

Beneficial effects of Galectin-9 on allergen-specific sublingual immunotherapy in a
Dermatophagoides farinae-induced mouse model of chronic asthma

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Short title: Galectin-9 in mouse SLIT model

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Abstract

Background: Allergen-specific sublingual immunotherapy is a potential disease-modifying treatment for allergic asthma. Galectin-9 (Gal-9), a β -galactoside-binding protein with various biologic effects, acts as an immunomodulator in excessive immunologic reactions by expanding regulatory T cells (Treg) and enhancing transforming growth factor (TGF)- β signaling. We investigated the efficacy of sublingually administered Gal-9 as an adjuvant to a specific allergen in a *Dermatophagoides farinae* (Df)-induced mouse model of chronic asthma. **Methods:** BALB/c mice were intranasally sensitized with Df extract 5 days/week for 5 weeks, and then sublingual Df-allergen extract for 2 weeks (5 days/week). Three days after the final sublingual treatment, mice were intranasally challenged with Df extract. The early asthmatic response (EAR) was evaluated 5 min after the last Df challenge. Airway hyperresponsiveness (AHR) was assayed and bronchoalveolar lavage (BAL) was performed 24 h after the last allergen challenge. Serum IgE and cytokine levels, and number of inflammatory cells in the BAL fluid (BALF) were analyzed. **Results:** Sublingual Df treatment in the presence of Gal-9, but not alone, significantly reduced AHR; EAR; number of eosinophils and interleukin-13 in the BALF; and serum IgE levels. BALF TGF- β 1 levels were significantly increased in the presence of Gal-9 compared with Df alone. Treg depletion blocked the inhibitory effects of Gal-9 on the EAR, AHR, eosinophilic airway inflammation, and Df-specific serum IgE levels, and suppressed BALF TGF- β 1 levels. **Conclusions:** Gal-9 exhibited beneficial effects of sublingual Df allergen-specific immunotherapy in a Df-induced mouse model of chronic asthma, possibly by Gal-9-induced TGF- β 1 production in the lung.

Key words: SLIT, galectin-9, mite allergen, TGF- β 1, mouse

Abbreviations: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; Df, *Dermatophagoides farinae*; EAR, early asthmatic response; Gal-9, galectin-9; SLIT, sublingual immunotherapy; Treg, regulatory T cells.

Introduction

Asthma is a common respiratory disease characterized by reversible airway obstruction, airway hyperresponsiveness (AHR), and chronic airway inflammation with eosinophils. The majority of patients with asthma are well controlled by combined treatment with inhaled corticosteroids, long-acting β -agonists, and leukotriene receptor antagonists¹⁻³. These drugs have anti-inflammatory effects on chronic airway inflammation, but do not cure asthma. While allergen-specific immunotherapy might cure asthma, some problems, such as the administration route of the allergen, must be overcome⁴. Recently, patients with allergic rhinitis and asthma induced by mite-allergen were treated with an allergen-specific sublingual immunotherapy (SLIT). The SLIT route could be beneficial toward curing these allergic airway diseases, but some difficulties remain^{5,6}.

Galectin-9 (Gal-9) is a β -galactoside binding animal lectin that induces various biologic reactions, such as cell chemoattraction, activation, and apoptosis⁷. Gal-9 functions as an immunomodulator in excessive immunologic reactions by expanding regulatory T cells (Treg) and immunosuppressive macrophages⁸⁻¹⁰. Furthermore, Gal-9 and CD44 interactions enhance the stability and function of adaptive Treg through smad3-dependent mechanisms¹¹. The role of Gal-9 in allergic respiratory diseases, however, remains unclear.

In the present study, we developed a SLIT model of mite allergen-induced chronic asthma using the *Dermatophagoides farinae* (Df)-induced murine model of chronic asthma that we previously developed¹². We also used recombinant stable human Gal-9¹³ as an adjuvant to the mite-allergen. The additive effects of Gal-9 in this SLIT model were evaluated by physiological examination, eosinophilic airway inflammation, and serum allergen-specific immunoglobulin levels, especially immunoglobulin IgE.

Methods

Animal model and sublingual immunotherapy

BALB/c mice were obtained from Charles River Laboratory (Yokohama, Japan). Female mice (8-12 weeks old) were intranasally sensitized with Df extract (40 µl of 1 mg/ml, LSL Co. Ltd, Tokyo, Japan) 5 days/week for 5 weeks (Fig. 1). Negative control animals were intranasally exposed to phosphate-buffered saline (PBS) in a similar manner. Following intranasal administration of the Df extract for 5 weeks, SLIT was performed 5 days/week, twice daily, for 2 weeks (Fig. 1). The SLIT agents were PBS, Df-allergen extract (250 µg/day, Torii, Co. Ltd, Tokyo, Japan), and Df-allergen extract with recombinant human stable Gal-9 (0.1, 0.3, or 1.0 µg [3 µM], GalPharma Co. Ltd, Takamatsu, Japan)¹³. For SLIT, the mouth of an anesthetized mouse was kept open by placing forceps under the tongue. We administered 10 µl of SLIT agent under the tongue with a micropipette that was kept in place for 1 min. In preliminary experiments, we used trypan blue to confirm that this method does not deliver the SLIT agent to the stomach or lung. Endotoxin contamination in the Df and Gal-9 solution was minimal, as previously described (< 0.25 EU/mg)^{13, 14}. In this SLIT model, intranasal Df exposure is stopped during the SLIT because it is important for the SLIT to prevent allergen contact in patients with atopic asthma, as previously described^{15, 16}. All experiments in the present study were performed under a protocol approved by the Institutional Animal Care and Use Committee of the Kawasaki Medical School.

Early asthmatic response (EAR) and airway hyperresponsiveness (AHR)

The early asthmatic response (EAR) was evaluated as the rate of increase (Δ sRaw) in airway resistance (sRaw) after the last Df challenge, compared with PBS exposure just before the last Df challenge in each mouse using a two-chambered, double-flow plethysmograph system (Pulmos; M.I.P.S, Osaka, Japan). We monitored Δ sRaw for 5 min after the last Df challenge. To investigate AHR, mice were forced to inhale PBS or acetyl- β -methylcholine chloride (Mch; Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 3, 6, or 12.5 mg/ml. We measured sRaw in awake mice using the two-chambered, double-flow plethysmograph system. Forced inhalation was administered for 3 min. After a 1-min rest, sRaw was measured for 2 min. AHR is expressed as the concentration of Mch required to provoke a doubling of sRaw (PC₂₀₀), like PC₂₀ in patients with asthma.

Collection of blood, bronchoalveolar lavage fluid (BALF), and lung tissue

After euthanasia, blood and bronchoalveolar lavage fluid (BALF) were collected, and the lungs were removed for pathologic evaluation by hematoxylin and eosin (HE) and Periodic acid-Schiff (PAS) staining. Mucus score was estimated by PAS-stained lung. A total of 10 airways of three mice from each group were categorized according to the abundance of PAS goblet cells, and assigned numerical scores (0 = <5% goblet cells; 1 = 5-25%; 2 = 25-50%; 3 = 50-75%; 4 = >75%). Bronchoalveolar lavage (BAL) was performed 24 h after the last allergen administration, and BALF was obtained by washing the lungs with 4 x 1 ml of PBS and centrifuged. The supernatant of the first wash was stored at -80°C until use. Cell pellets of all washes were collected and resuspended in 1 ml PBS. The number of BALF cells was counted using a cell counter. Cytospin slides were stained with Diff-Quik (Sysmex, Kobe, Japan). Differential cell counts were performed on at least 400 cells.

Enzyme-linked immunosorbent assay (ELISA)

Amounts of interleukin (IL) -5, IL-10, IL-13, interferon- γ (IFN- γ), and transforming growth factor (TGF)- β 1 (R&D Systems, Minneapolis, MN, USA) in the BALF were measured using an enzyme-linked immunosorbent assay (ELISA). The detection limits were 7.0 pg/mL for IL-5, 2.0 pg/mL for IL-10, 3.9 pg/mL for IL-13, 4.7pg/mL for IFN- γ , and 4.9 pg/mL for TGF- β 1. Concentrations below the detection limits were assumed to be zero for statistical analysis. Df-specific serum IgE was measured by ELISA as previously described ¹⁴. Briefly, diluted sera were incubated in Df extract-coated plates, followed by incubation with biotin-conjugated antibodies against IgE (Serotec, Raleigh, NC, USA) and streptavidin-horseradish peroxidase (Invitrogen, Carlsbad, CA, USA). The Df-specific serum IgE levels were expressed as relative absorbance units (optical density at 450 nm). For detection of Df-specific serum IgG1, and IgG2a, either biotinylated IgG1 (Southern Biotech, Birmingham, AL, USA) or IgG2a (BD Biosciences, San Jose, CA, USA) was used. Levels of Df-specific IgG1 and IgG2a were expressed as potency (%), which was calculated using the fluorescence intensity value, as previously described ¹⁷. The potency (%) = (sample – negative control)/(positive control – negative control) x 100. The pooled serum of Df-sensitized mice was used as a positive control.

Evaluation of Treg

Treg in the BALF and spleen were measured using a mouse Treg staining kit (eBioscience Inc, San Diego, CA, USA) using a FACSCalibur® flow cytometer (BD Biosciences, San Jose, CA, USA). For Treg depletion, mice were intraperitoneally injected with PC61

monoclonal antibody (mAb, 250 µg/mouse) 3 days before the starting SLIT (day 33). Treg depletion was confirmed by evaluating the spleen for CD4, CD25, and Foxp3 expression using flow cytometry. CD25 was detected using an anti-CD25 mAb that recognizes an epitope distinct from that of the PC61 mAb. Rat IgG was used as a negative control. As previously described ¹⁸, no CD4+CD25+Foxp3+ cells were detected in the spleen during SLIT for 2 weeks (day36 to day 51) after PC61-treatment (day 33), whereas these cells were detected in the spleen after Rat IgG-treatment (day 33). PC61 was obtained as a kind gift from Eiichi Nakayama (Kawasaki University of Medical Welfare, Kurashiki, Japan). Rat IgG was obtained from R&D systems.

