

CD44 is critical for airway accumulation of antigen-specific Th2, but not Th1, cells induced by antigen challenge in mice

Shigeki Katoh^{1,2}, Osamu Kaminuma³, Takachika Hiroi³, Akio Mori⁴, Takayuki Ohtomo⁴, Sachie Maeda⁵, Hiroki Shimizu¹, Yasushi Obase¹ and Mikio Oka¹

¹ Department of Respiratory Medicine, Kawasaki Medical School, Okayama, Japan

² Department of Integrated Medicine, Faculty of Medicine, Kagawa University, Kagawa, Japan

³ Department of Allergy and Immunology, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

⁴ Clinical Research Center for Allergy and Rheumatology, Sagamihara National Hospital, Kanagawa, Japan

⁵ CREST, Japan Science and Technology Agency, Osaka, Japan

CD44 is a cell adhesion molecule involved in lymphocyte infiltration of inflamed tissues. We previously demonstrated that CD44 plays an important role in the development of airway inflammation in a murine model of allergic asthma. In this study, we investigated the role of CD44 expressed on CD4⁺ T cells in the accumulation of T-helper type 2 (Th2) cells in the airway using CD44-deficient mice and anti-CD44 monoclonal antibodies. Antigen-induced Th2-mediated airway inflammation and airway hyperresponsiveness (AHR) in sensitized mice were reduced by CD44-deficiency. These asthmatic responses induced by the transfer of antigen-sensitized splenic CD4⁺ T cells from CD44-deficient mice were weaker than those from WT mice. Lack of CD44 failed to induce AHR by antigen challenge. Expression level and hyaluronic acid receptor activity of CD44, as well as Neu1 sialidase expression on antigen-specific Th2 cells, were higher than those on antigen-specific Th1 cells. Anti-CD44 antibody preferentially suppressed the accumulation of those Th2 cells in the airway induced by antigen challenge. Our findings indicate that CD44 expressed on CD4⁺ T cells plays a critical role in the accumulation of antigen-specific Th2 cells, but not Th1 cells, in the airway and in the development of AHR induced by antigen challenge.

Key words: Asthma · CD44 · CD44-deficient mice · Th2 cells



Supporting Information available online

Introduction

CD44 is a cell surface glycoprotein that participates in several physiologic and pathologic processes [1–3]. CD44 clearly functions as a receptor for hyaluronic acid (HA) [4]. CD44–HA

interactions can promote infiltration of activated T cells into the inflammatory tissue. This interaction involves the rolling of leukocytes over endothelial cells [5] and also regulates lymphocyte adhesion in vitro [6, 7]. We recently reported that CD44 receptor activity is induced by antigen stimulation in antigen-sensitized splenic CD4⁺ T cells [8].

Infiltration of antigen-activated CD4⁺ T cells into the airway might contribute to the development of asthma [9]. These CD4⁺ T cells can differentiate into functionally distinct effector subsets

Correspondence: Dr. Shigeki Katoh
e-mail: kshigeki@med.kawasaki-m.ac.jp

with different cytokine expression profiles. The T-helper type 1 (Th1) subset produces interferon (IFN)- γ , and the Th2 subset produces interleukin (IL)-4, IL-5, and IL-13 [10]. The Th1 response is often accompanied by the production of IgG2a/2c [11], whereas the Th2 response is often accompanied by the production of IgG1 and IgE. Both Th1- and Th2-specific cytokines can facilitate growth or differentiation of their own respective T-cell subset, but might also inhibit the development of the opposing subset [12]. We previously reported that adoptive transfer of in vitro-differentiated ovalbumin (OVA)-specific Th1 and Th2 cells conferred airway inflammation and airway hyper-responsiveness (AHR) to unprimed recipients [13].

In atopic asthma, Th2 immune responses might have a critical role in the development of allergen-induced airway eosinophilic inflammation and AHR [14, 15]. Therefore, the suppression of Th2 responses could be a potential target of immunotherapy for atopic asthma. We previously demonstrated that administration of anti-CD44 mAbs inhibits the development of airway inflammation and AHR in an *Ascaris suum* antigen-induced murine model of pulmonary eosinophilia [16]. Furthermore, we reported that treatment with anti-CD44 mAb reduces the number of T1/ST2⁺CD4⁺ T cells in the airway of mice immunized and challenged with *Dermatophagoides farinae* (Derf) [17]. Both Th1 and Th2 cells, however, express CD44 and use CD44 for their rolling on, and adhesion to, the intestinal endothelium [18]. Recently, Nagarkatti et al. reported that CD44 deficiency enhances the development of Th2 effectors in response to sheep red blood cells and chicken OVA [12]. Thus, the contribution of CD44 to Th1- and Th2-mediated allergic inflammation remains unclear.

In the present study, to directly clarify the role of CD44 in the development of asthma, airway inflammation and AHR were evaluated in a murine model of Derf-induced allergic asthma using CD44-deficient (CD44KO) mice. To further validate the role of CD44 expressed on CD4⁺ T cells in the induction of airway inflammation and AHR, antigen-sensitized splenic CD4⁺ T cells from CD44KO mice were transferred into unprimed mice. Finally, to clarify the selective contribution of CD44 among T-cell subsets, we analyzed the effect of anti-CD44 mAb on the accumulation of in vitro-differentiated OVA-specific Th1 and Th2 cells in the airway in OVA-challenged mice.

Results

Suppression of Derf-induced asthmatic reaction in the CD44-deficient mice

To investigate the contribution of CD44 in the development of asthma, we evaluated Derf-induced AHR and airway inflammation in the CD44KO mice compared with WT C57BL/6 mice in a murine model of allergic asthma. Two groups of mice were sensitized with either Derf in PBS or PBS alone, by intraperitoneal administration, according to the procedures described in Materials and Methods. AHR was evaluated 24 h after intranasal challenge with Derf by double-flow plethysmography. Derf

challenge induced a significant increase in airway reactivity to methacholine in comparison with PBS-treated controls in WT mice ($p = 0.0002$, Fig. 1A). Unlike in WT mice, AHR to methacholine after antigen challenge was not observed in CD44KO mice, and the degree of airway reactivity to methacholine was similar to that of PBS-exposed mice ($p = 0.5004$, Fig. 1A). The number of inflammatory cells in the BALF was evaluated 24 h after intranasal antigen challenge. After exposure to Derf, total leukocytes, macrophages, lymphocytes, eosinophils, and neutrophils were significantly increased in the BALF of Derf-exposed WT mice compared with PBS-exposed WT mice ($p < 0.05$, Fig. 1B). We compared the severity of inflammation in the airway between Derf-exposed CD44KO and WT mice. The numbers of total leukocytes, macrophages, and lymphocytes in the BALF of Derf-exposed CD44KO mice were lower than those of Derf-exposed WT mice ($p < 0.05$, Fig. 1B). The number of eosinophils in the BALF of Derf-exposed CD44KO mice was marginally lower than that of Derf-exposed WT mice ($p = 0.0963$, Fig. 1B). Furthermore, accumulation of Th1 and Th2 cells was investigated by counting the number of CD4⁺Tim-3⁺ and CD4⁺T1/ST2⁺ T cells, respectively, in the BALF. The accumulation of Th2 cells ($p = 0.0041$), but not Th1 cells ($p = 0.6911$), was suppressed in CD44KO mice compared with WT mice (Fig. 1C), after Derf challenge. Therefore, the lack of antigen-induced AHR in CD44KO mice might be caused by the down-regulation of Th2 cell accumulation in the lung.

