

Galectin-9 Inhibits CD44–Hyaluronan Interaction and Suppresses a Murine Model of Allergic Asthma

Shigeki Katoh¹, Naoki Ishii², Atsuya Nobumoto³, Keisuke Takeshita², Shu-Yan Dai³, Rika Shinonaga², Toshiro Niki², Nozomu Nishi⁴, Akira Tominaga⁵, Akira Yamauchi¹, and Mitsuomi Hirashima³

¹Department of Cell Regulation, Faculty of Medicine, Kagawa University, Kagawa, Japan; ²Research Division, GalPharma Company Limited, Kagawa, Japan; Departments of ³Immunology and Immunopathology, and ⁴Endocrinology, Faculty of Medicine, Kagawa University, Kagawa, Japan; and ⁵Division of Human Health and Medical Science, Graduate School of Kuroshio Science, Kochi University, Kochi, Japan

Rationale: Galectin-9 (Gal-9) belongs to the galectin family, which exhibits affinity for β -galactosides. Gal-9 has a variety of biological activities; however, its role in allergic inflammation is unknown.

Objectives: We evaluated the effect of a stable form of the human protein on allergic airway inflammation in a mite allergen-induced asthma model.

Methods: Human stable Gal-9 was given by intravenous injection to mice during antigen challenge. The effect of Gal-9 on airway inflammation and airway hyperresponsiveness (AHR) was then evaluated.

Measurements and Main Results: Gal-9 reduced AHR as well as Th2-associated airway inflammation. Furthermore, administration of Gal-9 as well as anti-CD44 monoclonal antibody inhibited the infiltration of peripheral blood Th2 cells into the airway. Interestingly, Gal-9 directly bound the CD44 adhesion molecule and inhibited interactions with hyaluronan (HA). Consistent with the concept that CD44–HA interactions mediate the migration of T cells into the lung, Gal-9 blocked CD44-dependent adhesion of BW5147 mouse T cells to HA.

Conclusions: We conclude that Gal-9 inhibits allergic inflammation of the airway and AHR by modulating CD44-dependent leukocyte recognition of the extracellular matrix.

Keywords: airway hyperresponsiveness; allergy; experimental model; galectin-9 ligand

Asthma is a complex disease, characterized by reversible airway obstruction, chronic airway inflammation, and airway hyperresponsiveness (AHR) (1). Accumulation of antigen-activated CD4⁺ T cells into the airway is believed to contribute to this disease (2). In the airways of individuals with asthma, CD4⁺ T cells release cytokines that regulate the recruitment of inflammatory cells, such as eosinophils and mast cells (3, 4).

CD44 is a highly glycosylated cell adhesion molecule that participates in lymphocyte adhesion to inflamed endothelium, hematopoiesis, tumor metastasis, and many other processes (5). The principal ligand for CD44 is hyaluronan (HA) (6). CD44 is expressed on a wide variety of cell types, but only a few of them actively bind HA. We previously reported that the glycosylation status of CD44 is an important determinant of ligand-binding ability (7). CD44–HA interactions can promote

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Galectin-9 (Gal-9) is a β -galactoside-binding protein that is involved in cell aggregation, adhesion, chemoattraction, activation, and apoptosis. Its role in allergic inflammation had not been reported.

What This Study Adds to the Field

Gal-9 inhibits allergic asthma in mice. The inhibitory effects of Gal-9 on allergen-induced airway inflammation appear to be due to modulation of CD44-dependent leukocyte recognition of the extracellular matrix.

extravasation and egress of antigen-activated lymphocytes on inflamed vascular beds. This interaction involves the rolling of leukocytes over endothelial cells (8). Furthermore, increased expression of HA was demonstrated on microvascular endothelial cells in response to proinflammatory stimuli, such as tumor necrosis factor- α , IL-1 β , and LPS *in vitro* (9). We recently found that anti-CD44 monoclonal antibody (mAb) treatment inhibits the development of antigen-induced airway inflammation and AHR (10).

Galectin-9 (Gal-9) is a β -galactoside-binding protein consisting of two carbohydrate recognition domains (CRD) connected by a linker peptide (11). Members of the galectin family are thought to be critical for regulating immune cell homeostasis and inflammation (12). Others have demonstrated, as have we, that Gal-9 has a variety of biological functions, such as cell aggregation, adhesion, chemoattraction, activation, and apoptosis (13–18). These findings suggest how Gal-9 may function as an immune modulator in physiologic and pathologic conditions. Recently, we have shown that Gal-9 induced apoptosis of activated but not resting human T cells (19). Zhu and colleagues have demonstrated that Gal-9 induced cell death in Th1 cells, but not in Th2 cells, in a T cell immunoglobulin domain and mucin domain (Tim)-3–dependent manner in mice (20). Furthermore, expression of Gal-9 is induced in a guinea pig model of allergic asthma (data not shown). Although exciting, these observations do not directly implicate Gal-9 in allergic inflammation of the airway.

We have now used a murine model of allergic asthma induced by transnasal administration of mite allergen and human stable Gal-9, G9NC (null), to evaluate the role of Gal-9 in these responses. By analyzing bronchoalveolar lavage fluid (BALF) and AHR, we found inhibitory effects of Gal-9 and possible mechanisms of action. Interestingly, Gal-9 specifically bound to CD44 and negatively controlled CD44–HA interactions. Consequently,

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Correspondence and requests for reprints should be addressed to Shigeki Katoh, M.D., Ph.D., Department of Cell Regulation, Faculty of Medicine, Kagawa University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan. E-mail: kshigeki@med.kagawa-u.ac.jp

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Gal-9 could represent a therapeutic target in allergic respiratory inflammation.

Some of the results of these studies have been previously reported in the form of abstracts (21, 22).

METHODS

Animal Model

Male 8-week-old BALB/c mice were sensitized by intraperitoneal injections of 500 μ g *Dermatophagoides farinae* allergen (Der) with 2 mg alum on Days 0 and 14, and were challenged by intranasal administration of 800 μ g of Der solution and aerosolized Der on Day 29. Negative control animals were injected with phosphate-buffered saline (PBS) plus alum and exposed to PBS in a similar manner. All experimental animals used in this study were under a protocol approved by the institutional animal care and use committee of Kagawa University.

BAL and AHR

BAL was performed 24 hours after the antigen challenge, and differential cell counts were performed. The specific airway resistance (sRaw) was measured (Pulmos; MIPS, Osaka, Japan) as previously described (10). AHR is expressed as the provocative concentration of methacholine (Mch) required to induce a doubling of sRaw.

Treatment

Human stable Gal-9, G9NC (null) (10, 30, or 100 μ g) or Gal-8, G8NC (null) (100 μ g) (23) was given by intravenous injection 24 hours and 1 hour before, and 8 hours after intranasal antigen challenge. Anti-CD4 mAb (GK1.5; 500 μ g) was administered by intraperitoneal injection 4 days before antigen challenge, and the depletion of CD4⁺ T cells was confirmed as previously reported (24). Anti-CD44 mAb IM7 (300 μ g) (25) or dexamethasone (3 mg/kg) were administered intraperitoneally 12 hours before intranasal antigen challenge, as previously described (10).

