

α -Galactosylceramide, a Ligand of Natural Killer T Cells, Inhibits Allergic Airway Inflammation

Hiroyuki Matsuda, Takafumi Suda, Jun Sato, Toshi Nagata, Yukio Koide, Kingo Chida, and Hirotohi Nakamura

Second Division, Department of Internal Medicine, and Department of Microbiology and Immunology, Hamamatsu University School of Medicine, Hamamatsu, Japan

α -Galactosylceramide (α -GalCer) is a specific ligand of natural killer T cells (NKT cells) that regulates the immune responses such as tumor rejection and autoimmunity by producing interferon (IFN)- γ and interleukin (IL)-4. However, it has not been determined whether α -GalCer-activated NKT cells modulate allergic inflammation. Because α -GalCer induces a large amount of IFN- γ production by NKT cells, we hypothesized that an *in vivo* administration of α -GalCer could inhibit allergic airway inflammation in mice. Strikingly, a single intraperitoneal injection of α -GalCer almost completely abrogated an infiltrate with eosinophils in the lung tissue as well as in the bronchoalveolar lavage. This inhibition of allergic inflammation was associated with a significant decrease in the levels of IL-4, IL-5, and IL-13 in bronchoalveolar lavage fluid and in the number of goblet cells. In addition, this ligand significantly inhibited airway hyperresponsiveness to inhaled methacholine and raised the serum levels of ovalbumin-specific IgG2a with a decrease in those of ovalbumin-specific IgE. In IFN- γ knockout mice, however, α -GalCer failed to exert such inhibitory effects in this asthma model. These results indicate that α -GalCer prevents allergic airway inflammation possibly through IFN- γ production by ligand-activated NKT cells, suggesting the potential therapeutic application of α -GalCer in asthma.

Keywords: asthma; natural killer T cell; α -galactosylceramide; interferon- γ

Chronic airway inflammation is currently considered to play a key role in the pathogenesis of asthma. The inflammatory component of this disease is recognized by increasing numbers of activated T-helper lymphocytes, eosinophils, and mast cells within the airway lumen and bronchial submucosa (1–5). Although an infiltrate with eosinophils in the airways was considered to be central to the pathophysiology of asthma, increasing evidence has suggested that activated CD4⁺ T cells that produce helper T cell type 2 (Th2) cytokines, such as interleukin (IL)-4, IL-5, and IL-13, orchestrate airway inflammation. Indeed, CD4⁺ T cell-producing Th2 cytokines increased in the airways and bronchoalveolar lavage fluid (BALF) of patients with asthma (3). In a murine model of asthma, Th2 cells were shown to induce the characteristic features of asthma, including airway hyperresponsiveness (AHR) and mucus metaplasia (6, 7). Adoptive transfer of Th2-type cells can also induce murine models of asthma (7), and CD4⁺ T cells regulate AHR independent of IL-4 and IL-5 (8), suggesting that Th2 cells are critically involved in this disease. The accumulating evidence suggests that patients with asthma have an immune polarization toward a Th2 pheno-

type, which profoundly contributes to the phenotype of asthma. Thus, the idea emerged that a shift in cytokine balance from a Th2 toward a helper T cell type 1 (Th1) profile could allow a more specific therapy against asthma. Indeed, several studies have demonstrated that administration of Th1 cytokines, such as interferon (IFN)- γ or IL-12, decreased eosinophil recruitment and AHR in murine models of asthma (9, 10).

Natural killer T cells (NKT cells) constitute a distinct lymphocyte subpopulation that has important immunoregulatory functions (11–13). NKT cells are characterized by the expression of invariant T cell receptor (TCR) encoded by V α 14 and J α 18 gene segments and V β 8, 7, 2 gene segments in mice (11, 13, 14). A similar population of cells, expressing homologous TCR chains (V α 24-J α Q and V β 11), has been identified in humans (11, 13, 15, 16). NKT cells are specific for glycolipid antigens that are presented by the nonpolymorphic major histocompatibility complex class I-like molecule CD1 d (11–13, 17). Although the natural ligand for NKT cells remains to be clarified, α -galactosylceramide (α -GalCer), a derivative of a marine sponge, specifically binds CD1 d and strongly stimulates NKT cells (18, 19). The most characteristic function of NKT cells is their capacity to rapidly secrete large amounts of cytokines, including IL-4 and IFN- γ , in response to TCR stimulation (11–13). Initially, NKT cells were believed to serve as a critical source of IL-4 for differentiation of Th2 cells. In murine models of autoimmune diabetes and encephalomyelitis, which are characterized by Th1-dominant immune responses, α -GalCer-activated NKT cells have been reported to prevent the onset and progression of these diseases by inducing a Th2 bias of the autoimmune T cells (20–22). However, α -GalCer was also shown to suppress the Th2 response against *Nippostrongylus brasiliensis* (23) and to enhance host resistance in mice infected with *Cryptococcus neoformans* (24) through IFN- γ production from NKT cells. Thus, the role of NKT cells in skewing the immunity to Th1 or Th2 response is still controversial. In asthma, recent studies have demonstrated that NKT cells are required for the development of antigen-specific AHR and allergic airway inflammation in a murine model of asthma (25, 26), but no data are available on the potential of specific ligands for NKT cells to modulate the Th2-biased immune response of asthma.

Here, we have investigated whether α -GalCer, a specific ligand for NKT cells, can modulate immune responses against inhaled antigen and protect mice against allergic airway inflammation. Our results demonstrate that a single administration of α -GalCer prevents AHR and allergic airway inflammation, such as an infiltrate with eosinophils, Th2 cytokine expression in BALF, and goblet cell hyperplasia. Studies with IFN- γ knockout (KO) mice revealed that IFN- γ was critical for α -GalCer-mediated protection against AHR and allergic airway inflammation. Taken together, our findings indicate that α -GalCer would be a novel therapeutic option for asthma.

MATERIALS AND METHODS

Mice

Female BALB/c wild-type mice were purchased from Japan SLC (Hamamatsu, Japan). IFN- γ KO mice, which were 15 times backcrossed

(Received in original form January 11, 2004 and in final form March 25, 2005)

T.S. was supported by a grant-in-aid for scientific research (13670595) from Japan Society for the Promotion of Science.

Correspondence and requests for reprints should be addressed to Takafumi Suda, M.D., Ph.D., Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, Shizuoka, 431-3192 Japan. E-mail: suda@hama-med.ac.jp

Am J Respir Cell Mol Biol Vol 33, pp 22–31, 2005

Originally Published in Press as DOI: 10.1165/rcmb.2004-0010OC on March 31, 2005

Internet address: www.atsjournals.org

from the B6 IFN- γ KO mouse onto the BALB/c strain, were obtained from The Jackson Laboratory (Bar Harbor, ME). These mice were bred under specific pathogen-free conditions. All mice were used at 8–10 wk of age. All animal experiments were performed according to the animal care guidelines of Hamamatsu University School of Medicine.

Lung Tissue Homogenate

Mice were killed at the various time points after α -GalCer injection. Lung was homogenized in 1 ml of phosphate-buffered saline (PBS) using a homogenizer. The homogenates were centrifuged and stored at -80°C before use.

Immunization and Challenge

Mice were immunized twice by intraperitoneal injection of 10 μg of ovalbumin (OVA, grade V; Sigma, St Louis, MO) absorbed to 50 μl (2.25 mg) of Inject Alum aluminum hydroxide solution (Pierce, Rockford, IL) with a 1-wk interval. Fourteen days after the last immunization, the primed mice were challenged by repeated exposure to an aerosol of OVA (10 mg/ml) dissolved in PBS using a nebulizer. The exposure was done for 30 min once a day for three consecutive days.

α -GalCer Treatment Protocol

α -GalCer was kindly provided by Kirin Brewery Co. (Gunma, Japan). α -GalCer was suspended in PBS supplemented with 0.5% polysorbate-20 (wt/vol), and the control vehicle was PBS supplemented with 0.5% polysorbate-20 (wt/vol). One hundred micrograms per kg body weight of α -GalCer or control vehicle was injected intraperitoneally on the first day of OVA challenge.

