A role for CD44 in an antigen-induced murine model of pulmonary eosinophilia

Shigeki Katoh,1 Nobuhiro Matsumoto,1 Kumiko Kawakita,1 Akira Tominaga,2 Paul W. Kincade,3 and Shigeru Matsukura1

1Third Department of Internal Medicine, Miyazaki Medical College, Miyazaki, Japan
2Department of Medical Biology, Kochi Medical School, Kochi, Japan
3Immunobiology and Cancer Program, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma, USA

Previous studies established that IL-5–producing CD4+ T cells play a pivotal role in allergic respiratory inflammation. It was also reported that CD4+ T cells express higher levels of CD44 in the airway than in peripheral blood of patients with allergic respiratory diseases. We have used experimental pulmonary eosinophilia induced in mice by *Ascaris suum* (Asc) extract to investigate the role of CD44 in the development of allergic respiratory inflammation. Intraperitoneal administration of anti-CD44 mAb prevented both lymphocyte and eosinophil accumulation in the lung. Anti-CD44 mAb also blocked antigen-induced elevation of Th2 cytokines as well as chemokines (CCL11, CCL17) in bronchoalveolar lavage fluid (BALF). Treatment with anti-CD44 mAb inhibited the increased levels of hyaluronic acid (HA) and leukotriene concentrations in BALF that typically result from antigen challenge. Anti-CD44 mAb also blocked antigen-induced airway hyperresponsiveness. An anti-CD44 mAb (IM7) inhibited the HA-binding ability of splenocytes associated with decreased levels of CD44. Soluble CD44 levels in serum were increased in Asc-challenged IM7–treated mice, but not in KM201-treated mice, compared with Asc-challenged rat IgG–treated mice. Ab’s that block CD44-HA binding reduced allergic respiratory inflammation by preventing lymphocyte and eosinophil accumulation in the lung. Thus, CD44 may be critical for development of allergic respiratory inflammation.

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ligand-binding site, directly blocks HA binding to CD44 (14), and the IM7 mAb induces substantial receptor shedding from the cell surface (9). We conclude that CD44 is mechanistically involved in allergic respiratory responses and could represent a therapeutic target.

**Methods**

**Animals.** C57BL/6 mice (8–12 weeks old) were obtained from CLEA Japan Inc. (Tokyo, Japan). All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of Miyazaki Medical College.

**Parasite extract.** Freeze-dried adult female *Ascaris suum* (Asc) worm preparations were kindly provided by Yukitumi Nawa (Miyazaki Medical College, Miyazaki, Japan). They were homogenized in PBS and centrifuged at 100,000 g for 20 min at 4°C. The supernatants were collected and adjusted to a protein concentration of 5 mg/ml for transnasal sensitization.

**Antigen challenge and BAL.** Asc sensitization was carried out according to the procedure described previously by Nogami et al. (15), with minor modification. Mice were anesthetized by diethyl ether, and 200 µg of the Asc solution was applied intranasally. The procedures were repeated for 2 consecutive days per week for 2 weeks. The mice were challenged by intranasal administration of 400 µg Asc solution on day 22 to induce the response. In some experiments, *Dermatophagoides farinae* allergen (Der) (GREER Laboratories Inc., Lenoir, North Carolina, USA) was used as antigen for transnasal sensitization instead of Asc. The mice were challenged with aerosolized Der (800 µg) on day 22 to induce the response. Twenty-four hours after the antigen challenge, mice were sacrificed by diethyl ether and peripheral blood was recovered by retro-orbital bleeding and the tracheas exposed for bronchoalveolar lavage (BAL). BALF was obtained by washing the lungs with 5 × 1 ml of PBS. Bone marrow was rinsed out of the right femur. Numbers of BALF cells and bone marrow cells were counted by cell counter. Cytospin or smear slides were stained with Diff-Quik (Midorijuji, Kobe, Japan). Differential cell counts were carried out on at least 500 cells.