ELISA

Amounts of IFN- γ , IL-5, IL-13, eotaxin/CCL11, thymus and activation-regulated chemokine (TARC)/CCL17, and total serum immunoglobulin (Ig) E were measured by ELISA. To measure the Der-specific serum IgE, diluted sera were incubated in Der extract (100 μ g/ml)-coated plates followed by incubation with biotin-conjugated rat anti-mouse IgE mAb. The Der-specific serum IgE levels were expressed as relative absorbance units (optical density at 450 nm [OD450]).

Flow Cytometry

BW5147 cells were tested for HA binding by flow cytometry, as previously described (26). They were preincubated with human Gal-1, human Gal-3, G8NC (null), G9NC (null), or human stable Gal-9R65D mutant, G9R65DNC (null) (23, 27). BALF and peripheral blood cells were stained with fluorescein isothiocyanate-anti-T1/ST2 as a Th2 cell surface marker (28), phycoerythrin-anti-CXCR3 as a Th1 cell surface marker (29), allophycocyanin-anti-CD4, and peridinin chlorophyll-a protein-anti-CD3. CD44-Ig fusion protein or human IgG1 was immobilized on Dynabeads protein A (Dyna Beads, Mannheim, Germany) as previously described (7). The coated beads were incubated with fluorescein isothiocyanate-HA (FL-HA) or FL-Gal-9, and analyzed with a flow cytometer. In some experiments, the beads were first incubated with anti-CD44 mAb, KM81 (25), or galectins.

Adhesion Assay

Cells were preincubated with the blocking mAb, KM81. In some experiments, cells were preincubated with G9NC (null) or G8NC (null). Then, 4×10^4 cells were seeded on HA-coated wells and incubated for 1 hour at 37°C. Adherent cells were evaluated by WST-1 assay (Roche Diagnostics, Mannheim, Germany), as described in the manufacturer's instructions.

Histochemical Analysis

Lung paraffin sections were stained with hematoxylin and eosin and toluidine-blue, and examined under light microscopy. Periodic acid-Schiff-stained lung sections were examined as previously described (30).

Statistical Analysis

For multiple comparison of different groups, the Kruskal-Wallis test for analysis of variance was used in addition to Scheffé *F* test for comparison between the individual groups.

Details are described in the online supplement.

RESULTS

An Inhibitory Effect of Human G9NC (Null) on Mite Allergen-induced AHR and Airway Inflammation in a Murine Model of Bronchial Asthma

A mite allergen-induced murine model of asthma was exploited to investigate the effect of Gal-9 on allergen-induced AHR and airway inflammation. Two groups of mice were sensitized with either Der in PBS or PBS alone, by intraperitoneal administration, according to procedures described in METHODS. Airway reactivity was evaluated 24 hours after intranasal challenge with Der by double flow plethysmography. In the Der group, AHR was seen after aerosolized Mch challenge compared with the PBS group (Figure 1A, Der vs. PBS). Gal-9, but not Gal-8, reduced AHR to aerosolized Mch in Der-treated mice in a dose-dependent manner (Figure 1A). Numbers of inflammatory cells in the BALF were evaluated 24 hours after the transnasal allergen challenge. After exposure to Der, numbers of total leukocytes, lymphocytes, eosinophils, and neutrophils were significantly increased in BALF compared with PBS-exposed mice (Figure 1B, Der vs. PBS). Administration of Gal-9 suppressed numbers of lymphocytes and eosinophils, but not macrophages and neutrophils, in a dose-dependent manner (Figure 1B). Gal-8 did not have a significant effect on numbers of inflammatory cells in BALF (Figure 1B). Evidence of inflammatory cell infiltration and the effect of Gal-9 in this model were further investigated by histologic examination of the lung staining with hematoxylin and eosin 24 hours after intranasal challenge. Transnasal Der challenge increased numbers of mononuclear cells, eosinophils, neutrophils, but not mast cells, in the peribronchial and perivascular tissue, and treatment with Gal-9 inhibited this leukocyte infiltration (Figure 1C). Mast cells could be detected in tracheal tissue from PBS, as well as Der-challenged mice, by staining with toluidine blue (data not shown). Furthermore, to evaluate the effect of Gal-9 on mucus secretion in the airway, periodic acid-Schiff-stained lung sections were examined. Allergen-induced mucus hypersecretion of goblet cells was suppressed by treatment with Gal-9 (Figure 1D). These observations suggest that Gal-9 reduced allergen-induced AHR and mucus hypersecretion. It also suppressed migration of leukocytes, particularly lymphocytes and eosinophils, into inflamed lungs.

Gal-9 Decreases Th2 Cytokines and Chemokines in BALF, but Not Serum IgE Antibodies

To investigate the mechanisms of antiinflammatory effects of Gal-9 in allergic airways, concentrations of Th1 (IFN- γ) and Th2 (IL-5 and IL-13) cytokines in BALF were measured by ELISA 24 hours after Der challenge. Increases in IL-5 and IL-13 levels in BALF after the transnasal Der administration were suppressed by Gal-9 in a dose-dependent manner (Figure 2A). IFN- γ levels in all BALF samples were below the detection limit (< 2 pg/ml) (data not shown). Next, we found that levels of eotaxin/CCL11 and TARC/CCL17 increased in BALF by transnasal Der administration. Gal-9 inhibited elevation of these chemokines (Figure 2B). These data suggest that treatment with Gal-9 alters the production of Th2 cytokines and chemokines required for AHR, as well as mucus hypersecretion and migration of eosinophils and Th2 cells into the lung.

