



Research Article

CD44 is critical for the enhancing effect of hyaluronan in allergen-specific sublingual immunotherapy in a murine model of chronic asthma

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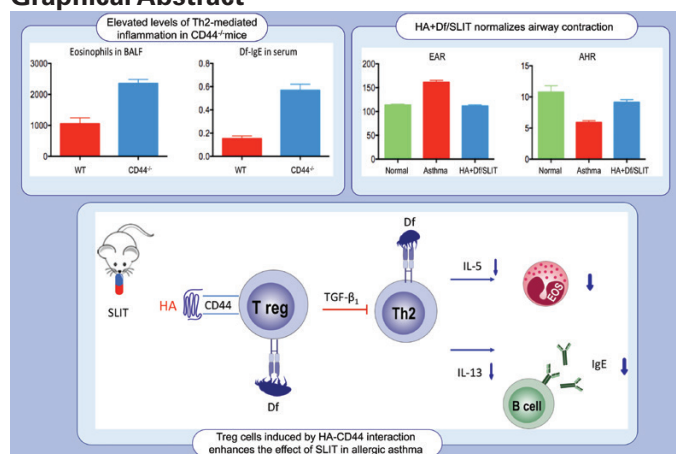
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Abstract

Allergen-specific sublingual immunotherapy (SLIT) is a potentially effective disease-modification treatment for patients with allergic asthma. Because CD44 signaling enhances regulatory T (Treg) cell-induction, administering CD44 ligands such as hyaluronan (HA) with allergen-specific SLIT may enhance the therapeutic effects. We evaluated the role of CD44 in Treg cell-induction in T helper type 2 (Th2)-mediated chronic airway inflammation using CD44^{-/-} mice and the efficacy of HA on SLIT in a *Dermatophagoides farinae* (Df)-induced murine model of chronic asthma. Th2 responses and Treg cell induction were evaluated in CD44^{-/-} mice. We devised a new SLIT model of Df-induced chronic asthma utilizing HA as an adjuvant. The effects of HA added to the new SLIT model were evaluated by the early asthmatic response (EAR) and airway hyperresponsiveness (AHR), eosinophilic airway inflammation, and serum Df-specific IgE levels. Th2-mediated chronic eosinophilic airway inflammation was worse in CD44^{-/-} mice compared with Df-sensitized wild-type (WT) mice. HA enhanced the effect of Df-induced Treg cells in a CD44-dependent manner. Sublingual Df treatment in combination with HA, but not alone, normalized EAR and AHR, and significantly reduced the serum IgE levels and the bronchoalveolar lavage fluid (BALF) eosinophil number. HA also induced Treg cells in a Df-sensitized spleen cell culture in a CD44-dependent manner. The treatment-enhancing effects of HA in this SLIT model were diminished in CD44^{-/-} mice. CD44 is a key contributor to Treg cell induction and critical for the enhancing effects of HA in a Df-induced murine model of chronic asthma.

Graphical Abstract



Th2-mediated chronic eosinophilic airway inflammation and serum levels of Df-specific IgE were increased in CD44^{-/-} mice. Sublingual Df treatment in the presence of HA normalized both EAR (early asthmatic response) and AHR (airway hyperresponsiveness). HA enhanced the effects of SLIT in a Df-induced mouse model of chronic asthma in a CD44-dependent manner.

Keywords: CD44, chronic asthma, hyaluronan, mouse, sublingual immunotherapy

Abbreviations: AHR: airway hyperresponsiveness; BALF: bronchoalveolar lavage fluid; Df: *Dermatophagoides farinae*; EAR: early asthmatic response; HA: hyaluronan; SLIT: sublingual immunotherapy; Treg: regulatory T.

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Introduction

Asthma is a common chronic allergic pulmonary disorder presenting as reversible airway obstruction, airway hyperresponsiveness (AHR), and eosinophilic airway inflammation. Approximately half of all asthma patients are sensitized to inhaled allergens, including house dust mites, as demonstrated by the presence of allergen-specific immunoglobulin E (IgE) in the serum. Inhalation drug therapy with corticosteroids, long-acting β -agonists, and leukotriene receptor antagonists, as well as the administration of biologics, either alone or in combination, provides generally good control for most asthma patients [1–4]. While these drugs effectively improve chronic airway inflammation, they cannot modify the disease over the long term, and chronic administration is often required. Allergen-specific immunotherapy provides a potential cure, but improvements to the efficacy, side effects, and patient adherence to the therapy are still needed [5, 6]. Over the last decade, allergen-specific sublingual immunotherapy (SLIT) has become the preferred treatment for patients suffering from allergic rhinitis and asthma induced by mite allergens [7, 8].

CD44, a cell adhesion molecule whose main ligand is hyaluronan (HA), is involved in several physiologic processes, including hemopoiesis, homing to mucosal lymphoid tissue, and lymphocyte infiltration into inflamed tissues [9]. We previously revealed that T helper type 2 (Th2)-mediated airway inflammation is critically mediated by interactions between CD44 and HA in a murine model of acute asthma using anti-CD44 monoclonal antibodies and CD44^{-/-} mice [10, 11]. We also found that galectin-9 (Gal-9) binds CD44 directly, thereby inhibiting the interactions between CD44 and HA [12]. We further revealed, both *in vivo* and *in vitro*, that Gal-9 induces Treg cells [13–15]. Wu *et al.* demonstrated that the Gal-9/CD44 interaction enhances the function of regulatory T (Treg) cells through transforming growth factor (TGF)- β -dependent mechanisms [16].

Because Treg cells are thought to contribute to the efficacy of SLIT [17], CD44 ligands may enhance the effect of allergen-specific SLIT in allergic asthma. Using a murine model of *Dermatophagoides farinae* (Df) antigen-induced chronic asthma, we recently found that Gal-9 enhances the therapeutic effects of SLIT in a Treg cell-dependent manner [18]. The details underlying how CD44 contributes to the development of chronic eosinophilic airway inflammation, however, remain unclear.

