

FRONTLINE:

Suppression of eosinophilic airway inflammation by treatment with α -galactosylceramide

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To clarify the essential role of NKT cells in allergy, we investigated the contribution of NKT cells to the pathogenesis of eosinophilic airway inflammation using α -galactosylceramide (α -GalCer), a selective ligand for NKT cells. Although continuous administration of α -GalCer during ovalbumin (OVA) sensitization increased OVA-specific IgE levels and worsened eosinophil inflammation, a single administration of α -GalCer at the time of OVA challenge completely prevented eosinophilic infiltration in wild-type mice. This inhibitory effect of α -GalCer was associated with a decrease in airway hyperresponsiveness, an increase in IFN- γ , and decreases in IL-4, IL-5 and IL-13 levels in the bronchoalveolar lavage fluids. Analysis of lung lymphocytes revealed that production of IFN- γ increased in NK cells, but not in T or NKT cells, following α -GalCer administration. Induction of vascular cell adhesion molecule-1 in the lungs of wild-type mice was also significantly attenuated by treatment with α -GalCer. These effects of α -GalCer were abrogated in J α 281^{-/-} mice, which lack NKT cells, and in wild-type mice treated with anti-IFN- γ Ab. Hence, our data indicate that α -GalCer suppresses allergen-induced eosinophilic airway inflammation, possibly by inducing a Th1 bias that results in inhibition of eosinophil adhesion to the lung vessels.

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Abbreviations: α -GalCer: α -galactosylceramide · AHR: airway hyperresponsiveness · GAPDH: glyceraldehyde-3-phosphate dehydrogenase · VCAM-1: vascular cell adhesion molecule-1

Introduction

Eosinophils have been accepted as having pivotal roles in allergic airway inflammation. They can release a variety of mediators, including preformed cationic proteins, oxygen radicals, lipid mediators, cytokines and growth factors, at the site of inflammation, and these can induce

airway hyperresponsiveness (AHR), airway injury, tissue remodeling, and asthma exacerbation [1–6]. Although the mechanism of generating eosinophilic airway inflammation is extremely complex, Th2 cytokines such as IL-4, IL-5 and IL-13 have been considered to play a role in eosinophil recruitment and activation.

NKT cell is a novel lymphoid lineage distinct from T, B or NK cells, and shows expression of both the TCR and NK cell receptors. In contrast to conventional T cells, which have a wide variety of TCR repertoires and recognize peptide antigens presented by polymorphic MHC class I or class II molecules, NKT cells in mice have a highly biased TCR containing an invariant $V\alpha 14$ $J\alpha 281$ and $V\beta 8$ TCR chain, and recognize glycolipid antigens in a CD1d-restricted manner [7]. NKT cells have unique characteristics such that they rapidly produce large amounts of immunoregulatory cytokines, such as IFN- γ and IL-4, after stimulation with either anti-CD3 Ab [8, 9] or glycolipid ligand [10, 11]. α -Galactosylceramide (α -GalCer), a synthetic glycolipid originally isolated from marine sponges, is specifically recognized by $V\alpha 14^+$ NKT cells. It has been demonstrated that exogenously administered α -GalCer induces several immune responses, including anti-tumor activity [12], host resistance to several microbial pathogens [12, 13], and antifibrotic activity [14], by activation of NKT cells to produce a large amount of IFN- γ , a representative Th1 cytokine. Moreover, IFN- γ is capable of amplifying its production by activating NK cells and T cells directly or *via* production of IL-12 [15, 16], and it is known to negatively regulate various asthmatic responses directly or by inhibiting Th2 cytokines. Indeed, mice lacking IFN- γ develop a significant increase in AHR, airway eosinophilia, and lung CD4 $^+$ T cells, compared with wild-type controls [17]. We therefore hypothesized that treatment with α -GalCer might reduce eosinophilic airway inflammation through production of IFN- γ .

To examine this hypothesis, in the present study we evaluated the effects of α -GalCer on the development of eosinophilic airway inflammation induced by allergen challenge using both wild-type and NKT cell-deficient mice. Possible molecular mechanisms for the suppression of eosinophilic inflammation were also investigated.

Results

α -GalCer has contradictory effects on allergen-induced eosinophilic airway inflammation

We first examined the effects of α -GalCer on OVA-induced eosinophilic airway inflammation and serum OVA-specific IgE levels in both wild-type and $J\alpha 281^{-/-}$

mice. Two different schedules of α -GalCer administration were used. In one, α -GalCer was given continuously during OVA sensitization, which worsened eosinophilic inflammation and increased serum OVA-specific IgE levels in wild-type mice. In the other, a bolus administration of α -GalCer was given at the time of challenge, which completely prevented eosinophil infiltration and did not change the IgE levels (Fig. 1A, C). The degree of eosinophil infiltration and the IgE levels were not altered by either method of α -GalCer administration in $J\alpha 281^{-/-}$ mice (Fig. 1B, C).

We next evaluated the effects of α -GalCer given by bolus injection on eosinophilic inflammation at the light microscopic level (Fig. 2A). In OVA-challenged wild-type mice, numerous eosinophils, which were positively stained by Congo red, were found to infiltrate into the lung interstitium around airways and blood vessels; however, treatment with α -GalCer almost completely eliminated the eosinophil infiltration. Marked infiltration of eosinophils was also observed in the lung interstitium of OVA-challenged $J\alpha 281^{-/-}$ mice, but in these mice α -GalCer did not suppress the degree of eosinophil infiltration at the same time point. In saline-challenged mice, the inflammatory cells were absent throughout the lungs in both genotypes.