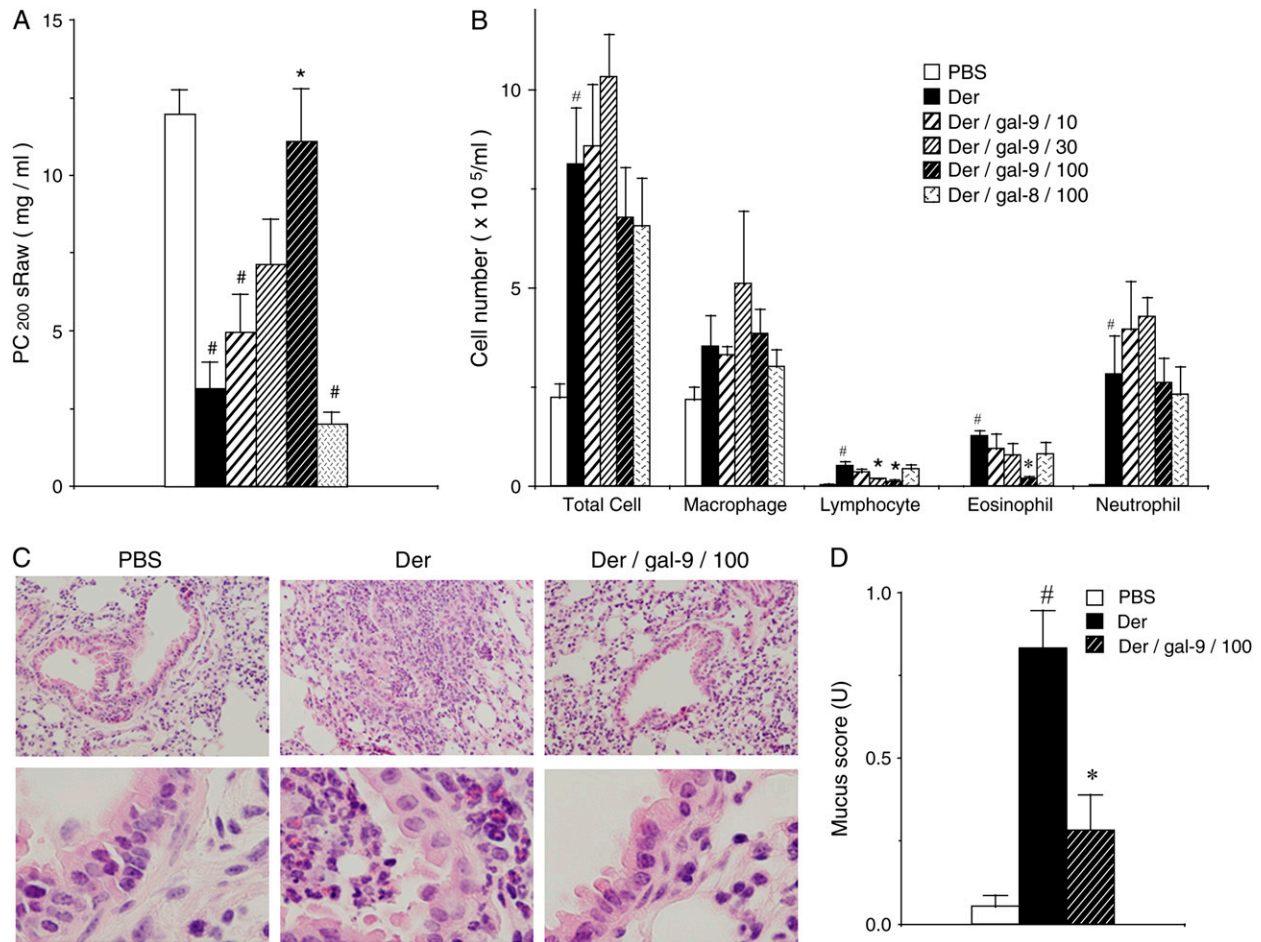


Figure 1. Galectin (Gal)-9 inhibits allergen-induced airway hyperresponsiveness (AHR) and airway inflammation. (A) The airway resistance (sRaw) to inhaled methacholine (Mch) was measured 24 hours after antigen challenge. AHR is expressed as provocative concentration of Mch required to induce a doubling of sRaw (PC₂₀₀) (mg/ml), as described in METHODS. (B) Numbers of inflammatory cells in bronchoalveolar lavage fluid were determined by Diff-Quick staining 24 hours after antigen challenge. (C) Histologic examination of formalin-fixed lung sections stained with hematoxylin and eosin (original magnification: upper panels, $\times 100$; lower panels, $\times 400$). (D) Mucus score was evaluated by examination of periodic acid-Schiff (PAS)-stained lung. A total of 50–75 airways from phosphate-buffered saline (PBS)- and *Dermatophagoides farinae* allergen (Der)-challenged mice from three independent experiments 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%). The sum of the airway scores from each lung was divided by the number of airways examined for the histologic goblet cell score (expressed as arbitrary units [U]). Data represent means $\pm$ SEM. The values shown are averaged from at least three independent experiments with 7–10 mice per group. * $p < 0.05$ compared with phosphate-buffered saline-challenged mice (PBS); * $p < 0.05$ compared with Der-challenged PBS treatment mice (Der). Der-challenged 10 μ g (Der/Gal-9/10); 30 μ g (Der/Gal-9/30); or 100 μ g (Der/Gal-9/100) of Gal-9 treatment mice; and Der-challenged 100 μ g of Gal-8 treatment mice (Der/Gal-8).

Total and Der-specific serum IgE levels were determined by ELISA in each group of mice. Those levels increased significantly in the Der group compared with the PBS group (total IgE: Der, 1449.8 ± 101.3 ng/ml; PBS, 65.9 ± 30.1 ng/ml; $p = 0.003$; Der-specific IgE: Der, 0.507 ± 0.010 OD450; PBS, 0.007 ± 0.004 OD450; $p = 0.014$). Gal-9 treatment of Der-challenged mice did not influence total (1307 ± 469.9 ng/ml) or Der-specific (0.531 ± 0.152 OD450) serum IgE levels. Ovalbumin (OVA)-specific IgE was not detected in the Der group, demonstrating the specificity of the Der-specific IgE ELISA (data not shown).

Critical Role of CD4⁺ T Cells in AHR and Airway Inflammation

CD4⁺ T cells are thought to play a critical role in this asthmatic animal model (24). Numbers of CD4⁺ T cells in BALF drastically increased (PBS, $1.6 \pm 0.8 \times 10^3$ /ml; Der, $30.9 \pm 8.4 \times 10^3$ /ml; $p < 0.01$), and treatment with Gal-9 suppressed (Der/Gal-9/100, $3.9 \pm 1.1 \times 10^3$ /ml; $p < 0.01$) this expansion. The dependence

of AHR and airway inflammation on CD4⁺ T cells was investigated by *in vivo* depletion of CD4⁺ T cells using the anti-CD4 mAb, GK1.5, as described in METHODS. Depletion of CD4⁺ T cells before antigen challenge prevented both AHR and accumulation of lymphocytes and eosinophils (Figures 3A and 3B; Der/anti-CD4mAb vs. Der/RlgG).

Others have demonstrated, as have we, that CD44 plays a critical role in the accumulation of T cells into inflammatory tissues (8, 10). Glucocorticoids remain one of the most effective antiinflammatory agents available for treatment of asthma (31), perhaps, in part, because they induce apoptosis of T cells (32). Anti-CD44 mAb and dexamethasone might have therapeutic potential for asthma by their use of different mechanisms; therefore, their effects on allergen-induced AHR and allergic airway inflammation were evaluated. Administration of anti-CD44 mAb, but not rat IgG, significantly suppressed numbers of lymphocytes and eosinophils in BALF and inhibited AHR.

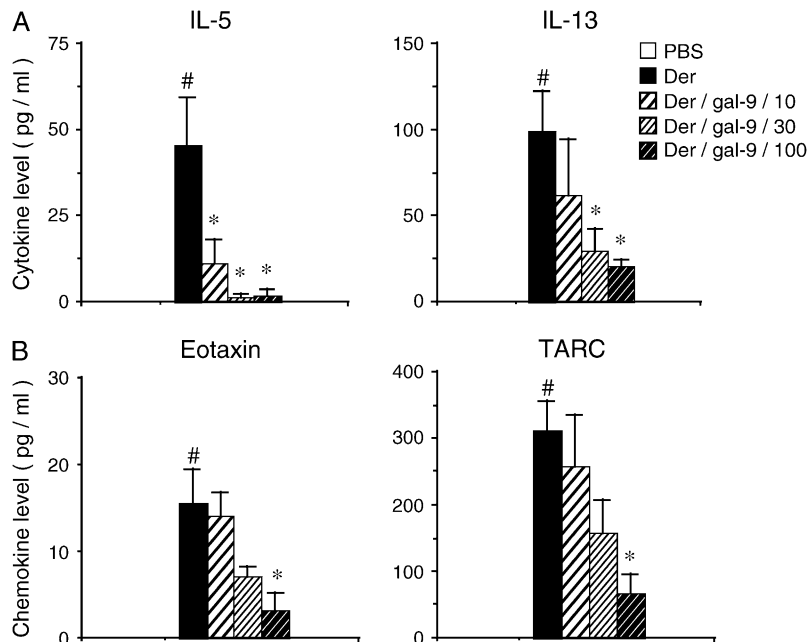


Figure 2. Gal-9 decreases Th2 cytokines and chemokines in bronchoalveolar lavage fluid (BALF). Concentrations of (A) IL-5 and IL-13, and (B) eotaxin and TARC were determined in BALF by ELISA. Data represent means $\pm$ SEM. The values shown are averaged from at least three independent experiments with 7–10 mice per group. [#] $p < 0.05$ compared with PBS-challenged mice (PBS); ^{*} $p < 0.05$ compared with Der-challenged PBS treatment mice (Der). Der-challenged 10 μ g (Der/Gal-9/10); 30 μ g (Der/Gal-9/30); or 100 μ g (Der/Gal-9/100) of Gal-9 treatment mice.