In the present study, we evaluated how CD44 contributes to the induction of Treg cells in Th2-mediated chronic airway inflammation using CD44^{-/-} mice and a Df-induced murine model of chronic asthma. Here, we describe a new SLIT model of Df-induced chronic asthma using HA as an adjuvant to the Df antigen. Enhanced effects of HA in the new SLIT model were examined by assessing the early asthmatic response (EAR) and AHR, eosinophilic airway inflammation, and serum levels of antigen-specific IgE. Finally, using CD44^{-/-} mice, we investigated the role of CD44 for the enhancing effects of HA in the new SLIT.

Methods

Murine model and sublingual immunotherapy

Female BALB/c and C57BL/6 mice (8–12 weeks of age) were obtained from Charles River Laboratory (Yokohama, Japan) and used in this study. CD44^{-/-} mice on a C57BL/6

background were bred in the experimental animal center of Kawasaki Medical School [10]. To produce a murine model of chronic eosinophilic airway inflammation, we transnasally administered a Df extract (40 μ g, 5 days/week for 5 weeks; Torii, Co. Ltd, Tokyo, Japan) to mice on a C57BL/6 background (Fig. 1a). As a negative control, mice were transnasally administered phosphate-buffered saline (PBS) using the same schedule. To produce a murine model of chronic asthma, we transnasally administered a Df extract (40 μ g, 5 days/week for 3 weeks) to mice on a BALB/c background, and performed SLIT 5 days/week, for 2 weeks (Fig. 1b). The SLIT agents were PBS, Df-antigen extract (125 μ g/day, Torii, Co. Ltd, Tokyo, Japan), or Df-antigen extract with HA (100 μ g/day, mean molecular weight: 4×10^4 Da, Lifecore Biomedical Co. Ltd, MN, USA). As previously reported [18], 10 μ l of the SLIT agent was administered to an anesthetized mouse. The Df and HA were confirmed to have minimal contamination by endotoxin (<0.25 EU/ml). For SLIT, the highest concentration of HA that can be used is 100 μ g/10 μ l owing to the high viscosity of the HA solution. In the preliminary experiment, we determined the effective concentration of HA for this SLIT model and found that 100 μ g/day of HA was more effective than 10 μ g/day of HA for decreasing the numbers of eosinophils in the BALF and the Df-specific IgE titers in the serum (data not shown). The Institutional Animal Care and Use Committee of the Kawasaki Medical School provided approval for the experimental protocols used in the present study (20-026, 21-003). The authors confirm that their institutional guidelines adhere to the ARRIVE guidelines.

Physiological examination of murine asthma

To evaluate the EAR, we used a two-chambered, double-flow plethysmograph system (Pulmos; M.I.P.S, Osaka, Japan) as previously described [18]. The EAR was assessed for 5 min after administering the last dose of Df. To evaluate the AHR, mice underwent forced inhalation of PBS or acetyl- β -methylcholine chloride (3, 6, 12.5, or 25 mg/ml; Millipore Sigma, St. Louis, MO, USA) for 3 min at 24 h after administering the last dose of Df. Airway resistance was measured in awake mice using the 2-chambered, double-flow plethysmograph system, as described before [18]. AHR is expressed as the concentration of acetyl- β -methylcholine chloride required to increase sRaw 2-fold (PC200), similar to the PC20 measure used for patients with asthma.

Collection of blood, and BALF

The mice were killed by carbon dioxide inhalation before collection of blood and BALF, as described previously [18]. In brief, 24 h after administration of the last dose of the antigen, the lungs were washed 4 times with 1 ml of PBS, and the supernatant of the first BALF collected was stored at -80°C until analyzed. The BALF cell pellets were then resuspended in 1 ml PBS and cells were counted as previously described [18].

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assays (ELISA) were applied to determine the BALF concentrations of interleukin (IL)-5, IL-13, IL-17, interferon- γ (IFN- γ), and TGF- β_1 (R&D Systems, Minneapolis, MN, USA). The ELISA detection limits were as follows: 7.0 pg/ml for IL-5, 1.5 pg/ml for IL-13, 5.0 pg/ml for IL-17, 4.7 pg/ml for IFN- γ , and 4.9 pg/ml for TGF- β_1 . For statistical analysis, values that were under the

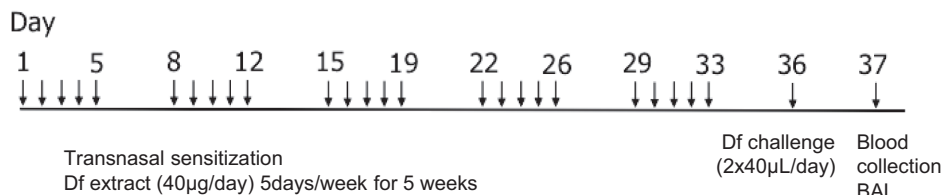
(a)

Mice : WT (C57BL/6)

CD44^{-/-}

Negative control : PBS

Chronic eosinophilic airway inflammation model : Df-extract (1mg/mL)



(b)