**Measurement of airway hyperresponsiveness.** The airway resistance (Raw) in conscious mice was measured with a two-chambered, double-flow plethysmograph system (Pulmos-I II III, MLPS, Osaka, Japan), as described previously (16). The specific Raw (sRaw) was measured through detection by the respective sensors of airflow supplied to the front and rear chambers according to the method described by Pennock et al. (17). Mice were challenged with aerosolized PBS or methacholine in increasing concentrations (3.125–50 mg/ml) for 2 min, and readings were taken and averaged for 2 min from 2 min after each nebulization.

**Administration of CD44 mAb’s.** Rat anti-mouse CD44 mAb’s KM201 (IgG1) (18) and IM7 (IgG2b) (19) were purified from their respective hybridomas using a protein G-Sepharose affinity column (Pharmacia Biotech AB, Uppsala, Sweden). Anti-CD44 mAb’s or control rat IgG (300 µg in 500 µl saline) was administered intraperitoneally 12 h before intranasal antigen challenge.

**Quantification of cytokines and chemokines in BALF.** Amounts of IL-4, IL-5 (Endogen Inc., Woburn, Massachusetts, USA), IFN-γ, eotaxin/CCL11, and thymus and activation-regulated chemokine (TARC; CCL17) (R & D Systems Inc., Minneapolis, Minnesota, USA) in BALF were measured using commercially available ELISA kits. Concentration of cysteinyl leukotrienes was assayed using leukotriene enzyme immunoassay (ELIA) kits (Pharmacia Biotech AB). The rat antiserum against leukotriene had the following cross-reactivities: LTC4 (100%), LTD4 (100%), LTE4 (70%), and LTB4 (0.3%), but not prostaglandin PGD2 (< 0.006), prostaglandin PGE2 (< 0.006), or thromboxane B2 (< 0.006). HA was measured by the sandwich-binding protein assay kit (Chugai Pharmaceutical Co. Ltd., Tokyo, Japan). The sensitivity of this kit was 10 ng/ml. The detection limits were 7.8, 7.8, 1.0, 7.8, 7.8, and 60 pg/ml for IL-4, IL-5, IFN-γ, TARC, eotaxin, and leukotrienes, respectively.

**Quantification of soluble CD44 in serum and BALF.** Amounts of soluble CD44 in serum and BALF were measured using ELISA as described previously with minor modifications (20). Briefly, after coating with 1 µg/ml of mAb KM114 at 4°C overnight, ELISA plates were washed with PBS containing 0.1% Tween and blocked with 5% nonfat dry milk. Standards or unknowns were then added in duplicate wells and incubated at 37°C for 30 min. Captured proteins were then incubated with biotinylated mAb IM7, and after washing, they were incubated with streptavidin-HRP. The ELISA was developed with substrate (sodium acetate buffer containing tetramethylbenzidine). Absorbance was then read at 450 nm with a plate reader. A soluble fusion protein containing the extracellular domain of murine hemopoietic CD44 and the hinge, CH2 and CH3 regions of human IgG1 (21), was used as a standard.

**Immunofluorescence and flow cytometry.** Splenocytes were removed from mice treated with anti-CD44 mAb’s, KM201 and IM7 (300 µg), or normal rat IgG (300 µg), and they were depleted of red blood cells. Cell surface expression of CD44 was examined by using anti-CD44 mAb, KM114-FITC (rat IgG1) (18). Epitope of CD44 detected by KM114 was not cross-reacted from those detected by IM7 or KM201 (data not shown). Rat IgG1-FITC was used as a isotype-matched control. Alveolar cells in BALF were obtained from mice treated with anti-CD44 mAb’s (KM201 and IM7) or normal rat IgG. They were stained with KM114-FITC and F4/80-PE (CalTag Laboratories Inc., Burlingame, California, USA), and then expression of CD44 on alveolar macrophages was compared among each experimental group gating on F4/80-positive cells. Cells were tested for HA binding by flow cytometry after staining with fluorescein-conjugated HA.
In the presence or absence of anti-CD44 mAb, IRAWB14, which is known to have an enhancing effect on the HA-binding ability of cells (23). As a specificity control, cells were also incubated with the blocking Ab KM114, followed by staining with FL-HA. Cell surface expression of CD44 and HA-binding were examined by direct immunofluorescence using an EPICS XL flow cytometer (Beckman Coulter Corp., Miami, Florida, USA). 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) on splenocytes.