Dexamethasone treatment also significantly reduced numbers of lymphocytes and eosinophils in BALF and inhibited AHR (Figures 3C and 3D).

Expression of CXCR3 and T1/ST2 on CD4⁺ T Cells

To further investigate antiinflammatory mechanisms of Gal-9, we examined CXCR3- and T1/ST2-bearing cells in the BALF and peripheral blood. These are markers of Th1 (29) and Th2 (28) cells, respectively. Flow cytometric analysis revealed that numbers of T1/ST2-expressing CD4⁺ T cells in BALF markedly

increased in the Der group compared with PBS control animals. Gal-9 treatment reduced numbers of Th2 cells in BALF. This inhibitory effect was also observed after treatment with either anti-CD44 mAb or dexamethasone (Figure 4A). Numbers of CXCR3-positive CD4⁺ T cells in BALF slightly increased in the Der group, and all of the treatments reduced numbers of these Th1 cells in BALF (Figure 4A). Interestingly, marked increases in percentages of Th2 cells were found in peripheral blood of Gal-9-treated, as well as anti-CD44 mAb-treated mice (Figure 4B). Gal-9 treatment, as well as anti-CD44 mAb treatment,

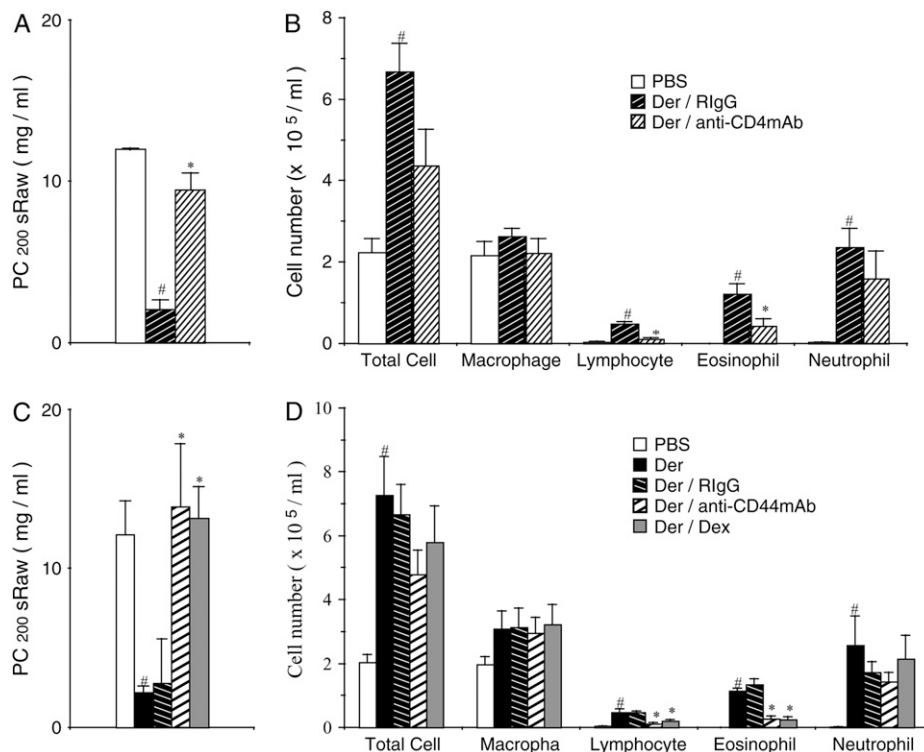


Figure 3. Effect of anti-CD4 monoclonal antibody (mAb) (GK1.5), anti-CD44 mAb (IM7), or dexamethasone on allergen-induced airway hyperresponsiveness (AHR) and accumulation of lymphocytes and eosinophils in bronchoalveolar lavage fluid (BALF). (A) and (C) AHR was measured and expressed in PC₂₀₀ sRaw (mg/ml), as described previously here. (B) and (D) Numbers of inflammatory cells in BALF were determined as described previously here. Data represent means $\pm$ SEM. The values shown are averaged from five independent experiments with 7–10 mice per group. [#] $p < 0.05$ compared with PBS-challenged mice (PBS); ^{*} $p < 0.05$ compared with Der-challenged rat immunoglobulin (Ig) G treatment mice (Der/RlgG). Der-challenged anti-CD4 mAb (Der/anti-CD4mAb); anti-CD44 mAb (Der/anti-CD44mAb); or dexamethasone (Der/Dex) treatment mice.

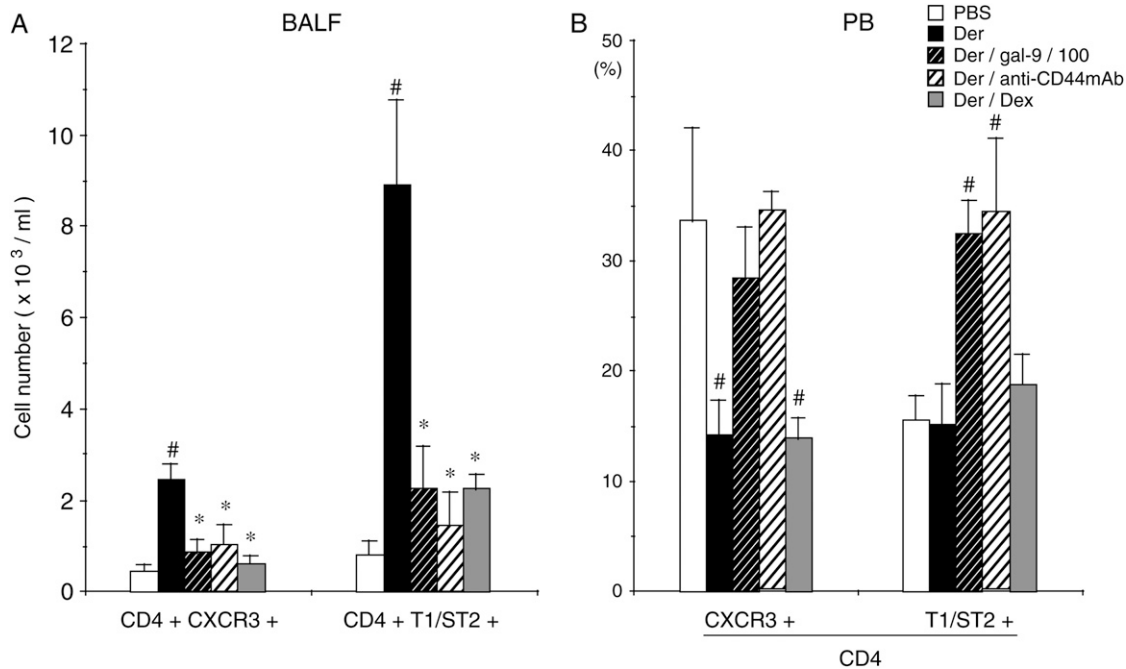


Figure 4. Expression of CXCR3 and T1/ST2 on CD4⁺ T cells in bronchoalveolar lavage fluid (BALF) and peripheral blood (PB). (A) The absolute numbers of CD4⁺ CXCR3⁺ T cells and CD4⁺ T1/ST2⁺ T cells in BALF, and (B) the frequency of CXCR3⁺ cells and T1/ST2⁺ cells on CD4⁺ T cells in PB, were determined by flow cytometry. BALF and PB cells were stained with fluorescein isothiocyanate-anti-T1/ST2 as a Th2 cell surface marker, phycoerythrin-conjugated anti-CXCR3 as a Th1 cell surface marker, allophycocyanin-conjugated anti-CD4, and peridinin chlorophyll-a protein-conjugated anti-CD3. Th1