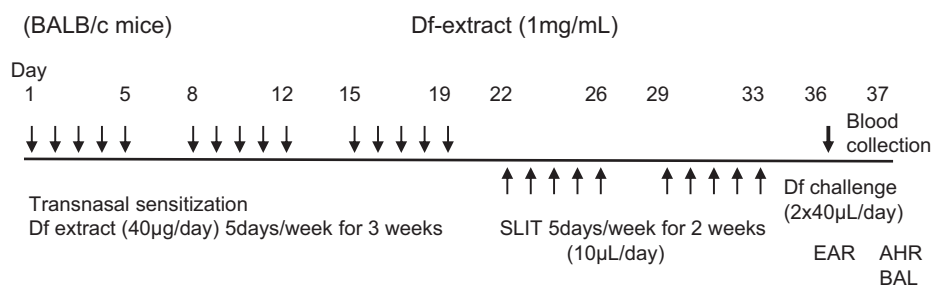


Figure 1: Murine disease models and sublingual immunotherapy. (a) To develop a chronic eosinophilic airway inflammation model, C57BL/6 or CD44^{-/-} mice were transnasally sensitized with Df extract (40 µg) 5 days a week for 5 weeks. Negative controls were transnasally administered phosphate buffered saline in a similar manner. The mice were then challenged by transnasal administration of 80 µg Df solution on Day 36. Blood was collected and then BAL was performed on Day 37. (b) To develop a murine model of chronic asthma, BALB/c mice were transnasally sensitized with Df extract (40 µg) 5 days a week for 3 weeks. Following transnasal sensitization, SLIT was performed 5 days a week, for 2 weeks. The EAR was examined on Day 36 just after the last Df administration. AHR and BAL were evaluated on Day 37, 24 h after the last administration of Df solution (80 µg).

detection limit were assigned a value of 0. ELISA was used to determine the serum levels of Df-specific IgE and IgG₁, as previously described [10]. The Df-specific serum IgE and IgG₁ levels were expressed as relative absorbance units (optical density at 450 nm).

Evaluation of Treg

Spleen cells from Df-sensitized mice were cultured with Df antigen (3 µg/ml) in the presence of HA (5 mg/ml) and anti-CD44 monoclonal antibodies (KM81), or isotype control, or HA alone for 24 h. Treg cells in both the BALF and cultured spleen cells were measured with a mouse Treg staining kit (eBioscience Inc, San Diego, CA, USA) using a FACSCalibur® flow cytometer (BD Biosciences, San Jose, CA, USA), as previously described [18].

Statistical analysis

The data are expressed as mean ± standard error (SEM). GraphPad Prism Software (version 6; San Diego, CA, USA) was used for all statistical analyses. For comparison of two groups, the Kruskal–Wallis test was used, followed by the Mann–Whitney *U* test or Dunn's multiple comparisons test. *P* < 0.05 was considered to indicate significance.

Results

Df-induced Th2-mediated chronic eosinophilic airway inflammation and Df-sensitization in CD44^{-/-} mice

To investigate the contribution of CD44 to chronic eosinophilic airway inflammation and Df-allergen sensitization, we evaluated Df-induced eosinophilic airway inflammation and Df-specific IgE levels in CD44^{-/-} mice compared with Df-specific IgE levels in C57BL/6 wild-type (WT) mice (Fig. 1a). Surprisingly, the total numbers of leukocytes and eosinophils, and the Th2 cytokine levels (IL-5 and IL-13) in the BALF were significantly increased in the Df-sensitized CD44^{-/-} mice compared with the Df-sensitized WT mice (*P* < 0.05, Fig. 2a and b). Further, Df-sensitized CD44^{-/-} mice had significantly higher serum titers of Df-specific IgE than Df-sensitized WT mice (*P* < 0.05, Fig. 2c).

Role of CD44 in the Df-induced Treg cells in Df-sensitized mice

CD44 contributes to the maintenance and activation of Foxp3+ Treg cells [16]. We evaluated Treg cells in the BALF to assess the mechanisms of the increase in the concentration of Th2 cytokines in the Df-sensitized CD44^{-/-} mice. The percentage of CD4+CD25+Foxp3+ Treg cells in the BALF was decreased in