We then assessed the recruitment of eosinophils separately in the airspace, lung interstitium, and peripheral blood (Fig. 2B). The number of eosinophils had significantly increased in the airspace and the lung interstitium in OVA-challenged wild-type mice; 32-fold and 80-fold increases in eosinophil number were found in the bronchoalveolar lavage (BAL) fluids and lung interstitium, respectively, compared with saline-challenged animals. Increases of 42-fold and 200-fold were observed in the number of eosinophils found in the BAL fluids and lung interstitium, respectively, in OVA-challenged $J\alpha 281^{-/-}$ mice, compared with those challenged with saline. Treatment with α -GalCer almost completely eliminated influx of eosinophils into both the airspace and lung interstitium in wild-type mice. In contrast, in $J\alpha 281^{-/-}$ mice, α -GalCer treatment did not influence the degree of eosinophil infiltration. The number of eosinophils in the peripheral blood was slightly elevated by OVA challenge in wild-type mice; however, no reversion of this trend was observed following α -GalCer administration.

α -GalCer reduces allergen-induced AHR

As shown in Fig. 3, our findings clearly demonstrate that OVA-challenged mice of both genotypes, wild-type and $J\alpha 281^{-/-}$ mice, developed increased AHR after methacholine injection, compared to saline-challenged mice. In contrast, OVA-challenged wild-type mice that were treated with α -GalCer developed decreased AHR,

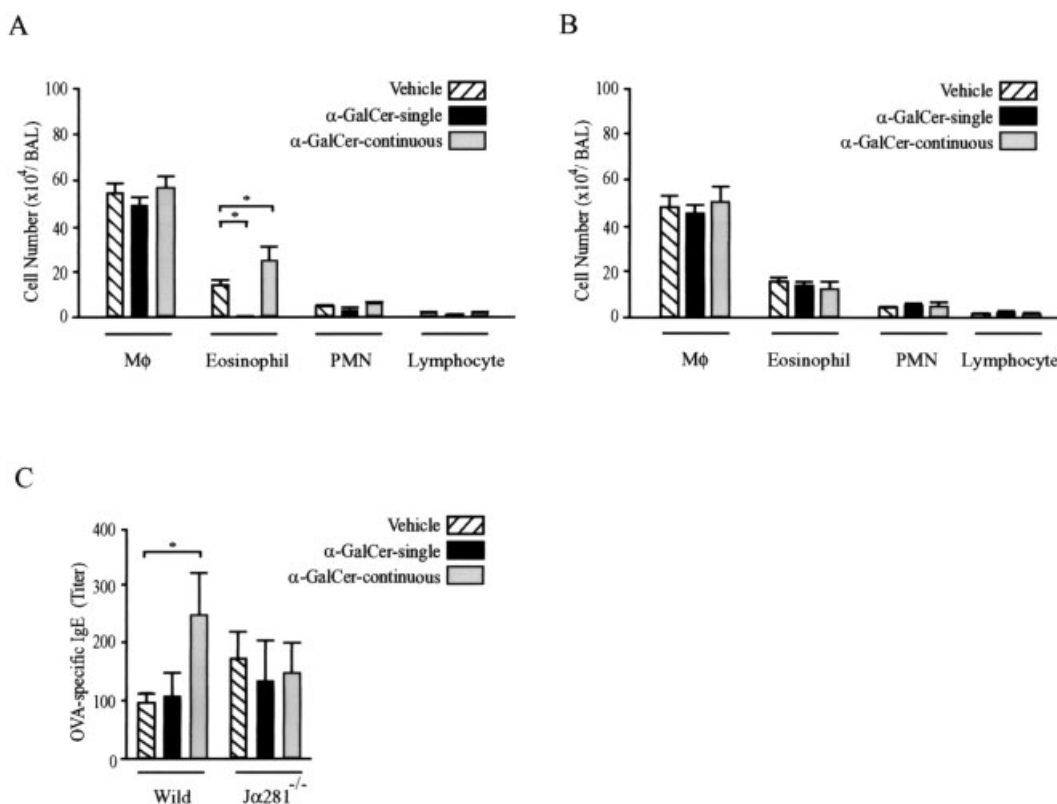


Figure 1. (A) A single administration of α -GalCer at the time of antigen challenge prevented eosinophilic inflammation, while continuous administration of α -GalCer during the antigen sensitization period increased eosinophil infiltration in wild-type mice. (B) The degree of eosinophil infiltration was not altered by either method of α -GalCer administration in $J\alpha 281^{-/-}$ mice. (C) OVA-specific IgE levels did not change with single administration of α -GalCer at the time of antigen challenge; however, those levels were significantly increased by continuous administration of α -GalCer from the antigen sensitization period in wild-type mice. Results are shown as means $\pm$ SEM from five to ten mice per group. * $p < 0.05$.

compared to the vehicle-treated group. α -GalCer did not change the severity of AHR in $J\alpha 281^{-/-}$ mice.

Expression of type I collagen mRNA is attenuated by α -GalCer

Since allergic airway diseases such as asthma are typically associated with airway remodeling, one of the characteristic features of which is the deposition of extracellular matrix proteins in the airway, we further monitored the expression of type I collagen mRNA. Using semi-quantitative RT-PCR, we found that type I collagen mRNA is induced by OVA challenge; however, its expression level was attenuated by α -GalCer administration in wild-type mice (Fig. 4). The expression of type I collagen was increased in OVA-challenged $J\alpha 281^{-/-}$ mice, regardless of administration of α -GalCer.

α -GalCer induces a shift towards IFN- γ and away from Th2 cytokines

To evaluate the effects of α -GalCer on the production of both Th1 and Th2 cytokines, the concentrations of IFN-

γ , IL-4, IL-5 and IL-13 in the BAL fluids collected 48 h after the challenge were determined by ELISA. The concentration of IFN- γ did not differ between OVA-challenged and saline-challenged mice of both genotypes (Fig. 5A). However, the concentrations of IL-4, IL-5 and IL-13 increased significantly in OVA-challenged mice, compared with saline-challenged animals of both genotypes (Fig. 5B–D). Treatment with α -GalCer significantly increased the concentration of IFN- γ (Fig. 5A) and reduced those of IL-4, IL-5 and IL-13 (Fig. 5B–D) in the BAL fluids of OVA-challenged wild-type mice. In $J\alpha 281^{-/-}$ mice, however, treatment with α -GalCer did not alter the concentrations of IFN- γ , IL-4, IL-5 and IL-13 in the BAL fluids (Fig. 5A–D). Similar results were obtained for BAL samples collected 24 h after OVA challenge (data not shown).