and Th2 cells were evaluated by CXCR3 and T1/ST2 expression gating on CD3⁺ and CD4⁺ cells. Data represent means $\pm$ SEM. The values shown are averaged from five independent experiments with 10 mice per group. [#] $p < 0.05$ compared with PBS-challenged mice (PBS); ^{*} $p < 0.05$ compared with Der-challenged PBS treatment mice (Der). Der-challenged Gal-9 100 μg (Der/Gal-9/100); anti-CD44 mAb (Der/anti-CD44mAb); or dexamethasone (Der/Dex) treatment mice.

restored the peripheral blood Th1 cells reduced by Der exposure (Figure 4B). These data demonstrate that treatments with Gal-9 and anti-CD44 mAb had similar effects on Th1 and Th2 cells in BALF or peripheral blood.

Gal-9 Specifically Binds CD44 and Inhibits CD44-HA Interactions

Carbohydrate residues of CD44 are important for its ligand (HA) recognition (7, 33), and CD44 has O-linked or N-linked glycosylation, which include possible binding sites for galectins (34). Therefore, we evaluated the effect of Gal-9 on CD44-HA interactions. BW5147 murine T cells, which expressed the hemopoietic form of CD44 (CD44H), can recognize HA in a CD44-dependent manner (26), and Gal-9 inhibited this interaction in a dose-dependent manner (Figure 5A). To examine the effect

of human galectins on soluble HA binding, BW5147 cells were pretreated with several human galectins before incubation with FL-HA. Interestingly, only Gal-9 significantly inhibited HA binding to BW5147 cells. Furthermore, this inhibitory effect of Gal-9 disappeared in the presence of lactose or by mutation of CRD in Gal-9 (Gal-9R65D) (Figure 5B).

The direct binding of Gal-9 to CD44H was examined using FL-Gal-9 and CD44-Ig fusion protein, which contain CD44H (7) (see METHODS in the online supplement). FL-Gal-9 bound to CD44-Ig-coated beads in a dose-dependent manner compared with human IgG1-coated beads, and this interaction was diminished in the presence of lactose (Figure 6A). Binding of FL-Gal-9 to CD44-Ig was inhibited in a dose-dependent fashion by addition of unlabeled Gal-9 (Figure 6B). Furthermore, binding of FL-HA to CD44-Ig-coated beads was inhibited by Gal-9

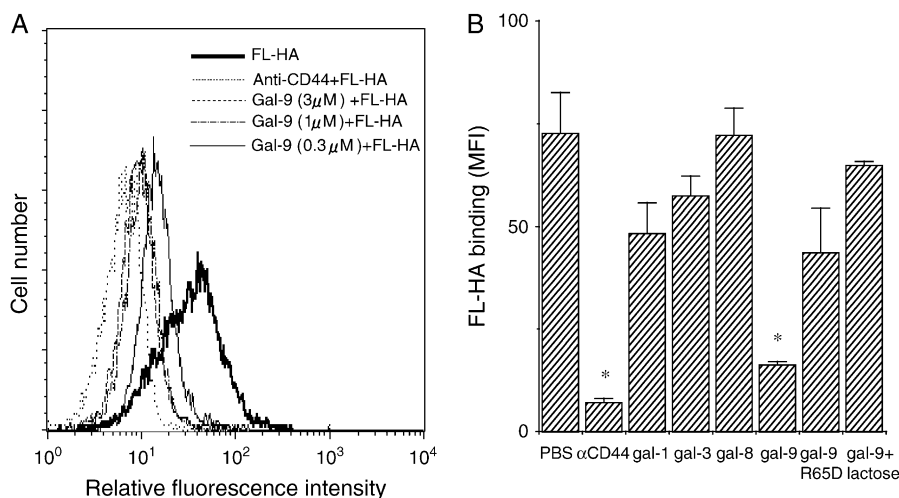


Figure 5. Gal-9 inhibits CD44-dependent soluble hyaluronan (HA) binding to BW5147 cells. HA-binding ability of BW5147 cells was evaluated by flow cytometry after staining with fluorescein isothiocyanate-conjugated HA (FL-HA). (A) Cells were incubated with the indicated concentrations of Gal-9, followed by staining with FL-HA. As a specificity control, the blocking anti-CD44 mAb (KM81) was used. These results are typical of those obtained in three independent experiments. (B) Values represent the means $\pm$ SEM of mean fluorescence intensity (MFI) from three independent experiments. ^{*} $p < 0.05$ compared with PBS treatment. Treatments: α CD44 (KM81, 1 μg), Gal-1, Gal-3, Gal-8, Gal-9, Gal-9R65D (1 μM), or Gal-9 (1 μM) + lactose (25 mM).