**Histochemical analysis.** Tissue sections, 5-mm thick, were affixed to microscope slides and deparaffinized. The slides were stained with hematoxylin and eosin and examined under light microscopy.

**Statistical analysis.** All data were expressed as mean plus or minus SEM. Differences between two experimental groups were examined for statistical significance using the two-tailed Mann-Whitney U test. For multiple comparison of different groups, the Kruskal-Wallis test for ANOVA was significant. We then used the Scheffe F test for comparison between the individual groups. Differences associated with P values less than 0.05 were considered significant.

**Results**

Anti-CD44 mAb's inhibit eosinophil and lymphocyte accumulation in BALF but do not change numbers of eosinophils in bone marrow. Two groups of mice were sensitized with either Asc in PBS or PBS transnasally according to procedures described in Methods. The number of inflammatory cells in BALF was evaluated 24 h after the last transnasal antigen administration. After exposure to Asc, total leukocytes, eosinophils, and lymphocytes were significantly increased in BALF compared with PBS-exposed mice. Administration of rat IgG (RIGG; 300 µg/mouse) was without influence (Figure 1a; Asc/RIGG), but anti-CD44 mAb’s (KM201 or IM7, 300 µg/mouse) given 12 h before the last Asc challenge significantly suppressed numbers of eosinophils and lymphocytes (P < 0.05) (Figure 1a; Asc/KM201 or Asc/IM7). Administration of a lower dose (30 µg/mouse) of IM7 partially inhibited the infiltration of eosinophils and lymphocytes (Figure 1a; Asc/IM7/30).

**Figure 1**

Anti-CD44 mAb blocks eosinophil and lymphocyte accumulation in BALF but does not affect numbers of eosinophils in bone marrow. The number of inflammatory cells in BALF (a) and percentages of eosinophils in bone marrow and peripheral blood (b) was determined 24 h after allergen challenge as described in Methods. Data represent mean ± SEM. The values shown are averaged from three to five independent experiments. *Significant differences (P < 0.05) between Asc-challenged mice (Asc) and PBS-challenged mice (PBS). *Significant differences (P < 0.05) between Asc-challenged anti-CD44 mAb-treatment mice (Asc/KM201, Asc/IM7, or Asc/IM7/30) and Asc-challenged RIGG-treatment mice (Asc/RIGG).

**Figure 2**

Anti-CD44 mAb blocks cytokine production in BALF. Concentrations of IL-4, IL-5, and IFN-γ in BALF were assessed 24 h after allergen challenge as described in Methods. Data represent mean ± SEM. The values shown are averaged from five independent experiments. *Significant differences (P < 0.05) between Asc-challenged mice (Asc) and PBS-challenged mice (PBS). *Significant differences (P < 0.05) between Asc-challenged anti-CD44 mAb-treatment mice (Asc/KM201 or Asc/IM7) and Asc-challenged RIGG-treatment mice (Asc/RIGG).
inflammatory cells in BALF (Figure 1a; Asc/IM7/30). Evidence of inflammatory cell infiltration and the effect of anti-CD44 were further investigated by histologic examination. Transnasal Asc treatment increased numbers of eosinophils and lymphocytes in the peribronchial and perivascular tissue, while treatment with anti-CD44 (KM201 or IM7), but not RlgG, inhibited this leukocyte infiltration (data not shown). Exposure to Asc significantly increased eosinophil percentages in bone marrow, and this response was unaffected by treatment with anti-CD44. Interestingly, marked eosinophilia was found in peripheral blood of KM201-treated, but not IM7- or RlgG-treated mice (P < 0.05) (Figure 1b). These observations suggest that migration of leukocytes, particularly lymphocytes and eosinophils, into inflamed lung tissue is a CD44-dependent process.