IFN- γ -producing NK cells increase after α -GalCer administration

We next evaluated the proportion of lung lymphocyte subsets and performed intracellular IFN- γ staining to assess IFN- γ production on a per-cell basis after 48 h in

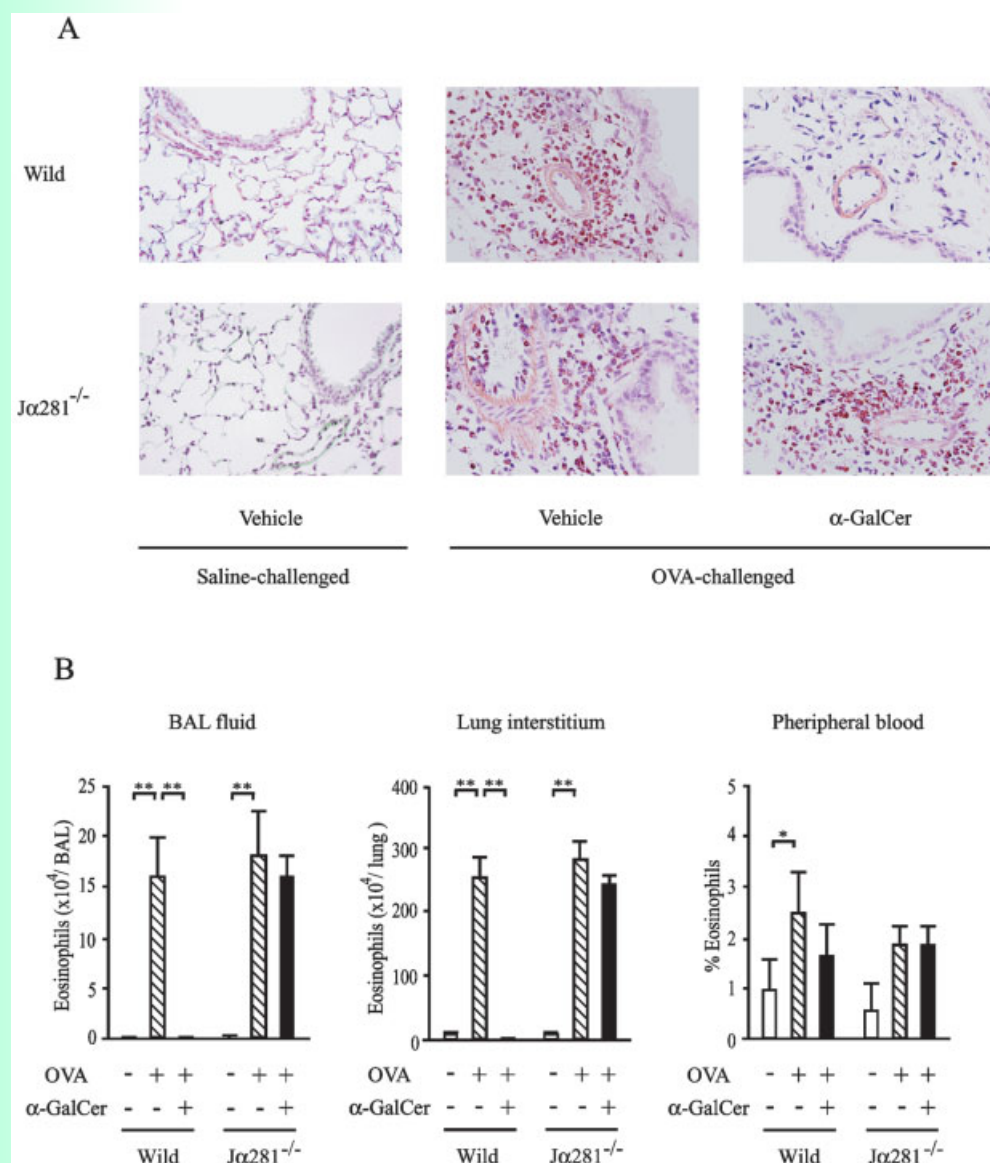


Figure 2. (A) Lung tissue sections from wild-type or $J\alpha 281^{-/-}$ mice were stained with Congo red. In OVA-challenged wild-type mice, α -GalCer completely suppressed the degree of eosinophil infiltration. (B) Treatment with α -GalCer almost completely eliminated influx of eosinophils into both the airspace and lung interstitium in wild-type mice. In contrast, in $J\alpha 281^{-/-}$ mice, α -GalCer treatment did not influence the degree of eosinophil infiltration. Results are shown as means $\pm$ SEM from five mice. * $p < 0.05$; ** $p < 0.01$.

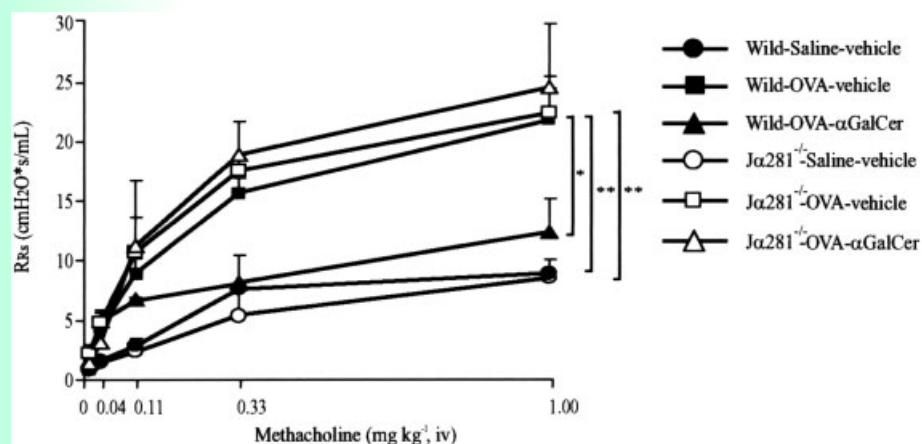


Figure 3. OVA-induced AHR was inhibited by α -GalCer in wild-type mice. Methacholine was intravenously injected at increasing doses (0.04–1 mg kg⁻¹), and the peak value of respiratory resistance (R_{rs}) for each dose was recorded. Results are shown as means $\pm$ SEM from three mice. Statistical analysis by repeated-measures ANOVA, * $p < 0.05$; ** $p < 0.01$.