Statistical analysis

All data are expressed as the mean \pm standard error (SEM). Statistical analysis was performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA, USA). The Kruskal-Wallis test with Dunn's multiple comparisons test or the Mann-Whitney U test was used to compare values between two groups. Differences with probability values of less than 0.05 were considered significant.

Results

Effects of Gal-9 on EAR and AHR in mite allergen-specific SLIT

To evaluate the effect of Gal-9 on SLIT, we develop a SLIT model using the previously reported Df-allergen and Df-induced mouse model of chronic asthma (Fig. 1). This model exhibits EAR, AHR, chronic eosinophilic airway inflammation, Df-specific IgE, and airway remodeling like chronic atopic asthma in human ¹². We investigated the efficacy of increasing concentrations of sublingually administered Gal-9 as an adjuvant to a specific allergen in this model. We first evaluated the EAR after exposure to a specific allergen. The airway resistance change from PBS exposure (%) at 5 min after intranasal Df challenge and AHR at 24 h after intranasal Df challenge were measured. Sublingual Df treatment alone did not significantly affect EAR and AHR, but the EAR and AHR levels tended to be lower compared with that after PBS treatment alone ($p=0.1840$ and $p=0.0534$, respectively, Fig. 2). Sublingual Df treatment in the presence of Gal-9 significantly reduced both EAR and AHR ($p<0.05$, Fig. 2). Further, sublingual Gal-9 treatment alone did not significantly affect EAR and AHR as compared with the PBS treatment alone group (Supplementary Fig. 1A).

Effects of Gal-9 on airway eosinophilic inflammation in mite allergen-specific SLIT

To evaluate the efficacy of Gal-9 on eosinophilic airway inflammation in the SLIT model, we examined inflammatory cell numbers in the BALF and histopathologic findings. Numbers of inflammatory cells in the BALF were counted 24 h after the intranasal allergen challenge. Numbers of total leukocytes and eosinophils, but not lymphocytes, neutrophils, and macrophages, were significantly reduced by SLIT in the presence of Gal-9 ($p<0.05$, Fig. 3A),

but not Df-alone. SLIT with Gal-9 by itself had no effect on eosinophilic airway inflammation (Supplementary Fig. 1B). Histopathologic examination of the lung tissue indicated that peribronchial and perivascular eosinophilic infiltration induced by Df-challenge was suppressed by SLIT, especially in the presence of Gal-9 (Fig. 3B). Furthermore, to examine the effect of SLIT on mucus secretion in the airway, we evaluated PAS-stained lung sections. Allergen-induced mucus-secreting goblet cells were relatively suppressed by SLIT with Gal-9, especially in a low dose of Gal-9 (0.1 μ g; $p=0.0008$, 0.3 μ g; $p=0.3683$, 1.0 μ g; $p=0.5483$, Fig. 3C).

Gal-9 decreases Th2 cytokine levels in the BALF as well as serum IgE levels in the SLIT model

To investigate the mechanisms of the anti-inflammatory effects of Gal-9 in the SLIT model, we measured the concentrations of IL-5 and IL-13 in the BALF by ELISA 24 h after Df-challenge. Elevated concentrations of IL-5 in the BALF were reduced by SLIT in the presence or absence of Gal-9 ($p<0.05$, Fig. 4A). The IL-13 concentration in the BALF was significantly reduced by SLIT in the presence of low and medium doses of Gal-9 ($p<0.05$, Fig. 4A). Furthermore, low and medium doses of Gal-9 suppressed the production of Df-specific IgE levels in the serum ($p<0.05$, Fig. 4B). Serum levels of Df-specific IgG1 tended to be lower in the presence of low ($p=0.1071$, Fig. 4B) and medium ($p=0.1826$, Fig. 4B) doses of Gal-9. The serum levels of Df-specific IgG2a did not differ significantly among treatments (Fig. 4B). Further, SLIT with Gal-9 by itself had no effect on the BALF Th2 cytokine and serum Df-specific IgE levels (Supplementary Fig. 1C). The production of TGF- β 1 in the BALF was significantly increased in this SLIT model. The TGF- β 1 level was higher in the presence of low and medium doses of Gal-9 than when Df alone was administered ($p=0.0526$

and $p=0.0061$, respectively, Fig. 5). IL-10 and IFN- γ were not detected in the BALF of this SLIT model (data not shown). The number of CD4+CD25+Foxp3^{high} Treg in the BALF was significantly increased by sublingual Df treatment in the presence of Gal-9 ($p<0.05$, Fig. 5), but not Df-alone.

Efficacy of Gal-9 on the SLIT model was blocked by Treg depletion

To deplete Treg during SLIT, PC61 anti-CD25 mAb was injected 3d before the starting SLIT, as described in the Methods. PC61 treatment diminished the inhibitory effects of Gal-9 on EAR and AHR ($p<0.05$, Fig. 6A). PC61 treatment blocked the Gal-9 SLIT-induced decrease in the number of eosinophils, IL-5 and IL-13 concentrations in the BALF, and serum IgE levels ($p<0.05$, Fig. 6B). PC61 treatment by itself had no effect on asthmatic responses in this SLIT model (Fig. 6A, B). Further, PC61 treatment suppressed the Gal-9 SLIT-induced increase in the concentration of TGF- β 1 in the BALF ($p<0.05$, Fig. 6C).

Discussion

The present findings demonstrated that Gal-9 enhanced the efficacy of SLIT in a mouse model of chronic asthma induced by the Df allergen. In this treatment strategy, novel SLIT using Gal-9 as an adjuvant improved the pathophysiology of asthma. The treatment in the presence of Gal-9, but not in the absence of Gal-9, significantly reduced both the EAR and AHR. Furthermore, both BALF IL-13 levels and serum Df-specific IgE levels were significantly reduced by Gal-9 as an adjuvant to the Df allergen. Gal-9 also inhibited the eosinophilic airway inflammation associated with decreased levels of the Th2 cytokines, IL-5 and IL-13.

Asthma is a common respiratory disease in humans, and more than half of patients with asthma have allergen-specific IgE³. House dust mites are one of the most common aeroallergens in asthma³. While inhaled corticosteroids control asthmatic symptoms well, they are not able to cure the disease. SLIT is a disease-modifying treatment for respiratory allergies¹⁹, but the benefit and effect may not be very substantial, and the treatment requires a long period of therapy and high amounts of the allergen. Further, it is difficult to diminish allergen-specific IgE production using SLIT^{20, 21}. Inhalation of a specific allergen might exacerbate asthma, even if asthmatic symptoms are well controlled. To cure asthma, it is important to suppress the production of allergen-specific IgE. Previous studies demonstrated the preventive effects of SLIT in a mite allergen-induced acute murine asthma model by several times (2 or 4 times) sensitization with or without alum^{15, 16}. In the present study, we use an established model of chronic severe asthma by 25 times intranasal sensitization without alum in mice similar to human chronic asthma in adults¹², and attempted to cure this disease model by SLIT using Gal-9 as an adjuvant. Recently, Shima et al. reported that SLIT using high-dose Df extract (5 mg/day, Torii, Co. Ltd) ameliorates AHR and eosinophilic airway inflammation, but not serum IgE levels in an asthmatic mouse model¹⁷. In the present

study, we demonstrated that our new SLIT using a low dose of the same allergen (250 µg/day, Torii, Co. Ltd) with Gal-9 is more effective in a similar asthmatic mouse model. Df-specific IgE levels in the serum were significantly reduced by sublingual Df treatment in the presence of Gal-9, but not in the absence of Gal-9. Further, allergen-induced EAR and AHR were significantly suppressed by Gal-9 as an adjuvant to the Df allergen. These findings suggest that administration of Gal-9 enhances the efficacy of SLIT in mite allergen-induced asthma and might contribute to cure this disease.

SLIT induces elevated levels of IL-10 and IgG4 in the serum of patients with allergic airway diseases ^{21, 22}. Treg are thought to play an important role in the SLIT. Recent studies demonstrated that Gal-9 acts an immunomodulator in excessive immunologic reactions by expanding Treg and enhancing TGF-β signaling ^{10, 11}. In the present study, we evaluated the regulation mechanism of the effect of Gal-9 on SLIT in addition to a specific allergen. Gal-9 may suppress eosinophilic airway inflammation by inhibiting IL-5 production in the lung or inducing apoptosis ^{23, 24}. Further, Gal-9 suppressed Df-specific IgE production by inhibiting IL-13 production, especially at low and medium doses. Next, we evaluated the levels of inhibitory cytokines in the BALF of the SLIT model. Levels of TGF-β1, but not IL-10 in the BALF were increased in sublingual Df-treated mice with low and medium doses of Gal-9 in this SLIT model. Further, numbers of CD4⁺CD25⁺Foxp3^{high} Treg in the BALF were also increased by SLIT in the presence of Gal-9. TGF-β1 and CD4⁺CD25⁺Foxp3^{high} Treg induced by Gal-9 in the lung might play an important role in the effects of Gal-9 on SLIT. Further studies are required to clarify the mechanisms of the adjuvant effects of Gal-9 in this SLIT model.

Gal-9 is a ligand for CD44 that blocks the binding between CD44 and its original ligand hyaluronan ^{25, 26}. Interestingly, Gal-9 binds CD44 expressed on Treg and enhances TGF-β signaling ¹¹. Previous studies demonstrated that a medium dose of Gal-9 (1 µM) is sufficient

to bind CD44 and a high dose of Gal-9 (3 μ M) induces apoptosis of Th1 cells through Tim-3 signaling, and induces apoptosis of IL-5 activated human eosinophils by an unknown ligand^{23, 26, 27}. The inhibitory effect of Gal-9 on IL-13 and Df-IgE production, and Gal-9 induction of TGF- β 1 were weaker at a high dose (3 μ M) than at low (0.3 μ M) and medium (1 μ M) doses. The effects of Gal-9 on inflammatory cells may differ depending on the Gal-9 concentration by acting on a different ligand.