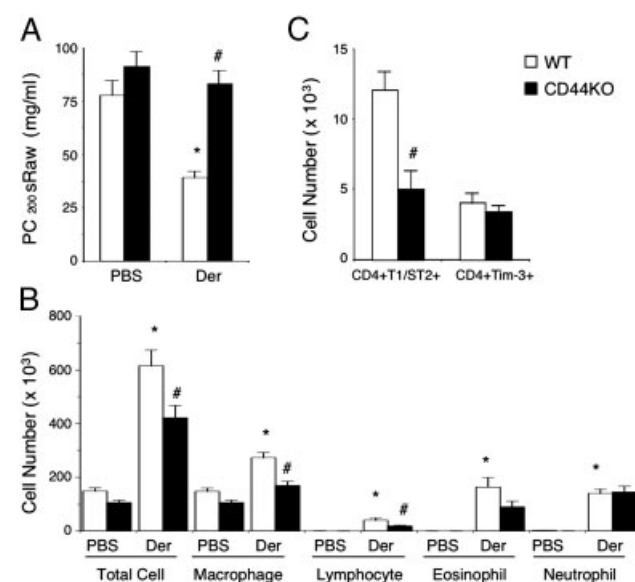


Figure 1. Suppression of Derf-induced AHR and airway inflammation in CD44KO mice. (A) AHR was measured and expressed in PC₂₀₀ sRaw (mg/ml) as described in Materials and Methods. (B) The absolute numbers of inflammatory cells in the BALF were estimated as described in Materials and Methods. (C) BALF cells were stained with FITC anti-T1/ST2, phycoerythrin-anti-Tim-3, PerCP anti-CD3, and APC anti-CD4. Th1 and Th2 cells were evaluated by Tim-3 and T1/ST2 expression gating on CD3⁺ and CD4⁺ T cells. (A–C) Data represent mean ± SEM. Mean values from 10 mice per group are shown, * $p < 0.05$ compared with PBS-challenged C57BL/6 WT mice; # $p < 0.05$ compared with Derf-challenged C57BL/6 WT mice. Significance was evaluated by the Mann-Whitney U test.

Role of CD44 in antigen-induced cytokine production in the lung

To investigate the possible roles of various cytokines and chemokines in allergic airway inflammation, concentrations in BALF of Th1 (IFN- γ) and Th2 (IL-5, IL-13) cytokines, and chemokines (TARC, IP-10, and eotaxin) were measured by ELISA. The levels of these cytokines and chemokines in the PBS group of both CD44KO and WT mice were under the detection limits (data not shown). Elevated levels of Th1 and Th2 cytokines were observed in both CD44KO and WT mice after Derf challenge. Th2 cytokine (IL-5 and IL-13) concentrations in the BALF of CD44KO mice were lower than those of WT mice ($p < 0.05$, Fig. 2A), while the amount of IFN- γ in the BALF of Derf-exposed CD44KO mice was higher than that of Derf-exposed WT mice ($p < 0.05$, Fig. 2A). Levels of TARC and eotaxin in the BALF of Derf-exposed CD44KO mice were similar to those of Derf-exposed WT mice, while the IP-10 concentration in the BALF of Derf-exposed CD44KO mice was higher than that in Derf-exposed WT mice ($p < 0.05$, Fig. 2B). These data demonstrate the possibility that CD44 deficiency not only suppresses Th2-mediated airway inflammation, but also facilitates Th1 development in Derf-sensitized and challenged mouse model.

To explore the role of CD44 in the development of Th1- or Th2-biased Th differentiation, antigen-specific antibody production, Derf-specific IgE, IgG1, IgG2c, and Th1, Th2 cytokine levels in the serum were determined by ELISA in Derf-immunized

CD44KO and WT mice before and after antigen challenge. Serum levels of Derf-specific IgE ($p = 0.3472$), IgG1 ($p = 0.1172$), and IgG2c ($p = 0.2948$) were not significantly different between CD44KO and WT mice before antigen challenge (Fig. 3A), whereas the serum levels of Derf-specific IgG2c ($p = 0.0109$), but not IgE ($p = 0.5589$) and IgG1 ($p = 0.8494$), were significantly higher in CD44KO mice compared with WT mice after Derf-challenge (Fig. 3B). Before antigen challenge, serum levels of IL-5 ($p = 0.2347$) and IL-13 ($p = 0.4189$) in CD44KO mice were similar to those in WT mice (Fig. 3C), while serum concentrations of IFN- γ in both CD44KO and WT mice were under the detection limit of this assay (data not shown). The IFN- γ concentration was higher in CD44KO mice than in WT mice after the antigen challenge ($p < 0.0001$, Fig. 3D). The serum level of IL-13 was lower in CD44KO mice than in WT mice (IL-13: $p = 0.0062$, Fig. 3D) and IL-5 level in the serum was marginally lower in CD44KO mice than in WT mice (IL-5: $p = 0.1288$, Fig. 3D).

CD4⁺ T cells from CD44KO mice induce reduced AHR and airway inflammation

To clarify the role of CD44 expressed on CD4⁺ T cells in antigen-induced airway inflammation, separately from antibody-mediated responses, we analyzed the asthmatic transfer model using antigen-sensitized splenic CD4⁺ T cells from CD44KO mice. Consistent with the results of antigen-sensitized mice (Fig. 1), transfer of splenic CD4⁺ cells from Derf-sensitized WT mice (B6/B6Der) ($p = 0.004$, Fig. 4A), but not CD44KO mice (CD44KO/B6Der) ($p = 0.657$, Fig. 4A), into unprimed WT mice significantly induced AHR to methacholine 24 h after Derf challenge. The numbers of lymphocytes, eosinophils, and neutrophils ($p < 0.05$, Fig. 4B), but not the numbers of total leukocytes ($p = 0.215$) and macrophages ($p = 0.691$), were significantly elevated in the BALF 24 h after intranasal allergen challenge in mice that received CD4⁺ T cells from Derf-sensitized WT mice (B6/B6Der). The number of lymphocytes ($p = 0.0243$), but not neutrophils ($p = 0.4527$) in the BALF, was significantly lower using CD4⁺ T cells from CD44KO mice (CD44KO/B6Der) than those from WT mice (B6/B6Der) (Fig. 4B). The number of eosinophils in the BALF was marginally lower using CD4⁺ T cells from CD44KO mice than those from WT mice ($p = 0.125$). Increased IL-5 and IL-13 levels in the BALF were significantly suppressed by using CD4⁺ T cells from CD44KO mice ($p = 0.0209$ and $p = 0.008$, respectively; Fig. 4C). On the other hand, IFN- γ levels in the BALF were significantly higher in CD44KO mice compared with WT mice ($p = 0.0091$, Fig. 4C). Furthermore, the number of Th2 cells ($p = 0.0017$, Fig. 4D), but not Th1 cells ($p = 0.2694$, Fig. 4D) in the BALF, was significantly lower in the transfer of CD4⁺ T cells from CD44KO mice (CD44KO/B6Der) compared with those from WT mice (B6/B6Der). These data suggest that the difference in airway inflammation including AHR between WT and CD44KO mice after antigen sensitization and challenge as shown in Fig. 1 was in part caused by the functional disparity of CD4⁺ T cells.