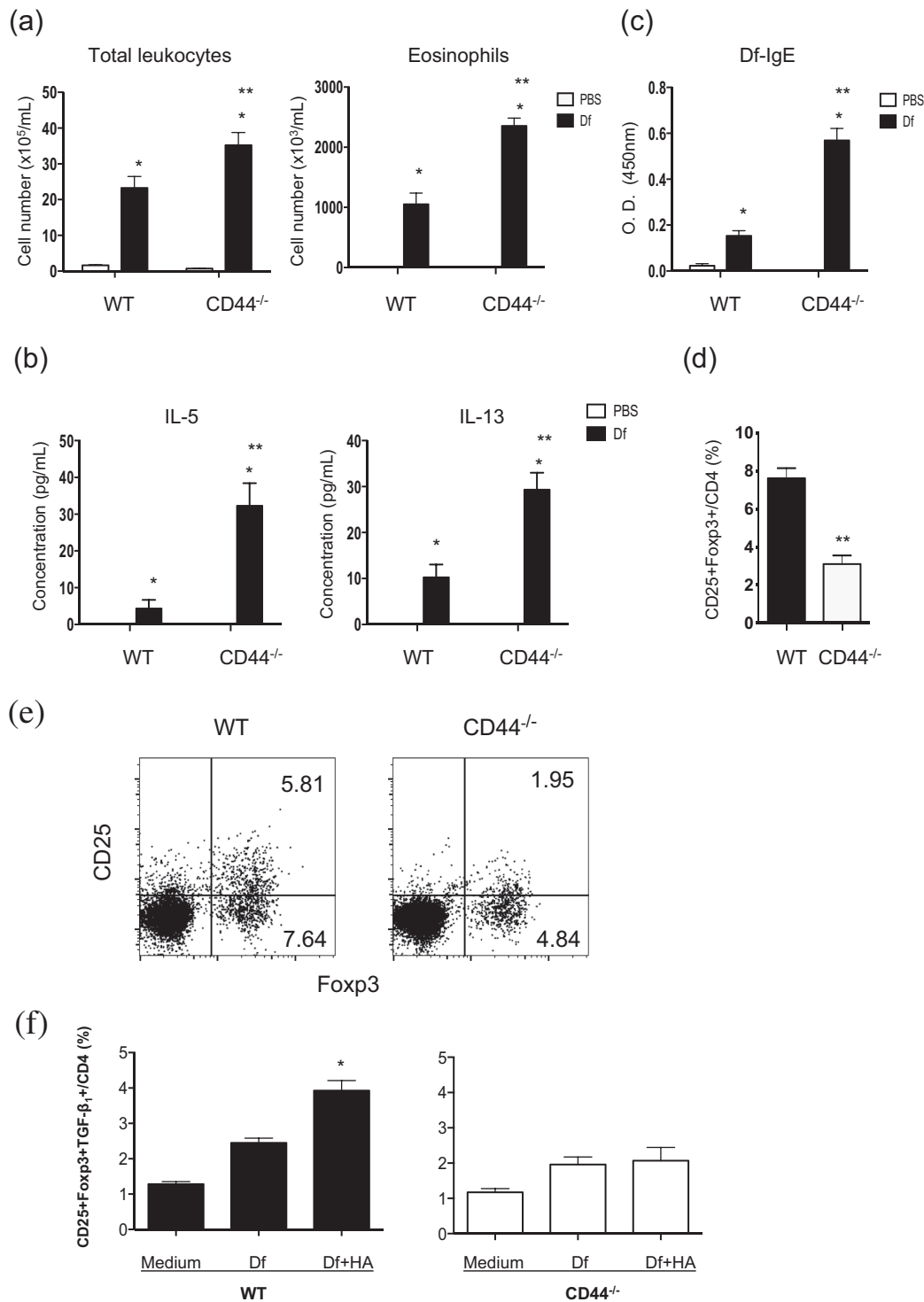


Figure 2: Characterization of Df-induced murine model of chronic airway inflammation in CD44^{-/-} mice. Absolute numbers of total leukocytes and eosinophils (a), and concentration of Th2 cytokines (IL-5 and IL-13) in the BALF were examined (b). Serum titers of Df-specific IgE were determined by enzyme-linked immunosorbent assay (c). Percentages of CD25+Foxp3+regulatory T cells in CD4 T cells in the BALF were estimated by flow cytometry (d, e). The measurement results are presented as the means ± SEM in eight mice. These results are typical of those obtained in three independent experiments. **P* < 0.05 compared with PBS, ***P* < 0.05 compared with WT mice. For comparison of two groups, the Kruskal–Wallis test, followed by the Mann–Whitney *U*-test, was used. Df sensitized spleen cells were cultured with Df-antigen in the presence or absence of HA for 24 h, and then induction of CD25+Foxp3+TGF-β₁+ Treg cells was estimated by flow cytometry in WT mice and CD44^{-/-} mice (f). **P* < 0.05 compared with medium alone. Measurement results are presented as the means ± SEM from three independent experiments. The Kruskal–Wallis test, followed by Dunn's multiple comparisons test, was used to compare values of different groups.

Df-sensitized CD44^{-/-} mice compared with Df-sensitized WT mice ($P < 0.05$, Fig. 2d and e). We stimulated Df-sensitized spleen cells with HA in the presence of the Df antigen and measured the percentage of Treg cells in cultured spleen cells. In the presence of HA, the percentage of antigen-induced CD4⁺CD25⁺Foxp3⁺TGF- β_1 ⁺ Treg cells was increased in WT mice, but not in CD44^{-/-} mice ($P < 0.05$, Fig. 2f).

Effects of HA on EAR and AHR in Df antigen-specific SLIT

To examine the effects of combining HA administration with allergen-specific SLIT, we developed a new SLIT method using a Df-induced murine chronic asthma model (Fig. 1b). This model exhibits features characteristic of allergic asthma in humans, including the EAR, AHR, chronic eosinophilic airway inflammation, and Df-specific IgE. The efficacy of sublingually administered HA in combination with the Df-antigen was investigated in the new SLIT model. First, we examined the EAR and AHR after exposure to Df-antigen, as described in the methods section. The EAR and AHR were not significantly suppressed by sublingual Df exposure compared with PBS exposure ($P = 0.059$ and $P = 0.163$, respectively, Fig. 3). In the presence of the allergen, sublingual HA treatment significantly suppressed the EAR compared with PBS exposure ($P < 0.001$, Fig. 3) and Df alone ($P = 0.012$, Fig. 3), and the AHR compared with PBS exposure ($P = 0.001$, Fig. 3), and normalized both EAR and AHR ($P = 0.4364$ and $P = 0.325$, respectively, vs the PBS-sensitized PBS-SLIT normal control group, Fig. 3). Further, sublingual HA treatment without Df exposure did not significantly suppress the EAR and AHR in comparison with the Df-sensitized PBS SLIT treatment group ($P > 0.999$ and $P = 0.923$, respectively, Fig. 3).

Effects of HA on eosinophilic airway inflammation and Df-sensitization in Df allergen-specific SLIT

We measured BALF cell numbers to evaluate eosinophilic airway inflammation using the new SLIT with HA.

Twenty-four hours after the transnasal Df challenge, we counted the number of leukocytes in the BALF. Lymphocyte and eosinophil numbers, but not neutrophil and macrophage numbers (data not shown), were significantly decreased by SLIT in combination with HA ($P < 0.05$, Fig. 4a), but not with Df alone. SLIT with HA alone did not affect eosinophilic airway inflammation (Fig. 4a). To examine the mechanisms underlying the anti-inflammatory effects of HA in the new SLIT, the IL-5, and IL-13 concentrations in the BALF were measured. The increased BALF IL-5 and IL-13 concentrations were suppressed by HA SLIT in combination with the Df allergen ($P < 0.05$, Fig. 4b). Furthermore, HA in combination with the Df allergen decreased the serum levels of Df-specific IgE and IgG₁ compared with PBS SLIT and Df-alone SLIT ($P < 0.05$, Fig. 4c). SLIT with HA alone, however, did not change either the BALF Th2 cytokine levels or the serum Df-specific IgE and IgG₁ levels (Fig. 4b and c).