Treg can be depleted by treatment with PC61 anti-CD25mAb¹⁸. To confirm the Treg-dependent inhibitory effects of Gal-9 in this SLIT model, Treg were depleted with PC61 pretreatment during SLIT as described in the Methods. As previously described¹⁸, during SLIT for 2 weeks, no CD4+CD25+Foxp3+ Treg were observed in the spleens of PC61-treated mice, whereas CD4+CD25+Foxp3+ Treg were observed in the spleens of Rat IgG-treated mice (data not shown). We used a low dose of Gal-9 to evaluate the contribution of Treg in the SLIT model, because a low dose of Gal-9 is more effective to induce TGF- β 1 in the BALF than a high dose. The increase TGF- β 1 levels in the BALF of Gal-9 SLIT mice was diminished by PC61 pretreatment. The inhibitory effects of Gal-9 on EAR and AHR, and eosinophilic airway inflammation in this SLIT model were also diminished by PC61 pretreatment compared with Rat IgG pretreatment. Finally, the inhibitory effects of Gal-9 on the production of Df-specific IgE in the serum were abolished by PC61-pretreatment. PC61 treatment may deplete not only CD4+CD25+Treg, but also CD4+CD25+ activated T cells. If the CD4+CD25+ activated T cells were mainly depleted by PC61 treatment, it would indicate that the Th2-mediated eosinophilic airway inflammation was decreased. The Th2 mediated eosinophilic airway inflammation, however, was increased by PC61 treatment. In this model, the effects of Treg depletion were mainly observed, even if activated T cells were also depleted. Further, PC61 treatment by itself and SLIT with Gal-9 by itself had no effect on asthmatic responses in this SLIT model. These data suggest that the inhibitory effects of Gal-

9 on asthmatic responses are Treg and Df allergen-dependent. Further studies are required to clarify the mechanisms of inhibitory effects of Gal-9 including TGF- β 1 production in detail.

In conclusion, Gal-9 inhibited not only Th2-related eosinophilic airway inflammation, but also allergen-induced EAR and allergen-specific IgE production in this SLIT model. SLIT using Gal-9 as an adjuvant might cure asthma more effectively than SLIT without Gal-9, and Gal-9 is an adjuvant that might shorten the therapy period and reduce the amount of allergen required in the SLIT.

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References

1. Busse WW, Lemanske RF Jr. Asthma. *N Engl J Med* 2001; **344**: 350-62.
2. Bousquet J, Mantzouranis E, Cruz AA, Aït-Khaled N, Baena-Cagnani CE, Bleecker ER, et al. Uniform definition of asthma severity, control, and exacerbations: document presented for the World Health Organization Consultation on Severe Asthma. *J Allergy Clin Immunol* 2010; **126**: 926-38.
3. Lambrecht BN, Hammad H. The immunology of asthma. *Nat Immunol* 2015; **16**: 45-56.
4. Moreno C, Cuesta-Herranz J, Fernandez-Tavora L, Alvarez-Cuesta E, Immunotherapy Committee, Sociedad Española de Alergología e Inmunología Clínica. Immunotherapy safety: a prospective multi-centric monitoring study of biologically standardized therapeutic vaccines for allergic diseases. *Clin Exp Allergy* 2004; **34**: 527-31.
5. Moingeon P, Batard T, Fadel R, Frati F, Sieber J, Van Overtvelt L. Immune mechanisms of allergen-specific sublingual immunotherapy. *Allergy* 2006; **61**: 151-65.
6. Frew AJ. Sublingual immunotherapy. *N Engl J Med* 2008; **358**: 2259-64.
7. Hirashima M, Kashio Y, Nishi N, Yamauchi A, Imaizumi TA, Kageshita T, et al. Galectin-9 in physiological and pathological conditions. *Glycoconj J* 2004; **19**: 593-600.
8. Seki M, Oomizu S, Sakata KM, Sakata A, Arikawa T, Watanabe K, et al. Galectin-9 suppresses the generation of Th17, promotes the induction of regulatory T cells, and regulates experimental autoimmune arthritis. *Clin Immunol* 2008; **127**: 78-88.

9. Arikawa T, Saita N, Oomizu S, Ueno M, Matsukawa A, Katoh S, et al. Galectin-9 expands immunosuppressive macrophages to ameliorate T-cell-mediated lung inflammation. *Eur J Immunol* 2010; **40**: 548-58.
10. Oomizu S, Arikawa T, Niki T, Kadowaki T, Ueno M, Nishi N, et al. Cellsurface galectin-9 expressing Th cells regulate Th17 and Foxp3⁺ Treg development by Galectin-9 secretion. *PLoS ONE* 2012; **7**: e48574.
11. Wu C, Thalhamer T, Franca RF, Xiao S, Wang C, Hotta C, et al. Galectin-9-CD44 interaction enhances stability and function of adaptive regulatory T cells. *Immunity* 2014; **41**: 270-82.
12. Ikeda M, Katoh S, Shimizu H, Oka M. Development of two types of mite-allergen induced murine model of chronic asthma with different severity. *Kawasaki Med J* 2016; **42**: 1-7.
13. Nishi N, Itoh A, Fujiyama A, Yoshida N, Araya S, Hirashima M, et al. Development of highly stable galectins: truncation of the linker peptide confers protease-resistance on tandem-repeat type galectins. *FEBS Lett* 2005; **579**: 2058-64.
14. Katoh S, Kaminuma O, Hiroi T, Mori A, Ohtomo T, Maeda S, et al. CD44 is critical for airway accumulation of antigen-specific Th2, but not Th1, cells induced by antigen challenge in mice. *Eur J Immunol* 2011; **41**: 3198-207.
15. Yu H-Q, Li X-H, Guo H, Liu Z-G, Ran P-X, Ji K-M, et al. Sublingual immunotherapy efficacy of *Dermatophagoides farinae* vaccine in a murine asthma model. *Int Arch Allergy Immunol* 2010; **152**: 41-8.
16. Hagner S, Rask C, Brimnes J, Andersen PS, Raifer H, Renz H, et al. House dust mite-specific sublingual immunotherapy prevents the development of allergic inflammation in a mouse model of experimental asthma. *Int Arch Allergy Immunol* 2016; **170**: 22-34.

17. Shima K, Koya T, Tsukioka K, Sakagami T, Hasegawa T, Fukano C, et al. Effects of sublingual immunotherapy in a murine asthma model sensitized by intranasal administration of house dust mite extracts. *Allergol Int.*(in press).
18. Setiady YY, Coccia JA, Park PU. In vivo depletion of CD4+FOXP3+ Treg cells by the PC61 anti-CD25 monoclonal antibody is mediated by FcγRIII+ phagocytes. *Eur J Immunol* 2010; **40**: 780-6.
19. Durham SR, Penagos M. Sublingual or subcutaneous immunotherapy for allergic rhinitis? *J Allergy Clin Immunol* 2016; **137**: 339-49.
20. Bergmann KC, Demoly P, Worm M, Fokkens WJ, Carrillo T, Tabar AI, et al. Efficacy and safety of sublingual tablets of house dust mite allergen extracts in adults with allergic rhinitis. *J Allergy Clin Immunol* 2014; **133**: 1608-14.
21. Nolte H, Maloney J, Nelson HS, Bernstein DI, Lu S, Li Z, et al. Onset and dose-related efficacy of house dust mite sublingual immunotherapy tables in an environmental exposure chamber. *J Allergy Clin Immunol* 2015; **135**: 1494-501.
22. Burks AW, Calderon MA, Casale T, Cox L, Demoly P, Jutel M, et al. Update on allergy immunotherapy: American Academy of Allergy, Asthma & Immunology/European Academy of Allergy and Clinical Immunology/PRACTALL consensus report. *J Allergy Clin Immunol* 2013; **131**: 1288-96.e3.
23. Katoh S, Nobumoto A, Matsumoto N, Matsumoto K, Ehara N, Niki T, et al. Involvement of galectin-9 in lung eosinophilia in patients with eosinophilic pneumonia. *Int Arch Allergy Immunol* 2010; **153**: 294-301.
24. Katoh S, Oomizu S, Niki T, Shimizu H, Obase Y, Korenaga M, et al. Possible regulatory role of galectin-9 on *Ascaris suum*-induced eosinophilic lung inflammation in mice. *Int Arch Allergy Immunol* 2012; **158 (suppl 1)**: 58-65.

25. Miyake K, Underhill CB, Lesley J, Kincade PW. Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. *J Exp Med* 1990; **172**: 69-75.
26. Katoh S, Ishii N, Nobumoto A, Takeshita K, Dai SY, Shinonaga R, et al. Galectin-9 inhibits CD44-hyaluronan interaction and suppresses a murine model of allergic asthma. *Am J Respir Crit Care Med* 2007; **176**: 27-35.
27. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, et al. The tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol* 2005; **6**: 1245-52.

Figure legends

Fig. 1. Animal model and sublingual immunotherapy. BALB/c mice were intranasally sensitized with *Dermatophagoides farinae* (Df) extract (40 µl of 1 mg/ml) 5 days/week for 5 weeks. Negative control animals were intranasally exposed to PBS in a similar manner. Following intranasal administration of Df extract for 5 weeks, sublingual immunotherapy (SLIT) was performed 5 days/week, twice daily, for 2 weeks. Early asthmatic response (EAR) was evaluated on day 50 just after the last Df challenge. Airway hyperresponsiveness (AHR) and bronchoalveolar lavage (BAL) were evaluated on day 51, 24 h after the last Df (80 µl: 40 µl twice of 1 mg/ml) challenge.

Figure 2. Effects of Gal-9 on early asthmatic response (EAR) and airway hyperresponsiveness (AHR) in mite allergen-specific SLIT. EAR was evaluated and expressed as airway resistance change from PBS exposure (%) as described in the Methods. Airway hyperresponsiveness (AHR) was measured and expressed as PC₂₀₀ sRaw (mg/ml). Data represent means \pm SEM. Values shown are means from 12 mice per group. These results are typical of those obtained in three independent experiments. The Kruskal-Wallis test was used to compare values of different groups followed by Dunn's multiple comparisons test. * $p < 0.05$ compared with Df-sensitization (-), SLIT: Df (-) Gal-9 (-), and Df-challenge (-) group. # $p < 0.05$ compared with Df-sensitization (+), SLIT: Df (-) Gal-9 (-), and Df-challenge (+) group.