Anti-CD44 mAb's decrease Th2 cytokines and chemokines in BALF. To investigate the possible roles of various cytokines in allergic airway inflammation, concentrations in BALF of Th1 (IFN-γ) and Th2 (IL-4 and IL-5) cytokines were measured by ELISA 24 h after the last Asc challenge. After the last transnasal Asc administration, the increases in IL-4 and IL-5 production in BALF were significantly suppressed by anti-CD44 (KM201 or IM7) treatment (P < 0.05). Control RlgG had no effect. IFN-γ production in BALF was unchanged by Asc challenge and anti-CD44 treatment (Figure 2). Next, we found that levels of eotaxin and TARC were induced in BALF by transnasal Asc treatment. Anti-CD44 mAb's (KM201, IM7), but not RlgG–inhibited production of these chemokines (P < 0.05) (Figure 3); therefore, treatment with these reagents altered the production of factors required for both production and migration of eosinophils.

Anti-CD44 mAb's decrease HA and leukotriene production. Levels of HA, a major ligand of CD44 in BALF, were determined by ELISA 24 h after the last challenge. Asc treatment of mice resulted in significant increases in HA concentrations in BALF compared with PBS-treated mice. Administration of anti-CD44 (KM201 and IM7), but not RlgG, significantly decreased HA production (P < 0.05) (Figure 4). This suggests that production of HA in the lung tissue may be increased in allergic inflammation, and anti-CD44 treatment may inhibit antigen-induced HA production in the lung.

Potential mechanisms through which anti-CD44 inhibits leukocyte accumulation. To investigate the mechanisms of anti-inflammatory effects of mAb's, the expression and HA-binding ability of CD44 on the surface of splenocytes and alveolar macrophages in mice treated with anti-CD44 mAb's were examined as described in Methods. Flow-cytometric analysis revealed a marked decrease in HA concentrations in BALF of mice treated with anti-CD44 mAb's compared with PBS-treated mice. Administration of anti-CD44 (KM201 and IM7), but not RlgG, significantly decreased HA production (P < 0.05) (Figure 4). This suggests that production of HA in the lung tissue may be increased in allergic inflammation, and anti-CD44 treatment may inhibit antigen-induced HA production in the lung. BALF levels of leukotriene that can induce the accumulation of eosinophils into the airway (24) were also determined by EIA 24 h after the last challenge, and allergen-treated mice had increased amounts. Administration of anti-CD44 mAb's (KM201 or IM7), but not RlgG, 12 h before the last challenge significantly (P < 0.05) decreased leukotriene production (Figure 4). Inhibition of leukotriene production could result in decreased migration of eosinophils into the lung.

Effect of anti-CD44 mAb's on allergen-induced airway hyperresponsiveness to methacholine. Airway reactivity was evaluated 24 h after the last intranasal challenge with Asc by double-flow plethysmography as described in Methods. In the Asc/RlgG group, airway hyperresponsiveness was seen after aerosolized methacholine challenge (50 mg/ml), with significant increases in airway resistance compared with the PBS group (Figure 5, P < 0.05; Asc/RlgG versus PBS). Anti-CD44 mAb's (KM201 or IM7) significantly reduced the airway hyperresponsiveness to aerosolized methacholine in Asc-treated mice compared with RlgG (Figure 5, P < 0.05; Asc/KM201 or Asc/IM7 versus Asc/RlgG).

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reduction in percentages of CD44-expressed splenocytes of mice treated with IM7 as compared with those injected with either RIgG or KM201 (*P* < 0.05). Furthermore, administration of IM7 but not KM201 reduced the proportions of HA-binding cells in the presence of anti-CD44 mAb, IRAWB14, as compared with mice treated with RIgG (*P* < 0.05) (Figure 6a). Anti-CD44 mAb’s did not affect the expression and HA-binding ability of CD44 on alveolar macrophages in BALF of Asc-treated mice (Figure 6b). The influence of anti-CD44 mAb’s on soluble CD44 levels in serum and BALF of Asc-treated mice was also examined. Interestingly, soluble CD44 levels in serum were increased in Asc-challenged IM7-treated mice (Asc/IM7), but not in KM201-treated mice (Asc/KM201), compared with Asc-challenged RIgG-treated mice (Asc/RIgG). Anti-CD44 mAb’s did not affect soluble CD44 levels in BALF of Asc-treated mice (Figure 7). The inhibitory effect of anti-CD44 mAb, IM-7, on migration of leukocytes into inflamed lung tissue may be due to shedding of CD44 on the cell surface as well as reduction of the HA-binding ability of leukocytes.