BAL

At 6 h or 48 h after the last antigen challenge, mice were killed and the BALF was collected. BAL was performed by cannulation of the trachea, and the whole lungs were lavaged. To measure the levels of cytokines in BALF, BAL was done with PBS (0.5 ml twice) and the fluid was stored at -80°C until assayed. To analyze the cellular components of BALF, BAL was performed with PBS (1 ml four times) and the recovered cells were enumerated using a hemocytometer. Cell differential counts were determined on Diff-Quik (International reagent, Kobe, Japan)-stained cytocentrifuge preparations of the cells recovered from BAL.

Airway Responsiveness

Airway responsiveness was assessed as previously described (27), with a minor modification. Briefly, 48 h after the last OVA challenge, conscious mice were placed into a whole-body plethysmograph chamber (Buxco Electronics, Sharon, CT). Airflow obstruction was expressed as enhanced pause (Penh), calculated as: $\text{Penh} = [\text{Te (expiratory time)} / \text{Tr (relaxation time)} - 1] \times [\text{Pef (peak expiratory flow)} / \text{Pif (peak inspiratory flow)}]$. Penh were measured after 3 min of aerosol administration of PBS (PenhPBS) and 3.125 mg/ml methacholine (Mch; Sigma) (PenhMch) delivered for 3 min. The values of Penh expressed per 15 s were averaged from four determinations recorded every 5 s. Percentage of Penh was calculated as the fold increase of PenhMch compared with PenhPBS: $\% \text{Penh} = \text{PenhMch} / \text{PenhPBS}$.

Lung Histology and Goblet Cell Hyperplasia

The lungs were harvested after BAL and inflated with 0.2 ml of 10% formalin instilled through a tracheostomy tube. Formalin-preserved lung tissue was stained with hematoxylin-eosin (H&E) or periodic acid-Schiff (PAS). Goblet cell hyperplasia was determined by counting the number of PAS⁺ cells surrounding the central airway on a digital image under $\times 200$ magnification. PAS⁺ cells with nuclei within the sections were counted, and four sections were evaluated per lung. The circumference of the airway at the basement membrane was measured by NIH image. The results are expressed as the number of PAS⁺ cells per unit length (mm) of basement membrane.

Measurement of OVA-Specific Antibodies in Serum

At 48 h after the last antigen challenge, blood was drawn from the inferior vena cava for measurement of OVA-specific serum IgE and IgG2a antibodies. OVA-specific antibodies were measured by enzyme-

linked immunosorbent assay (ELISA). Briefly, 96-well plates (Nalge Nunc International, Rochester, NY) were coated with 200 $\mu\text{g}/\text{ml}$ of OVA diluted in 0.1 M NaHCO_3 (pH 8.3). After overnight incubation at 4°C , the plates were washed five times and blocked with 30% Block Ace (Dainippon Seiyaku, Osaka, Japan) in PBS for 30 min at room temperature. Serum samples were serially diluted in 10% Block Ace in PBS/Tween-20 (0.05%) for 1 h at room temperature. The plates were then washed, followed by incubation with alkaline phosphatase-conjugated rat anti-mouse IgE and IgG2a antibodies (Southern Biotechnology, Birmingham, AL). After washing, *p*-nitrophenyl phosphate substrate (Sigma) buffer was added to the wells and the absorbance was measured at 405 nm with an EL340 I automatic plate reader (Bio-tec instruments, Winooski, VT).

Cytokine Assay

Mouse IL-4, IL-5, IL-12, IL-13, and IFN- γ levels in BALF, serum, and lung homogenate, were measured by ELISA (R&D Systems, Minneapolis, MN).

Statistical Analysis

Data are expressed as the mean $\pm$ SEM unless otherwise indicated. A Mann-Whitney U test and two-way repeated measures ANOVA were used for statistical analysis with the Stat View statistical program (Abacus Concept, Berkeley, CA). A *P* value of less than 0.05 was considered statistically significant.

RESULTS

NKT Cell Activation by α -GalCer *In Vivo*

Previous studies have demonstrated that α -GalCer stimulates NKT cells to produce both IL-4 and IFN- γ (18, 19). To ascertain that the amount of α -GalCer (100 $\mu\text{g}/\text{kg}$) is relevant for activating NKT cells *in vivo*, we measured the serum cytokine levels at different time points after injection of α -GalCer in naïve wild-type mice. As shown in Figure 1A, intraperitoneal administration of α -GalCer induced a rapid rise of IL-4 with the peak value (3.88 ng/ml) at 3 h and a delayed elevation of IFN- γ with the peak value (6.26 ng/ml) at 24 h in the serum. The elevations of IL-4 and IFN- γ in α -GalCer-injected mice were consistent with those of a previous study (28). The time course of IFN- γ response was much longer than that of IL-4. As well as in the sera, a rapid rise of IL-4 and prolonged elevation of IFN- γ concentration were found in the lung tissue, although a rise IFN- γ was more rapid (6 h) in the lung tissue than in the sera (Figure 1B). Because antigen-presenting cells have been shown to produce IL-12 after α -GalCer administration (29), we also measured the levels of IL-12 protein in the sera and the lung homogenates. In the sera, its level was transiently elevated in a single injection of α -GalCer in wild-type mice, but the peak value was much lower than that of IFN- γ or IL-4 (Figure 1A). In addition, a very small increase in IL-12 protein was found in the lung homogenates (Figure 1B).

Effects of α -GalCer on Lung Histology

To determine whether α -GalCer could modulate antigen-induced airway inflammation, we administered α -GalCer into mice at the first challenge of OVA. In sham-sensitized, sham- or OVA-challenged mice treated with α -GalCer or vehicle, there were no inflammatory cell infiltrates, including eosinophils, in the lung histology (Figures 2A–2D). In OVA-sensitized mice treated with vehicle, OVA challenge induced a marked eosinophilic infiltration around the airways and vessels (Figure 2E). Strikingly, a single administration of α -GalCer (100 $\mu\text{g}/\text{kg}$) almost completely abrogated the eosinophilic inflammation in the lungs of OVA-challenged mice (Figure 2F).

Effects of α -GalCer on BAL Cells

Next, we examined the effect of α -GalCer on the cell number and components of the BALF. In OVA-sensitized, OVA-challenged

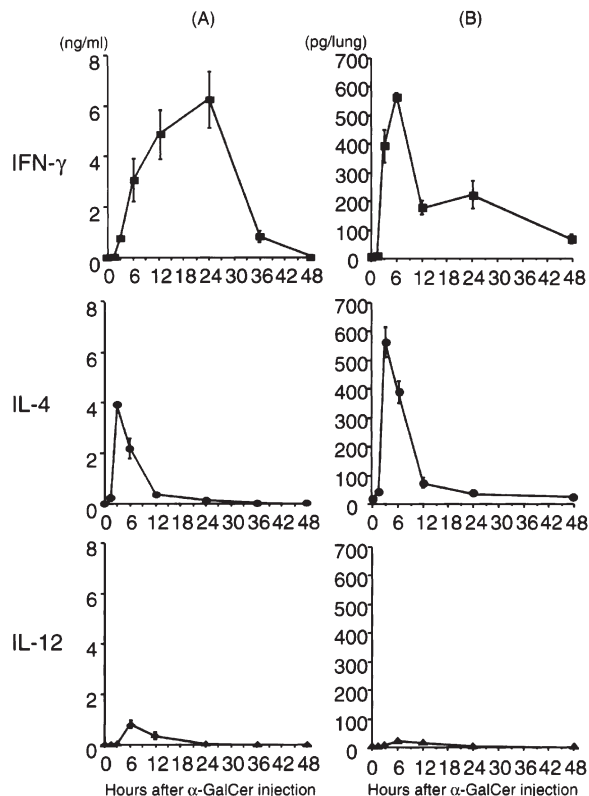


Figure 1. Elevation of serum and lung tissue cytokines in naïve BALB/c mice treated with α -GalCer. Each dot in the figure shows the cytokine concentration in a pooled sample from three or four mice. Data are expressed as means $\pm$ SEM.

mice, a marked increase in the numbers of eosinophils and lymphocytes, as well as total cells, was observed 6 h and 48 h after the last challenge, compared with sham-sensitized, sham- or OVA-challenged mice with vehicle administration (Figures 3A and 4A). Treatment of α -GalCer (100 μ g/kg) significantly decreased the numbers of eosinophils, lymphocytes, and total cells in the BALF, compared with OVA-sensitized, OVA-challenged mice treated with vehicle 6 h and 48 h after the last OVA exposure (Figures 3A and 4A). α -GalCer caused a dose-dependent inhibition of antigen-induced airway inflammation, giving a plateau at the dose of 100 μ g/kg, as evaluated by eosinophil counts in the BALF (Figure 5). Treatment with the dose of less than 0.1 μ g/kg α -GalCer did not alter the number and differential of the cells obtained in the BALF (Figure 5).