Mechanisms of HA+Df/SLIT

The BALF TGF- β_1 level was increased significantly by Df-SLIT in the presence of HA compared with the Df-sensitized PBS-SLIT group ($P < 0.05$, Fig. 5a). In this study, SLIT did not affect the BALF IL-17 levels (Fig. 5a), and no IFN- γ was detected in the BALF (data not shown). HA in the presence of the Df antigen led to an increased number of CD4⁺Foxp3⁺TGF- β_1 ⁺ Treg cells and the expression levels of Foxp3 in the Df-sensitized spleen cells in a CD44-dependent manner ($P < 0.05$, Fig. 5b and c).

Efficacy of HA in the SLIT model was diminished in CD44^{-/-} mice

We confirmed the critical role of CD44 for the efficacy of HA in this SLIT model using CD44^{-/-} mice. We developed a SLIT model using C57BL/6 (WT) mice with 3 weeks of transnasal sensitization (Fig. 1b). The BALF eosinophil numbers were significantly decreased by Df-SLIT in the presence of HA in WT mice, but not in CD44^{-/-} mice (Fig. 6a). Further,

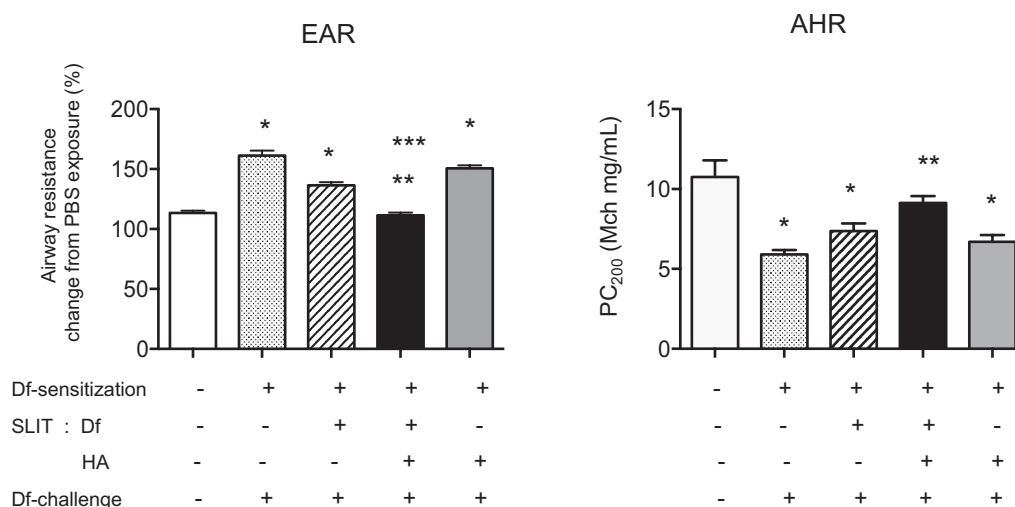


Figure 3: Effects of HA on the EAR and AHR in Df-allergen-specific SLIT. The EAR was examined and expressed as airway resistance change from PBS exposure (%), and AHR was examined and expressed as PC₂₀₀ sRaw (mg/ml), as described in the methods section. Data represent means $\pm$ SEM. Values shown are means from 12 mice per group. The Kruskal–Wallis test, followed by Dunn's multiple comparisons test, was used to compare values of different groups. * $P < 0.05$ compared with Df-sensitization (–), SLIT: Df (–) HA (–), and Df-challenge (–) group. ** $P < 0.05$ compared with Df-sensitization (+), SLIT: Df (–) HA (–), and Df-challenge (+) group. *** $P < 0.05$ compared with Df-sensitization (+), SLIT: Df (+) HA (–), and Df-challenge (+) group.

