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Critical Role for Galectin-3 in Airway Inflammation and Bronchial Hyperresponsiveness in a Murine Model of Asthma

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Galectin-3 is a member of a β-galactoside-binding animal lectin family. Previous *in vitro* studies have demonstrated that galectin-3 is involved in a number of activities; however, the roles of this lectin in physiological and pathological processes *in vivo* remain to be elucidated. Herein, we show, in a murine model of ovalbumin (OVA)-induced asthma that 1) peribronchial inflammatory cells expressed large amounts of galectin-3; 2) bronchoalveolar lavage fluid from OVA-challenged mice contained significantly higher levels of galectin-3 compared to control mice; and 3) macrophages in bronchoalveolar lavage fluid were the major cell type that contained galectin-3. We investigated the role of galectin-3 in the allergic airway response by comparing galectin-3-deficient ($gal3^{-/-}$) mice and wild-type ($gal3^{+/+}$) mice. OVA-sensitized $gal3^{-/-}$ mice developed fewer eosinophils and lower goblet cell metaplasia, after airway OVA challenge compared to similarly treated $gal3^{+/+}$ mice. In addition, the OVA-sensitized $gal3^{-/-}$ mice developed significantly less airway hyperresponsiveness after airway OVA challenge compared to $gal3^{+/+}$ mice. Finally, $gal3^{-/-}$ mice developed a lower Th2 response, but a higher Th1 response, suggesting that galectin-3 regulates the Th1/Th2 response. We conclude that galectin-3 may play an important role in the pathogenesis of asthma and inhibitors of this lectin may prove useful for treatment of this disease. *(Am J Pathol 2004, 165:2045–2053)*

Human asthma is characterized by airway inflammation, reversible bronchoconstriction, and bronchial hyperreactivity.[1] Recent advancements have led to the identification of a number of cellular and molecular components that contribute to airway inflammation and hyperreactivity.[2,3] T cells play a central role in asthmatic inflammation and cytokines produced by Th2 cells contribute to the initiation of the inflammatory response.[4,5] Mast cells and eosinophils are important effector cells and the peptide and lipid inflammatory mediators that they secrete contribute to the bronchial response.[3,6] A number of chemokines together with various cell adhesion molecules function in concert to cause migration of inflammatory cells to the lung.[2,7] Animal models of human asthma have become instrumental in the elucidation of mechanisms of allergic airway inflammation and airway hyperresponsiveness (AHR) as well as in testing therapeutic agents. The use of these models, especially in genetically altered animals, has provided significant insights into the roles of various cell types and molecules in asthmatic reactions.[8]

Molecular studies of the components of the asthmatic response have traditionally focused on protein-protein interactions. In recent years, it has become increasingly evident that protein-carbohydrate interactions represent an important means of information transfer within and between cells, as well as between cells and the cellular matrix,[9] in normal[10] and pathological processes.[11] In the airways, resident tissues and cells, as well as migratory inflammatory cells, are all decorated by a large array of oligosaccharide structures, which are well suited to be recognized by members of mammalian lectin families.[12] Although the role of some C-type lectins, such as selectins and the low-affinity IgE receptor (FcεRII/CD23), in

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asthma has been studied in detail,[13–16] much remains to be learned about other lectins, including galectins.

Galectins are members of a β-galactoside-binding animal lectin family.[17–22] Galectin-3 is one of the best characterized members and is composed of a carboxyl-terminal lectin domain connected to an amino-terminal nonlectin part consisting primarily of short tandem repeats. It is a pleiotropic protein widely distributed in tissues and found in epithelial cells, dendritic cells, and inflammatory cells. Various extracellular and intracellular activities of galectin-3 have been demonstrated *in vitro*.[23,24] Examples of extracellular activities are modulation of cell adhesion,[25–27] chemoattraction of monocytes/macrophages (including alveolar macrophages)[28] as well as endothelial cells,[29] and angiogenesis.[29] Other examples include activation of various cell types, such as mast cells,[30] neutrophils,[31] monocytes/macrophages,[32] and lymphocytes,[33] through binding to cell surface glycoconjugates. The protein has also been implicated in suppression of T-cell activation through restriction of T-cell receptor lateral motility.[34] An example of intracellular activities is the regulation of cell survival, possibly through a mechanism involving interactions with Bcl-2 family members, with which this lectin shares sequence similarity.[35,36] These findings suggest that galectin-3 may play an important role in inflammatory responses, but direct *in vivo* evidence for such a role has so far been lacking. The development of genetically engineered galectin-3-deficient (gal3$^{-/-}$) mice has provided the much-needed tool to elucidate the physiological and pathological functions of this protein *in vivo*. Recently, we and others have shown that gal3$^{-/-}$ mice exhibit reduced peritoneal inflammation in response to thioglycollate treatment.[37,38]

Based on the demonstrated functions of galectin-3, we hypothesized that this lectin might play a role in the development of airway inflammation, as occurs in asthma. In the present study, we have analyzed the expression and function of galectin-3 in a murine model of asthma, in which mice were sensitized systemically with ovalbumin (OVA) and then challenged with OVA through the airways. These mice developed an asthmatic response in the lungs, characterized by the accumulation of eosinophils, production of Th2 cytokines, and development of AHR.[39] We found that the expression of galectin-3 was up-regulated in the inflamed airways. More importantly, when compared to wild-type mice, gal3$^{-/-}$ mice exhibited significantly diminished airway inflammation and hyperresponsiveness.

Materials and Methods

Mice

Gal3$^{-/-}$ mice were developed as described.[38] These mice were backcrossed to C57BL/6 mice for nine generations and interbreeding of gal3$^{+/-}$ F9 resulted in gal3$^{+/+}$ and gal3$^{-/-}$ mice in the C57BL/6 background, which were used throughout this study.