Figure 3. Effects of Gal-9 on airway inflammation and remodeling in mite allergen-specific SLIT. Absolute numbers of inflammatory cells in the bronchoalveolar fluid (BALF) were estimated (A). Data represent means \pm SEM. Values shown are means from 12 mice per

group. These results are typical of those obtained in three independent experiments. (B). Histologic examination of formalin-fixed lung sections stained with hematoxylin and eosin (original magnification: upper panels, $\times 100$; lower panels, $\times 1000$). Arrows indicate eosinophils. These results are typical of those obtained in three independent experiments. Mucus score was determined as described in the Methods. A total of 10 airways of three mice from each group were analyzed (C). The Kruskal-Wallis test was used to compare values of different groups followed by Dunn's multiple comparisons test. * $p < 0.05$ compared with Df-sensitization (-), SLIT: Df (-) Gal-9 (-), and Df-challenge (-) group. # $p < 0.05$ compared with Df-sensitization (+), SLIT: Df (-) Gal-9 (-), and Df-challenge (+) group.

Figure 4. Effects of Gal-9 on Th2 cytokine levels in the BALF and serum immunoglobulin levels in the SLIT model. IL-5 and IL-13 levels in the BALF (A), and Df-specific IgE, IgG1, and IgG2a titers in the serum (B) were determined by ELISA. The detection limit was 7.0 pg/mL for IL-5 and 3.9 pg/mL for IL-13. Concentrations below the detection limits were assumed to be zero for statistical analysis. Data represent means \pm SEM. Values shown are means from 12 mice per group. These results are typical of those obtained in three independent experiments. The Kruskal-Wallis test was used to compare values of different groups followed by Dunn's multiple comparisons test. * $p < 0.05$ compared with Df-sensitization (-), SLIT: Df (-) Gal-9 (-), and Df-challenge (-) group. # $p < 0.05$ compared with Df-sensitization (+), SLIT: Df (-) Gal-9 (-), and Df-challenge (+) group.

Figure 5. Effects of Gal-9 on Treg in the BALF in the SLIT model. TGF- β 1 concentration and CD4⁺CD25⁺Foxp3^{high} cell number in the BALF were determined by ELISA and flowcytometry, respectively. The detection limit was 4.9 pg/mL for TGF- β 1. Concentrations

below the detection limits were assumed to be zero for statistical analysis. Data represent means \pm SEM. Values shown are means from 12 mice per group. These results are typical of those obtained in three independent experiments. The Kruskal-Wallis test was used to compare values of different groups followed by Dunn's multiple comparisons test. * $p < 0.05$ compared with Df-sensitization (-), SLIT: Df (-) Gal-9 (-), and Df-challenge (-) group. # $p < 0.05$ compared with Df-sensitization (+), SLIT: Df (-) Gal-9 (-), and Df-challenge (+) group.

Figure 6. Efficacy of Gal-9 on the SLIT model was blocked by Treg depletion. Early asthmatic response (EAR) and airway hyperresponsiveness (AHR) were evaluated (A). Number of eosinophils and concentration of IL-5, and IL-13 in the BALF and Df-specific IgE levels in the serum were determined (B). Concentration of TGF- β 1 in the BALF was determined (C). Data represent means \pm SEM. Values shown are means from 8 mice per group. These results are typical of those obtained in three independent experiments. Between two-group comparisons were examined by Mann-Whitney U test.