Effects of α -GalCer on Cytokine Profile of BALF

Furthermore, we determined whether administration of α -GalCer altered the cytokine profile in the BALF. In OVA-sensitized, OVA-challenged mice with vehicle administration, high amounts of Th2 cytokines, including IL-4, IL-5, and IL-13, were detected (Figures 3B and 4B). Administration of α -GalCer (100 μ g/kg) significantly reduced the levels of IL-4, IL-5, and IL-13 at 6 h and at 48 h in the BALF (Figures 3B and 4B). In sham-sensitized, sham- or OVA-challenged mice treated with vehicle or α -GalCer, Th2 cytokines were not detected in the sera. IFN- γ protein was not detected in the BALF of the control mice, including sham-sensitized, sham- or OVA-challenged mice, and OVA-sensitized, OVA-challenged mice with vehicle or α -GalCer (data not shown).

Effects of α -GalCer on Airway Responsiveness

Because AHR is a hallmark and distinguishing feature of asthma, we next determined whether α -GalCer administration would affect the airway responsiveness in mice. As shown in Figure 6A, α -GalCer administration significantly reduced the airway responsiveness in OVA-sensitized, OVA-challenged mice compared with vehicle administration, and the airway responsiveness did not increase in sham-sensitized, OVA-challenged mice with α -GalCer or vehicle treatment.

Effects of α -GalCer on Goblet Cell Hyperplasia

Airway mucus hypersecretion is a prominent feature of asthma (30) and animal models of this disease reveal goblet cell hyperplasia in the airway (31, 32). Because mucin productions in goblet cells can be visualized by PAS staining, we used this staining to quantify goblet cell hyperplasia. In OVA-sensitized, OVA-challenged mice, exposure of OVA increased the number of PAS⁺ cells in the airway epithelia. However, α -GalCer treatment significantly inhibited goblet cell hyperplasia compared with vehicle treatment (Figure 6B). Goblet cell hyperplasia was not seen in the control mice treated with α -GalCer or vehicle (Figure 6B).

Effects of α -GalCer on OVA-Specific IgE and IgG2a

It has been well documented that IgE responses are mediated by antigen-specific Th2 cells, whereas IgG2a responses depend on Th1 cells (33). Thus, we assessed whether α -GalCer treatment influenced OVA-specific IgE and IgG2a production. In OVA-sensitized, OVA-challenged mice with vehicle or α -GalCer treatment, OVA-specific IgE and IgG2a were readily detected by ELISA (Figure 6C). Mice administered α -GalCer had significantly higher levels of OVA-specific IgG2a than those given vehicle (Figure 6C). The levels of OVA-specific IgE tended to be lower in α -GalCer-treated than vehicle-treated mice, but the difference was not significant (Figure 6C). In the control mice, OVA-specific IgE and IgG2a were not detected (Figure 6C).

Effects of α -GalCer on OVA-Induced Airway Inflammation in IFN- γ KO Mice

Because ligand-activated NKT cells secrete a large amount of IFN- γ , we hypothesized that IFN- γ production from NKT cells stimulated by α -GalCer might be essential for the inhibition of OVA-induced airway inflammation. To clarify this, we examined the effect of α -GalCer on airway inflammation in IFN- γ KO mice. The protocol used in wild-type mice could induce OVA-specific airway inflammation even in IFN- γ KO mice. In sham-sensitized, OVA-challenged IFN- γ KO mice, no eosinophilic infiltration was found (Figures 7A and 7B), whereas OVA-sensitized, OVA-challenged IFN- γ KO mice showed allergic airway inflammation (Figure 7C). The percentage of BALF eosinophils was significantly higher in OVA-sensitized, OVA-challenged IFN- γ KO mice with vehicle treatment than in OVA-sensitized, sham-challenged IFN- γ KO mice with vehicle treatment ($25.4 \pm 2.8\%$ versus 0.0% , respectively, $P = 0.0002$). Contrary to wild-type mice, in OVA-sensitized, OVA-challenged IFN- γ KO mice, α -GalCer treatment did not inhibit cellular infiltration of the lung (Figure 7D) nor reduce the numbers of BALF eosinophils, lymphocytes, or total cells (Figure 8A). No significant difference in the levels of BALF IL-4, IL-5, or IL-13 was found between vehicle- and α -GalCer-treated IFN- γ KO mice, although these cytokines tended to be higher in α -GalCer-treated IFN- γ KO mice (Figure 8B). No significant difference in the airway responsiveness was found between α -GalCer- and vehicle-treated IFN- γ KO mice, although the airway responsiveness tended to be higher in α -GalCer-treated IFN- γ KO mice (Figure 9A). Further, administration of α -GalCer had no effect on the numbers

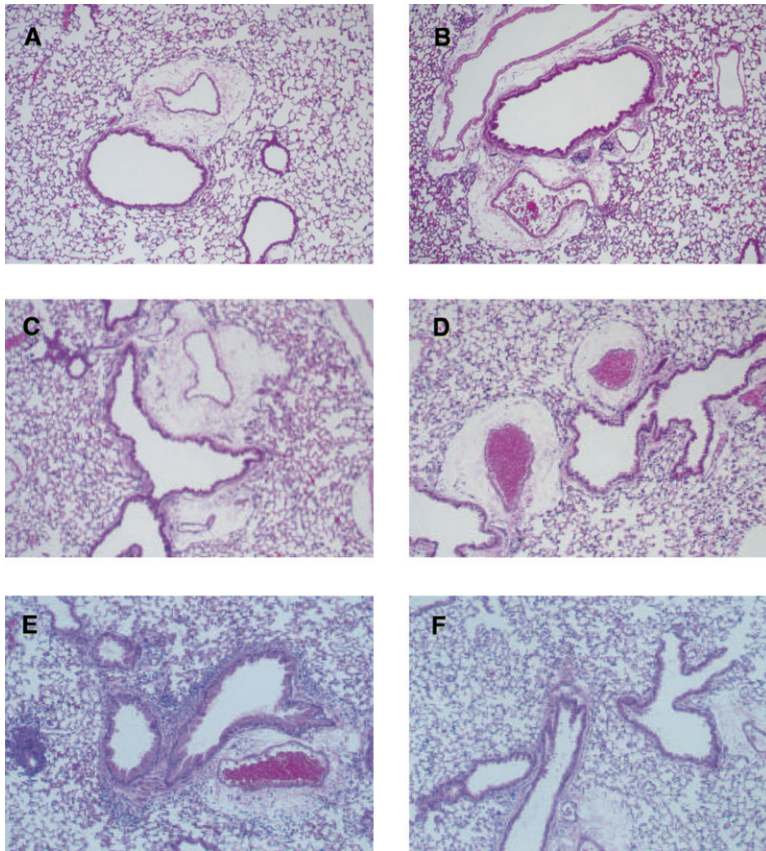


Figure 2. Decrease of lung inflammatory cell infiltration in OVA-sensitized, OVA-challenged BALB/c mice by α -GalCer injection. A lung tissue section of sham-sensitized, sham-challenged mice treated with vehicle (A) or α -GalCer (B), sham-sensitized, OVA-challenged mice treated with vehicle (C) or α -GalCer (D), and OVA-sensitized, OVA-challenged mice treated with vehicle (E) or α -GalCer (F). The magnifications were $\times 200$.

of PAS⁺ cells in the airway epithelia (Figure 9B), or OVA-specific IgE in the sera (Figure 9C). IgG2a was not detected in either α -GalCer-treated or vehicle-treated IFN- γ KO mice (data not shown). These results indicate that α -GalCer could not inhibit the allergic airway inflammation in IFN- γ KO mice, suggesting that the inhibitory effect of α -GalCer is mediated by IFN- γ production.