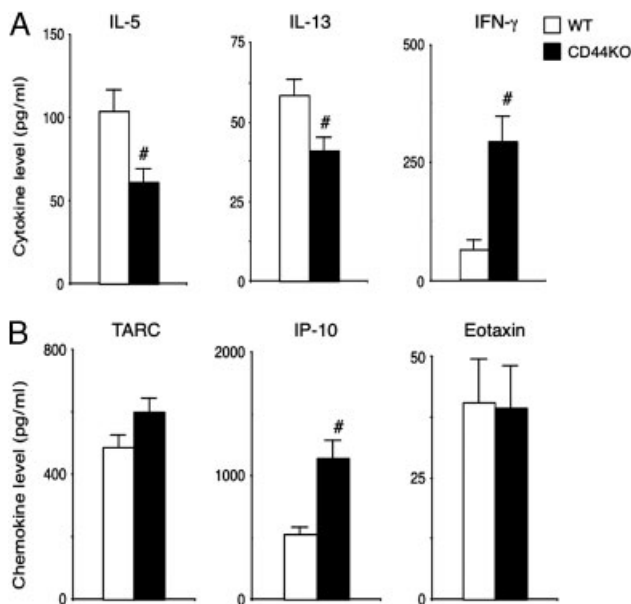


Figure 2. Cytokine production in the BALF of CD44KO mice. Concentrations of (A) IL-5, IL-13, IFN- γ and (B) TARC, IP-10, and eotaxin in the BALF were determined by ELISA. Data represent mean $\pm$ SEM. Mean values from 18 mice per group are shown, $^{\#}p < 0.05$ compared with Derf-challenged C57BL/6 WT mice. Significance was evaluated by the Mann-Whitney U test.

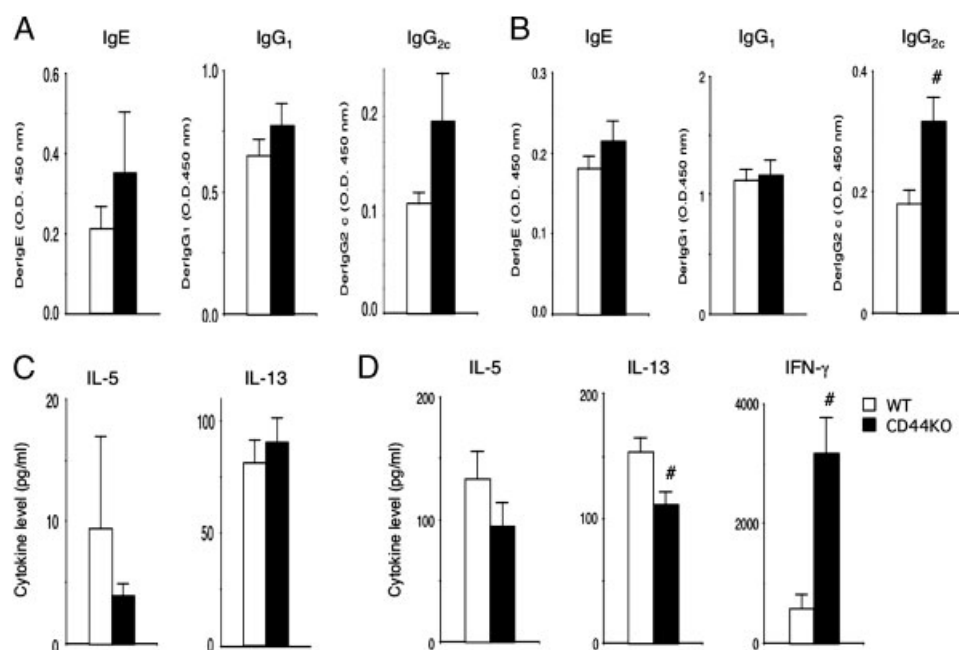


Figure 3. Evaluation of anti-Derf Ab levels and serum cytokine production in CD44KO mice. Mice were immunized with Derf as described in Materials and Methods. Sera were collected 24 h before (A, C) and after (B, D) Derf challenge. Derf-specific IgE, IgG₁, IgG_{2c} (A, B), and IL-5, IL-13, IFN-γ (C, D) were assessed by ELISA. Data represent mean ± SEM. Mean values from 15 to 18 mice per group are shown, **p* < 0.05 compared with Derf-challenged C57BL/6 WT mice. Significance was evaluated by the Mann–Whitney *U* test.

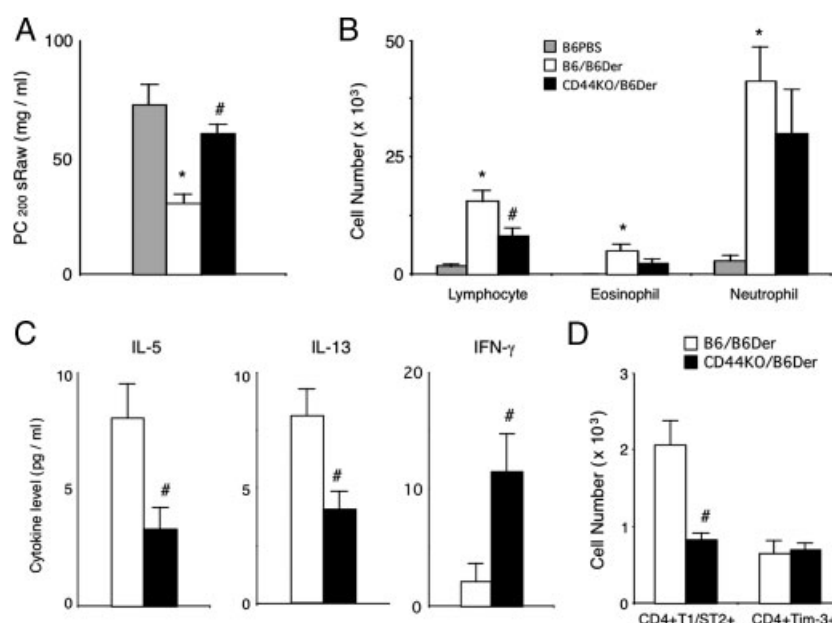


Figure 4. Airway inflammation induced by antigen-sensitized splenic CD4⁺ T cells from CD44KO mice. Derf-immunized splenic CD4⁺ T cells from C57BL/6 (B6/B6Der) and CD44KO mice (CD44KO/B6Der) were transferred to normal B6 mice by intravenous injection. Twenty-four hours after antigen challenge, (A) AHR was measured and expressed in PC₂₀₀ sRaw (mg/mL) and BAL was performed as described in Materials and Methods. (B) The absolute numbers of inflammatory cells in the BALF were estimated as described in Materials and Methods. (C) Concentrations of IL-5, IL-13, and IFN-γ in the BALF were determined by ELISA. (D) CD4⁺T1/ST2⁺ T cells and CD4⁺Tim-3⁺ T cells in the BALF were determined by flow cytometry. BALF cells were stained with FITC anti-T1/ST2, phycoerythrin-anti-Tim-3, PerCP anti-CD3, and APC anti-CD4. Th1 and Th2 cells were evaluated by Tim-3 and T1/ST2 expression gating on CD3⁺ and CD4⁺ T cells. (A–D) Data represent mean ± SEM of six independent experiments. **p* < 0.05 compared with PBS-immunized and challenged B6 mice (B6PBS); #*p* < 0.05 differences in CD4⁺ T cells from between CD44KO (CD44KO/B6Der) and WT (B6/B6Der) mice. PBS immunized and challenged B6 mice were used as the negative control (B6PBS). Significance was evaluated by the Mann–Whitney *U* test.