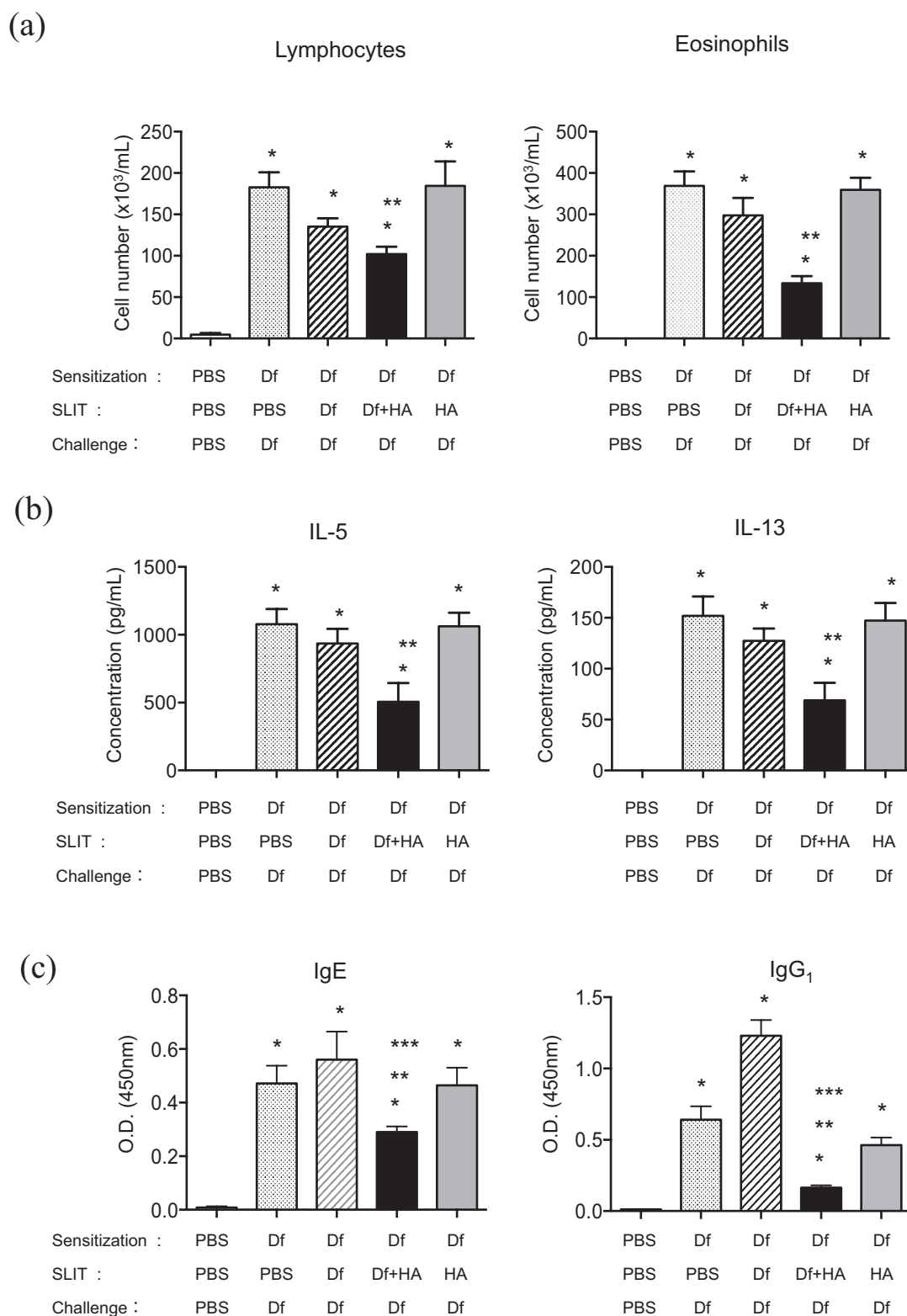


Figure 4: Effects of HA on the airway inflammation and serum immunoglobulin levels in Df allergen-specific SLIT. Absolute numbers of lymphocytes and eosinophils in the BALF were evaluated (a). IL-5 and IL-13 levels in the BALF (b), and Df-specific IgE, and IgG₁ titers in the serum (c) were examined by ELISA. Data represent means $\pm$ SEM. Values shown are means from 12 mice per group. The Kruskal–Wallis test followed by Dunn’s multiple comparisons test, was used to compare values of different groups. * $P < 0.05$ compared with PBS-sensitization, SLIT: PBS, and PBS-challenge group. ** $P < 0.05$ compared with Df-sensitization (+), SLIT: PBS, and Df-challenge (+) group. *** $P < 0.05$ compared with Df-sensitization (+), SLIT: Df (+) HA (–), and Df-challenge (+) group.