Immunization and Airway Antigen Challenge

The mice were immunized with 10 μg of OVA (grade V; Sigma, St. Louis, MO) in 2 mg of aluminum hydroxide gel intraperitoneally. The mice were placed in a Plexiglas chamber, 10 to 14 days later, and subjected to aerosolized OVA (10 mg/ml) in saline administered by a nebulizer for 30 minutes each day for 3 to 6 days, as specified for the experiments described in the figure legends. The control mice in all experiments received nonpyrogenic saline (Baxter, Deerfield, IL) at corresponding time points. In some experiments, OVA from Sigma was compared with endotoxin-free OVA (ET-free OVA; kindly provided by Dr. E. Janssen, La Jolla Institute of Allergy and Immunology, San Diego, CA). This OVA was prepared by collecting chicken albumin aseptically and freeze-drying it in pyrogen-free vials. When macrophages were cultured with ET-free OVA, tumor necrosis factor-α secretion was not detectable, indicating absence of endotoxin.

For the measurement of AHR, an intraperitoneal injection of 10 μg of OVA mixed with 1 mg of aluminum hydroxide gel was administered on day 0 and an identical booster injection was given on day 7. Starting 7 days later, the mice were treated with aerosolized OVA (60 mg/ml) dissolved in phosphate-buffered saline (PBS, pH = 7.4), or PBS, for 20 minutes per day in each of the subsequent 7 days. Control mice were treated with PBS alone. Treatment was initialized with an ultrasonic nebulizer (model 5000; DeVilbiss, Somerset, PA) into a plastic chamber that was 23 × 23 × 11 cm. The aerosol was delivered by providing ~1 liter per minute (LPM) airflow at the nebulizer and excess aerosol escaped the box through a series of holes opposite the aerosol entry port.

Bronchoalveolar Lavage (BAL)

BAL was performed 3 hours after the last airway antigen challenge. The BAL fluid obtained was centrifuged at 400 × *g* to collect cells. The supernatant fluid was then centrifuged at 1000 × *g* to remove cellular debris and stored at −70°C until evaluated. Total viable cell numbers were determined by trypan blue exclusion. Differential cell counts were determined by staining cytospins with either Wright-Giemsa (Sigma) or Leukostat staining kit (Fisher Scientific Co., Pittsburgh, PA).

Histology

Lung tissue samples were fixed in 10% zinc-formalin (Biochemical Sciences, Inc., Swedesboro, NJ) and paraffin-embedded. Goblet cells were stained and counted as previously described.[40] Briefly, 1 ml of 10% zinc-formalin (Fisher Scientific) was administered into the lungs via cannulated trachea. The small right lobe of the lung was dissected out, fixed in zinc-formalin, paraffin-embedded, and then sectioned, dewaxed, hydrated, stained with periodic acid-Schiff (PAS) stain, and counterstained with hematoxylin Gill no. 2 (Sigma). The goblet

cells (both PAS+ and PAS−) around both the large and small bronchioles in each section were counted.

Immunohistochemistry was also performed with the paraffin-embedded sections. The endogenous peroxidase activity as well as nonspecific protein binding was sequentially blocked using 0.3% hydrogen peroxide and 5% normal goat serum, respectively. The sections were incubated with affinity-purified rabbit anti-galectin-3 antibody[41] or normal rabbit IgG antibody (control) at 10 μg/ml for 30 minutes at room temperature and were then washed five times in PBS. Bound antibody was detected by sequential incubation with biotinylated goat anti-rabbit antibody and streptavidin-horseradish peroxidase followed by 3,3-diaminobenzidine (Biogenex Laboratories, San Ramon, CA). Slides were then washed in water and counterstained with hematoxylin Gill no. 2 (Sigma). For immunocytochemistry, cytospins of BAL fluid cells were stained according to a previously described method,[32] except that affinity-purified rabbit anti-galectin-3 antibody was used followed by steps as described above.

Quantitation of Galectin-3

Galectin-3 levels in BAL fluid were quantitated by enzyme-linked immunosorbent assay (ELISA) using a procedure similar to that described for human galectin-3.[32] Reagents used were affinity-purified goat anti-galectin-3 antibody as the capture antibody, affinity-purified rabbit anti-galectin-3 antibody[41] as the primary detection antibody, horseradish peroxidase-coupled goat anti-rabbit antibody (Zymed Laboratories, South San Francisco, CA) as the secondary detection antibody, and *o*-phenylenediamine dihydrochloride as the substrate. Recombinant mouse galectin-3 (a kind gift of Dr. J. Wang, Michigan State University, East Lansing, MI) was used as the standard.

Quantitation of Interleukin (IL)-4, Interferon (IFN)-γ, IgE, IgG_1, and IgG_{2a}

IL-4 and IFN-γ levels in BAL fluid were measured by ELISA using commercial reagents (PharMingen, San Diego, CA) according to the manufacturer's protocol. Total IgE levels in BAL fluid and sera were determined by ELISA using affinity-purified goat and rabbit anti-IgE antibodies.[42] The OVA-specific IgG_1 and IgG_{2a} antibodies in BAL fluids were detected on microtiter plates coated overnight with OVA at 10 μg/ml. The plates were blocked with 1% bovine serum albumin in PBS containing 0.05% Tween 20 for 2 hours at room temperature. Incubation of BAL fluid samples in OVA-coated wells was followed by biotin-labeled rabbit anti-mouse IgG_1 and IgG_{2a} antibodies (Zymed Laboratories) each for 2 hours at room temperature. The plates were then incubated with horseradish peroxidase-avidin (Bio-Rad, Richmond, CA) followed by the horseradish peroxidase substrate *o*-phenylenediamine dihydrochloride (Sigma-Aldrich, St. Louis, MO) each for 30 minutes and read at 490 nm. The concentration of each Ig subclass in the samples was determined with the computer program SoftMaxPro provided with the plate reader (Molecular Devices, Sunnyvale, CA) and was read off a standard curve generated by incubating several concentrations of purified mouse IgG_1 or IgG_{2a} in wells coated either with rat anti-mouse IgG_1 or rat anti-mouse IgG_{2a}, respectively (CalTag Laboratories, Burlingame, CA) followed by biotinylated antibodies as above.