Anti-CD44 mAb preferentially suppresses accumulation of Th2 cells in the airway

In antigen sensitization and CD4⁺ T-cell-transfer models, the accumulation of Th2 cells, but not Th1 cells, was reduced by CD44 deficiency (Figs. 1C and 4D). Therefore, to directly evaluate the comparative role of CD44 in the accumulation of antigen-specific Th1 and Th2 cells in the lung, in vitro-differentiated OVA-specific Th1 and Th2 cells were used for asthmatic adoptive transfer model using DO11.10 mice [13]. We confirmed the expression of Th-specific chemokine receptor (Th1: CXCR3, Th2: CCR4) on these cells. OVA-specific Th1 cells preferentially expressed CXCR3, whereas OVA-specific Th2 cells preferentially expressed CCR4 [13]. Levels of CD44 expressed on OVA-specific Th2 cells were higher than those on OVA-specific Th1 cells, whereas expression levels of CD49d were similar between these Th1 and Th2 cells (Fig. 5A, Fig. S1). Furthermore, receptor activity of CD44 was higher on OVA-specific Th2 cells than Th1 cells (Fig. 5A, Fig. S1). CD44 consists of a numerous number of variant isoforms generated by alternative splicing of ten variant exons [19]. To investigate the differential expression of CD44 isoforms on Th1 and Th2 cells, the expression of representative transcript variant 1, 3, 5, and 6, as well as total CD44 was determined by quantitative real-time RT-PCR. In accordance with the surface expression of CD44 (Fig. 5A), mRNA levels of total CD44 and all its variants examined in this study were significantly higher in Th2 cells than Th1 cells (Fig. 5B). We have demonstrated that HA-binding activity of CD44 is negatively regulated by its sialylation [20]. Therefore, the expression of several sialidases in Th1 and Th2 cells was investigated. The expression of sialidases Neu1 and Neu3 was significantly higher in Th2 cells than Th1 cells (Fig. 5C). Therefore, relative potent activation and participation of CD44 in the accumulation of Th2 cells may be caused, at least in part, by higher expression of these sialidases.

We then developed a Th1- and Th2-mediated airway inflammation model using the previously described methods [13]. To investigate the role of CD44 in this model, anti-CD44 mAb, IM7 was injected with these in vitro-differentiated Th1 or Th2 cells, as compared with anti-CD49d mAb, PS2. In mice that underwent transfer of Th1 or Th2-polarized DO11.10 T cells, accumulation of antigen-specific T cells in the airway was detectable upon inhalation challenge with OVA (Fig. 6A). Subsequently, the migration of eosinophils, neutrophils, and lymphocytes was significantly induced in both Th1- and Th2-cell-transferred mice. The migration of these cells was dependent on infused T cells and their specific antigen, because they failed to infiltrate the lungs of bovine serum albumin-challenged mice. Neither IM7 nor PS2 affected the infiltration of inflammatory cells into the lung in Th1-transferred mice. On the other hand, IM7, but not PS2, suppressed antigen-induced accumulation of lymphocytes in Th2-transferred mice ($p = 0.0494$). Interestingly, infiltration of Th2-polarized DO11.10 T cells, but not Th1-polarized DO11.10 T cells, into the lung was significantly suppressed by IM7 ($p = 0.009$). PS2 treatment had no effect on the infiltration of

these Th cells into the lung (Fig. 6A). These findings suggest that both Th1 and Th2 cells could migrate in the lung upon antigen challenge, though CD44 specifically participates in the accumulation of Th2 cells.

Finally, we investigated the antigen-induced AHR in this Th1- or Th2-transferred model. Both Th1 and Th2 cells could induce AHR and IM7 suppressed Th2 cell-induced AHR, but not Th1 cell-induced AHR (Fig. 6B).

Discussion

CD44 is a widely distributed cell adhesion molecule involved in lymphocyte infiltration into the inflammatory tissue [1–3]. We recently reported that HA-binding ability of CD44 could be induced by antigen stimulation in antigen-sensitized splenic CD4⁺ T cells [8]. Antigen-stimulated CD4⁺ T cells in the airway are believed to contribute to the development of asthma [9]. In the present study, CD4⁺ Derf-immunized splenic T cells were used for an asthmatic transfer model. The lack of CD44 on antigen-sensitized CD4⁺ T cells suppressed antigen-induced Th2-mediated airway inflammation and failed to induce AHR. Taken together with findings from a previous study, CD44 expressed on CD4⁺ T cells plays an important role in the development of murine model of allergic asthma.

To clarify the comparative role of CD44 among T-cell subsets, we used in vitro-differentiated OVA-specific Th1 and Th2 cells for an asthmatic adoptive transfer model. We demonstrated that OVA-transgenic splenic CD4⁺ T cells could induce allergic airway inflammation using a Th cell-transfer model to unprimed recipients. In vitro-differentiated OVA-specific Th1 cells induced massive accumulation of neutrophils, whereas eosinophil infiltration was specifically induced by in vitro-differentiated OVA-specific Th2 cells after antigen challenge, consistent with the previous findings [21, 22]. Anti-CD44 mAb specifically inhibited the infiltration of Th2-differentiated DO11.10 T cells, but not Th1-differentiated DO11.10 T cells, into the airway. Previous studies demonstrated that stimulated Th1 cells bind to P-selectin and infiltrate into the inflammatory tissue, whereas Th2 cells do not [23, 24]. HA-binding capacity was consistently larger in Th2 than Th1 cells in vitro, while the inhibition of CD44 reduced rolling, and adhesion to the intestinal vasculature similarly in Th1 and Th2 cells in vivo [18]. In this study, the expression level of CD44 and HA-binding ability were greater on in vitro-differentiated OVA-specific Th2 than Th1 cells, but the expression level of CD49d on OVA-specific Th2 cells was similar to that on OVA-specific Th1 cells. Treatment of these Th cells with anti-CD44 mAb, but not with anti-CD49d mAb, preferentially inhibited the accumulation of in vitro-differentiated OVA-specific Th2 cells into the airway compared with Th1 cells. As demonstrated in the previous studies [25, 26], antigen-induced AHR was induced in mice transferred with not only Th2 cells, but also Th1 cells. However, AHR mediated by Th2 but not Th1 was suppressed by the CD44-blocking Ab. These findings suggest that antigen-specific Th2 cells could preferentially use CD44 expressed on