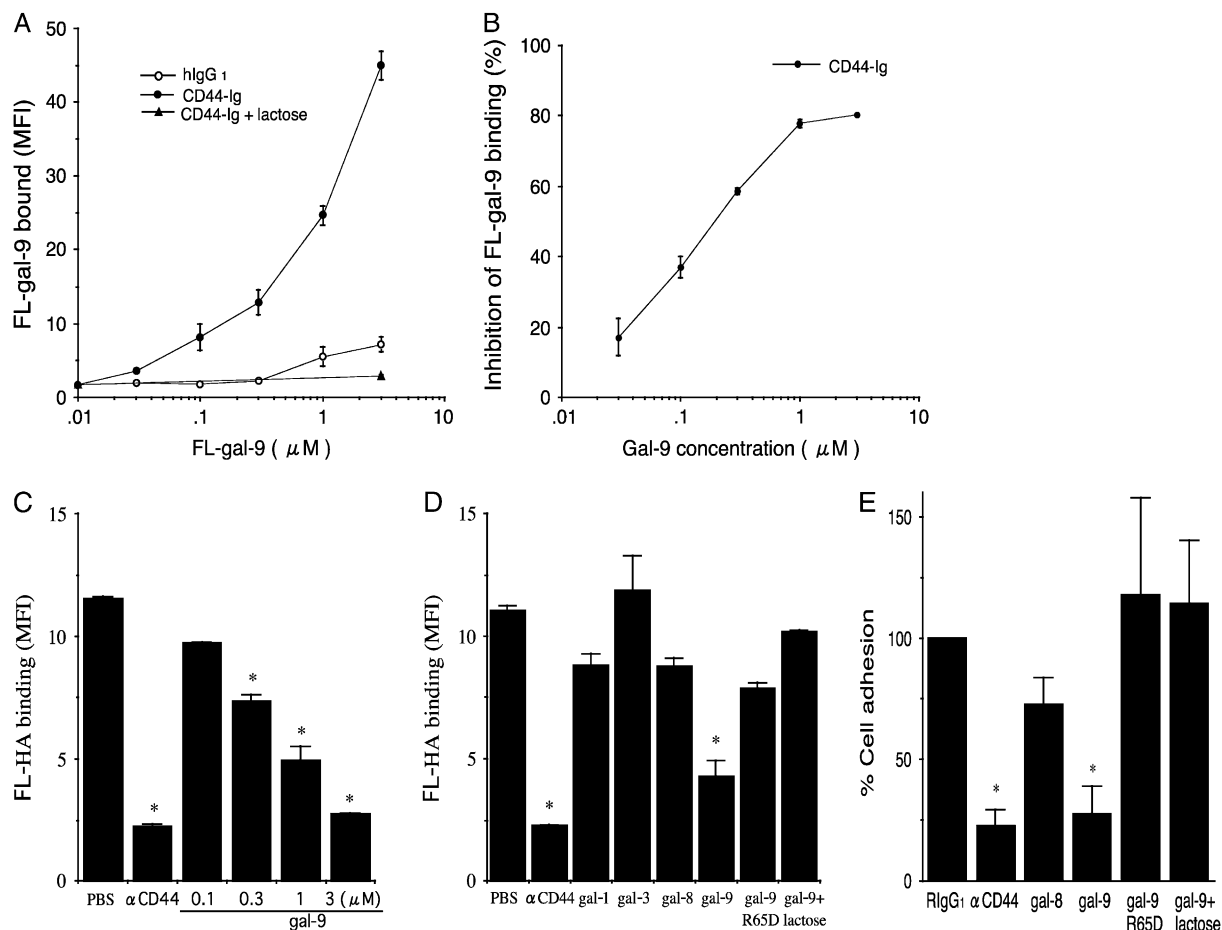


Figure 6. Gal-9 specifically binds CD44-Ig fusion protein-coated beads and inhibits CD44-Ig interaction to soluble HA and the binding of BW5147 murine T cells to immobilized HA. (A) Fluorescein isothiocyanate-conjugated Gal-9 (FL-Gal-9) was tested at the indicated concentrations for the binding to CD44-Ig-coated beads in the presence or absence of 25 mM lactose by flow cytometry. Human IgG1-coated beads (hlgG1) were used as a negative control. (B) CD44-Ig beads were first incubated with the indicated concentrations of nonlabeled Gal-9, followed by additional incubation with FL-Gal-9 (1 μM). Data represent means $\pm$ SEM. The values shown were averaged from three independent experiments. (C) CD44-Ig beads were treated with the indicated concentrations of Gal-9 and then stained with FL-HA. As a specificity control, the blocking anti-CD44 mAb (KM81) was used. * $p < 0.05$ compared with PBS treatment. Values represent the means $\pm$ SEM of MFI from three independent experiments. (D) Pretreated CD44-Ig beads were stained with FL-HA and analyzed by flow cytometry. Values represent the means $\pm$ SEM of MFI from three independent experiments. * $p < 0.05$ compared with PBS treatment. Pretreatment: αCD44 (KM81, 1 μg), Gal-1, Gal-3, Gal-8, Gal-9, Gal-9R65D (1 μM), or Gal-9 (1 μM) + lactose (25 mM). (E) BW5147 cells were tested for their adhesion to HA-coated plates in the presence or absence of the indicated pretreatment. Adherent cells were evaluated by WST-1 assay, as described in the manufacturer's instructions. * $p < 0.05$ compared with rat IgG1 (RlgG1) treatment. Pretreatment: αCD44 (KM81, 0.05 μM), Gal-8 (0.03 μM), Gal-9 (0.03 μM), Gal-9R65D (0.03 μM), or Gal-9 (0.03 μM) + lactose (25 mM).

in a dose-dependent manner (Figure 6C). Again, only Gal-9 significantly inhibited HA binding to CD44-Ig-coated beads, and this inhibitory effect of Gal-9 disappeared in the presence of lactose or mutation of CRD (Gal-9R65D) (Figure 6D).

Consistent with the concept that HA-CD44 interactions mediate the migration of T cells, BW5147 cells showed significant CD44-dependent adhesion to HA-coated plates. Gal-9, but not Gal-8, markedly blocked BW5147 adhesion to HA. Furthermore, this inhibitory effect was diminished by addition of lactose, and Gal-9R65D did not inhibit the adhesion of BW5147 cells to HA-coated plates (Figure 6E).

DISCUSSION

We are now able to report that a stable form of Gal-9 can dramatically modulate allergic asthma in an experimental animal model. That is, treatment significantly reduced AHR and prevented

eosinophil or lymphocyte infiltration into the airway. Gal-9 inhibited eosinophilic airway inflammation associated with decreased levels of the Th2 cytokines, IL-5 and IL-13, as well as the Th2-related chemokines, eotaxin and TARC. Furthermore, we show that Gal-9 can specifically recognize CD44, a molecule previously described as proinflammatory. Gal-9 interaction with CD44 interferes with CD44 binding of HA, suggesting two lectins can have opposing actions. Treatment of mice with either Gal-9 or an anti-CD44 mAb inhibited accumulation of Th2 cells into the lung.

Asthma is a syndrome with marked heterogeneity, characterized by chronic, immune-mediated responses in the lung, reversible airflow limitations, and AHR (1). CD4⁺ T cells, which are believed to be present in the airways of patients with asthma, secrete cytokines, such as IL-5 and IL-13 (2, 3). CD4⁺ T cells recognize exogenous antigens and initiate allergic inflammation

in the lung; elimination of CD4⁺ T cells abrogates the development of AHR in murine asthma (2, 24). CD4 is expressed not only by conventional CD4⁺ T cells, but also by natural killer T (NKT) cells. Recently, Akbari and colleagues demonstrated an essential role for NKT cells in producing IL-4 and IL-13 during allergen-induced AHR (35). We found that numbers of CD4⁺ T cells increased in the BALF of our experimental animals. Furthermore, depletion of CD4⁺ T cells before allergen challenge inhibited the induction of AHR and eosinophilic airway inflammation (Figures 3A and 3B). Therefore, CD4⁺ T lymphocytes and NKT cells may both contribute to the pathogenesis of asthma.

Although CD44 is expressed on most blood cells, few of them actively use it to bind HA under normal circumstances. However, the HA-binding ability of CD44 is inducible by activation of T cells (36). Expression of HA on microvascular endothelial cells is induced by proinflammatory stimuli (9), and CD44 is involved in the rolling and firm adhesion of leukocytes on endothelial cells (37). Furthermore, Bonder and colleagues demonstrated that both CD4⁺ Th1 and Th2 cells use CD44 to roll and adhere on HA immobilized on endothelium in CD44-deficient mice (38). We recently demonstrated that administration of anti-CD44 mAb suppressed AHR and inhibited accumulation of lymphocytes and eosinophils into the lung (10). We have now confirmed the beneficial effect of antibody therapy in a mite allergen-induced murine model of allergic asthma. Both anti-CD44 mAb and Gal-9 inhibited Th2 cell-mediated airway inflammation and AHR (Figures 1–3).