Measurement of Airway Responsiveness

Mice were anesthetized by an intraperitoneal injection of pentobarbital (180 mg/kg). After a surgical plane of anesthesia was achieved, the trachea was cannulated with a 19-gauge tubing adaptor attached to polyethylene tubing that passed through the plethysmograph chamber and was attached to a four-way connector, which was connected to a rodent ventilator (model 683; Harvard Apparatus, South Natick, MA) and pressure transducer. The ventilator was set to provide 150 breaths/minute with tidal volumes of 5 to 6 ml/kg and a positive end expiratory pressure of 3 to 4 cm H_2O. An internal jugular vein was cannulated with a saline-filled silicone catheter (0.021 cm OD, 6 to 8 cm in length, $<$0.005 ml volume) and attached to a 0.1-ml micro syringe. A 5 $\times$ 2-mm thoracotomy incision was made in a manner that allowed pleural pressure to equal body surface pressure. Flow was calculated by differentiation of the volume signal, transpulmonary pressure was measured as the difference of tracheal cannula and box pressure, and lung resistance was calculated as reported previously.[43] Lung resistance (R_L) was measured before and after each dose (26 to 34 μl volume) of intravenous methacholine (MCh). Percent baseline R_L was calculated by dividing the greatest R_L value obtained after MCh injection by the baseline value obtained immediately before and multiplying the result by 100.

Statistical Analysis

Statistical analysis of control and experimental groups was accomplished by Student's *t*-test using the software Statview 4.01 (SAS Institutes, Cary, NC). Changes in lung resistance to increasing concentrations of MCh were compared in mice using a two-factor repeated measures analysis of variance with the genetic strain and dose of MCh as the group factors. A *P* value less than 0.05 was considered significant.

Results

Galectin-3 Expression in the Airways Is Up-Regulated during Allergic Airway Inflammation

Lung tissue and BAL fluid from OVA-sensitized C57BL/6 mice challenged with either nebulized OVA or saline were analyzed. As expected, in contrast to the normal lungs from the control mice (Figure 1A), the inflamed lungs (Figure 1B) contained prominent peribronchial inflammatory cell infiltrations. Immunohistochemical analysis of galectin-3 expression showed that there was an increase in