**Discussion**

Preclinical efficacies have been documented for CD44 inhibitors in several inflammatory models that include allergic contact dermatitis (9) and collagen-induced arthritis (6, 7). Here we have studied the effects of anti-CD44 treatment in a murine model of allergen-induced airway inflammation. Injection of anti-CD44 significantly prevented eosinophil and lymphocyte infiltration into the airways without altering numbers of eosinophils in the bone marrow. Anti-CD44 inhibited not only accumulation of eosinophils and lymphocytes, but also airway hyperresponsiveness in mite allergen-treated mice. Mite antigen is a common allergen of human bronchial asthma, and Asc antigen is not a typical antigen of allergic inflammation of the airway. We therefore examined the effect of anti-CD44 mAb (IM7) on Der-induced allergic inflammation of the airways of mice. Two groups of mice were sensitized with either Der in PBS or PBS transnasally, according to procedures described in Methods. BAL was performed 24 h after the last antigen challenge. After exposure to Der, numbers of total leukocytes, macrophages, lymphocytes, and eosinophils were significantly increased in BALF compared with PBS-exposed mice. Administration of anti-CD44 mAb (IM7) given 12 h before the last Der challenge significantly suppressed numbers of lymphocytes and eosinophils (*P* < 0.05) (Figure 8a). Airway resistance was also evaluated 24 h after the last Der challenge. In the Der group, airway hyperresponsiveness was seen after aerosolized methacholine challenge (25 and 50 mg/ml), with significant increase in airway resistance compared with the PBS group (Figure 8b, *P* < 0.05; Der/RIgG versus PBS). Anti-CD44 mAb (IM7) significantly reduced the airway hyperresponsiveness to aerosolized methacholine in Der-treated mice compared with RIgG (Figure 8b, *P* < 0.05; Der/IM7 versus Der/RIgG).
pulmonary eosinophilic inflammation, associated with decreased levels of Th2 cytokines, IL-4 and IL-5, as well as the chemokines eotaxin and TARC. Furthermore, increases in concentrations of HA and leukotrienes in BALF were suppressed by anti-CD44 treatment. Finally, allergen-induced airway hyperresponsiveness was reduced by anti-CD44 treatment.

CD44 has been used as a marker of memory T cells (25). The principal ligand for CD44 is HA (2), on which activated T cells can roll under physiologic shear-stress conditions (3). HA-binding ability could be induced by activation of T cells in vivo or in vitro (26, 27). There is evidence to suggest that CD44 mediates lymphocyte migration and increased expression of CD44 on CD4+ T cells in BALF in a murine model of asthma (11). These findings suggest that the recruitment of T lymphocytes into the lung after antigen challenge may be the result of augmented expression and activation of CD44. CD44 is also expressed on eosinophils, but the possible contribution of CD44 to adhesion and migration of eosinophils has not been described. We have reported previously that alveolar eosinophils express higher levels of CD44 than eosinophils in peripheral blood of patients with eosinophilic pneumonia (12). Another group reported that CD44 high eosinophils were enriched in sputum relative to peripheral blood in patients with bronchial asthma (28). HA has a pronounced effect on eosinophil survival by GM-CSF production that is partially mediated by CD44 (13). Taken together, CD44 on eosinophils may play an important role in allergic diseases of respiratory tracts. In this study, infiltration of both eosinophils and T cells was inhibited by administration of anti-CD44 mAb’s (Figure 1a and Figure 8a). Anti-CD44 mAb’s may interrupt CD44 and HA binding. We used KM201, which is known to block HA binding by CD44 (14). The mechanism underlying the effects of the CD44 Ab appears linked to the suppression of the recruitment of eosinophils or lymphocytes, or both, into the airways. Cumulatively, the findings suggest that CD44 takes part in allergen-induced pathophysiologic inflammatory responses, likely by recruiting both eosinophils and T lymphocytes that express this adhesion molecule. Increased expression of HA on microvascular endothelial cells has been reported by proinflammatory stimuli (5). CD44-HA interactions involve the rolling of leukocytes on endothelial cells (3). Our data suggest increased production of HA in the lung during allergic inflammation and inhibitory effect on HA production of anti-CD44 mAb. Anti-CD44 mAb may interrupt binding leukocytes and endothelial cells.