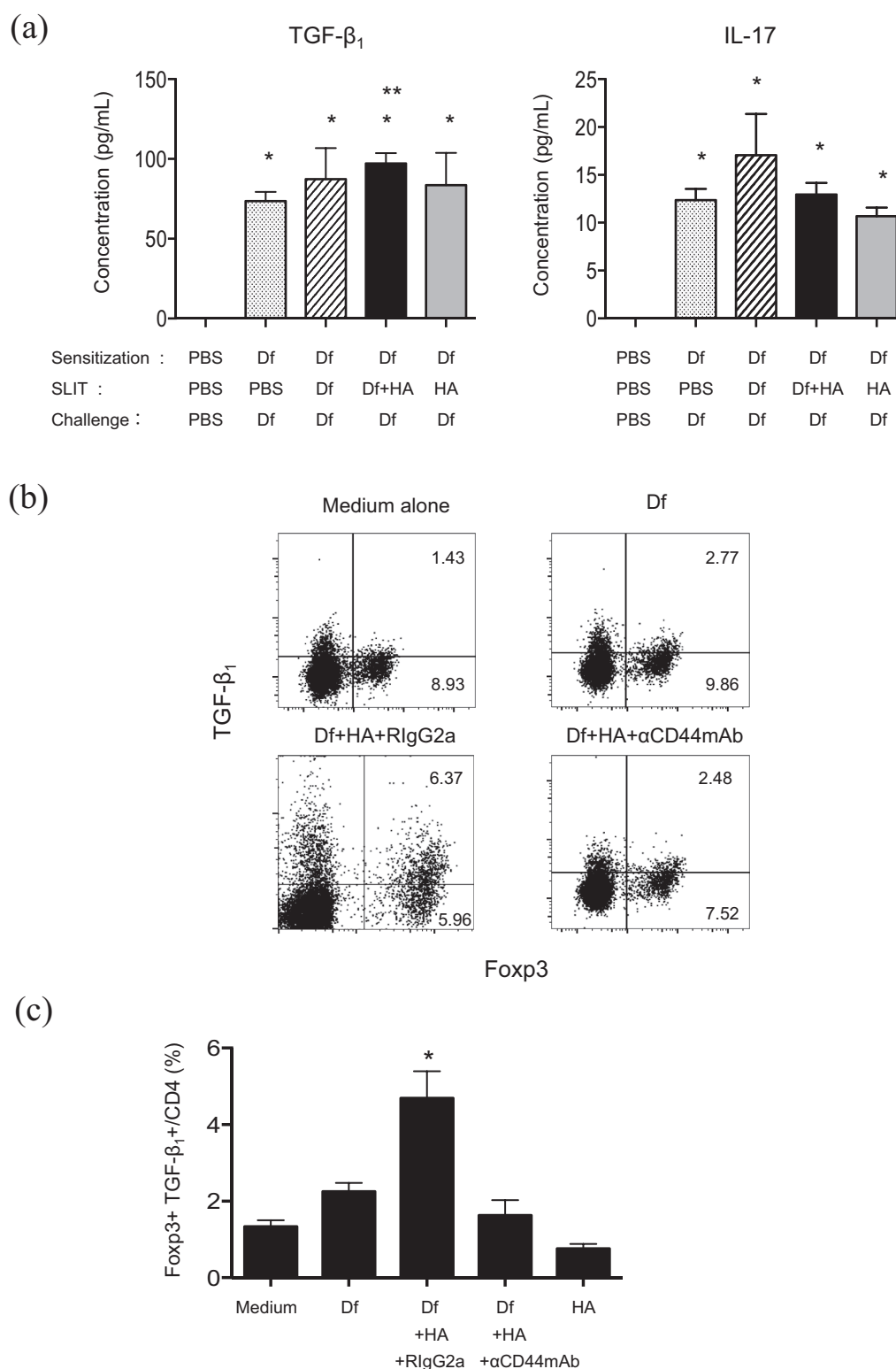


Figure 5: Effects of HA on TGF- β_1 and IL-17 levels in the BALF (a) and on induction of regulatory T cells in the culture of Df-sensitized spleen cells (b, c). TGF- β_1 and IL-17 levels in the BALF were examined by ELISA. Data represent means $\pm$ SEM. Values shown are means from 12 mice per group (a). The Kruskal–Wallis test was used to compare values of different groups followed by Dunn’s multiple comparisons test. * $P < 0.05$ compared with PBS-sensitization, SLIT: PBS, and PBS-challenge group. ** $P < 0.05$ compared with Df-sensitization (+), SLIT: PBS, and Df-challenge (+) group. (b) TGF- β_1 + Treg cells were estimated by flow cytometry. (c) Data represent means $\pm$ SEM. Values shown are means from five mice per group. * $P < 0.05$ compared with medium alone. These results are typical of those obtained in three independent experiments. The Kruskal–Wallis test, followed by Dunn’s multiple comparisons test, was used to compare values of different groups.

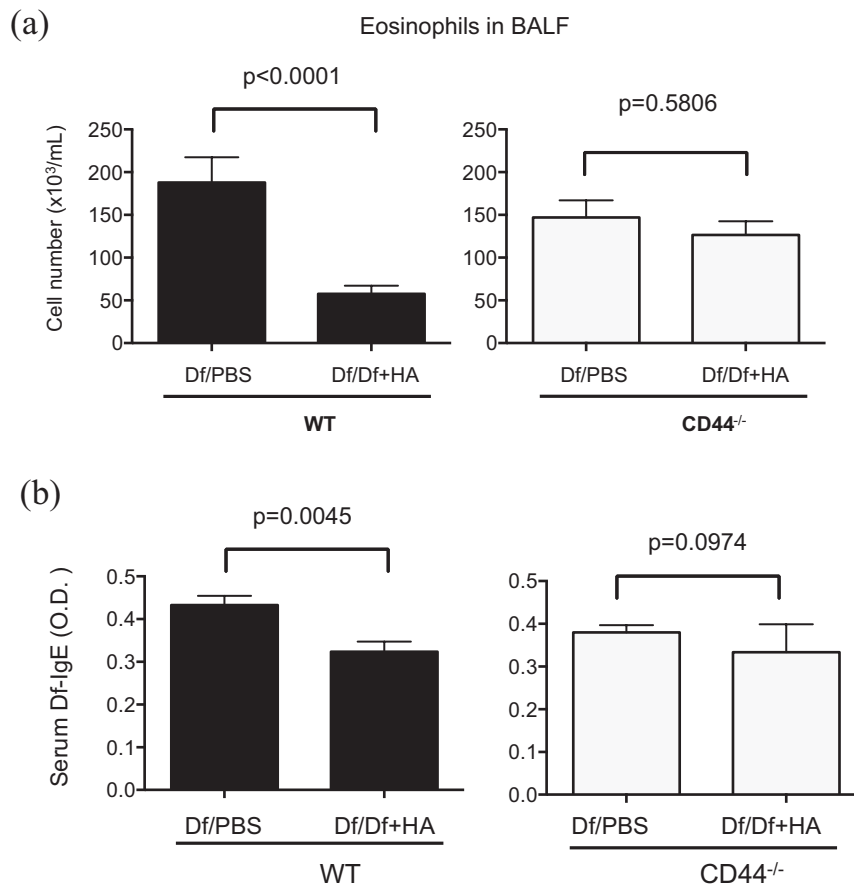


Figure 6: Effects of HA on chronic eosinophilic airway inflammation and serum Df-specific IgE levels in the CD44^{-/-} mouse SLIT model. Absolute numbers of eosinophils in the BALF were examined (a). Df-specific IgE levels in the serum were estimated (b). Data represent means $\pm$ SEM. Values shown are means from 12 mice per group. For comparison of two groups, the Kruskal–Wallis test, followed by the Mann–Whitney *U*-test, was used.

Df-SLIT in the presence of HA suppressed the serum levels of Df-specific IgE in WT mice, but not in CD44^{-/-} mice (Fig. 6b).

Discussion

The findings of the present study revealed that CD44 contributes to the induction of Treg cells in the presence of Df antigen *in vivo* and *in vitro*. In a model of chronic eosinophilic airway inflammation induced by Df sensitization in CD44^{-/-} mice, we observed increased Th2-mediated eosinophilic airway inflammation and serum Df antigen-specific IgE levels, and decreased numbers of CD4⁺CD25⁺Foxp3⁺ Treg cells in the BALF, as compared with WT mice. Further, the enhanced effect of HA on Df antigen-induced Treg cells was diminished in CD44^{-/-} mice *in vitro*. The addition of HA to Df allergen-specific SLIT enhanced the efficacy of Df allergen-specific SLIT in this murine model of chronic asthma. Treatment with this novel SLIT and HA might provide a cure for allergic asthma because Df antigen treatment in the presence of HA normalized both the EAR and AHR. In addition, the BALF IL-13 and serum Df-specific IgE levels were significantly decreased by HA when used as an adjuvant to the Df antigen. The addition of HA also inhibited Df-induced eosinophilic airway inflammation related to low IL-5 levels. The enhancing effects of HA on the Df allergen-specific SLIT, however, were diminished in CD44^{-/-} mice.