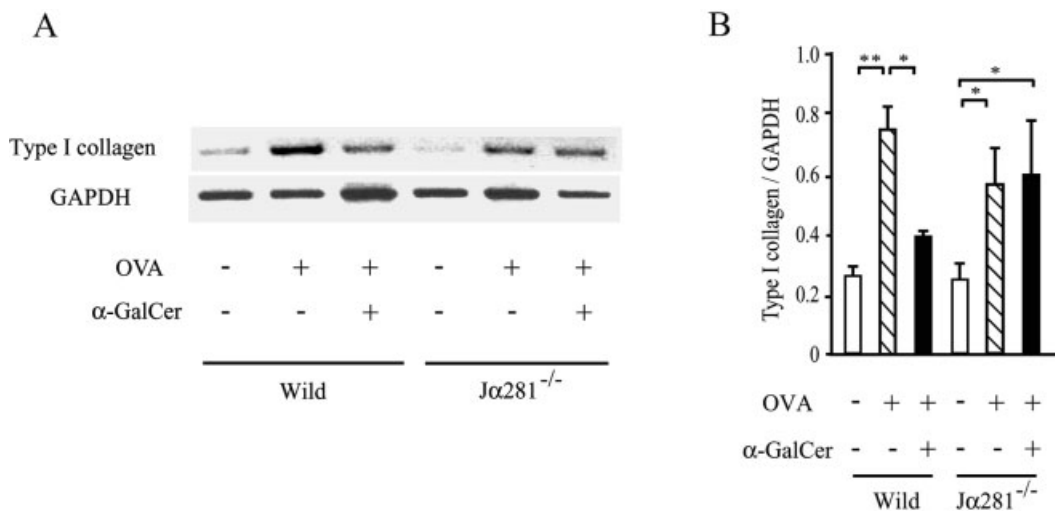


Figure 4. Semi-quantitative RT-PCR demonstrates the decreased expression of type I collagen in the lungs of OVA-challenged wild-type mice treated with α -GalCer. The levels of GAPDH mRNA are shown as loading controls. (A) Representative examples are shown. (B) Quantitative analysis of the bands was carried out using imaging software. Results are shown as means $\pm$ SEM from five mice. * $p < 0.05$; ** $p < 0.01$.

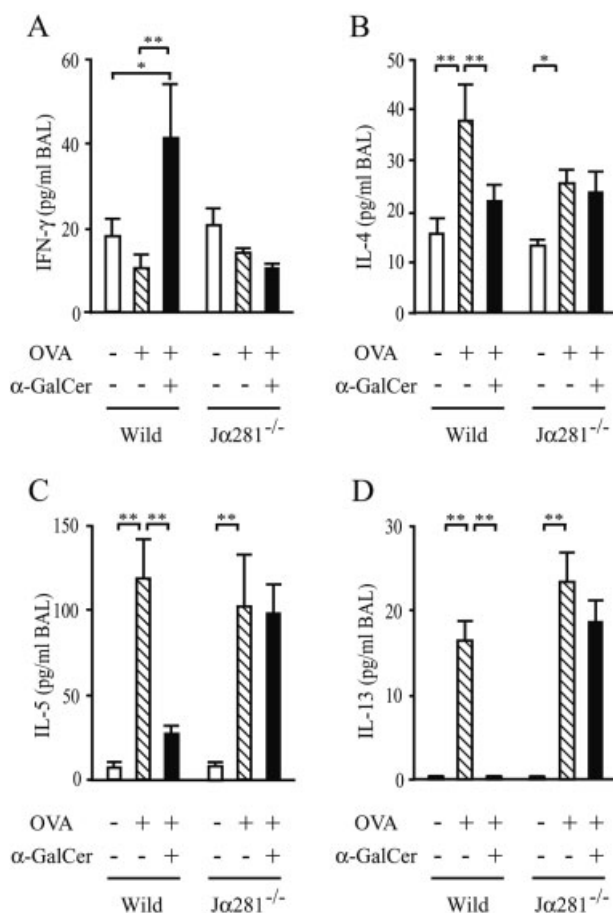


Figure 5. Treatment with α -GalCer significantly increased the concentration of IFN- γ (A) and reduced those of IL-4, IL-5 and IL-13 (B–D) in the BAL fluids of OVA-challenged wild-type mice. In *Jα281*^{-/-} mice, however, treatment with α -GalCer did not alter the concentrations of these cytokines. Results are shown as means $\pm$ SEM from five mice. * $p < 0.05$; ** $p < 0.01$.

OVA-challenged mice. The proportion of lung NKT cells, defined as TCR β ⁺ α -GalCer-CD1d tetramer⁺ lymphocytes, was 0.99% and 0.73% in vehicle-treated and α -GalCer-treated wild-type mice, respectively (Table 1), and IFN- γ -positive NKT cells were undetectable in both groups (Fig. 6A). The proportion of lung T cells, defined as TCR β ⁺ DX5⁻ lymphocytes, was not significantly different between vehicle-treated and α -GalCer-treated mice (Table 1), and the proportion of IFN- γ -positive T cells also did not differ among the groups (Fig. 6B). On the other hand, the proportion of lung NK cells, defined as TCR β ⁻ DX5⁺ lymphocytes, was increased by treatment with α -GalCer in wild-type mice (Table 1). The proportion of NK cells that were IFN- γ positive also increased significantly in wild-type mice following treatment with α -GalCer at the time of challenge (Fig. 6C). In *Jα281*^{-/-} mice, however, α -GalCer did not alter the proportion of both total NK cells and IFN- γ -positive NK cells at the same time point (Table 1, Fig. 6C). We also evaluated the intracellular production of IL-4, along with that of IFN- γ , and found that the proportion of IL-4-positive T cells and NKT cells did not differ significantly among the groups (data not shown).