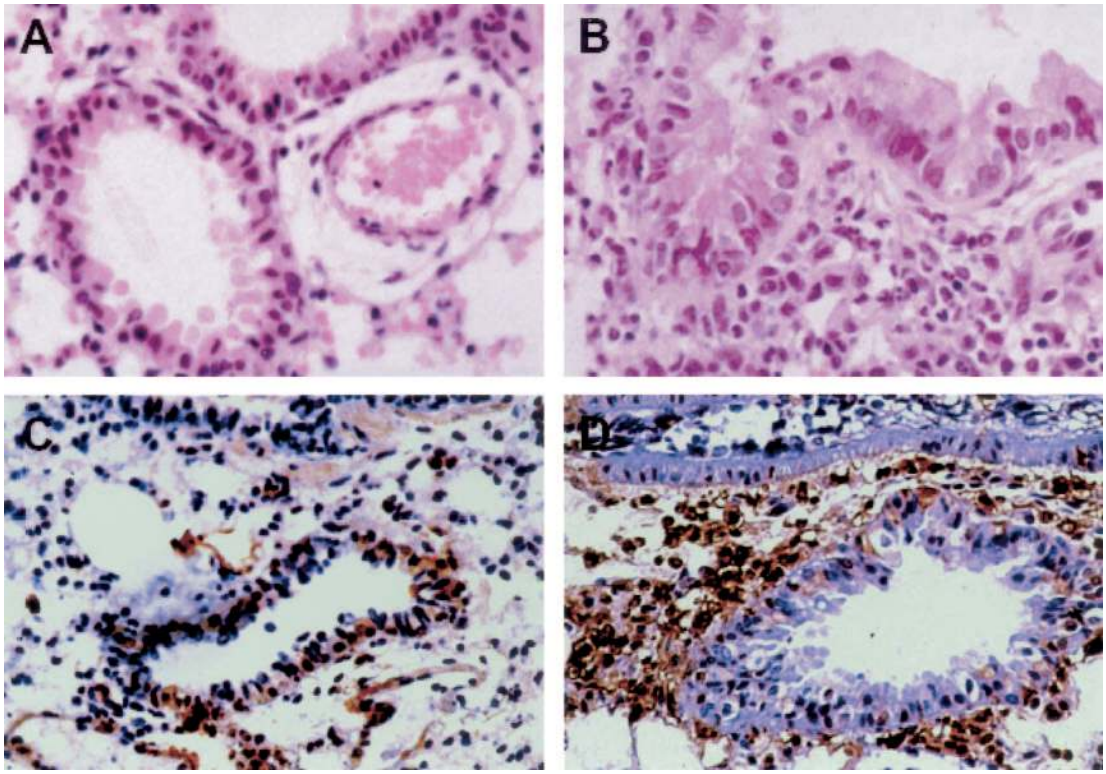

Figure 1. Immunochemical staining for galectin-3 in the lung tissue and BAL fluid from mice with allergic airway inflammation. C57BL/6 mice were immunized with OVA in aluminum hydroxide gel intraperitoneally and then challenged 14 days later with aerosolized OVA 30 minutes a day for 6 days; control mice were treated with aerosolized saline. The mice were sacrificed 3 hours after the last antigen challenge. H&E staining of a lung section from control (**A**) and experimental (**B**) mice, and immunohistochemical staining for galectin-3 of a lung section from control (**C**) and experimental (**D**) mice were performed as described in Materials and Methods. Brown staining in **C** and **D** represents positive reactivity. No staining was observed when normal rabbit IgG was used instead of rabbit anti-galectin-3 antibody (data not shown).

galectin-3 staining in the inflamed lungs (Figure 1D) compared to the normal lungs (Figure 1C). The increased staining is mostly because of infiltrating cells. Also as expected, inflammatory cells in BAL fluid from mice with inflamed airways were mostly eosinophils, but monocytes/macrophages and a few lymphocytes were also present (Figure 2A). Immunocytochemical staining for galectin-3 showed that macrophages were strongly stained, whereas eosinophils were not stained (Figure 2B). Finally, galectin-3 levels in BAL fluid from mice challenged with aerosolized OVA were significantly higher than that from mice treated with aerosolized saline (Figure 2C). The specificity of the anti-galectin-3 antibody used in these analyses was confirmed by the fact that lung tissues and lavaged cells from gal3$^{-/-}$ mice were not stained at all by this antibody (data not shown). To determine whether galectin-3 release into the airway secretions was influenced by presence of endotoxin in OVA, mice were challenged either with saline (group 1), regular OVA (group 2), or ET-free OVA (group 3). The results showed that mice from both groups 2 and 3 developed comparable levels of airway inflammation as indicated by the amount of cellular infiltration. In addition, galectin-3 levels in BAL fluids obtained from both groups were similar and higher than that from group 1 (data not shown). The results indicate that galectin-3 release by airway cells was not because of low levels of endotoxin in OVA.

Gal3$^{-/-}$ Mice Exhibit Significantly Reduced Airway Inflammatory Responses

To determine whether galectin-3 contributes to the airway inflammatory response, we compared gal3$^{-/-}$ with gal3$^{+/+}$ mice. Previously, we have shown that gal3$^{-/-}$ mice do not exhibit any overt defects and the total numbers of lymphocytes, ratios of CD_4^+/CD_8^+ cells, and numbers of CD_3^+ cells in various lymphoid organs are comparable between gal3$^{-/-}$ and gal3$^{+/+}$ mice.[38] Mice were systemically immunized with OVA in aluminum hydroxide gel, then challenged with aerosolized OVA or saline, and the inflammatory response was assessed by enumerating cells in BAL fluid. Both genotype controls challenged with aerosolized saline showed only a small number of cells in BAL fluid that were mostly monocytes (data not shown). However, on challenging with aerosolized OVA, both genotypes mounted an inflammatory response, but gal3$^{-/-}$ mice consistently showed significantly lower numbers of total inflammatory cells in BAL fluid compared to similarly challenged gal3$^{+/+}$ mice (Figure 3A). The difference was primarily because of eosinophils (Figure 3B), but also partly because of neutrophils (Figure 3B, inset), which represent only a small fraction of the leukocytes in BAL fluid. The numbers of monocytes/