DISCUSSION

In the present study, we demonstrate that α -GalCer, a specific ligand for NKT cells, prevents AHR and allergic airway inflammation in a murine model of asthma. A single administration of α -GalCer almost completely abrogated an infiltrate with eosinophils in the lung and reduced AHR, together with the decrease in BALF Th2 cytokine expression and goblet cell hyperplasia. This protection was accompanied by a significant increase in the serum levels of antigen-specific IgG2a and a decrease in those of antigen-specific IgE. By contrast, the same ligand failed to exert such preventive effect in IFN- γ KO mice. These data suggest that the specific activation of NKT cells by α -GalCer inhibits an antigen-specific Th2 response and AHR possibly by IFN- γ production. This is the first report to demonstrate a potential therapeutic role of α -GalCer against asthma.

Recent studies have demonstrated that administration of Th1 cytokines, such as IFN- γ or IL-12, inhibits antigen-induced airway inflammation, AHR, and Th2 cytokine expression in a murine model of asthma (9, 10), providing a basis for Th1-predisposing immunotherapy against asthma. In the present study, we found that administration of α -GalCer elicited a prolonged rise in IFN- γ as well as a rapid peak of IL-4 in the sera as well as in the lung tissues, and that the production of IFN- γ was much longer than that of IL-4. Regarding IL-12, α -GalCer

cannot activate NKT cells to produce IL-12. However, a recent study demonstrated that α -GalCer administration stimulates APCs to secrete IL-12 through CD40-CD40L ligation between ligand-activated NKT cells and APCs (29). In the present study, however, we found only a negligible increase of IL-12 protein in the lung with a transient, low peak of serum IL-12 in the α -GalCer-injected mice. Recently, the protective effects of α -GalCer against infections as well as tumors have been shown to be mediated by IFN- γ , not dependent on IL-12 (34–36). Collectively, these results suggest that IFN- γ play a major role in the protection of allergic airway inflammation by α -GalCer. This idea is strongly supported by the observation that α -GalCer failed to prevent asthmatic phenotypes in IFN- γ KO mice. Possibly, IFN- γ production elicited by α -GalCer markedly reduced Th2 cytokine production in the lung, which, in turn, might decrease AHR, eosinophilic infiltration, and goblet cell hyperplasia in the airway. In addition, increased IFN- γ production, together with the reduced Th2 cytokine expression, is likely to cause a significant elevation in the serum levels of OVA-specific IgG2a corresponding to a Th1 response, and a decrease in those of OVA-specific IgE, which indicates a shift of antigen-specific responses from a Th2-dominant type toward a Th1-dominant type.

In our models of allergic airway inflammation, IFN- γ KO mice showed weaker response to OVA challenge than wild-type mice. The counts of total cells, eosinophils, and lymphocytes in the BALF were lower in OVA-sensitized, OVA-challenged IFN- γ KO mice than in OVA-sensitized, OVA-challenged wild-type mice. A less efficient allergic response to OVA was reported in IFN- γ KO mice than in wild-type mice (37). Because IFN- γ KO mice was found to have reduced major histocompatibility complex class II expression on macrophages (38), it is possible

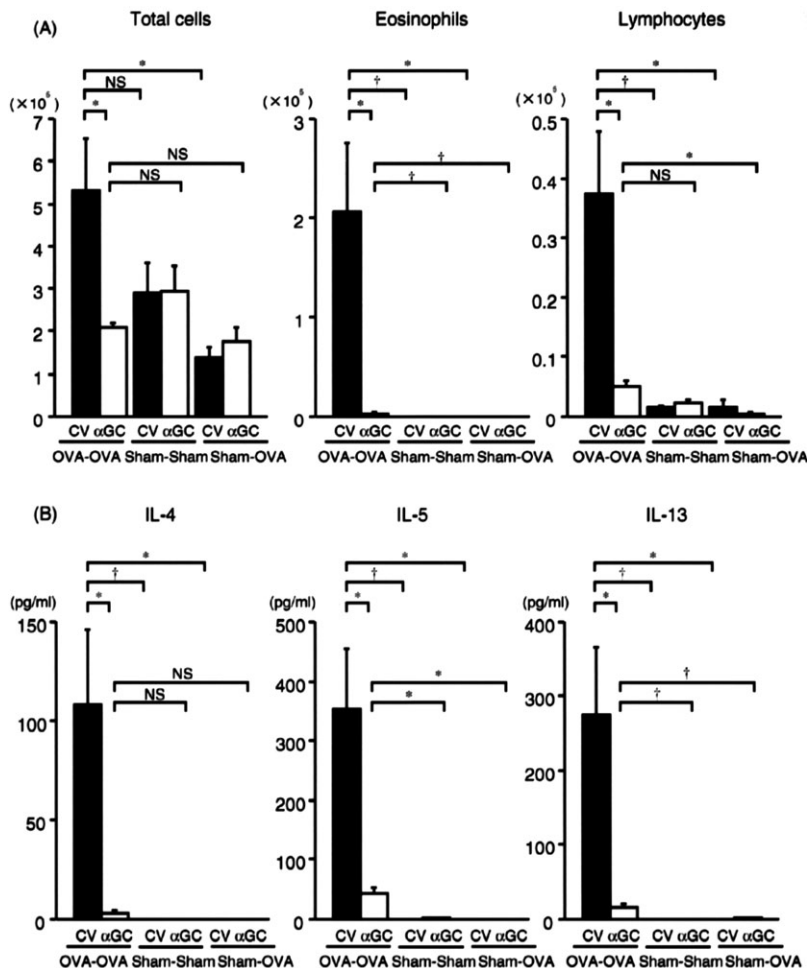


Figure 3. α -GalCer inhibits allergic airway inflammation in OVA-challenged BALB/c mice 6 h after the last OVA exposure. Black columns (CV) and open columns (α GC) indicate mice treated with control vehicle and α -GalCer, respectively. (A) Numbers of total cells, eosinophils and lymphocytes in BALF. (B) Cytokine concentration in BALF. Data are expressed as means $\pm$ SEM. (OVA-sensitized, OVA-challenged mice treated with control vehicle, $n = 9$; α -GalCer, $n = 12$; sham-sensitized, OVA-challenged mice treated with control vehicle, $n = 4$; α -GalCer, $n = 4$; sham-sensitized, sham-challenged mice treated with control vehicle, $n = 4$; α -GalCer, $n = 4$. * $P < 0.01$; † $P < 0.05$; NS, not significant.)

that antigen presentation by antigen-presenting cells would be less efficient, resulting in a weak allergic response. However, allergic airway inflammation did occur in IFN- γ KO mice based on the differential counts of BALF cells and the lung histology. Indeed, the percentage of BALF eosinophils was significantly higher in OVA-sensitized, OVA-challenged IFN- γ KO mice with vehicle treatment than in OVA-sensitized, sham-challenged IFN- γ KO mice with vehicle treatment ($25.4 \pm 2.8\%$ versus 0% , respectively), and the lung histology of OVA-sensitized, OVA-challenged IFN- γ KO mice, but not OVA-sensitized, sham-challenged IFN- γ KO mice, showed an infiltrate with eosinophils. Interestingly, this allergic airway inflammation could not be inhibited by α -GalCer in IFN- γ KO mice. Although IFN- γ KO mice might have a less efficient allergic response, several studies have demonstrated that an inhibitory effect of certain ligands on allergic airway inflammation can be evaluated even in those mice (39, 40). For example, Rodriguez and coworkers and Kline and colleagues independently showed that specific ligands of Toll-like receptors, lipopolysaccharide, and CpG oligodeoxynucleotides dramatically suppressed allergic airway inflammation in wild-type mice (39, 40). Further, to clarify whether this suppression is dependent on IFN- γ , they performed the experiments using IFN- γ KO mice, and found that lipopolysaccharide and CpG oligodeoxynucleotides could almost completely inhibit allergic airway inflammation even in IFN- γ KO mice, leading them to conclude that the suppression by these ligands is not mediated by IFN- γ . In sharp contrast to those observations, the present study showed that α -GalCer failed to inhibit allergic

airway inflammation in IFN- γ KO mice. To interpret our data, there is a limitation that the allergic response to OVA observed in IFN- γ KO mice was not comparable to that in wild-type mice. Taken together our results with those of the previous studies; however, the inhibitory effect of α -GalCer is mediated, at least in part, by IFN- γ .