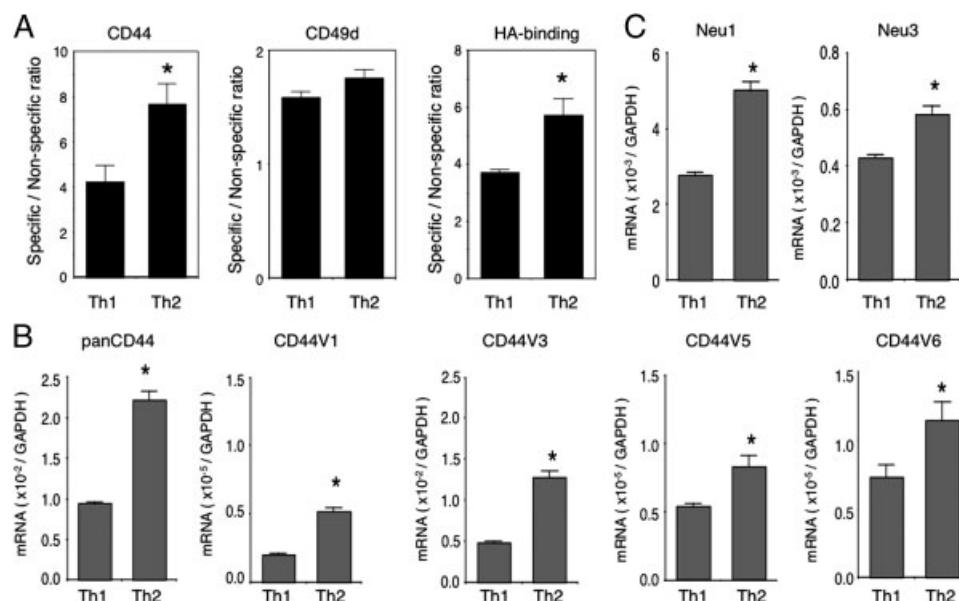


Figure 5. CD44 expression, HA-binding, and sialidase expression by in vitro-differentiated OVA-specific Th1 and Th2 cells. OVA-specific Th1 and Th2 cells were obtained as described in Materials and Methods. (A) Expression of CD44 and CD49d on these Th1 and Th2 cells and CD44-dependent HA-binding ability of Th cells were evaluated by flow cytometry. Harvested cells were stained with FL-HA in the presence or absence of KM81, an anti-CD44 blocking antibody. Data represent mean ± SEM of three independent experiments. (B) Expression of total CD44 and its variants, and (C) expression of sialidases in Th1 and Th2 cells. Messenger RNA expression in in vitro-differentiated OVA-specific Th1 and Th2 cells was determined by quantitative real-time PCR (RT-PCR). Data are expressed as mean ± SEM of mRNA abundance normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The results shown are representative of two separate experiments ($n = 4$). Asterisks indicate significant differences ($p < 0.05$) between the indicated groups. Significance was evaluated by the Mann–Whitney U-test.

themselves for infiltration and resultant exhibition of their pathogenic functions in the airway induced by an antigen.

In the present study, we first developed a murine model of allergic asthma using CD44KO mice. In CD44KO mice, the lack of CD44 decreased Derf-induced Th2-mediated airway inflammation and failed to induce AHR. Nagarkatti et al. demonstrated that CD44-deficiency triggers a Th2-biased Th development using OVA immunization with a Th1-skewing adjuvant CFA without airway antigen challenge [12]. In the present study, we used Th2-skewing adjuvant aluminum hydroxide for Derf-immunization. Before antigen challenge, the levels of Th2 cytokines, Derf-specific IgE, and IgG1 in the serum of CD44KO mice were similar to those in WT mice, while IFN- γ was not detected in the serum of both CD44KO and WT mice, and the serum level of Derf-specific IgG2c was similar between CD44KO and WT mice. These data suggested that the lack of CD44 did not influence the Th1- or Th2-biased Th development in the sensitization phase of this model. After antigen challenge, the number of Th2 cells and the levels of Th2 cytokines in the BALF of CD44KO mice were lower than those in WT mice, while the levels of Th2 chemokine (TARC) in the BALF of CD44KO mice were similar to those in WT mice. Finally, we demonstrated that anti-CD44 mAb inhibited the infiltration of OVA-specific in vitro-differentiated Th2, but not Th1, cells into the airway after antigen challenge. These data suggested that CD44 plays a critical role in the infiltration of Th2 cells into the airway induced by antigen challenge, in large part, as an adhesion molecule. Anti-CD44 mAb significantly reduced airway

accumulation of eosinophils and the concentration of eotaxin in the BALF in murine models of pulmonary eosinophilia [17, 18]. Consistently, the number of eosinophil in the BALF of CD44KO mice was marginally lower than those in WT mice, although the level of eotaxin in the BALF of CD44KO mice was similar to that of WT mice in Derf-sensitized and challenged mouse asthmatic model in this study. Even though exact reason for such discrepancy is unclear at present, it may be caused by differences of antigen, mouse strain, and the way of antigen administration.

Increased levels of both Th1 and Th2 cytokines in the serum were observed after antigen challenge. Increased levels of Th2 cytokines in the BALF reflect the elevated levels of Th2 cytokines in the serum of WT mice after antigen challenge. Higher levels of IFN- γ in the BALF and serum in CD44KO mice might be caused by lower levels of Th2 cytokines in the BALF and serum in CD44KO mice compared with WT mice after antigen challenge, because IFN- γ was not detected in the serum of both CD44KO and WT mice, while the serum levels of Th2 cytokines were similar between CD44KO and WT mice before the antigen challenge. Higher levels of IFN- γ might contribute to the higher levels of Derf-specific IgG2c in serum of CD44KO mice after antigen challenge.

The number of macrophages in the BALF was not significantly different between CD44KO and WT mice at baseline, as previously described [27]. In this Derf-induced asthmatic model, CD44KO mice had significantly fewer macrophages compared with WT mice 24 h after antigen challenge. However, the number