House dust mites are the most common allergen in allergic asthma, with exposure leads to the production of mite-allergen specific IgE [2]. Inhaled corticosteroids effectively control asthmatic symptoms, but cannot cure allergic asthma. Although SLIT is a well-known disease-modifying treatment for respiratory allergies, the benefits are limited [19]. To cure allergic asthma, it is critical to suppress the EAR, allergen-specific IgE production, and Th2-mediated inflammation, but it is challenging to reduce allergen-specific IgE production and Th2-mediated inflammation with ongoing SLIT [20, 21]. Preventive effects of SLIT were demonstrated in a murine model of mite allergen-induced acute asthma, induced by multiple sensitizations with or without alum [22, 23]. Here, we used a previously established murine model of chronic asthma induced by long-term (15 times) transnasal sensitization without alum that produces features characteristic of human chronic asthma and performed a SLIT treatment using HA as an adjuvant. Shima *et al.* demonstrated that SLIT using a high-dose of Df extract (5 mg/day, Torii, Co. Ltd), while not affecting serum IgE levels, improved AHR and eosinophilic airway inflammation in a murine asthma model [24]. We recently demonstrated that Gal-9 in addition to Df allergen, but not allergen alone (250 μ g/day, Torii, Co. Ltd) suppressed the EAR, AHR, eosinophilic airway inflammation, Th2 cytokine production, and the serum titer of Df-specific IgE in a similar model of murine asthma [18]. Our newly developed SLIT using a low dose of the same antigen

(125 µg/day, Torii, Co. Ltd) in combination with HA (molecular weight: 40 000, 100 µg/day, Lifecore Biomedical, Co. Ltd) described herein completely inhibited EAR and AHR in a murine model of chronic asthma. Furthermore, sublingual Df treatment in the presence of HA led to significantly lower levels of serum Df-specific IgE, and BALF IL-5 and IL-13. Therefore, administering HA to enhance the efficacy of SLIT in Df antigen-induced asthma might not only contribute to curing the disease but also to reducing the side effects of the therapy such as itching and inflammation in the mouth. HA may be considered a safety adjuvant because HA, but not Gal-9 has already been approved for use as a medicine in humans.

Treg cells have a crucial role in SLIT [20, 21]. TGF-β₁ regulates antigen-specific CD4 T cell responses, and allergen-specific Foxp3⁺ Treg cells in allergen-specific immunotherapy ameliorate allergic asthma [25, 26]. Recent studies demonstrated that CD44 expressed on Treg cells immunomodulates disproportionate immunologic responses by expanding the number of Treg cells and enhancing TGF-β signaling, and CD44 costimulation promotes the function of Foxp3⁺ Treg cells by inducing the production of TGF-β [13, 14, 27]. Further, we demonstrated the effect of Gal-9 on SLIT in addition to Df-antigen in a Treg cell-dependent manner [17]. In the present study, we investigated the mechanisms underlying the effects of HA on SLIT in combination with Df-antigen. The suppression of eosinophilic airway inflammation observed by adding HA to Df-antigen SLIT may be due to its effect to inhibit the production of IL-5 in the lung [15, 28]. We further found that HA reduced Df-specific IgE production by inhibiting IL-13 production. Subsequent evaluation of the levels of helper T cell-mediated cytokines in the BALF of Df-treated mice following treatment with the new SLIT using HA revealed increased levels of TGF-β₁, but not IL-17 or IFN-γ. The numbers of CD4⁺ Foxp3⁺ TGF-β₁⁺ Treg cells in the Df-sensitized spleen cell culture were also increased by Df stimulation in the presence of HA in a CD44-dependent manner.

We previously demonstrated that CD44 positively regulates the accumulation of Th2 cells in the airway and the critical role of CD44 expressed on antigen sensitized-CD4⁺ T cells or antigen-specific Th2 cells infiltrating the lung using an acute model of allergic asthma [10, 11]. These models are developed by fewer than four sensitizations in the presence or absence of alum, and one antigen challenge 2 weeks after the sensitization to evaluate the role of CD44 in the infiltration of Th2 cells into the lung. In the present study, we developed a chronic Th2-mediated eosinophilic airway inflammation model using 25 transnasal sensitizations without alum (Fig. 1a). In this model, eosinophilic airway inflammation and IgE production were negatively regulated by CD44-induced Treg cells. Acute and chronic eosinophilic inflammation models in CD44^{-/-} mice differ with respect to the lung eosinophilia and serum IgE titers.

To confirm the critical role of CD44 for the enhancing effect of HA in the new SLIT, we used CD44^{-/-} mice. Df-allergen-specific SLIT in the presence of HA suppressed eosinophilic airway inflammation and serum levels of Df-specific IgE in C57BL/6 WT mice, but not in CD44^{-/-} mice. We clearly demonstrated that CD44 is necessary for improving eosinophilic airway inflammation and Df-sensitization in this SLIT model using HA in combination with the Df-antigen. Further, Treg cells are induced by HA in the presence of the allergen in a CD44-dependent manner *in vitro*. The CD44-HA interaction enhances the induction of

Treg cells, which might contribute to cure the murine model of chronic asthma induced by the Df antigen.

In conclusion, administration of HA as an adjuvant in this Df-allergen specific SLIT model inhibited Th2-associated eosinophilic airway inflammation as well as the production of the antigen-induced EAR and antigen-specific IgE, and might be an effective cure for allergic asthma in humans.

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Conflict of interest

I declare that none of the authors has any potential financial conflict of interests related to this manuscript.

Author contributions

SK, KO-D, and TO participated in the design of the study. TU, HT, HM, and SK carried out the mouse studies, ELISA, and flow cytometry. SK drafted the manuscript.

Ethics approval

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 (20-026, 21-003).

The authors confirm that their institutional guidelines adhere to the ARRIVE guidelines.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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