Activated NKT cells by α -GalCer produce a variety of cytokines, including IFN- γ , IL-4, and IL-13 (11–13). Recently, NKT cells have been shown to contribute to the regulation of different types of immune responses such as tumor immunity, autoimmunity, and infection (11–13). An important unresolved issue concerning NKT cells is that they could be able to show flexibility in their cytokine response and become polarized in the Th1 or Th2 direction. Originally, ligand-activated NKT cells were reported to favor the development of a Th2 response, because they were thought to be primary IL-4-producing cells in the initial phase of immune responses. Consistent with this, α -GalCer was shown to protect animal models of autoimmune diseases, which are mediated by a Th1-dominant response, such as type1 diabetes (21, 22) and experimental autoimmune encephalomyelitis (20, 41) by inducing Th2 polarization. However, it has become clear that NKT cells are not essential for establishing a Th2 response, as evidenced by the fact that intact Th2 responses can be retained in CD1 d- or β_2 -microglobulin-deficient mice, which are devoid of NKT cells (23, 42). Recent studies demonstrated that α -GalCer-activated NKT cells suppressed the Th2 response against *Nippostrongylus brasiliensis* (23) and protected mice against malaria infection through their IFN- γ production (35).

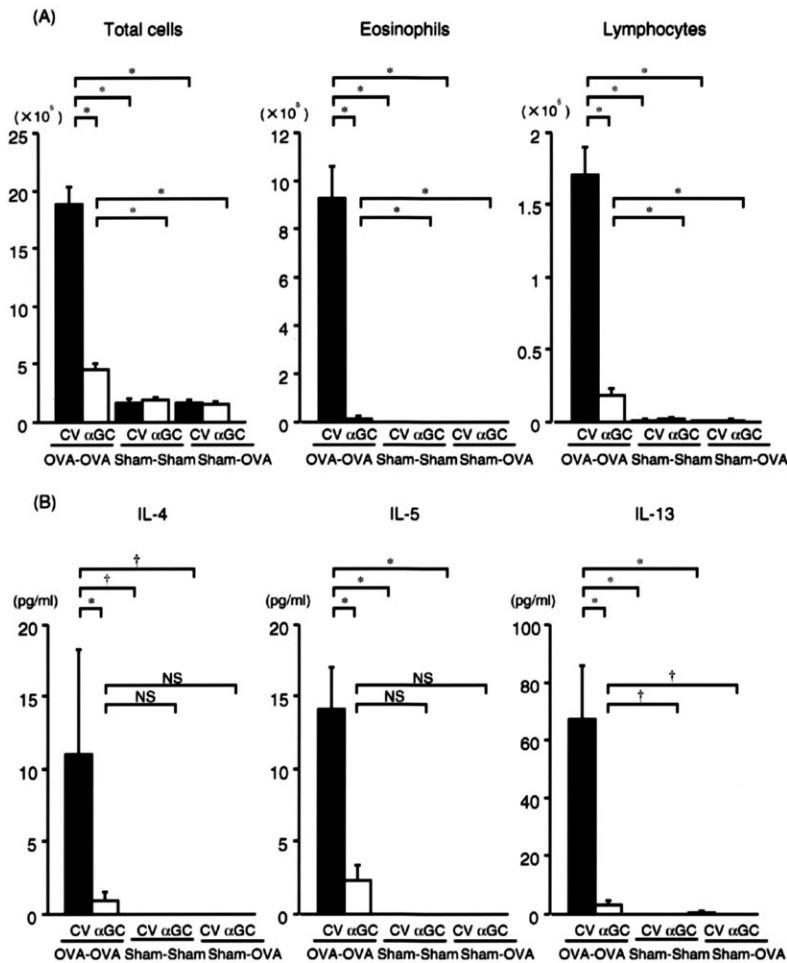


Figure 4. α -GalCer inhibits allergic airway inflammation in OVA-challenged BALB/c mice 48 h after the last OVA exposure. Black columns (CV) and open columns (α GC) indicate mice treated with control vehicle and α -GalCer, respectively. (A) Numbers of total cells, eosinophils, and lymphocytes in BALF. (B) Cytokine concentration in BALF. Data are expressed as means $\pm$ SEM. (OVA-sensitized, OVA-challenged mice treated with control vehicle, $n = 13$; α -GalCer, $n = 8$; sham-sensitized, OVA-challenged mice treated with control vehicle, $n = 4$; α -GalCer, $n = 4$; sham-sensitized, sham-challenged mice treated with control vehicle, $n = 4$; α -GalCer, $n = 4$. * $P < 0.01$; † $P < 0.05$; NS, not significant.)

More recently, administration of α -GalCer was shown to elicit prolonged IFN- γ production from NKT cells in mice infected with *Cryptococcus neoformans*, resulting in the augmentation of local host resistance to this fungus (24). These data suggest that ligand-activated NKT cells also contribute to Th1-mediated responses. Collectively, the role of NKT cells in promoting a Th1 or Th2 response is still debatable. The contradictory evi-

dence might be partially related to differences in the site, dosage, and frequency of the ligand administration.

In allergic airway inflammation, there have been few studies investigating the role of NKT cells. Interestingly, using NKT cell-deficient mice two recent studies by Akbari and coworkers and Lisbonne and colleagues demonstrated the requirement of NKT cell for the development of the characteristic features of

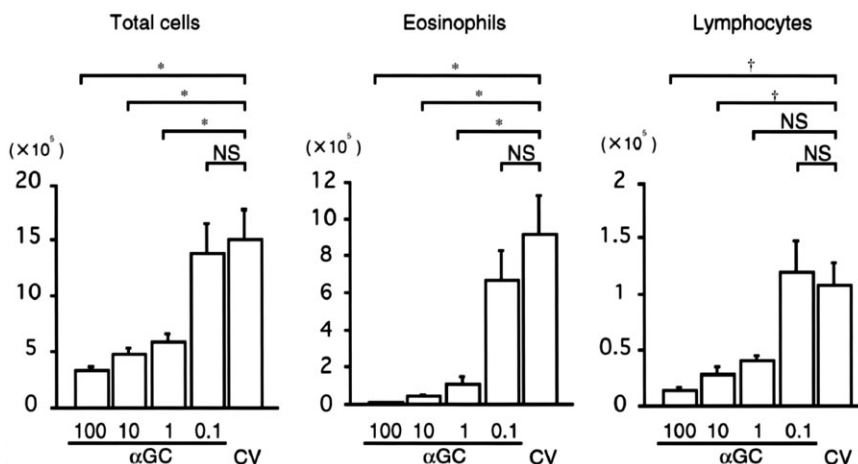


Figure 5. α -GalCer causes a dose-dependent inhibition of antigen-induced airway inflammation. Data are expressed as means $\pm$ SEM of five to eight mice per group. (* $P < 0.01$ compared with control vehicle-treated mice. † $P < 0.05$ compared with control vehicle-treated mice. NS, not significant compared with control vehicle-treated mice.)