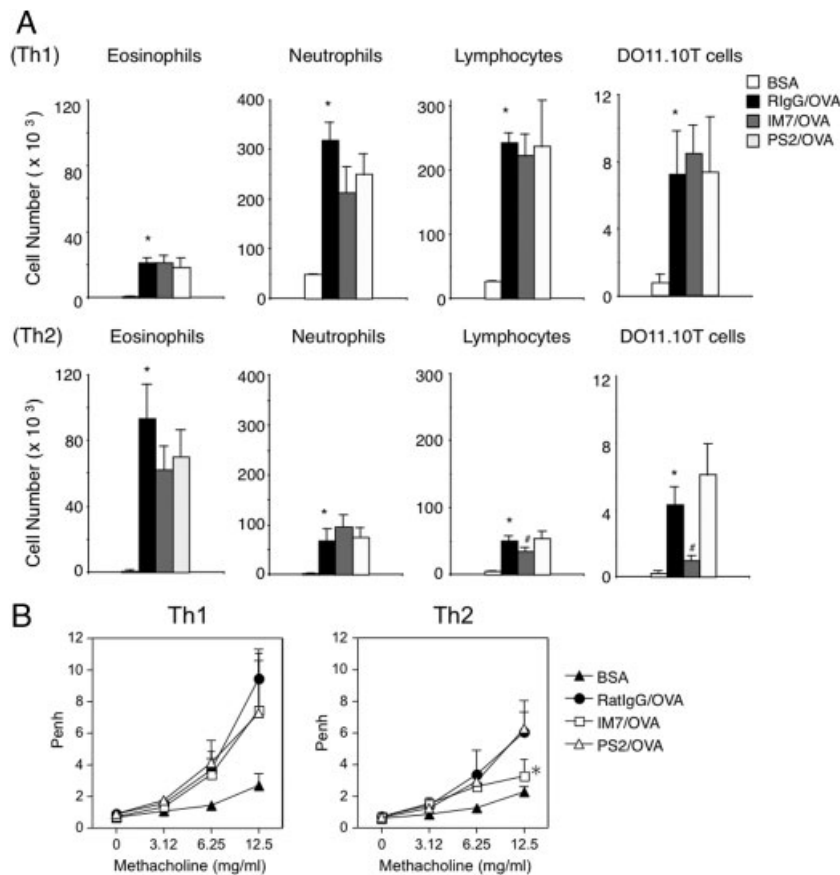


Figure 6. Effects of anti-CD44 antibody on antigen-induced cellular infiltration and AHR in the lung of Th1- and Th2-transferred mice. Differentiated Th1 and Th2 (3×10^7) cells were stained with CFSE and transferred to normal mice by intravenous injection as described in Materials and Methods. After 24 h, mice were challenged with inhaled 10% OVA or bovine serum albumin (BSA) for 60 min. BAL was performed 48 h after challenge and (A) the numbers of eosinophils, neutrophils, and lymphocytes in BALF were counted in Th1 (upper panel)- and Th2 (lower panel)-transferred mice, respectively. The number of CFSE-positive (DO11.10 T) cells was also determined by flow cytometry. (B) Three hundred micrograms of anti-CD44 mAb (IM7) (IM7/OVA), anti-CD49d mAb (PS2/OVA), or RatIgG (RlgG/OVA) was injected with differentiated Th1 and Th2 cells. AHR was evaluated by Penh as described in Materials and Methods. Data represent mean $\pm$ SEM. Mean values from 7 to 10 mice per group are shown, * $p < 0.05$ compared with BSA-challenged mice; # $p < 0.05$ compared with RatIgG-treated/OVA-challenged mice. Significance was evaluated by the Mann–Whitney U-test.

of macrophages in the BALF was similar in adoptive transfer model between WT and CD44KO CD4⁺ cells. Defects in CD44-deficient macrophages migration to the lung were previously described following intranasal infection with *Mycobacterium tuberculosis* [28] and exposure to inhaled lipopolysaccharides [27]. Taken together, recruitment of macrophages to the lung is, in part, dependent on CD44.

Although most blood cells are CD44⁺, only small numbers use it to recognize HA. We recently reported that HA-binding activity of CD44 is regulated by sialidase Neu1. In accordance with this finding, antigen-activated Th2 cells that more effectively bound HA expressed higher levels of Neu1 as compared with Th1 cells. In addition, the CD44KO mice used in this study could express truncated CD44 molecule. However, they were generated by deletion of exon 2 and exon 3 containing possible HA-binding site [29, 30], suggesting that the ability of the truncated CD44 potentially expressed in those mice to bind HA is gone. Therefore, our presented findings using CD44KO mice and Th1-/Th2-transferred mice strongly suggest that not only the expression,

but also the HA-binding ability of CD44, is important for the accumulation of Th2 cells in the lung.

In conclusion, our findings indicate that CD44 expressed on Th2 cells plays a critical role in the accumulation of Th2 cells in the lung and the resulting airway inflammation including the development of AHR induced by antigen challenge. Our observation suggests that CD44 could be a target molecule for the treatment of Th2-mediated airway inflammation including allergic asthma. Further investigations are required to clarify the role of CD44 in chronic airway inflammation.

Materials and methods

Animal model

BALB/c and C57BL/6 mice (female, 8–12 wk old) were obtained from Charles River Laboratory (Yokohama, Japan). DO11.10

transgenic mice (BALB/c background) were from Jackson Laboratory (Bar Harbor, ME). CD44-deficient mice on a C57BL/6 background were generated at Amgen Institute (Toronto, Canada; generously provided by Dr. Tak W. Mak from the University Health Network in Toronto, Canada) and were characterized previously [29, 31]. We used female mice, 8–12 wk old, bred in the experimental animal center of Kagawa University and Kawasaki Medical School. Mice were sensitized by intraperitoneal injections of 500 µg Derf allergen (GREER Laboratories, Lenoir, NC) with 2 mg alum on day 0 and day 14. The mice were then challenged by intranasal administration of 800 µg Derf solution on day 29. Negative control animal was injected with phosphate-buffered saline (PBS) plus alum and exposed to PBS in a similar manner. All experiments in this study were approved by the institutional animal care and use committee of Kagawa University and Kawasaki Medical School.

Bronchoalveolar lavage and AHR

Bronchoalveolar lavage was obtained by washing the lungs with 4×1 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 re-suspended in 1 mL of PBS. The number of BALF cells was counted by cell counter. Cytospin slides were stained with Diff-Quik (Sysmex, Kobe, Japan). Differential cell counts were carried out on at least 400 cells. Since many techniques have been developed to evaluate airway function in murine models, we employed two methods among them that enable to use conscious mice to investigate AHR. The airway resistance (sRaw) in conscious mice was measured with a two-chambered, double-flow plethysmograph system (Pulmos; M.I.P.S, Osaka, Japan) as previously described [16]. Enhanced pause (Penh) was measured with unrestrained whole body plethysmography as described previously (WBP system, Buxco, Wilmington, NC) [32]. Mice were challenged with aerosolized PBS or acetyl- β -methylcholine chloride (Mch) (Sigma, St. Louis, MO) in increasing concentrations (1.5–50 or 3.12–12.5 mg/mL) for 3 min and readings were taken and averaged for 3 min from 1 min after each nebulization. AHR was expressed as the concentration of methacholine required to provoke a doubling of sRaw (PC200).

Cell transfer and challenge procedures

CD4⁺ T cells were prepared from spleen cells of Derf-immunized C57BL/6- or CD44-deficient mice using a CD4⁺ T cell positive selection isolation kit (Miltenyi Biotec, Gladbach, Germany). The purity of the obtained CD4⁺ T cells was over 95%. Five million CD4⁺ T cells were intravenously injected into the tail vein of naïve C57BL/6 recipient mice. Twenty-four hours after cell transfer, the recipient mice were challenged by intranasal administration of 800 µg Derf solution. In some experiments, Th1- and Th2-polarized cells were used for a Th transfer model. Th1 and Th2 cells were obtained as described previously [13]. Briefly,

OVA-specific naïve CD4⁺ T cells were isolated from the spleen of mice expressing the transgene for DO11.10 TCR $\alpha\beta$ using a CD4⁺ T cell isolation kit (Miltenyi Biotec). Cells were cultured in the presence of 100 µg/mL OVA, 10 U/mL IL-2 (BD Biosciences, San Jose, CA), and X-ray-irradiated splenocytes of BALB/c mice. For Th1 phenotype development, IL-12 (10 U/mL, PeproTech, Rocky Hill, NJ) and anti-IL-4 mAb (1 µg/mL, BD Biosciences) were added, and for Th2 phenotype development, IL-4 (10 U/mL, PeproTech) and anti-IL-12 mAb (1 µg/mL, BD Biosciences) were used. To determine the integrity of polarization, cells were activated by anti-CD3 mAb (1455-2C11; BD Biosciences), and cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA) in the resulting culture supernatants. Before transfer, polarized Th1 and Th2 cells were stained with a fluorescein-based dye, 5-(and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Invitrogen, Carlsbad, CA), as described previously [13]. Twenty-four hours after cell transfer, mice were challenged with aerosolized 10% OVA dissolved in saline. For blocking studies, before transfer, Th cells were pre-incubated with 300 µg rat anti-mouse CD44 mAb (IM7) [33], rat anti-mouse CD49d mAb (PS2) [34], or control rat IgG.