Fig. 1

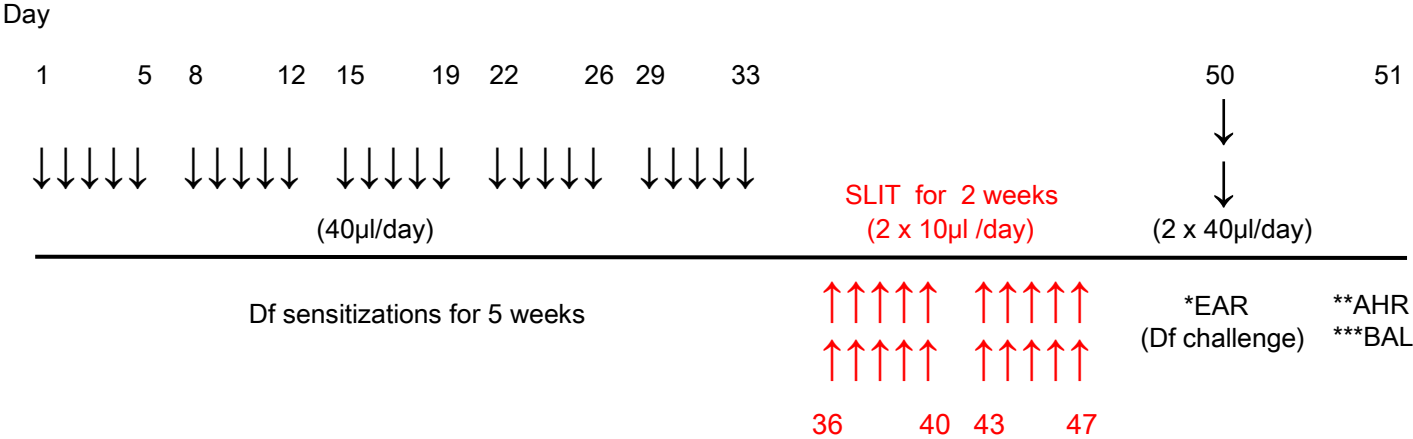


Fig. 2

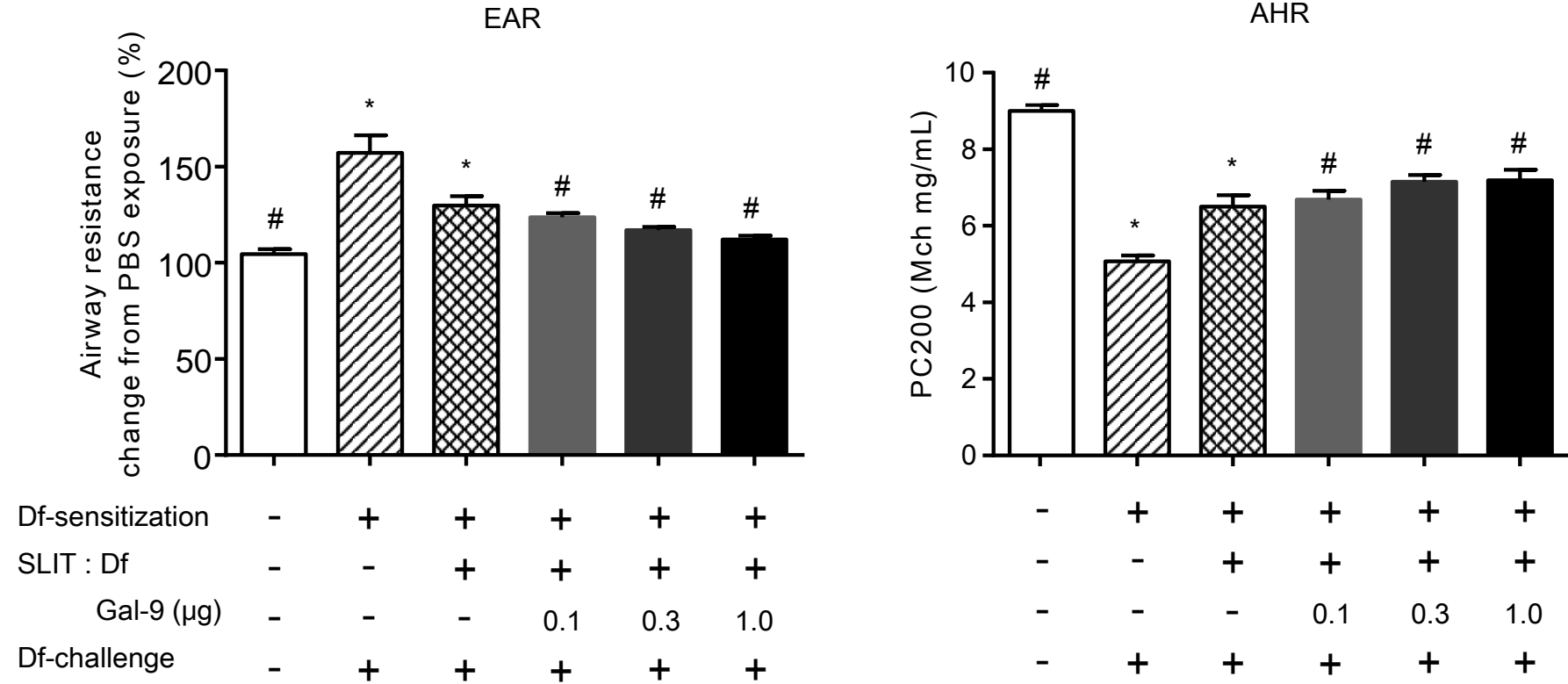


Fig. 3A

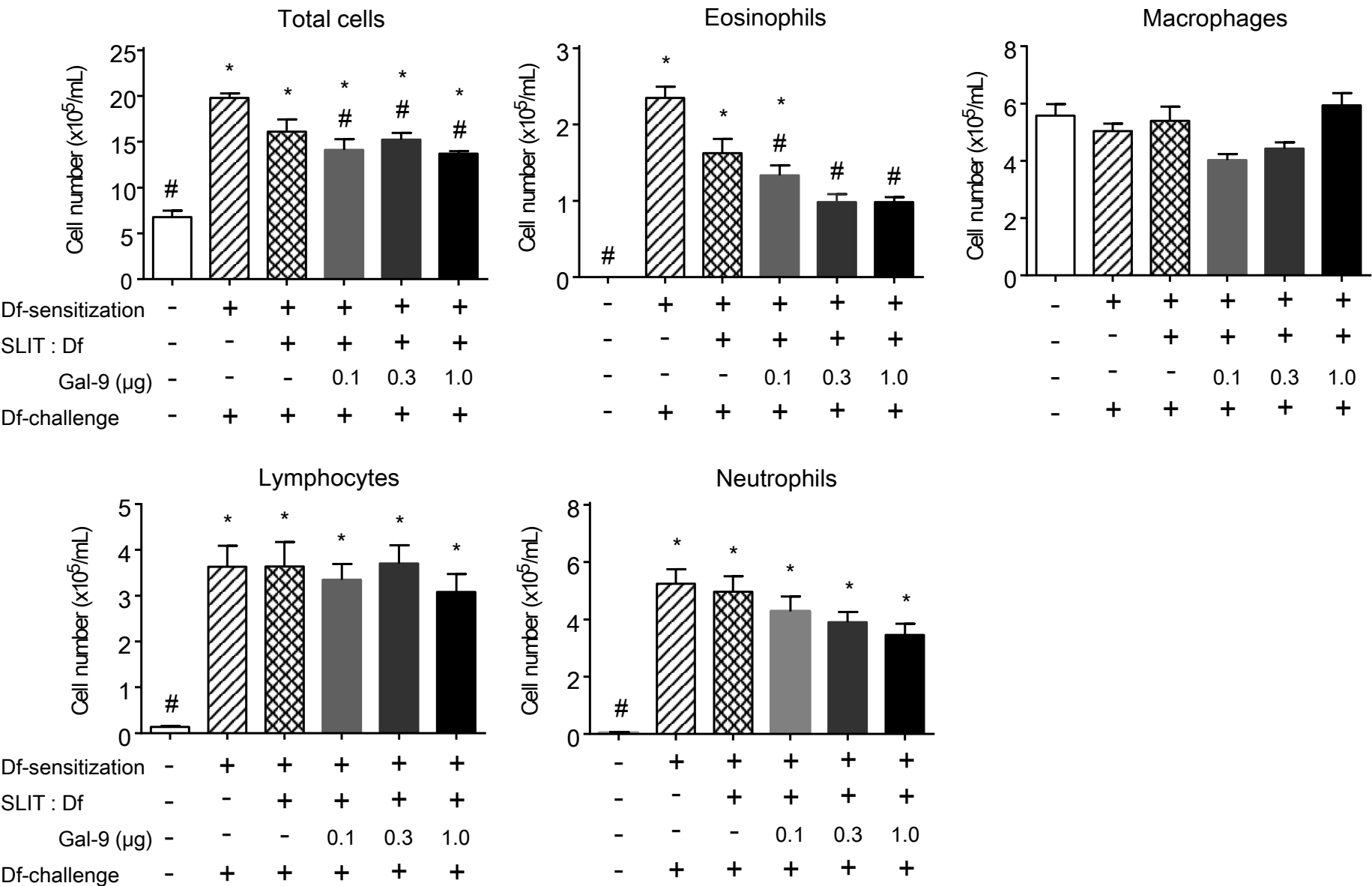
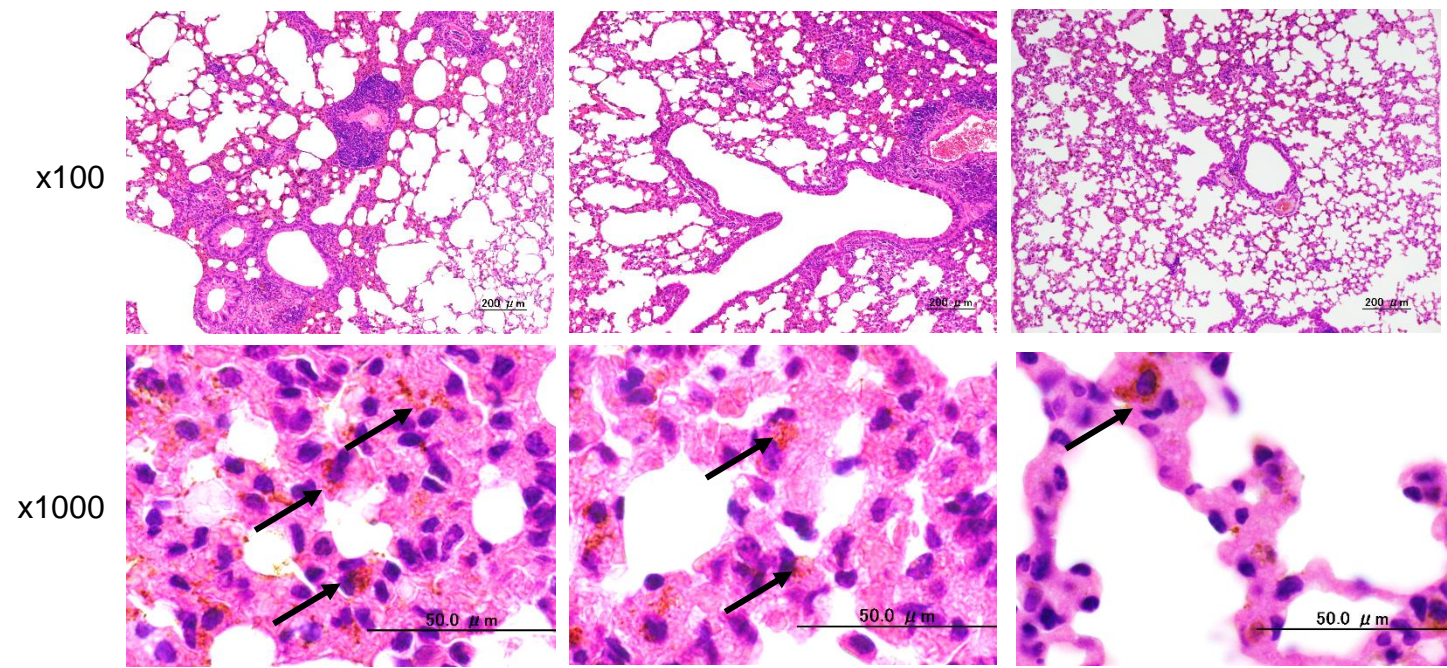