TARC is a lymphocyte-directed CC chemokine that specifically chemoattracts CC chemokine receptor 4–positive Th2 cells (29). TARC is constitutively expressed in the lung and was upregulated in allergic inflammation (30). Production of TARC in bronchial epithelial cells is upregulated by IL-4 (31). The CC chemokine eotaxin is a potent and specific chemoattractant for...
eosinophil (32). This protein has been shown to play an important role in the infiltration of eosinophils to tissues in an asthmatic animal model (33). Production of eotaxin in bronchial epithelial cells was also upregulated in allergic inflammation by IL-4 (34). Our data suggest that anti-CD44 mAb's inhibited the increased concentrations of TARC and eotaxin in BALF of antigen-treated mice by reduction of IL-4 production in the airways. Furthermore, because these chemokines can influence eosinophil and lymphocyte migration, recruited eosinophils and lymphocytes may be triggered to release additional cytokines in an autocrine manner. Anti-CD44 mAb's could block this cascade.

It has been reported that cysteinyl leukotrienes cause eosinophil accumulation in the airway (24). In this study, the reduction in eosinophil numbers was accompanied by a marked decline in leukotriene concentrations in BALF of anti-CD44-treated mice. It is possible that Asc treatment resulted in the recruitment and activation of eosinophils that released the leukotrienes. Anti-CD44 may inhibit migration of eosinophils that is thought to produce leukotriene. Furthermore, cysteinyl leukotrienes also have been shown to be involved in the bronchial hyperresponsiveness induced by allergen exposed in sensitized mice and asthmatics (35, 36). In this study, anti-CD44 inhibited allergen-induced airway hyperresponsiveness to methacholine (Figure 5 and Figure 8b). Decrease in the production of cysteinyl leukotrienes in BALF is one of the possible mechanisms of inhibitory effect on allergen-induced airway hyperresponsiveness.

Asc is not a common antigen in airway allergic inflammation; therefore, we assessed the inhibitory effect of IM7 on eosinophilic inflammation and airway hyperresponsiveness induced by mite allergen, a common antigen of human bronchial asthma. IM7 anti-CD44 inhibited eosinophilic inflammation and airway hyperresponsiveness induced by not only Asc antigen but also mite antigen (Figure 8).

To further investigate the mechanisms of anti-CD44 mAb's to inhibit the accumulation of lymphocytes and eosinophils, we assessed expression of CD44 and its HA-binding ability on splenocytes and alveolar macrophages. KM201, which is known to block HA binding to CD44, had no effect on expression levels of CD44 on splenocytes. Percentages of eosinophils were increased not only in peripheral blood but also in bone marrow of KM201-treated antigen-challenged mice compared with PBS-exposed mice. These results suggest that KM201 inhibits infiltration of eosinophils from peripheral blood to the airway. The other Ab, IM7, is known to be able to induce shedding of CD44 from cell surfaces. IM7 does inhibit the HA-binding ability of splenocytes associated with the decreased levels of CD44 expression on these cells, while IM7 had no effect on the HA binding of alveolar macrophages. Anti-CD44 could affect the inflammatory cells in peripheral blood but not in the alveolar space, because mAb's injected in the peritoneal cavity may not permeate this area. IM7 might inhibit infiltration of lymphocytes and eosinophils into the airway by means of reduced CD44 before migration into inflamed tissue. Percentages of eosinophils were increased in bone marrow but not in peripheral blood of IM7-treated mice compared with RlgG-treated mice. Therefore, CD44 might be necessary to move eosinophils into circulation from bone marrow. IM7 can transmit signals through CD44 into the cytoplasm (37). While our observations do not reveal detailed mechanisms, they do show that CD44 participates in airway inflammation and indicate that particular Ab's could have therapeutic potential.

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