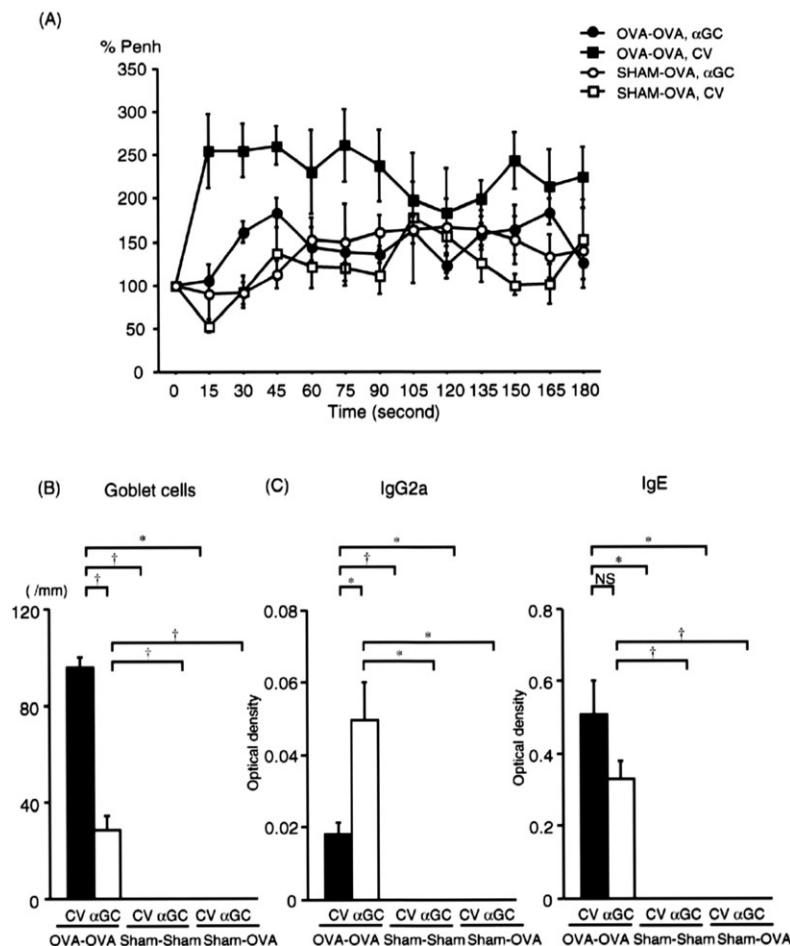


Figure 6. α -GalCer is effective in prevention of AHR, goblet cell hyperplasia, and alteration of the immunoglobulin production. (A) Airway responsiveness. Data are expressed as means $\pm$ SEM for each group ($n = 4$). Significant differences ($P < 0.01$) are found between curve of α -GalCer-treated OVA-sensitized, OVA-challenged mice and vehicle-treated OVA-sensitized, OVA-challenged mice or sham-sensitized, OVA-challenged mice. (B) Goblet cell hyperplasia of the lungs. (C) Serum OVA-specific IgG2a and IgE levels. Data are expressed as means $\pm$ SEM. (B and C; OVA-sensitized, OVA-challenged mice treated with control vehicle [CV, black columns], $n = 13$; α -GalCer [α GC, open columns], $n = 8$; sham-sensitized, OVA-challenged mice treated with control vehicle, $n = 4$; α -GalCer, $n = 4$; sham-sensitized, sham-challenged mice treated with control vehicle, $n = 4$; α -GalCer, $n = 4$. * $P < 0.01$; $\dagger P < 0.05$; NS, not significant.)

asthma (25, 26). $V\alpha 14i$ NKT cell-deficient mice were shown to develop decreased AHR and OVA-induced airway inflammation, but adoptive transfer of $V\alpha 14i$ NKT cells producing IL-4 and IL-13 fully restored them. They considered that administra-

tion of nominal antigen into the lungs stimulates $V\alpha 14i$ NKT cells through mechanisms that are independent of α -GalCer. However, the precise mechanisms by which antigen administration into the lungs stimulates $V\alpha 14i$ NKT cells are unclear,

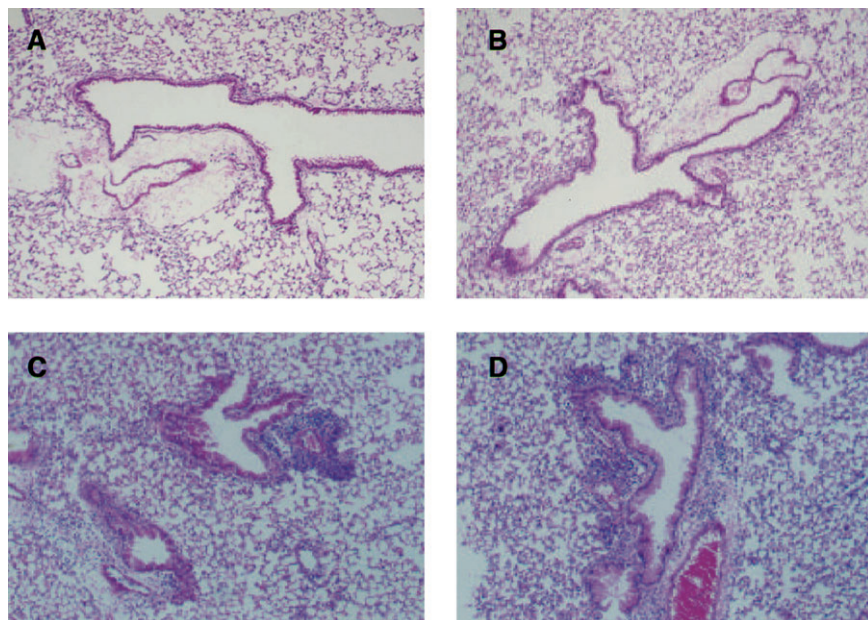


Figure 7. α -GalCer does not alter the lung inflammatory cell infiltration in OVA-challenged IFN- γ KO mice. A lung section of sham-sensitized, OVA-challenged IFN- γ KO mice treated with vehicle (A) or α -GalCer (B), and OVA-sensitized, OVA-challenged IFN- γ KO mice treated with vehicle (C) or α -GalCer (D). The magnifications were $\times 200$.

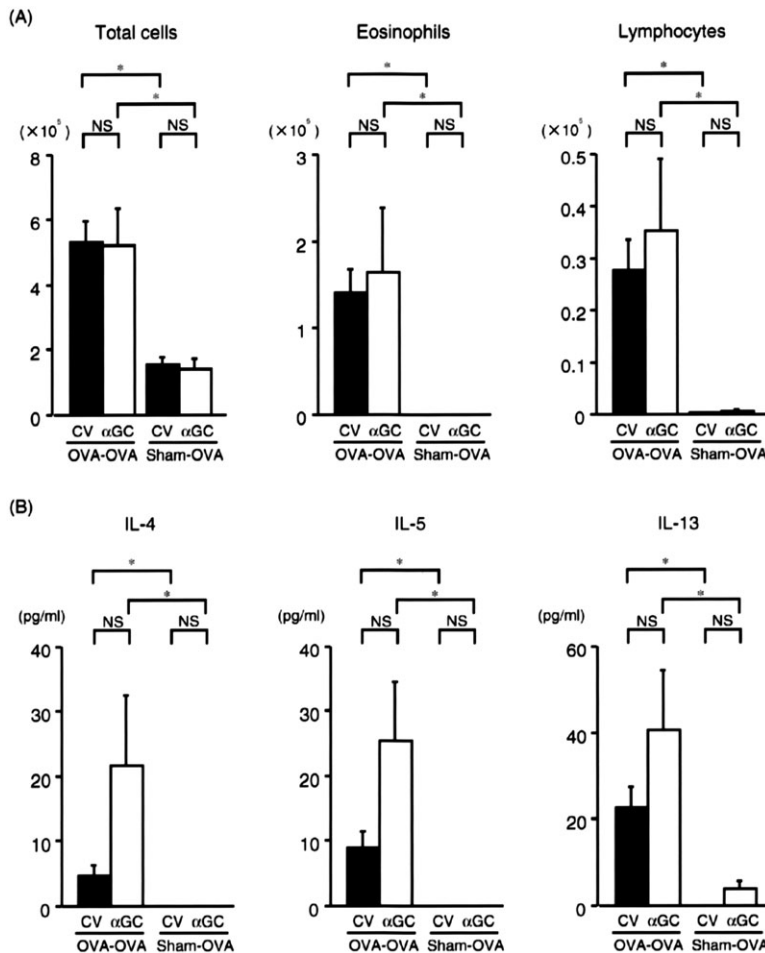


Figure 8. α -GalCer does not inhibit allergic airway inflammation in OVA-challenged IFN- γ KO mice 48 h after the last OVA exposure. Black columns (CV) and open columns (α GC) indicate IFN- γ KO mice treated with control vehicle and α -GalCer, respectively. (A) Numbers of total cells, eosinophils, and lymphocytes in BALF. (B) Cytokine content in BALF. Data are expressed as means $\pm$ SEM. (OVA-sensitized, OVA-challenged mice treated with control vehicle, $n = 12$; α -GalCer, $n = 11$; sham-sensitized, OVA-challenged mice treated with control vehicle, $n = 4$; α -GalCer, $n = 4$). * $P < 0.01$; $^{\dagger}P < 0.05$; NS, not significant.)

because OVA itself is unable to activate V α 14i NKT cells. Both Akbari and colleagues and Lisbonne and coworkers proposed that unknown self antigens, presumably glycolipids, that are exposed by antigen challenge into the lung could bind CD1 d, which is recognized by V α 14i NKT cells, thereby stimulating these cells to produce IL-4 and IL-13. Thus, they concluded that V α 14i NKT cells in the lungs play a crucial role in the development of asthma, suggesting that suppression of NKT cell function might be a therapeutic strategy for the treatment of asthma. In contrast to these observations, we herein show that stimulation of NKT cells by α -GalCer can indeed inhibit asthmatic phenotypes, such as AHR and allergic airway inflammation. Possibly, this discrepancy is explained by the different natures and doses of ligands that are recognized by NKT cells. Miyamoto and colleagues recently demonstrated that analogs of α -GalCer synthesized by replacing sugar moiety and/or truncating the aliphatic chains induced a predominant production of IL-4, but not IFN- γ , by NKT cells, thus preventing autoimmune encephalomyelitis (41). This evidence clearly indicates that NKT cells differently respond to different ligands in terms of their profiles of cytokine production. Thus, α -GalCer and putative endogenous ligands exposed by antigen challenge into the lung may differ with the capacities to modulate the immune response toward Th1- or Th2-type through ligand-activation of NKT cells, but further study will be needed to clarify this. Collectively, although NKT cells may be required for developing asthma, α -GalCer does inhibit AHR and allergic airway inflammation probably through large IFN- γ production from ligand-activated

NKT cells, providing important evidence for designing a therapeutic strategy targeting NKT cells using α -GalCer.