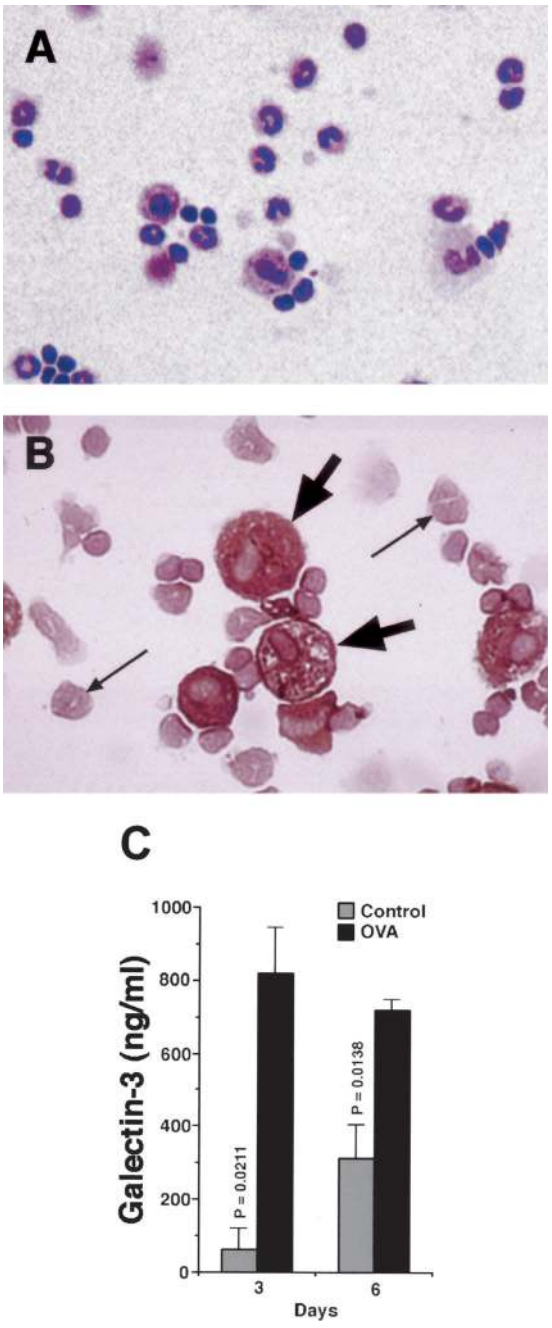

Figure 2. Detection of galectin-3 in cells and supernatants from BAL fluid. BAL fluid was obtained 3 hours after the last airway treatment in the experiments described in Figure 1. H&E staining of cells (**A**) in BAL fluid and immunocytochemical staining for galectin-3 in these cells (**B**) were performed as described in Materials and Methods. Macrophages are indicated by **broad arrows** and eosinophils are indicated by **thin arrows**. Brown staining in **B** represents positive reactivity. No staining was observed when normal rabbit IgG was used instead of rabbit anti-galectin-3 antibody. **C:** Three hours after the last antigen challenge, BAL fluid was obtained and galectin-3 levels were determined by ELISA. Each data point represents the mean ± SEM of results from three mice; similar results were obtained in three separate experiments.

macrophages in BAL fluid were not significantly different between gal3$^{+/+}$ and gal3$^{-/-}$ mice (Figure 3B).

A characteristic feature of the murine model of asthma is goblet cell metaplasia with an accompanying increase in mucin production giving rise to mucous plugs in the

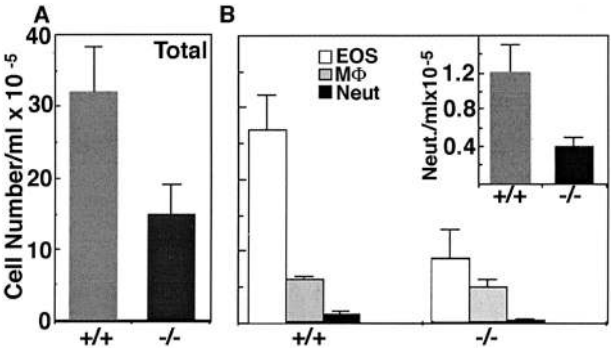

Figure 3. Quantitation of leukocyte in BAL fluid from gal3$^{+/+}$ and gal3$^{-/-}$ mice with allergic airway inflammation. Gal3$^{+/+}$ and gal3$^{-/-}$ mice were immunized with OVA in aluminum hydroxide gel intraperitoneally and 14 days later were challenged with aerosolized OVA 30 minutes a day for 3 days. BAL fluid was obtained 3 hours after the last challenge and total leukocytes (**A**) and subpopulations of leukocytes (**B**) in the fluid were enumerated. The data for neutrophil recoveries are also presented in the **inset** in **B**. *P* values for the differences between gal3$^{-/-}$ and gal3$^{+/+}$ mice: total cells, <0.027; eosinophils, <0.011; macrophages, NS; neutrophils, <0.0278.

airways.[44] When we stained the lung tissue for mucin, we found that the goblet cells of gal3$^{+/+}$ mice stained more intensely than those from gal3$^{-/-}$ mice, indicating higher mucin production per goblet cell in the former (Figure 4A). In addition, the number of mucin-producing goblet cells in the lungs was significantly higher in gal3$^{+/+}$ mice than gal3$^{-/-}$ mice (Figure 4B).

Galectin-3-Deficient Mice Are Defective in the Development of AHR

Development of AHR is another feature of human asthma consistently manifested in the murine model.[8] The OVA-sensitized mice were challenged with aerosolized OVA repeatedly and lung resistance (R_L) was measured before and after each dose of intravenous MCh. We found that gal3$^{-/-}$ mice developed a significantly lower degree of lung resistance in response to MCh challenge, compared to gal3$^{+/+}$ mice (Figure 5), suggesting that AHR to airway antigen challenge is ameliorated in mice with galectin-3 deficiency.

Gal3$^{-/-}$ Mice Develop a Lower Th2 Response but a Higher Th1 Response

To understand better the basis for the lower airway responses because of galectin-3 deficiency, we compared the Th1 versus Th2 responses between gal3$^{+/+}$ and gal3$^{-/-}$ mice. First, we examined the levels of cytokines in BAL fluid. As shown in Figure 6A, IL-4 levels in BAL fluid from gal3$^{-/-}$ mice were significantly lower than those from gal3$^{+/+}$ mice. In contrast, the opposite results were observed for IFN-γ (Figure 6B). Previously, we found that BAL fluid from mice with allergic airway inflammation contained significant amounts of IgE, including antigen-specific IgE, which correlated well with the degree of airway inflammation.[45] Measurement of IgE levels in the BAL fluid thus represents a convenient and reliable way for assessing allergic airway inflammation. As shown