Fig. 3B



Df-sensitization	+	+	+
SLIT : Df	-	+	+
Gal-9 (µg)	-	-	0.1
Df-challenge	+	+	+

Fig. 3C

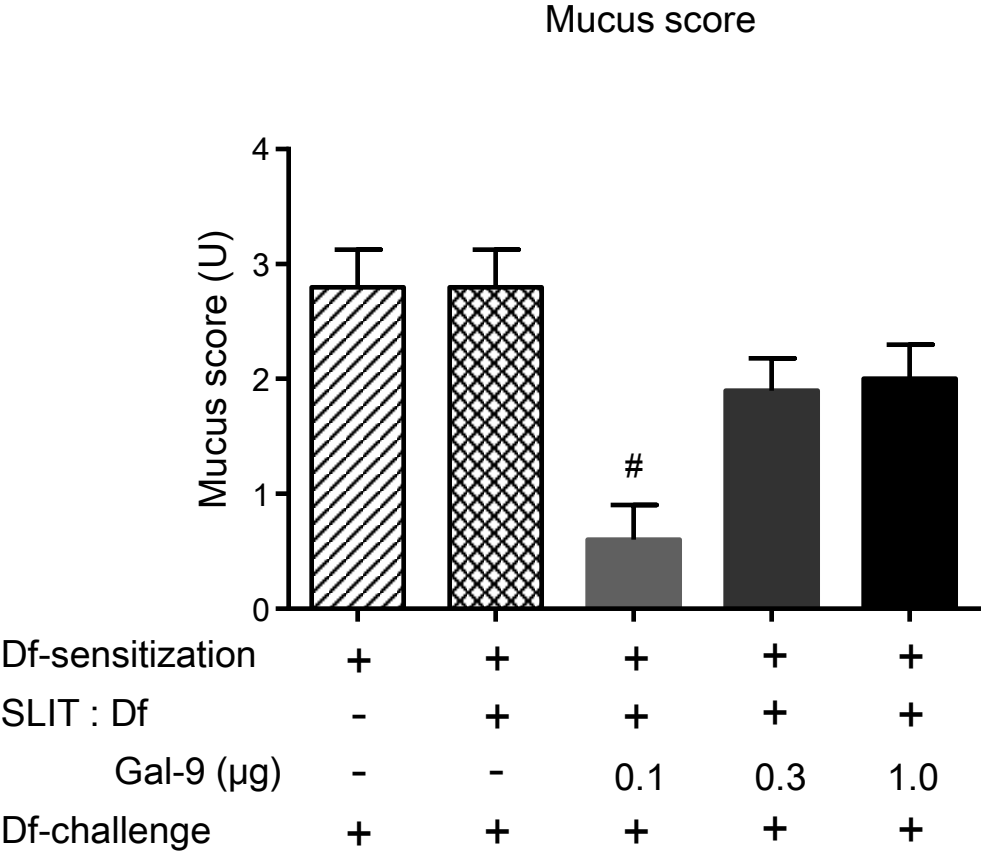


Fig. 4A

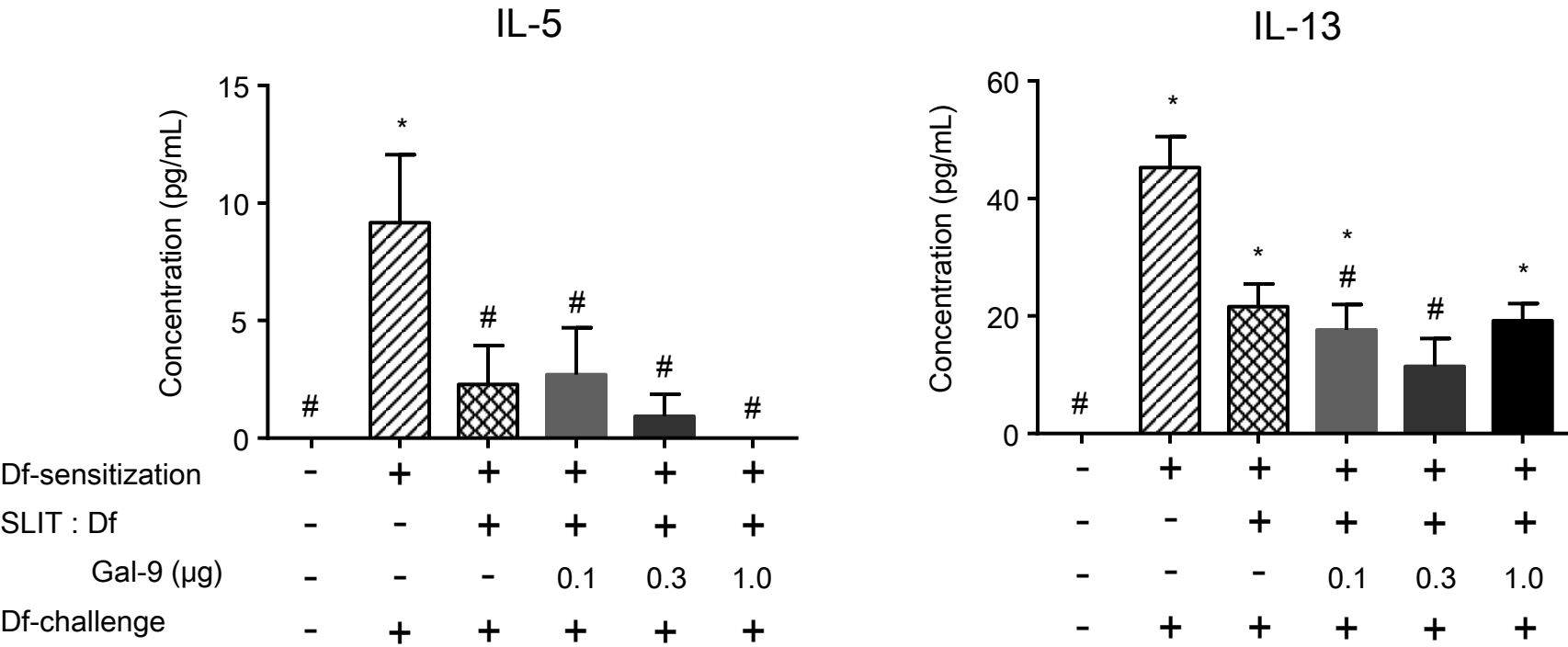


Fig. 4B

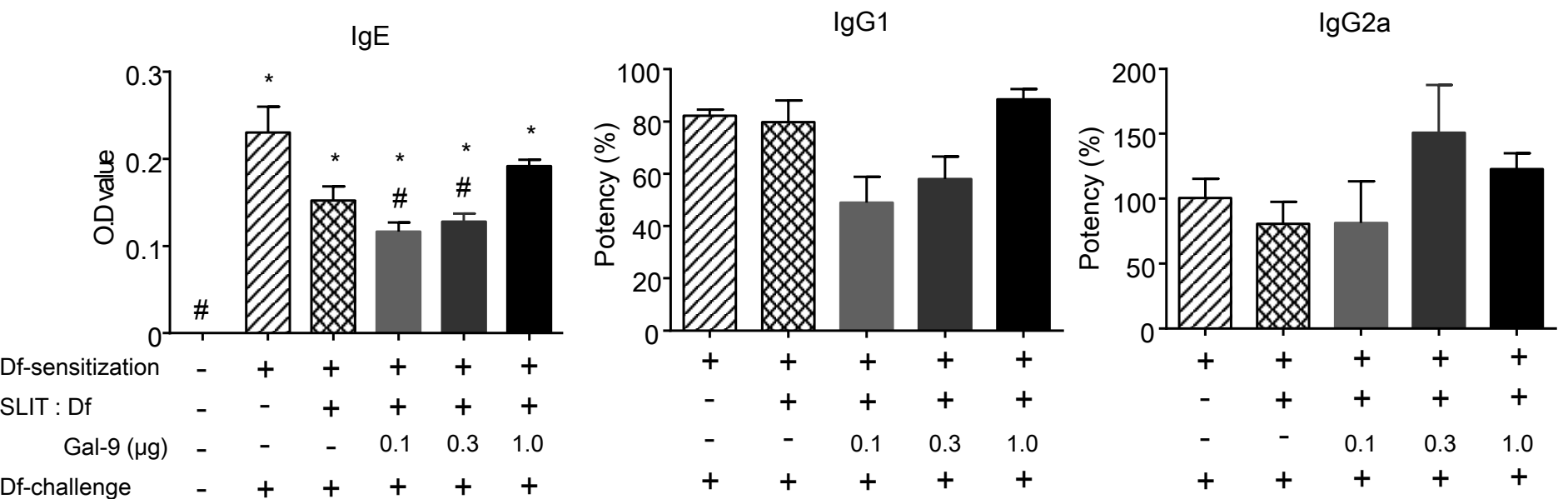


Fig. 5

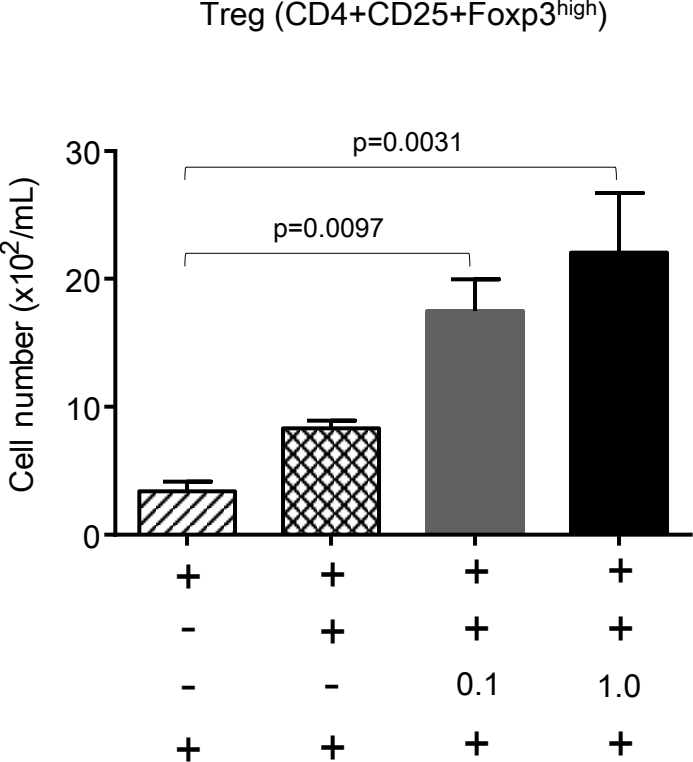
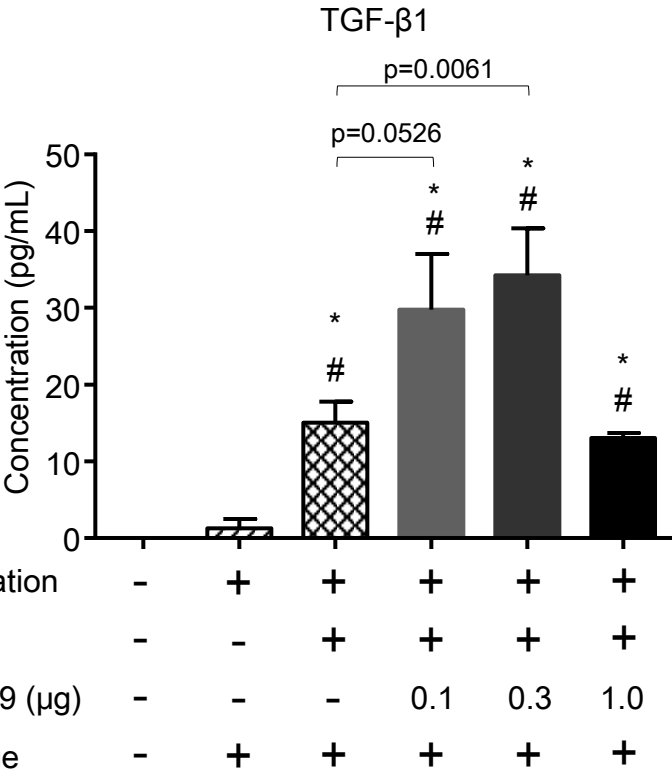