Because recognition of α -GalCer by NKT cells is highly conserved among species, α -GalCer can bind both mouse and human CD1 d and activate NKT cells of either species. Thus, the present data provide the rationale for an immunotherapy with α -GalCer for human asthma. Effective immunotherapy with Th1 cytokines, such as IFN- γ or IL-12, was shown to need daily consecutive injections in animal models of asthma (10, 43). In the practical setting for humans, daily administration of these Th1 cytokines is difficult. In addition, their adverse effects have hampered clinical applications of Th1 cytokines (44). In contrast, the present results demonstrate that only a single injection of α -GalCer is sufficient to protect against airway inflammation, because it induced a sustained increase of serum and lung IFN- γ . Recently, a phase I study of α -GalCer in patients with solid tumors was undertaken (45). In this study, the patients were treated with weekly intravenous injections of α -GalCer, and this ligand was well tolerated over a wide range of doses. Taken together, these data suggest a practical use of α -GalCer for therapeutic intervention in asthma.

In conclusion, the present study documents a novel approach for protecting allergic airway inflammation by targeting NKT cells with α -GalCer, a synthetic ligand specific for NKT cells. It is intriguing that a single application of α -GalCer effectively prevents AHR and OVA-induced allergic airway inflammation through IFN- γ production, which suggests the therapeutic potential of α -GalCer in the treatment of asthma.

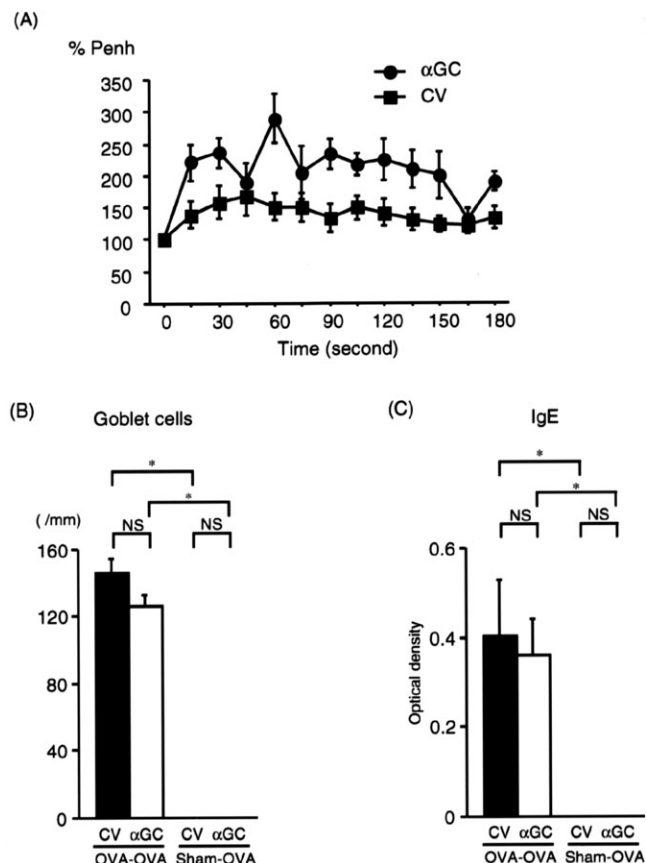


Figure 9. Administration of α -GalCer has no effect of airway responsiveness, goblet cell hyperplasia, or IgE production. (A) Airway responsiveness. Data are expressed as means $\pm$ SEM for each group ($n = 4$). No significant difference between curve of α -GalCer- and vehicle-treated mice. (B) Goblet cell hyperplasia. (C) Serum OVA-specific IgE levels. Data are expressed as means $\pm$ SEM. (B and C: OVA-sensitized, OVA-challenged mice treated with control vehicle [CV, black columns], $n = 12$; α -GalCer [α GC, open columns], $n = 11$; sham-sensitized, OVA-challenged mice treated with control vehicle, $n = 4$; α -GalCer, $n = 4$. * $P < 0.01$; $^{\dagger}P < 0.05$; NS, not significant.)

Conflict of Interest Statement: H.M. has no declared conflicts of interest; T.S. has no declared conflicts of interest; J.S. has no declared conflicts of interest; T.N. has no declared conflicts of interest; Y.K. has no declared conflicts of interest; K.C. has no declared conflicts of interest; and H.N. has no declared conflicts of interest.

References

- Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu Rev Immunol* 1999;17:255–281.
- Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, Bentley AM, Corrigan C, Durham SR, Kay AB. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. *N Engl J Med* 1992;326:298–304.
- Robinson D, Hamid Q, Bentley A, Ying S, Kay AB, Durham SR. Activation of CD4⁺ T cells, increased TH2-type cytokine mRNA expression, and eosinophil recruitment in bronchoalveolar lavage after allergen inhalation challenge in patients with atopic asthma. *J Allergy Clin Immunol* 1993;92:313–324.
- Seminario MC, Gleich GJ. The role of eosinophils in the pathogenesis of asthma. *Curr Opin Immunol* 1994;6:860–864.
- Busse WW, Lemanske RF Jr. Asthma. *N Engl J Med* 2001;344:350–362.
- Cohn L, Tepper JS, Bottomly K. IL-4-independent induction of airway hyperresponsiveness by Th2, but not Th1, cells. *J Immunol* 1998;161:3813–3816.
- Cohn L, Homer RJ, Marinov A, Rankin J, Bottomly K. Induction of airway mucus production by T helper 2 (Th2) cells: a critical role for

interleukin 4 in cell recruitment but not mucus production. *J Exp Med* 1997;186:1737–1747.

