF contributed to data collection. KS, TKo, TS, TH, KOD, SW, ES and TKi performed the statistical analysis and interpretation of the results. All authors read and approved the final manuscript.

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