ELISA

Amounts of IFN- γ , IL-5, IL-13, eotaxin (CCL11), TARC (CCL17), and IP-10 (CXCL10) (R&D Systems, Minneapolis, MN) in the BALF were measured using ELISA. The detection limits were 2.0, 2.0, 1.5, 3.0, 5.0, and 4.2 pg/mL for IFN- γ , IL-5, IL-13, eotaxin, TARC, and IP-10, respectively. The Derf-specific serum IgE, IgG1, and IgG2c were measured by ELISA as previously described [17], using biotin-conjugated antibodies against IgE (Serotec, Raleigh, NC), IgG1 (Bethyl, Montgomery, TX), or IgG2c (Bethyl), and streptavidin-horse radish peroxidase (Invitrogen, Carlsbad, CA). The ELISA was developed with tetramethylbenzidine substrate. The Derf-specific serum Ab levels were expressed as relative absorbance units (optical density at 450 nm). Serum dilutions used in these ELISA were $\times 50$ for IgE, $\times 10\,000$ for IgG1, and $\times 100$ for IgG2c.

Messenger RNA expression

Total RNA was extracted from in vitro-differentiated OVA-specific Th1 and Th2 cells. After reverse transcription using oligo(dT)12–18 primer and ReverTra ACE (Toyobo, Osaka, Japan), quantitative real-time RT-PCR was performed using Assay-on-Demand™ Gene Expression Products (TaqMan® MGB probes) with an ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA). To detect the expression of mRNA for total CD44, CD44 transcript variant 1, 3, 5, and 6, a primer/probe set harboring exon 2 to 3, 7 to 8, 5 to 16, 5 to 13, and 5 to 14 was employed, respectively. Primer/probe sets harboring exon 3 to 4 of sialidase 1 and exon 1 to 2 of sialidase 3 were also used.

Flow cytometry

Th cells were tested for HA binding by flow cytometry after staining with fluorescein-conjugated HA (FL-HA) [20]. As a specificity control, cells were also incubated with the CD44 blocking antibody KM81 (Cedarlane, Ontario, Canada), followed by staining with FL-HA. Cell surface expression of CD44 and CD49d was examined by direct immunofluorescence using a flow cytometer. Flow cytometric analysis was performed by gating the lymphocyte population on the basis of their relative size (forward light scatter) and granularity (side angle scatter). BALF cells were stained with fluorescein isothiocyanate-anti-T1/ST2 (MD Biosciences, Zurich, Switzerland) as a Th2 cell surface marker [35], phycoerythrin-anti-CXCR3 (BD Biosciences), or phycoerythrin-anti-Tim-3 (cBioscience, San Diego, CA) as a Th1 cell surface marker [36, 37], allophycocyanin (APC)-anti-CD4 (BD Biosciences), and peridinin–chlorophyll–protein complex (PerCP) anti-CD3 (BD Biosciences). The number of CFSE-positive cells was also determined by flow cytometry.

Statistical analysis

All data are expressed as mean $\pm$ standard error (SEM). The Kruskal–Wallis test was used to compare values of different groups. In cases with a significant difference between groups, inter-group comparisons were assessed using the Mann–Whitney *U* test. Differences with probability values of less than 0.05 were considered significant.

Acknowledgements: CD44-deficient mice on a C57BL/6 background were generously provided by Dr. Tak W. Mak from the University Health Network in Toronto, Canada. This work was supported in part by Core Research for Evolutional Science and Technology of the Japan Science and Technology Agency: Grants-in-Aid for Scientific Research, Japan: and the Project Research Grants from Kawasaki Medical School.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

- Lesley, J., Hyman, R. and Kincade, P. W., CD44 and its interaction with the extracellular matrix. *Adv. Immunol.* 1993. **54**: 271–335.
- DeGrendele, H. C., Estess, P., Picker, L. J. and Siegelman, M. H., CD44 and its ligand hyaluronate mediate rolling under physiologic flow: a novel lymphocyte-endothelial cell primary adhesion pathway. *J. Exp. Med.* 1996. **183**: 1119–1130.
- Kincade, P. W., Zheng, Z., Katoh, S. and Hanson, L., The importance of cellular environment to function of the CD44 matrix receptor. *Curr. Opin. Cell Biol.* 1997. **9**: 635–641.
- Miyake, K., Underhill, C. B., Lesley, J. and Kincade, P. W., Hyaluronate can function as a cell adhesion molecule and CD44 participates in hyaluronate recognition. *J. Exp. Med.* 1990. **172**: 69–75.
- DeGrendele, H. C., Estess, P. and Siegelman, M. H., Requirement for CD44 in activated T cell extravasation into an inflammatory site. *Science* 1997. **278**: 672–675.
- Mohamadzadeh, M. H., DeGrendele, H., Arizpe, H., Estess, P. and Siegelman, M. H., Proinflammatory stimuli regulate endothelial hyaluronan expression and CD44/HA-dependent primary adhesion. *J. Clin. Invest.* 1998. **101**: 97–108.
- Nandi, A., Estess, P. and Siegelman, M. H., Hyaluronan anchoring and regulation on the surface of vascular endothelial cells is mediated through the functionally active form of CD44. *J. Biol. Chem.* 2000. **275**: 14939–14948.
- Katoh, S., Maeda, S., Fukuoka, H., Wada, T., Moriya, S., Mori, A., Yamaguchi, K. et al., A crucial role of sialidase Neu1 in hyaluronan receptor function of CD44 in T helper type 2-mediated airway inflammation of murine acute asthmatic model. *Clin. Exp. Immunol.* 2010. **161**: 233–241.
- Wills-Karp, M., Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* 1999. **17**: 255–281.
- Mosmann, T. R. and Coffman, R. L., TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 1989. **7**: 145–173.
- Martin, R. M., Brady, J. L. and Lew, A. M., The need for IgG2c specific antiserum when isotyping antibodies from C57BL/6 and NOD mice. *J. Immunol. Methods* 1998. **212**: 187–192.
- Guan, H., Nagarkatti, P. S. and Nagarkatti, M., Role of CD44 in the differentiation of Th1 and Th2 cells: CD44-deficiency enhances the development of Th2 effectors in response to sheep RBC and chicken ovalbumin. *J. Immunol.* 2009. **183**: 172–180.
- Mori, A., Ogawa, K., Someya, K., Kunori, Y., Nagakubo, D., Yoshie, O., Kitamura, F. et al., Selective suppression of Th2-mediated airway eosinophil infiltration by low-molecular weight CCR3 antagonists. *Int. Immunol.* 2007. **19**: 913–921.
- Robinson, D., Hamid, Q., Bentley, A., Ying, S., Kay, A. B. and Durham, S. R., Activation of CD4+T cells, increased Th2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. *J. Allergy Clin. Immunol.* 1993. **92**: 313–324.
- Umetsu, D. T., McIntire, J. J., Akbari, O., Macaubas, C. and DeKruyff, R. H., Asthma: an epidemic of dysregulated immunity. *Nat. Immunol.* 2002. **3**: 715–720.
- Katoh, S., Matsumoto, N., Kawakita, K., Tominaga, A., Kincade, P. W. and Matsukura, S., A role for CD44 in an antigen-induced murine model of pulmonary eosinophilia. *J. Clin. Invest.* 2003. **111**: 1563–1570.
- Katoh, S., Ishii, N., Nobumoto, A., Takeshita, K., Shu-Yan, D., Shinonaga, R., Niki, T. 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.
- Bonder, C. S., Clark, S. R., Norman, M. U., Johnson, P. and Kubes, P., Use of CD44 by CD4+Th1 and Th2 lymphocytes to roll and adhere. *Blood* 2006. **107**: 4798–4806.
- Tolc, C., Hofmann, M., Herrlich, P., and Ponta, H., Splicing choice from ten variant exons establishes CD44 variability. *Nucleic Acids Res.* 1993. **21**: 1225–1229.
- Katoh, S., Miyagi, T., Taniguchi, H., Matsubara, Y.-I., Kadota, J.-I., Tominaga, A., Kincade, P. W. et al., An inducible sialidase regulates the