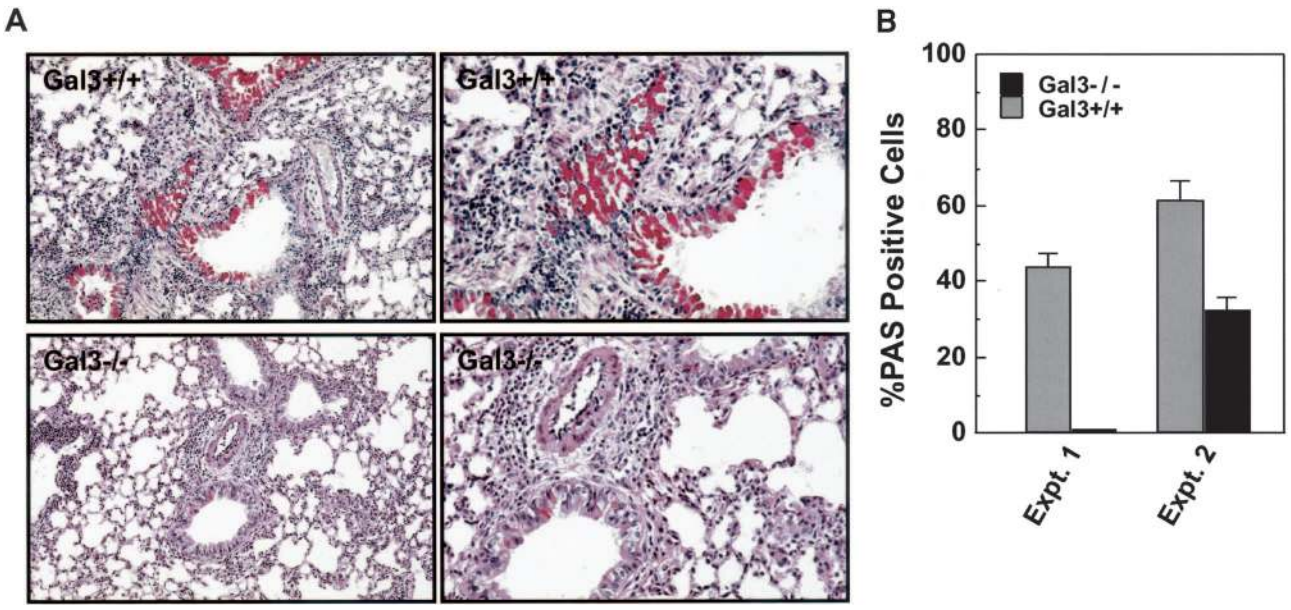

Figure 4. Comparison of goblet cell mucin production by gal3$^{+/+}$ and gal3$^{-/-}$ mice. OVA-sensitized mice were challenged 14 days later with aerosolized OVA given 30 minutes each day for 6 days. Three hours after the last aerosolized antigen challenge, the lungs were fixed and processed for PAS stain as described under Materials and Methods. **A:** Representative areas of the lungs from gal3$^{+/+}$ and gal3$^{-/-}$ mice under magnification with ×10 (**left**) and ×20 (**right**) objectives in which mucin-producing goblet cells are stained red. **B:** Comparison of percentages of PAS^{+} goblet cells between gal3$^{+/+}$ and gal3$^{-/-}$ mice (four mice for each genotype). The number of mucin-producing goblet cells in the lungs of gal3$^{+/+}$ mice was significantly higher than in gal3$^{-/-}$ mice (expt.1, $P < 0.014$; expt.2, $P < 0.0001$).

in Figure 6C, BAL fluid from OVA-challenged gal3$^{-/-}$ mice contained significantly lower concentrations of IgE compared to identically treated gal3$^{+/+}$ mice. We also measured the ratio of OVA-specific IgG_{2a} (a Th1 antibody) to IgG_1 (a Th2 antibody) and noted that gal3$^{-/-}$ mice have a higher ratio (Figure 6D). In addition, we obtained cells from the lungs and the spleen from the OVA-challenged mice and cultured them in the presence of OVA. We noted that cells from gal3$^{-/-}$ mice produced significantly higher amounts of IFN-γ (a Th1 cytokine) and lower amounts of IL-4 (a Th2 cytokine), compared to gal3$^{+/+}$ mice (data not shown). The results suggest that gal3$^{-/-}$ mice have lower Th2 but higher Th1 responses compared to gal3$^{+/+}$ mice.

Galectin-3-Deficient Mice Exhibit a Lower IgE Response

We compared the IgE response in gal3$^{+/+}$ and gal3$^{-/-}$ mice and found that gal3$^{-/-}$ mice sensitized with OVA and then challenged by aerosolized OVA exhibited lower serum IgE levels compared to similarly treated gal3$^{+/+}$ mice (Figure 7A). To determine whether the two genotypes differ in their IgE response to systemic immunization, we treated the mice intraperitoneally with OVA in aluminum hydroxide gel and then challenged them intraperitoneally with the same antigen in aluminum hydroxide gel three times and evaluated the IgE levels in sera after the second through fourth immunizations. We found that gal3$^{-/-}$ mice mounted a significantly lower IgE response after the secondary boost compared with gal3$^{+/+}$ mice (Figure 7B). The former continued to show suppressed IgE levels after each of the subsequent antigen challenges, although the differences became less pronounced at later time points.

Discussion

We have demonstrated here that galectin-3 contributes significantly to allergic airway inflammation and AHR in a murine model of asthma. Firstly, galectin-3 expression is increased in the airways of mice developing allergic inflammation, both in the peribronchial inflammatory cells (in particular monocytes/macrophages) and in fluid lining the airways. Secondly, gal3$^{-/-}$ mice, sensitized to OVA systemically and then challenged with the same antigen through the airways, exhibit significantly reduced allergic airway inflammation, as measured by the number of eosinophils, compared to similarly treated gal3$^{+/+}$ mice. Thirdly, gal3$^{-/-}$ mice also manifest lower goblet cell metaplasia compared to gal3$^{+/+}$ mice. Finally, gal3$^{-/-}$ mice develop significantly less airway responsiveness to MCh compared to gal3$^{+/+}$ mice, on airway antigen challenge.