- Hogan SP, Matthei KI, Young JM, Koskinen A, Young IG, Foster PS. A novel T cell-regulated mechanism modulating allergen-induced airways hyperreactivity in BALB/c mice independently of IL-4 and IL-5. *J Immunol* 1998;161:1501–1509.
- Nakajima H, Iwamoto I, Yoshida S. Aerosolized recombinant interferon- γ prevents antigen-induced eosinophil recruitment in mouse trachea. *Am Rev Respir Dis* 1993;148:1102–1104.
- Iwamoto I, Nakajima H, Endo H, Yoshida S. Interferon gamma regulates antigen-induced eosinophil recruitment into the mouse airways by inhibiting the infiltration of CD4⁺ T cells. *J Exp Med* 1993;177:573–576.
- Bendelac A, Rivera MN, Park SH, Roark JH. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu Rev Immunol* 1997;15:535–562.
- Godfrey DI, Hammond KJ, Poulton LD, Smyth MJ, Baxter AG. NKT cells: facts, functions and fallacies. *Immunol Today* 2000;21:573–583.
- Kronenberg M, Gapin L. The unconventional lifestyle of NKT cells. *Nat Rev Immunol* 2002;2:557–568.
- Lantz O, Bendelac A. An invariant T cell receptor α chain is used by a unique subset of major histocompatibility complex class I-specific CD4⁺ and CD48⁺ T cells in mice and humans. *J Exp Med* 1994;180:1097–1106.
- Porcelli S, Yockey CE, Brenner MB, Balk SP. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD48⁺ α/β T cells demonstrates preferential use of several V β genes and an invariant TCR α chain. *J Exp Med* 1993;178:1–16.
- Dellabona P, Casorati G, Friedli B, Angman L, Sallusto F, Tunncliffe A, Roosneek E, Lanzavecchia A. In vivo persistence of expanded clones specific for bacterial antigens within the human T cell receptor α/β CD48⁺ subset. *J Exp Med* 1993;177:1763–1771.
- Bendelac A, Lantz O, Quimby ME, Yewdell JW, Bennink JR, Brutkiewicz RR. CD1 recognition by mouse NK1⁺ T lymphocytes. *Science* 1995;268:863–865.
- Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, et al. CD1d-restricted and TCR-mediated activation of V α 14 NKT cells by glycosylceramides. *Science* 1997;278:1626–1629.
- Burdin N, Brossay L, Koezuka Y, Smiley ST, Grusby MJ, Gui M, Taniguchi M, Hayakawa K, Kronenberg M. Selective ability of mouse CD1 to present glycolipids: α -galactosylceramide specifically stimulates V α 14⁺ NK T lymphocytes. *J Immunol* 1998;161:3271–3281.
- Jahng AW, Maricic I, Pedersen B, Burdin N, Naidenko O, Kronenberg M, Koezuka Y, Kumar V. Activation of natural killer T cells potentiates or prevents experimental autoimmune encephalomyelitis. *J Exp Med* 2001;194:1789–1799.
- Sharif S, Arreaza GA, Zucker P, Mi QS, Sondhi J, Naidenko OV, Kronenberg M, Koezuka Y, Delovitch TL, Gombert JM, et al. Activation of natural killer T cells by α -galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes. *Nat Med* 2001;7:1057–1062.
- Hong S, Wilson MT, Serizawa I, Wu L, Singh N, Naidenko OV, Miura T, Haba T, Scherer DC, Wei J, et al. The natural killer T-cell ligand α -galactosylceramide prevents autoimmune diabetes in non-obese diabetic mice. *Nat Med* 2001;7:1052–1056.
- Cui J, Watanabe N, Kawano T, Yamashita M, Kamata T, Shimizu C, Kimura M, Shimizu E, Koike J, Koseki H, et al. Inhibition of T helper cell type 2 cell differentiation and immunoglobulin E response by ligand-activated V α 14 natural killer T cells. *J Exp Med* 1999;190:783–792.
- Kawakami K, Kinjo Y, Yara S, Koguchi Y, Uezu K, Nakayama T, Taniguchi M, Saito A. Activation of V α 14⁺ natural killer T cells by α -galactosylceramide results in development of Th1 response and local host resistance in mice infected with *Cryptococcus neoformans*. *Infect Immun* 2001;69:213–220.
- Akbari O, Stock P, Meyer E, Kronenberg M, Sidobre S, Nakayama T, Taniguchi M, Grusby MJ, DeKruyff RH, Umetsu DT. Essential role of NKT cells producing IL-4 and IL-13 in the development of allergen-induced airway hyperreactivity. *Nat Med* 2003;9:582–588.
- Lisbonne M, Diem S, de Castro Keller A, Lefort J, Araujo LM, Hachem P, Fournneau JM, Sidobre S, Kronenberg M, Taniguchi M, et al. Cutting edge: invariant V α 14 NKT cells are required for allergen-induced airway inflammation and hyperreactivity in an experimental asthma model. *J Immunol* 2003;171:1637–1641.
- Russo M, Nahori MA, Lefort J, Gomes E, de Castro Keller A, Rodriguez D, Ribeiro OG, Adriauch S, Gallois V, de Faria AM, et al. Suppression

- of asthma-like responses in different mouse strains by oral tolerance. *Am J Respir Cell Mol Biol* 2001;24:518–526.
28. Kitamura H, Iwakabe K, Yahata T, Nishimura S, Ohta A, Ohmi Y, Sato M, Takeda K, Okumura K, Van Kaer L, *et al.* The natural killer T (NKT) cell ligand α -galactosylceramide demonstrates its immunopotentiating effect by inducing interleukin (IL)-12 production by dendritic cells and IL-12 receptor expression on NKT cells. *J Exp Med* 1999;189:1121–1128.
 29. Tomura M, Yu WG, Ahn HJ, Yamashita M, Yang YF, Ono S, Hamaoka T, Kawano T, Taniguchi M, Koezuka Y, *et al.* A novel function of V α 14⁺CD4⁺NKT cells: stimulation of IL-12 production by antigen-presenting cells in the innate immune system. *J Immunol* 1999;163:93–101.
 30. Fahy JV. Goblet cell and mucin gene abnormalities in asthma. *Chest* 2002;122:320S–326S.
 31. Henderson WR Jr, Lewis DB, Albert RK, Zhang Y, Lamm WJ, Chiang GK, Jones F, Eriksen P, Tien YT, Jonas M, *et al.* The importance of leukotrienes in airway inflammation in a mouse model of asthma. *J Exp Med* 1996;184:1483–1494.
 32. Zuhdi Alimam M, Piazza FM, Selby DM, Letwin N, Huang L, Rose MC. Muc-5/5ac mucin messenger RNA and protein expression is a marker of goblet cell metaplasia in murine airways. *Am J Respir Cell Mol Biol* 2000;22:253–260.
 33. Coffman RL, Lebman DA, Rothman P. Mechanism and regulation of immunoglobulin isotype switching. *Adv Immunol* 1993;54:229–270.
 34. Smyth MJ, Crowe NY, Pellicci DG, Kyriassoudis K, Kelly JM, Takeda K, Yagita H, Godfrey DI. Sequential production of interferon-gamma by NK1.1⁺ T cells and natural killer cells is essential for the antimetastatic effect of α -galactosylceramide. *Blood* 2002;99:1259–1266.
 35. Gonzalez-Aseguinolaza G, de Oliveira C, Tomaska M, Hong S, Bruna-Romero O, Nakayama T, Taniguchi M, Bendelac A, Van Kaer L, Koezuka Y, *et al.* α -galactosylceramide-activated V α 14 natural killer T cells mediate protection against murine malaria. *Proc Natl Acad Sci USA* 2000;97:8461–8466.
 36. Duthie MS, Kahn SJ. Treatment with α -galactosylceramide before Trypanosoma cruzi infection provides protection or induces failure to thrive. *J Immunol* 2002;168:5778–5785.
 37. Hofstra CL, Van Ark I, Hofman G, Nijkamp FP, Jardieu PM, Van Oosterhout AJ. Differential effects of endogenous and exogenous interferon- γ on immunoglobulin E, cellular infiltration, and airway responsiveness in a murine model of allergic asthma. *Am J Respir Cell Mol Biol* 1998;19:826–835.
 38. Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A, Stewart TA. Multiple defects of immune cell function in mice with disrupted interferon- γ genes. *Science* 1993;259:1739–1742.
 39. Rodriguez D, Keller AC, Faquim-Mauro EL, de Macedo MS, Cunha FQ, Lefort J, Vargaftig BB, Russo M. Bacterial lipopolysaccharide signaling through Toll-like receptor 4 suppresses asthma-like responses via nitric oxide synthase 2 activity. *J Immunol* 2003;171:1001–1008.
 40. Kline JN, Krieg AM, Waldschmidt TJ, Ballas ZK, Jain V, Businga TR. CpG oligodeoxynucleotides do not require T_H1 cytokines to prevent eosinophilic airway inflammation in a murine model of asthma. *J Allergy Clin Immunol* 1999;104:1258–1264.
 41. Miyamoto K, Miyake S, Yamamura T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing T_H2 bias of natural killer T cells. *Nature* 2001;413:531–534.
 42. Zhang Y, Rogers KH, Lewis DB. β 2-microglobulin-dependent T cells are dispensable for allergen-induced T helper 2 responses. *J Exp Med* 1996;184:1507–1512.
 43. Kips JC, Brusselle GJ, Joos GF, Peleman RA, Tavernier JH, Devos RR, Pauwels RA. Interleukin-12 inhibits antigen-induced airway hyperresponsiveness in mice. *Am J Respir Crit Care Med* 1996;153:535–539.
 44. Bryan SA, O'Connor BJ, Matti S, Leckie MJ, Kanabar V, Khan J, Warrington SJ, Renzetti L, Rames A, Bock JA, *et al.* Effects of recombinant human interleukin-12 on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* 2000;356:2149–2153.
 45. Giaccone G, Punt CJ, Ando Y, Ruijter R, Nishi N, Peters M, von Blomberg BM, Scheper RJ, van der Vliet HJ, van den Eertwegh AJ, *et al.* A phase I study of the natural killer T-cell ligand α -galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* 2002;8:3702–3709.