Fig. 6A

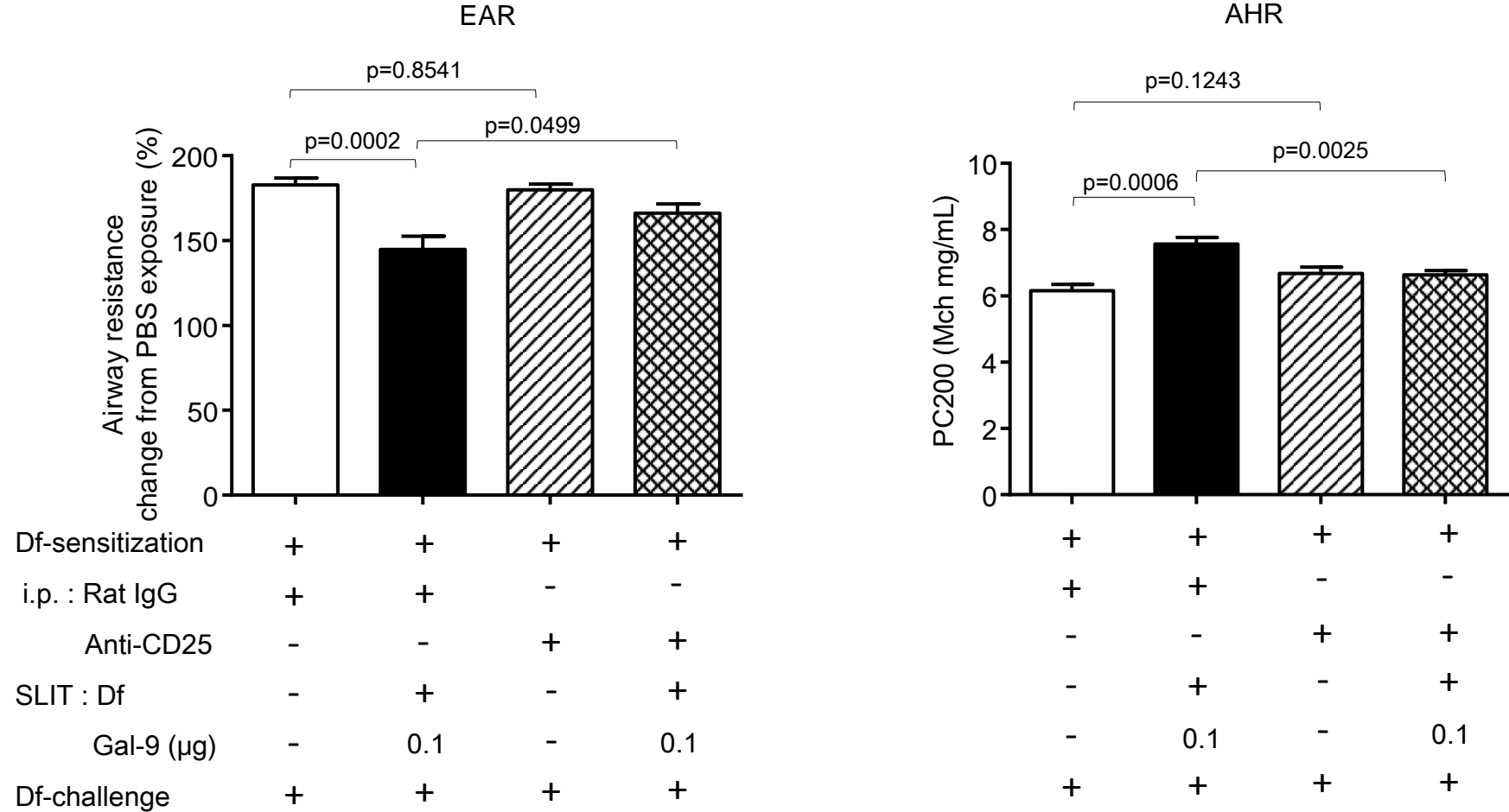


Fig. 6A

Fig. 6B

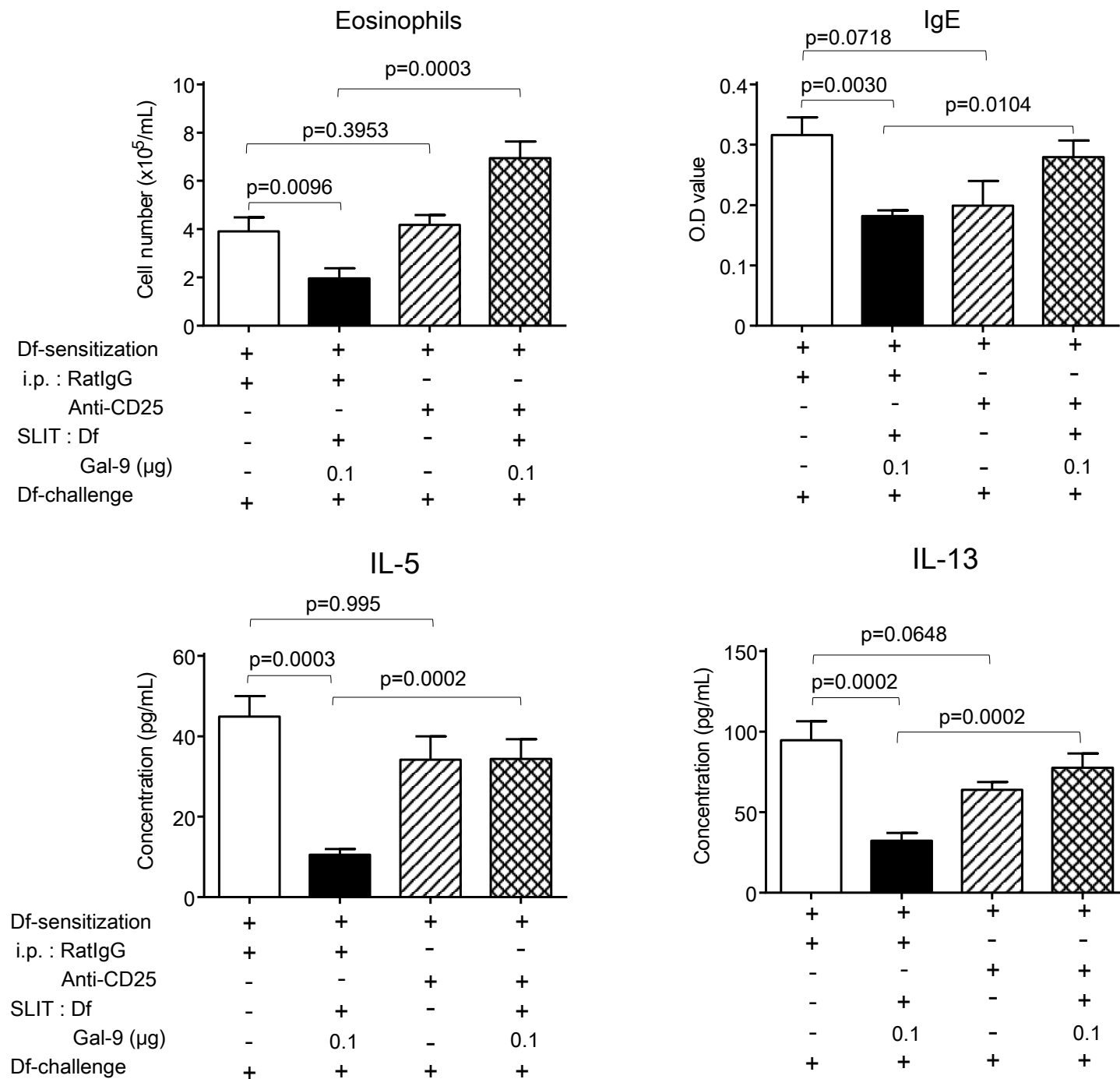
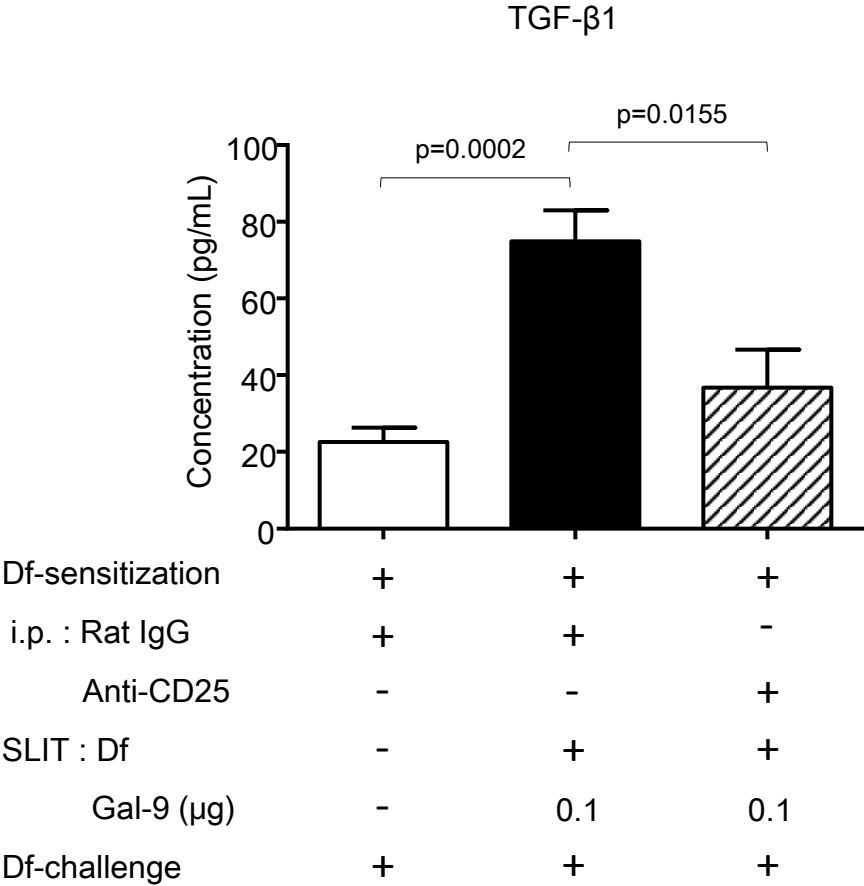