Intraperitoneal administration of anti-CD44 mAb (300 µg/body) significantly inhibited asthmatic responses *in vivo* (Figures 3C and 3D), whereas T cell adhesion to HA-coated plates was inhibited in the presence of anti-CD44 mAb (0.05 µM) *in vitro* (Figure 6E). On the other hand, intravenous injection of 100 µg of human G9NC (null) three times during the antigen challenge significantly inhibited accumulation of lymphocytes and eosinophils in BALF and AHR (Figures 1A and 1B), whereas 0.03 µM (1 µg/ml) of human G9NC (null) inhibited T cell adhesion to HA-coated plates (Figure 6E). Human G9NC (null) and anti-CD44 mAb were stoichiometrically similar in at least *in vitro* inhibitory potential. Physiologic concentration of Gal-9 was 31.4 (± 3.5) ng/ml in peripheral blood of normal BALB/c mice (n = 6) (data not shown). Pharmacokinetic study of intravenous administration of human G9NC (null) demonstrated that the concentration of human Gal-9 in peripheral blood 3 hours after injection of 100 µg of human G9NC (null) was 0.952 (± 0.088) µg/ml in mice (data not shown). The amounts of human G9NC (null) used in this study are reasonable for the concentration of Gal-9, which can inhibit the interaction of CD44 and immobilized HA. Intravenous administration of 100 µg of human G9NC (null) had no significant effect on peripheral blood counts at least 6 and 24 hours after the injection (*see METHODS* in the online supplement).

OVA is not a common antigen in human allergic asthma. Therefore, we used Der antigen for a murine model of allergic asthma to assess the therapeutic effect of Gal-9. The number of eosinophils in the airway of mite antigen-induced mouse asthma is often lower than that of the OVA-induced asthma model, including this study (39, 40). There was a minimum contamination of endotoxin in the Der antigen (< 0.25 EU/mg) (*see METHODS* in the online supplement). Protease activity of the Der antigen might have different effects from OVA on the airway inflammation (41).

A number of observations indicate that Gal-9 is involved in T-cell apoptosis. We found that Gal-9 induced programmed death of CD3-stimulated peripheral human CD4⁺ T cells. As with glucocorticoids, this apoptosis used the calcium-calpain-

caspase-1 pathway (19). On the other hand, Zhu and colleagues demonstrated that Gal-9 preferentially induced apoptosis of Th1 cells in a Tim-3-dependent manner (20). To investigate how Gal-9 inhibits Th2 cell-mediated airway inflammation, we examined the expression of Th1 and Th2 cell-associated markers (CXCR3 and T1/ST2, respectively) on CD4⁺ T cells in BALF and peripheral blood. Dexamethasone reduced numbers of T1/ST2-expressing CD4⁺ T cells in BALF without increasing percentages of Th2 cells in peripheral blood. Treatment with Gal-9 or anti-CD44 mAb decreased Th2 cells in BALF, while increasing percentages of Th2 cells in peripheral blood. On the other hand, dexamethasone reduced numbers of CXCR3-expressing CD4⁺ T cells in BALF, while decreasing percentages of peripheral blood Th1 cells. Treatment with Gal-9 or anti-CD44 mAb decreased Th1 cells in BALF without reducing circulating Th1 cells (Figure 4). These data suggest that dexamethasone induces apoptosis of both Th1 and Th2 cells. Treatment with either anti-CD44 mAb or Gal-9 inhibits infiltration of Th1 and Th2 cells into the airway. Therefore, Gal-9 may have therapeutic potential for asthma by using mechanisms distinct from corticosteroids.

Gal-9 inhibited not only Th2-related airway inflammation, but also AHR. Previous studies demonstrated that IL-13 induces mucus overproduction of bronchial epithelial cells and plays an essential role in allergen-induced AHR (30, 42). The inhibitory effect of Gal-9 on AHR in this study could have resulted from reduced IL-13 production and mucus secretion in the airway (Figures 1D and 2A).

Mast cells recognize allergen through high-affinity IgE receptors, and IgE is believed to build an allergen-containing complex. Allergen cross-linking of receptors leads to the activation of mast cells, with the release of numerous mediators and cytokines (4). Recent studies have demonstrated that mast cells are important for AHR (43). We evaluated mast cells in our asthmatic model, but toluidine-blue staining mast cells were not found in sites of lung inflammation (data not shown). Furthermore, Gal-9 treatment during allergen challenge did not influence serum levels of total and Der-specific IgE. Mast cells may not play an important role in the development of AHR and airway inflammation in this model.

The CD44 core protein is predicted to be 42 kD, but it has at least five motifs for asparagine-linked carbohydrate attachment, and 10 possible sites for O-linked glycosylation (34). The mature protein on most blood cells is about 90 kD, and the acidic charge is largely contributed by sialic acids (5, 44). Differential glycosylation of this molecule is sufficient to influence ligand binding, and we previously demonstrated that glycosylation of CD44 negatively regulates its interaction with HA (7, 33). Particular CD44 variants also interact with fibronectin, collagen, serglycin, and osteopontin (45–47). Carbohydrate on native Gal-9 might be recognized by CD44, but the recombinant Gal-9 used in our experiments was expressed in *Escherichia coli*. Gal-9 specifically recognized CD44-Ig fusion protein, but not human IgG1 (Figure 6A). Such a difference in the binding affinity is reasonable, because galectin has sugar (β-galactoside)-binding specificity by enhancing affinity to either branched or repeated glycans (48). In any case, we have demonstrated recognition and functional competition between these two lectins. Gal-9, but not other members of the galectin family, modulates the binding of HA to CD44H, and the addition of lactose inhibited this effect of Gal-9 (Figures 5 and 6). This is significant when considered with the large body of evidence indicating that CD44–HA interactions are proinflammatory and can mediate tumor metastasis (9, 49, 50).

We conclude that Gal-9 may have unique therapeutic potential because of its ability to modulate CD44-mediated functions, although Gal-9 could bind to other glycoproteins.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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Galectin-9 inhibits CD44-hyaluronan interaction and suppresses a murine model of allergic asthma

Shigeki Katoh, Naoki Ishii, Atsuya Nobumoto, Keisuke Takeshita, Shu-Yan Dai, Rika Shinonaga, Toshiro Niki, Nozomu Nishi, Akira Tominaga, Akira Yamauchi, and Mitsuomi Hirashima

Online Data Supplement

Methods

Animals

Male BALB/c mice (6-8 weeks old) were obtained from Charles River laboratories Japan (Yokohama, Japan). All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of Kagawa University.

Antigen Sensitization and Challenge

Mite *Dermatophagoides farinae* Allergen (Der) (GREER laboratories, Inc. Lenoir, NC) was used as antigen for this experimental model of murine asthma. LPS concentration of the Der preparation was < 0.25 EU/mg of Der (limulus turbimetric kinetic assay using a Toxnometer ET-2000, Wako, Japan). Mice were actively sensitized by intraperitoneal injections of 500 µg Der with 2 mg alum on day 0 and day 14. They were challenged by intranasal administration of 800 µg of Der solution and aerosolized Der on day 29 to induce the response. Negative control animals were injected with PBS plus alum and exposed to PBS in a similar manner.