α -GalCer attenuates the expression of vascular cell adhesion molecule-1 in the lung

Since inhibition of eosinophil recruitment occurred at sites in the airway and lung interstitium, we examined the effects of α -GalCer on the expression of eosinophil chemoattractants and adhesion molecules. Although the concentrations of the CC chemokines eotaxin and MCP-1 in the BAL fluids were increased in both wild-type and

Table 1. Proportions of NKT cells, T cells and NK cells in lung lymphocytes evaluated by flow cytometric analysis^{a)}

Treatment	TCR β^+ α -GalCer-CD1d ⁺ NKT cells, %	TCR β^+ DX5 ⁻ T cells, %	TCR β^- DX5 ⁺ NK cells, %
Wild type-vehicle	0.99 $\pm$ 0.01	35.91 $\pm$ 2.35	10.97 $\pm$ 0.93
Wild type- α -GalCer	0.73 $\pm$ 0.13	39.36 $\pm$ 3.80	18.73 $\pm$ 0.73*
<i>Jα281^{-/-}</i> -vehicle	not detected	33.67 $\pm$ 2.41	13.59 $\pm$ 1.18
<i>Jα281^{-/-}</i> - α -GalCer	not detected	29.79 $\pm$ 1.33	11.80 $\pm$ 1.01

^{a)} The percentages of TCR β^+ α -GalCer-CD1d tetramer⁺ NKT cells, TCR β^+ DX5⁻ T cells and TCR β^- DX5⁺ NK cells in lung lymphocytes are shown as means $\pm$ SEM from three to five wild-type or *J α 281^{-/-}* mice. **p* < 0.01, compared to vehicle-treated wild-type mice.

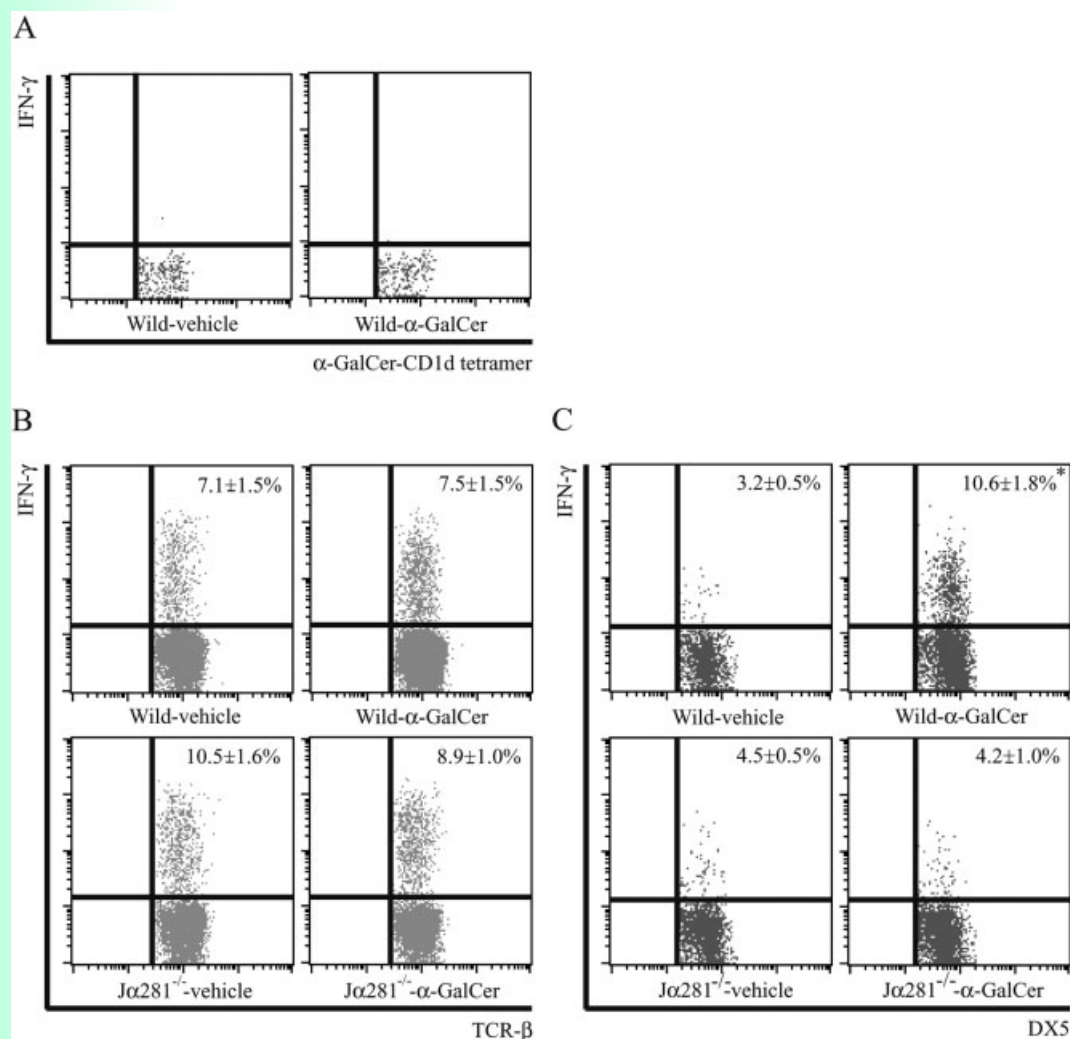


Figure 6. TCR β^+ α -GalCer-CD1d tetramer⁺ NKT cells (A), TCR β^+ DX5⁻ T cells (B) and TCR β^- DX5⁺ NK cells (C) were gated and analyzed for the presence of intracellular IFN- γ in wild-type mice and *J α 281^{-/-}* mice. The proportion of IFN- γ -positive NK cells increased significantly in wild-type mice following treatment with α -GalCer. Results are shown as means $\pm$ SEM from three to five mice. **p* < 0.01, compared to vehicle-treated wild-type mice.