- hyaluronic acid binding ability of CD44-bearing human monocytes. *J. Immunol.* 1999. **162**: 5058–5061.
- 21 Kaminuma, O., Mori, A., Ogawa, K., Nakata, A., Kikkawa, H., Naito, K., Suko, M. et al., Successful transfer of late phase eosinophil infiltration in the lung by infusion of helper T cell clones. *Am. J. Respir. Cell Mol. Biol.* 1997. **16**: 448–454.
 - 22 Huang, T. J., MacAry, P. A., Eynott, P., Moussavi, A., Daniel, K. C., Askenase, P. W., Kemeny, D. M. et al., Allergen-specific Th1 cells counteract efferent Th2 cell-dependent bronchial hyperresponsiveness and eosinophilic inflammation partly via IFN- γ . *J. Immunol.* 2001. **166**: 207–217.
 - 23 Blander, J. M., Visintin, I., Janeway, C. A., Jr. and Medzhitov, R., $\alpha(1,3)$ -fucosyltransferase VII and $\alpha(2,3)$ -sialyltransferase IV are up-regulated in activated CD4 T cells and maintained after their differentiation into Th1 and Migration into inflammatory sites. *J. Immunol.* 1999. **163**: 3746–3752.
 - 24 Xu, H., Manivannan, A., Jiang, H.-R., Liversidge, J., Sharp, P. F., Forrester, J. V. and Crane, I. J., Recruitment of IFN- γ -producing (Th1-like) cells into the inflamed retina in vivo is preferentially regulated by P-selectin glycoprotein ligand 1:P/E-selectin interactions. *J. Immunol.* 2004. **172**: 3215–3224.
 - 25 Takaoka, A., Tanaka, Y., Tsuji, T., Jinushi, T., Hoshino, A., Asakura, Y., Mita, Y. et al., A critical role for mouse CXCL chemokine(s) in pulmonary neutrophilia during Th type 1-dependent airway inflammation. *J. Immunol.* 2001. **167**: 2349–2353.
 - 26 Cui, J., Pazdziorko, S., Miyashiro, J. S., Thakker, P., Pelker, J. W., Declercq, C., Jiao, A. et al., TH1-mediated airway hyperresponsiveness independent of neutrophilic inflammation. *J. Allergy Clin. Immunol.* 2005. **115**: 309–315.
 - 27 Hollingsworth, J. W., Li, Z., Brass, D. M., Garantziotis, S., Tomberlake, S. H., Kim, A. I., Hossain, I. et al., CD44 regulates macrophage recruitment to the lung in lipopolysaccharide-induced airway disease. *Am. J. Respir. Cell Mol. Biol.* 2007. **37**: 248–253.
 - 28 Leemans, J. C., Florquin, S., Heikens, M., Pals, S. T., van der Neut, R. and van der Poll, T., CD44 is a macrophage binding site for *Mycobacterium tuberculosis* that mediates macrophage recruitment and protective immunity against tuberculosis. *J. Clin. Invest.* 2003. **111**: 681–689.
 - 29 Schmits, R., Filmus, J., Gerwin, N., Senaldi, G., Kiefer, F., Kundig, T., Wakeham, A. et al., CD44 regulates hematopoietic progenitor distribution, granuloma formation, and tumorigenicity. *Blood* 1997. **90**: 2217–2233.
 - 30 Bajorath, J., Greenfield, B., Munro, S. B., Day, A. J., and Aruffo, A., Identification of CD44 residues important for hyaluronan binding and delineation of the binding site. *J. Biol. Chem.* 1998. **273**: 338–343.
 - 31 Rafi-Janajreh, A. Q., Chen, D., Schmits, R., Mak, T. W., Grayson, R. L., Spontenberg, D. P., Nagarkatti, M. et al., Evidence for the involvement of CD44 in endothelial cell injury and induction of vascular leak syndrome by IL-2. *J. Immunol.* 1999. **163**: 1619–1627.
 - 32 Hamelmann, E., Schwarze, J., Takeda, K., Oshiba, A., Larsen, G. L., Irvin, C. G., Gelfand, E. W., Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 1997. **156**: 766–775.
 - 33 Zheng, Z., Katoh, S., He, Q., Oritani, K., Miyake, K., Lesley, J., Hyman, R. et al., Monoclonal antibodies to CD44 and their influence on hyaluronan recognition. *J. Cell Biol.* 1995. **130**: 485–495.
 - 34 Miyake, K., Weissman, I. L., Greenberger, J. S. and Kincade, P. W., Evidence for a role of the integrin VLA-4 in lympho-hemopoiesis. *J. Exp. Med.* 1991. **173**: 599–607.
 - 35 Lohning, M., Stroehmann, A., Coyle, A. J., Grogan, J. L., Lin, S., Gutierrez-Romos, J.-C., Levinson, D. et al., T1/ST2 is preferentially expressed on murine Th2 cells, independent of interleukin 4, interleukin 5, and interleukin 10, and important for Th2 effector function. *Proc. Natl. Acad. Sci. USA* 1998. **95**: 6930–6935.
 - 36 Bonecchi, R., Bianchi, G., Panina-Borgignon, P., D'Ambrosio, D., Lang, R., Borsatti, A., Sozzani, S. et al., Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1 s) and Th2 s. *J. Exp. Med.* 1998. **187**: 129–134.
 - 37 Zhu, C., Anderson, A. C., Schubart, A., Xiong, H., Imitola, J., Khoury, S. J., Zheng, X. X. et al., The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat. Immunol.* 2005. **6**: 1245–1252.

Abbreviations: AHR: airway hyperresponsiveness · Derf: *Dermatophagoides farinae* · HA: hyaluronic acid

Full correspondence: Dr. Shigeki Katoh, Department of Respiratory Medicine, Kawasaki Medical School, 577, Matsushima, Kurashiki, Okayama 701-0192, Japan
Fax: +81-(86)-464-1041
e-mail: kshigeki@med.kawasaki-m.ac.jp

Received: 20/2/2011
Revised: 3/7/2011
Accepted: 9/8/2011
Accepted article online: 23/8/2011