On the basis of the results from previous studies by this and other laboratories, several possibilities may be considered for the proinflammatory role of galectin-3. The first possibility is that galectin-3 released into the extracellular space under inflammatory conditions may activate inflammatory cells associated with allergic responses or contribute to the retention of these cells to extracellular matrix glycoproteins at the sites of inflammation.[25,30,32,46] The second possibility is that galectin-3 is known to have anti-apoptotic properties,[35,36,47,48]

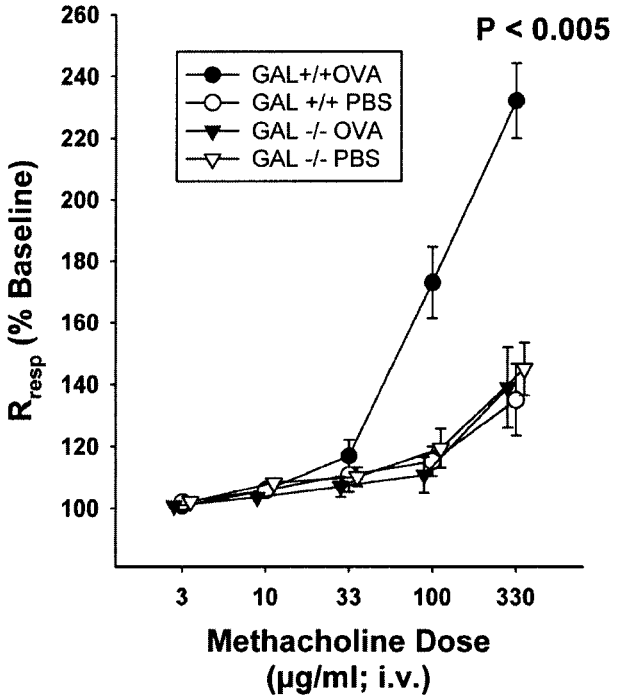

Figure 5. Comparison of AHR between gal3$^{+/+}$ and gal3$^{-/-}$ mice. Five gal3$^{+/+}$ and eight gal3$^{-/-}$ mice were immunized twice with OVA and then challenged with aerosolized OVA, as described in Materials and Methods. Five mice for each genotype were exposed to aerosolized PBS instead of OVA. The airway response to MCh was measured by whole body plethysmography as described in Materials and Methods. Lung resistance (R_L) was measured before and after each dose of intravenous MCh. Percent baseline R_L was calculated by dividing the greatest R_L value obtained after MCh injection by the baseline value obtained immediately before and multiplying the result by 100. $P < 0.005$.

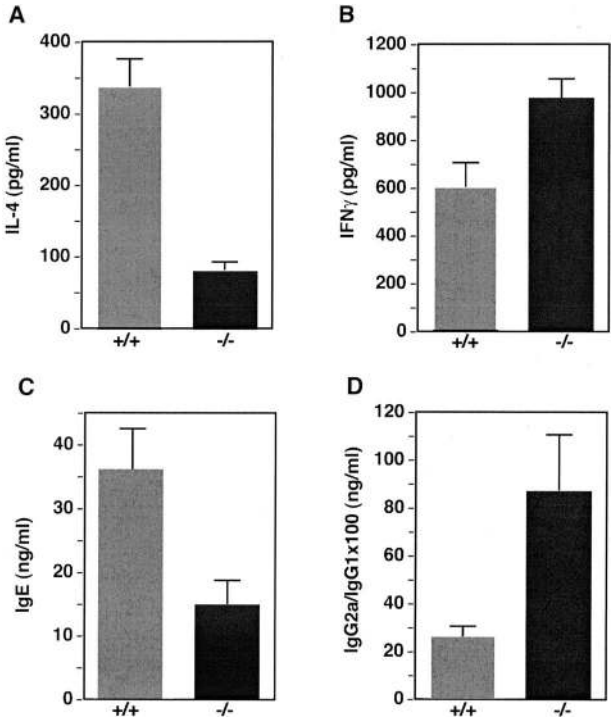

Figure 6. Quantitation of cytokines and immunoglobulin in BAL fluid. Gal3$^{+/+}$ and gal3$^{-/-}$ mice were immunized and then challenged with OVA as described in Figure 3. The levels of IL-4 (**A**), IFN-γ (**B**), total IgE (**C**), and ratio of OVA-specific IgG_{2a} to IgG_1 (**D**) in BAL fluid were determined by ELISA. The results represent the mean ± SEM of data from a total of 12 mice for each genotype for IL-4 and IgE, 7 mice each for IFN-γ, and 23 mice each for IgG_{2a}/IgG_1. The *P* values are: IL-4, <0.027; IFN-γ, <0.0227; IgE, <0.05; and IgG_{2a}/IgG_1, <0.014.

which could render the inflammatory cells less sensitive to apoptotic stimuli thereby enhancing their survival in the inflamed airways.

The third possibility concerns the chemotactic activity of galectin-3. Galectin-3 has been shown to directly attract human alveolar macrophages *in vitro* and recruit monocytes *in vivo* in a mouse air pouch model, as well as attract eosinophils by indirect mechanisms in this *in vivo* model.[28] However, galectin-3 does not appear to function as a monocyte/macrophage chemoattractant in the murine asthma model, because the amount of monocytes/macrophages in BAL fluid from OVA-challenged gal3$^{+/+}$ and gal3$^{-/-}$ mice was not significantly different. On the other hand, we have recently shown that galectin-3 plays a critical role in phagocytosis by macrophages through an intracellular mechanism,[49] suggesting that galectin-3 may influence the state of activation of resident airway cells that express galectin-3. Because the recruitment of eosinophils is dependent on several factors, including the production of chemoattractants by resident airway cells, it is possible that galectin-3 affects this process through regulating the airway production of proallergic chemokines.