J α 281^{-/-} mice by OVA challenge, treatment with α -GalCer did not change eotaxin and MCP-1 levels in the BAL fluids of either genotype (Fig. 7A, C). The concentration of RANTES was not altered either by OVA challenge or by α -GalCer administration in both wild-type and *J α 281^{-/-}* mice (Fig. 7B).

We next evaluated the pulmonary expression of vascular cell adhesion molecule-1 (VCAM-1), an important adhesion molecule for eosinophil adhesion, by Western blot analysis (Fig. 8A, B). Only a small amount of VCAM-1 protein was expressed in the lungs of both wild-type and *J α 281^{-/-}* mice when challenged with

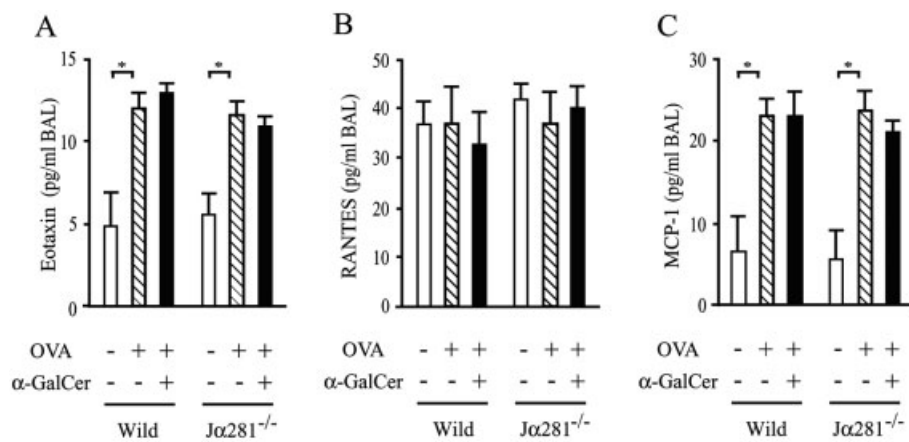


Figure 7. The concentrations of eotaxin (A) and MCP-1 (C) in the BAL fluids were increased in both wild-type and $J\alpha 281^{-/-}$ mice by OVA challenge; however, treatment with α -GalCer did not change these levels in the BAL fluids of either genotype. The concentration of RANTES (B) was not altered either by OVA challenge or by α -GalCer administration. Results are shown as means $\pm$ SEM from five mice. * $p < 0.01$.

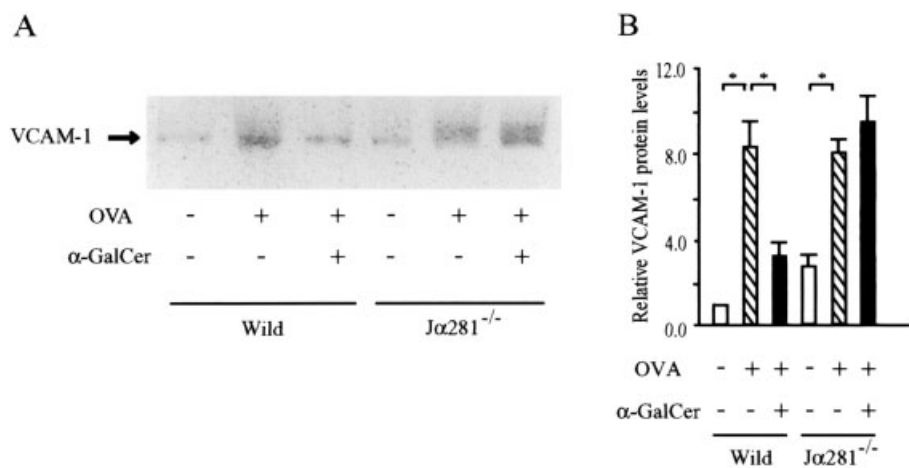


Figure 8. Western blot analysis demonstrated increased expression of VCAM-1 in response to OVA challenge in wild-type mice and $J\alpha 281^{-/-}$ mice. Treatment with α -GalCer markedly reduced the expression of VCAM-1 in the lungs of OVA-challenged wild-type mice. (A) Representative examples are shown. (B) Quantitative analysis of the blots was carried out using imaging software. Results are shown as means $\pm$ SEM from three mice. * $p < 0.05$.

saline. Expression of VCAM-1 was significantly increased after challenge with OVA in both mouse genotypes, and treatment with α -GalCer markedly reduced the expression of VCAM-1 in the lungs of wild-type mice. However, α -GalCer did not reduce the VCAM-1 expression in the lungs of $J\alpha 281^{-/-}$ mice.

Neutralization of IFN- γ abrogates the effects of α -GalCer

To determine the role of IFN- γ in α -GalCer-mediated suppression of the eosinophilic airway response, anti-IFN- γ Ab was administered to α -GalCer-treated wild-type mice and was shown to abrogate the effects of α -GalCer. Hence, the number of eosinophils in BAL fluids was significantly increased in mice treated with both α -

GalCer and anti-IFN- γ Ab, compared with mice treated with α -GalCer and isotype Ig (Fig. 9A). Next, we examined whether anti-IFN- γ Ab could induce VCAM-1 expression, and we were able to show that reduction of VCAM-1 expression by α -GalCer was also abrogated by administration of anti-IFN- γ Ab (Fig. 9B).