Bronchoalveolar Lavage (BAL)

To evaluate airway inflammation, BAL was performed 24h after the antigen challenge as previously described (10). Numbers of BALF cells were 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.

Measurement of airway hyper-responsiveness (AHR)

The airway resistance (Raw) in conscious mice was measured with a two-chambered, double-flow plethysmograph system (Pulmos-I II III; M.I.P.S, Osaka, Japan) as previously described (10). Mice were challenged with aerosolized PBS or acetyl- β -methylcholine chloride (Mch) (sigma, St. Louis, MO) in increasing concentrations (1.5 to 50 mg/ml) for 3 min and readings were taken and averaged for 2 min from 1 min after each nebulization. Airway responsiveness is expressed as the provocative concentration of Mch required to induce a doubling of sRaw (PC₂₀₀).

Treatment with gal-9, anti-CD4 monoclonal antibody (mAb), anti- CD44 mAb, and dexamethasone

Human stable gal-9, G9NC (null) (10, 30, or 100 μ g) or gal-8, G8NC (null) (100 μ g) (23) was given by intravenous injection 24 h and 1h before, and 8h after intranasal antigen challenge. As a negative control, PBS was injected in the same manner. The effect of intravenous administration of gal-9 (100 μ g) on peripheral blood counts was examined 6h and 24h after injection. There were no significant change in the blood cell number and composition (data not shown). 500 μ g of anti-CD4 mAb (GK1.5, American Tissue Culture Collection) or control Rat

IgG was administered by intraperitoneal injection 4 days before antigen challenge as previously described (24). We confirmed the depletion of CD4⁺ T cells in spleen 4 days after GK1.5 treatment and 24h after antigen challenge using anti-CD4 mAb and flow cytometric analysis as previously reported (24). Rat anti-mouse CD44 mAb IM7 (25), control rat IgG (300 µg in 500 µl PBS), or dexamethasone (3mg/kg) were administered intraperitoneally 12h prior to intranasal Ag challenge as previously described (10). Endotoxin was eliminated using Cellufine ET clean L. The level of LPS in the all galectin and antibody preparations used in this study were < 0.3 EU/ml (< 0.03 ng/ml) in limulus turbimetric kinetic assay using a Toxnometer ET-2000.

Cytokine ELISA and IgE assay

Amounts of IFN-γ (Pierce Endogen, Rockford, IL), IL-5, IL-13, eotaxin/CCL11, and thymus and activation-regulated chemokine (TARC/CCL17) (R&D Systems, Minneapolis, MN) in BALF were measured using commercially available enzyme linked immunosorbent assay (ELISA) kits. Total serum IgE was measured by ELISA kit (Yamasa, Tokyo, Japan). To measure the Der-specific serum IgE, diluted sera were first incubated in Der extract (100 µg/ml), or ovalbumin (Sigma) (used as a control antigen, 100 µg/ml)-coated ELISA plates followed by incubation with biotin-conjugated rat anti-mouse IgE mAb (Serotec, Oxford, UK). Antibody was detected with streptavidin-horseradish peroxidase (HRP). The color reaction was developed using tetramethylbenzidine (TMB) as a substrate. The optical density (OD) of the reaction was determined using an automatic ELISA plate reader at 450 nm. The total serum IgE levels were expressed in ng/ml. The Der-specific serum IgE levels were expressed as relative

absorbance units (OD450).

Immunofluorescence and Flow Cytometry

BW5147 cells which expressed hematopoietic form of CD44 (CD44H) were tested for HA binding by flow cytometry after staining with fluorescein isothiocyanate (FITC)-conjugated HA (FL-HA) for 15 min at 4 °C (26). As a specificity control, cells were also incubated with the blocking anti-CD44mAb KM81 (25) for 15 min at 4 °C, followed by staining with FL-HA. To evaluate the effect of galectins on HA binding of these cells, they were pre-incubated with human gal-1, human gal-3, human stable gal-8, G8NC (null), G9NC (null), or human stable gal-9R65D mutant, G9R65DNC (null) (23, 27) for 15 min at 4 °C, followed by staining with FL-HA. In some experiments, cells were incubated with G9NC (null) in the presence of 25 mM lactose. HA-binding were examined by direct immunofluorescence (MFI; mean fluorescence intensity) using a FACSCalibur® flow cytometer (BD Biosciences). BALF and peripheral blood cells were stained with FITC-anti-T1/ST2 (MD Biosciences, Zurich, Switzerland) as a Th2 cell surface marker (28), phycoerythrin (PE)-conjugated anti-CXCR3 (R&D systems) as a Th1 cell surface marker (29), allophycocyanin (APC)-conjugated anti-CD4, and peridinin chlorophyll-a protein (PerCP)-conjugated anti-CD3 (BD Biosciences). Th1 and Th2 cells were evaluated by CXCR3 and T1/ST2 expression gating on CD3-, and CD4-positive cells.

Binding of HA and galectin-9 to Immobilized CD44-Ig

125 µg of CD44-Ig fusion protein (CD44-Ig) which contain CD44H (7) was agitated with 100 µl of Dynabeads Protein A (DynaL Biotech, Oslo, Norway) for 1 h at 4 °C. As a negative control, human IgG₁ was used instead of CD44-Ig. After washing with PBS, the coated beads

were blocked with 1% FCS/PBS and then incubated with FL-HA or FL-gal-9 for 15 min at 4 °C in the presence or absence of 25 mM lactose. In some experiments, the beads were first incubated with KM81, or galectins for 15 min at 4 °C and then stained with FL-HA or FL-gal-9, as described above. After a final wash, the beads were analyzed with FACSCalibur®.

Cell adhesion assay

96-well culture plates were coated with 50 µl of HA (1 mg/ml; Sigma) for 24h at 4 °C. The plates were washed with PBS three times and blocked with PBS containing 1% BSA for 2 h at 37 °C. They were then washed three times with RPMI containing 0.02 % BSA and used immediately. Cells were pre-incubated with the blocking mAb, KM81 for 30 min at RT. As a negative control, iso-type matched Rat IgG₁ was used. In some experiments, cells were pre-incubated with G9NC (null) or G8NC (null). Then 4×10^4 cells were seeded on HA-coated wells and incubated for 1 h at 37 °C. Adherent (viable) cells were evaluated by WST-1 assay (Roche Diagnostics, Mannheim, Germany). Absorbance was read at 450 nm with a plate reader as described in the manufacturer's instructions.

Histochemical analysis

Tissue sections were affixed to microscope slides, and deparaffinized. The slides were stained with hematoxylin-eosin, Giemsa-May-Grunwald, toluidine-blue, and examined under light microscopy. Periodic acid-Schiff (PAS)-stained lung sections were examined as previously described (30). Briefly, fifty to 75 airways from PBS- and Der-challenged mice from three independent experiments were categorized according to the abundance of PAS goblet cells and assigned numerical scores (0: < 5% goblet cells; 1: 5 to 25%; 2: 25 to 50%; 3: 50 to 75%; 4: >

75%). The sum of the airway scores from each lung was divided by the number of airways examined for the histologic goblet cell score (expressed as arbitrary units; U).

Statistical Analysis

For multiple comparison of different groups, the Kruskal-Wallis test for ANOVA (analysis of variance) was used in addition to the Scheffe *F* test for comparison between the individual groups. The results are expressed as mean $\pm$ SEM, and p values less than 0.05 were considered statistically significant.