Results obtained in the present studies suggest another possibility: galectin-3 may contribute to regulation of the Th1/Th2 response. We noted that gal3$^{-/-}$ mice mounted a lower airway Th2 response, but a higher Th1 response compared to gal3$^{+/+}$ mice (Figure 6). Moreover, the IgE response to systemic immunization is also lower in the former mice (Figure 7). In addition, spleen and lung cells from the OVA-challenged gal3$^{-/-}$ mice produced higher amounts of IFN-γ (a Th1 cytokine) and lower amounts of IL-4 (a Th2 cytokine), compared to gal3$^{+/+}$ mice when stimulated with OVA *in vitro* (data not shown). Therefore, the lower airway responses in gal3$^{-/-}$

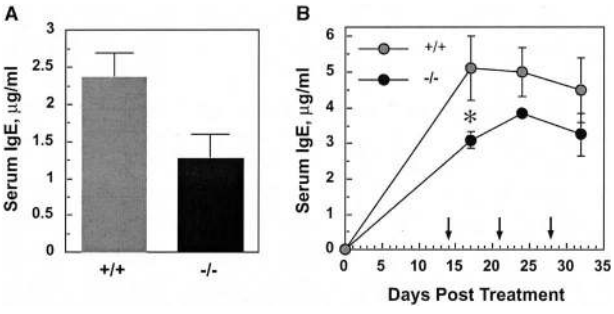

Figure 7. Quantitation of total IgE in sera from gal3$^{+/+}$ and gal3$^{-/-}$ mice. **A:** Gal3$^{+/+}$ and gal3$^{-/-}$ mice were treated as described in Figure 3. **A:** The total serum IgE levels were determined by ELISA. The results are the mean ± SEM from four experiments with three mice for each genotype in each experiment. $P < 0.046$ for the differences between gal3$^{-/-}$ and gal3$^{+/+}$ mice. **B:** Gal3$^{+/+}$ and gal3$^{-/-}$ mice were inoculated with 10 µg of OVA in aluminum hydroxide gel intraperitoneally four times on days 0, 14, 21, and 28 and the total IgE levels from sera obtained on days 1, 17, 24, and 32 were determined by ELISA. The **arrows** indicate the days the mice were immunized. The data are presented as the mean ± SEM from one of two experiments with four mice for each genotype in each experiment. *, $P < 0.029$; responses between gal3$^{+/+}$ and gal3$^{-/-}$ throughout the entire period are significantly different by analysis of variance ($P < 0.0242$).

mice may be explained by the deviation of the immune response to Th1. Two possibilities need to be considered. First, because galectin-3 has been implicated in down-modulating TCR responsiveness,[34] loss of galectin-3 expression may result in increased sensitivity of Th1 cells to antigen, and thus higher production of Th1 cytokines. Second, the alteration in the cytokine profile in gal3$^{-/-}$ mice could be secondary to the reduction in airway eosinophils, because eosinophils are able to secrete various cytokines.[50] It should be pointed out that Th1 cells can exacerbate airway inflammation.[51,52] Thus, our observed overall phenotype of gal3$^{-/-}$ mice may reveal that reduction in the Th2 response exceeds any increase in the Th1 response.

We found that galectin-3 is necessary for the expression of allergen-induced changes in AHR, suggesting that galectin-3 plays an important role in the development of AHR. This may be partly related to the effects of galectin-3 on the inflammatory response. However, the most intriguing possibility is that galectin-3 may contribute directly or indirectly to airway abnormal smooth muscle contractile responses. The induction of allergic airway inflammation is associated with the appearance of monocytes/macrophages in close proximity to the airways and our data indicate that galectin-3 is highly expressed by these cells (Figures 1 and 2). These cells are located near structural and inflammatory cells that are known to participate in airway responsiveness and the alteration in airway structure that are characteristic of asthma. As stated above, galectin-3 can influence the state of activation of the resident airway cells, and thus may indirectly affect the airway responsiveness. Alternatively, the secretion of galectin-3 may be required for altered airway smooth muscle function and the genesis of AHR. In either case, our results suggest that after allergen sensitization and challenge, galectin-3 is expressed mainly by a mononuclear cell population that is near the airways and that galectin-3 is required for the development of AHR. These are novel findings with mechanistic implications.

Recently, it has been shown that intranasal delivery of plasmids containing galectin-3 cDNA into rats resulted in reduced eosinophil infiltration and AHR in response to airway antigen challenge.[53] The findings demonstrate that application of pharmacological concentrations of a galectin-3-expression construct to the airway epithelial surface can have suppressive effects on airway responses that are clearly different from the potentiative role of physiological concentrations of this protein as suggested by the present studies. It is also to be noted that galectin-3 has been shown to inhibit the production of IL-5 in eosinophils and T cells,[54] and thus can potentially down-regulate eosinophil response, because IL-5 can enhance eosinophil maturation. Thus, the effects of galectin-3 may depend on the cell types expressing this protein and its concentration as well as its temporal and spatial distribution in the lung.

In summary, endogenous galectin-3 is a proinflammatory molecule and a potentiator of AHR in a murine model of asthma. Existing information in the literature suggests that galectin-3 can contribute to allergic airway inflammation and AHR through a number of different mechanisms. Because galectin-3 is highly expressed by monocytes/macrophages that accumulate in the airways during allergic airway inflammation, it is highly likely that galectin-3 contributes to the airway response by controlling the activities of these cells, or galectin-3 secreted by these cells induces the airway response directly. In addition, galectin-3 can contribute to the allergic airway response by directing the immune response toward Th2 through a yet unidentified mechanism(s). Additional studies will be required to further elucidate the mechanisms as well as determine whether this protein similarly contributes to the asthmatic response in humans. Future studies may show that galectin-3 is a potential target for therapeutic intervention in human asthma.

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