Discussion

The most striking aspect of NKT cells is their capacity to secrete large quantities of both Th1 and Th2 cytokines after stimulation with a specific ligand [8–11]. NKT cells are believed to have an ability to manipulate the nature of the immunological process towards either a Th1-biased or Th2-biased response; therefore, these cells are

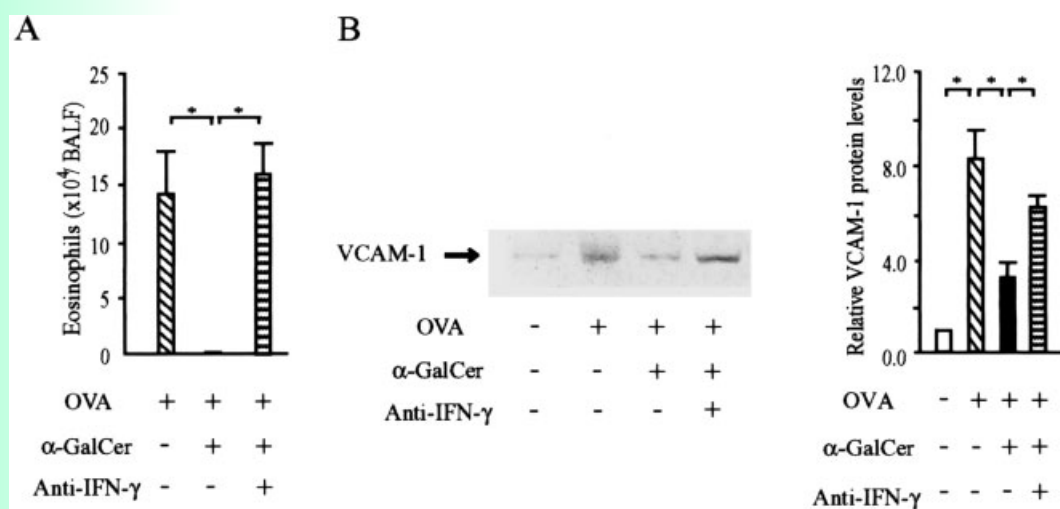


Figure 9. Neutralization of IFN- γ abrogated the effects of α -GalCer. (A) The number of eosinophils in BAL fluids was significantly increased in mice treated with both α -GalCer and anti-IFN- γ Ab, compared with mice treated with α -GalCer and isotype Ig. (B) The reduction of VCAM-1 expression by α -GalCer was also abrogated by administration of anti-IFN- γ Ab. Results are shown as means $\pm$ SEM from three to five mice. * p < 0.01.

considered to be crucial cells in promoting various immunological responses, including those to cancer [12, 18, 19], infection [12, 13], and autoimmune disease [20, 21]. However, the regulatory role of NKT cells in the allergic response still remains controversial. Recent studies have demonstrated that both airway eosinophilia and AHR do not develop in NKT cell-deficient mice, suggesting that NKT cells are essential for the development of an allergic reaction [22, 23]. Nevertheless, in the present study, we demonstrated that airway eosinophilia, OVA-specific IgE levels and AHR were not different between wild-type mice and *Ja281*^{-/-} mice, which lack NKT cells, supporting the results of previous studies showing that an allergic response can develop in the absence of NKT cells [24, 25]. Possible explanations for these discrepancies might be the differences in the genetic background of mice, the experimental protocol for OVA immunization, and the time point for evaluation. We have used the BALB/c strain, which is considered to be a Th2-dependent strain, whereas the C57BL/6 strain used in other studies [23] is considered to be Th1 dependent. Additionally, we have sensitized the mice with OVA more frequently than in other studies, and we evaluated the airway inflammation 48 h after OVA challenge since we could not detect a sufficient number of inflammatory cells in the BAL fluids 24 h after the challenge, the time schedule chosen in previous experiments (data not shown) [22, 23].

Our results provide intriguing findings that α -GalCer, a selective ligand for NKT cells, has both stimulatory and strong negative regulatory functions in the development of antigen-specific eosinophilic airway inflammation. Eosinophilic inflammation worsened and serum OVA-

specific IgE levels increased when α -GalCer was continuously administered during antigen sensitization, whereas inflammation was completely prevented when α -GalCer was administered once at the time of challenge. It has been indicated that activation of NKT cells with α -GalCer results in generation of Th2 cells and increases in serum IgE levels [26, 27], whereas Cui *et al.* demonstrated that activation of NKT cells by α -GalCer suppresses antigen-specific IgE production *in vivo* and inhibits Th2 cell differentiation *in vitro* [28]. In addition, Burdin *et al.* proved that α -GalCer induces a burst of IFN- γ secretion in the early phase after administration, while repeated administration of α -GalCer induces a Th2 response [29]. Based on these observations and our results, the timing of ligand stimulation might have a key role in determining whether the immune response takes a Th1 or Th2 direction. In this sense, NKT cells can work both ways, as a double-edged sword, and in this paper we have focused on the negative regulatory effects of α -GalCer-activated NKT cells on the allergic response, which might provide for a new therapeutic approach to asthma.

A single dose of α -GalCer at the time of OVA challenge abrogated the presence of eosinophils around the airway, and this was correlated with a reduction in AHR and down-regulation of the expression of type I collagen. Thus, α -GalCer appears to have effects on inflammation, airway tone, and collagen deposition. Since these phenomena were not observed in *Ja281*^{-/-} mice, we concluded that NKT cells may play a principal role in these suppressive effects of α -GalCer on antigen-induced airway inflammation. We also administered α -GalCer by the intranasal route and evaluated the effects

on eosinophilic airway inflammation; however, the degree of airway inflammation did not significantly change (data not shown). Hence, since the population of NKT cells is usually very low in lung tissue, systemic administration of α -GalCer may be required to prevent airway eosinophilia.