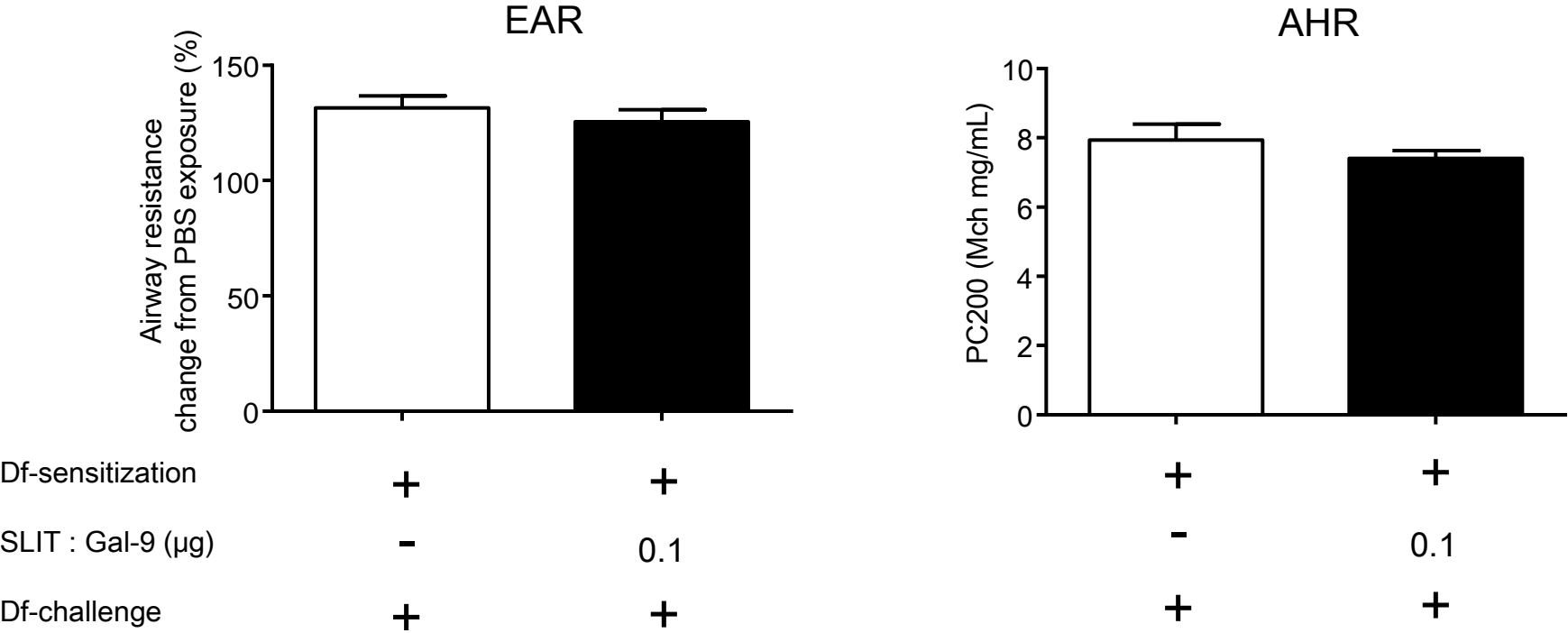
Fig. 6C



Supplementary figure legends

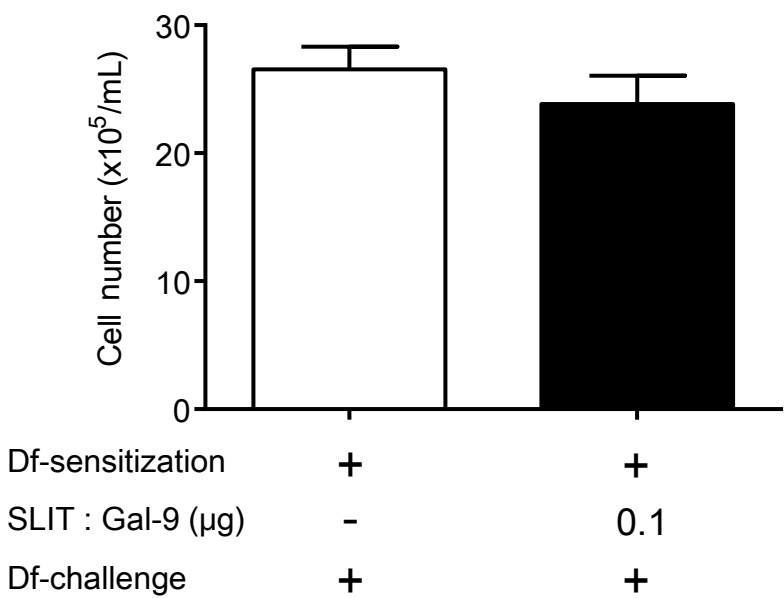
Supplementary Fig. 1 Effects of sublingually administered Gal-9 on asthmatic responses, eosinophilic airway inflammation, and serum allergen-specific IgE in mouse model of chronic asthma. Early asthmatic response (EAR) was evaluated and expressed as airway resistance change from PBS exposure (%). Airway hyperresponsiveness (AHR) was measured and expressed as PC200 sRaw (mg/ml) (A). Absolute numbers of inflammatory cells in the bronchoalveolar fluid (BALF) were estimated (B). IL-5 and IL-13 levels in the BALF, and Df-specific IgE titers in the serum were determined by ELISA. The detection limits were 7.0 pg/mL for IL-5 and 3.9 pg/mL for IL-13. Concentrations below the detection limits were assumed to be zero for statistical analysis (C). Data represent means \pm SEM. Values shown are means from 9 mice per group. Comparisons between two groups were examined by Mann-Whitney U test.

Supplementary Fig. 1A

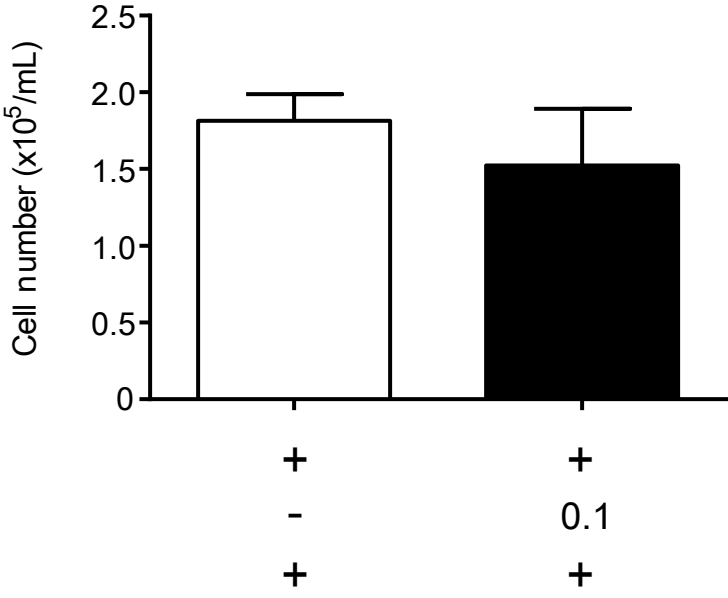


Supplementary Fig. 1B

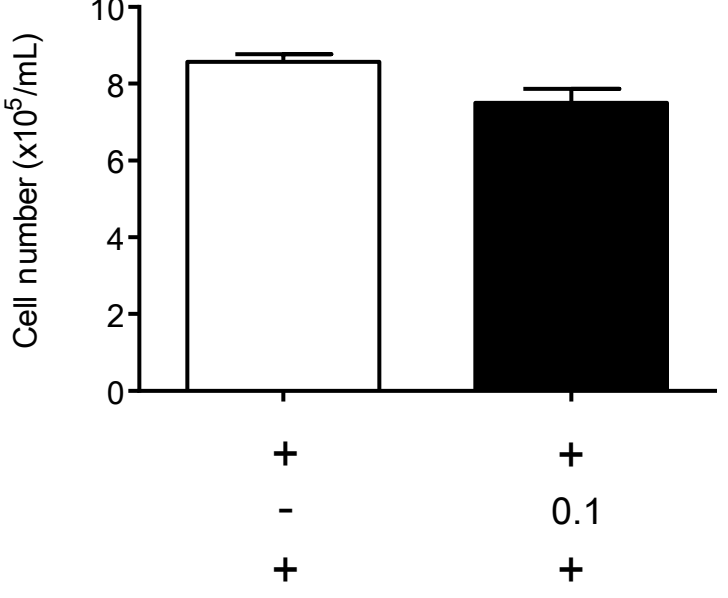
Total cells



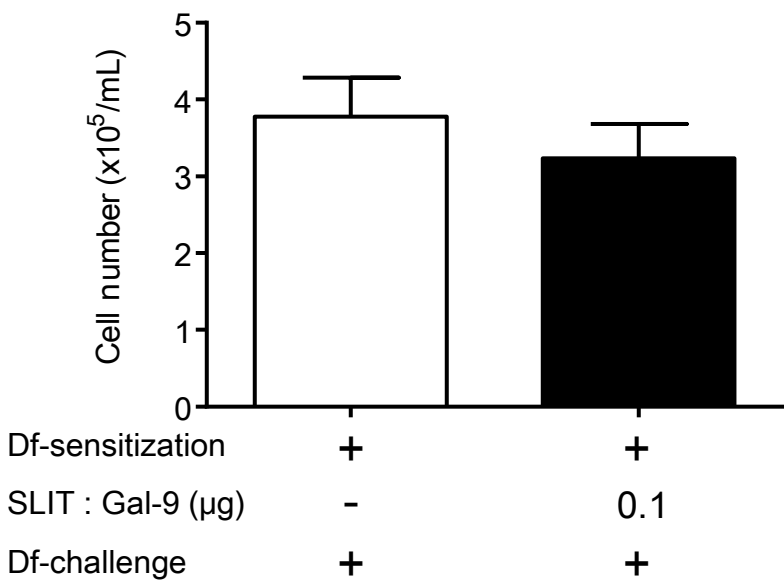
Eosinophils



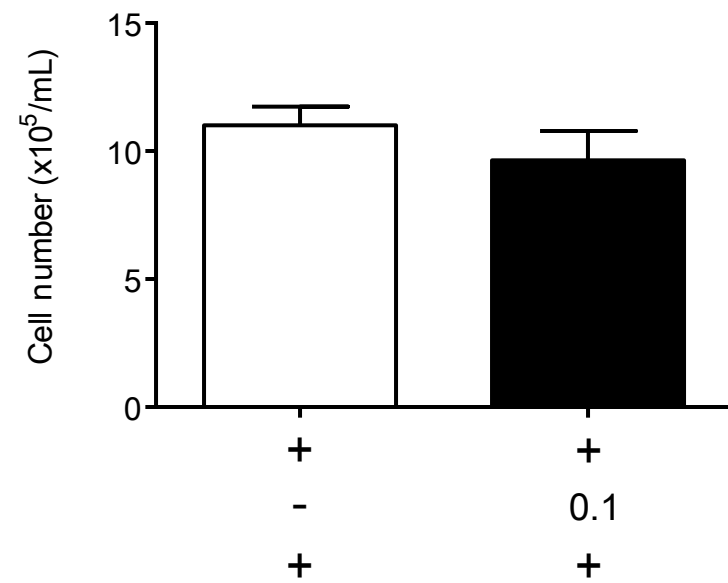
Macrophages



Lymphocytes



Neutrophils



Supplementary Fig. 1C