The cytokine profile was also affected by α -GalCer; the production of IFN- γ was increased and, conversely, the IL-4, IL-5 and IL-13 levels were decreased. Moreover, neutralization of IFN- γ abrogated the inhibitory effects of α -GalCer on airway eosinophilia in our murine model. Several immune responses, including protective activities against *C. neoformans* and malaria infections [12, 13], antimetastatic activities against melanoma [12], and antifibrotic effects in bleomycin-induced pulmonary fibrosis [14] are also known to be accompanied by an increase in the IFN- γ level after administration of α -GalCer. It is well known that IFN- γ has antagonistic actions against allergic responses [30, 31], and administration of IFN- γ into the airways of allergic mice has been demonstrated to attenuate Th2 responses, including airway eosinophilia [32, 33]. Taken together, these data allow us to suggest that the inhibitory effects of α -GalCer on eosinophilic inflammation are, at least in part, mediated by IFN- γ .

In the analysis of lung lymphocyte subsets, the proportion of NKT cells in lung lymphocytes was very small in OVA-challenged wild-type mice, and IFN- γ -positive NKT cells were undetectable. In addition, treatment with α -GalCer did not affect the number of NKT cells. These findings suggest that NKT cells might initially trigger suppressive effects on the allergic airway, but may not be the major cell source of IFN- γ . T cells are also known to orchestrate inflammatory responses and to be a major cell source of IFN- γ and IL-4; however, we could not detect any significant difference in the proportion of T cells and that of cytokine-producing T cells between vehicle-treated and α -GalCer-treated wild-type mice. In contrast, the proportion of NK cells in lung lymphocytes and their production of IFN- γ were increased by administration of α -GalCer to OVA-challenged wild-type mice. NKT cells clearly participate in the stimulation of NK cells, because no increase in the proportion of NK cells or in their production of IFN- γ was observed in *Ja281*^{-/-} mice. Matsuda *et al.* have demonstrated that NKT cells produce large amounts of IFN- γ and IL-4 within 2 h after stimulation with α -GalCer, but then disappear by 5 h and do not reappear until 1 wk later [34]. Kawakami *et al.* have also suggested that α -GalCer treatment in mice infected with *C. neoformans* firstly induces activation of NKT cells, then NK cells, and lastly Th1 cells to produce IFN- γ [13]. That is to say, NKT cells may secrete cytokines at the very outset of the immune response after α -GalCer administration, thereby inducing NK

cells, and not T cells, to become one of the major sources of IFN- γ in the subsequent phase.

It was of interest that inhibition of eosinophil recruitment occurred at sites in the airway and lung interstitium. Eosinophils infiltrate into the airway in several steps, including rolling along the vascular wall, firm adhesion to the endothelium, and transmigration into the extravascular space. Therefore, we examined the effects of α -GalCer on the expression of eosinophil chemoattractants and adhesion molecules, which are considered to play a key role in the infiltration process. Contrary to our expectations, the concentrations of CC chemokines, including eotaxin, RANTES and MCP-1, in the BAL fluids were not affected by α -GalCer treatment. On the other hand, VCAM-1 expression in the lung homogenates was reduced by administration of α -GalCer in wild-type mice. The expression of VCAM-1 has been found to increase in asthmatic airways [35] and during treatment using a mAb against VCAM-1 to prevent antigen-induced airway eosinophilia [36]. In our study, the reduction of VCAM-1 expression caused by α -GalCer administration was abrogated in *Ja281*^{-/-} mice or in wild-type mice treated with IFN- γ -neutralizing Ab. This suggests that IFN- γ production caused by α -GalCer-stimulated NKT cells has an important role in attenuating VCAM-1 expression. Furthermore, it is well known that Th2 cytokines stabilize expression of VCAM-1 [37, 38] and regulate the proliferation and life span of eosinophils. Thus, down-regulation of such Th2 cytokines might also be involved in modulating eosinophil homing.

In summary, we have provided evidence that ligand-activated NKT cells may participate in attenuating eosinophilic airway inflammation by inducing Th1 bias, which results in decreased expression of VCAM-1. Our data suggest that exogenous administration of the synthetic glycolipid, α -GalCer, may have potential as a therapeutic option for preventing airway eosinophilia in allergic inflammation.

Materials and methods

Animals

Wild-type BALB/c mice were purchased from Charles River Japan (Yokohama, Japan). *Ja281*^{-/-} mice of the same BALB/c background were established by specific deletion of the *Ja281* gene segment [10]. All animals used in the study were 7–12 wk of age and were maintained in our animal facility under specific pathogen-free conditions. All animal studies were approved by the Institutional Review Board at our institution.

Experimental protocols

Mice were sensitized to 100 µg of OVA (Sigma Chemical Co., St. Louis, MO) emulsified in 40 mg of Al(OH)₃ in 0.4 mL saline by subcutaneous injection on day 1. On days 10 and 19, mice received the same dose of OVA as boosters. On day 28, these mice were challenged with 10 µg OVA dissolved in 50 µL saline, which was delivered by an intranasal route under anesthesia with 4% halothane (Takeda Chemical Industries, Ltd., Osaka, Japan). The saline-challenged group received the same dose of OVA on days 1, 10, and 19, and was challenged with saline on day 28. A synthetic form of α -GalCer, KRN7000, was used as a specific ligand for NKT cells. α -GalCer at 10 µg, or an appropriate amount of control vehicle, diluted in 0.4 mL saline, was injected subcutaneously at the same time as the challenge with OVA on day 28. We performed a preliminary examination of cytokine levels in BAL fluids after administration of 2 µg and 10 µg α -GalCer and concluded that a dose of 10 µg α -GalCer was most appropriate for evaluation of both the degree of eosinophilic airway inflammation and cytokine alterations. In some mice, Alzet Osmotic Pumps (Durect Corp., Cupertino, CA) were subcutaneously implanted on day 1, and either α -GalCer (delivered at a rate of 2 µg/kg/24 h) or the appropriate amount of vehicle was administered throughout the experiment. Except for the measurement of airway responsiveness, analysis was performed 48 h after OVA challenge.

Assessment of eosinophils

The number of eosinophils was assessed in the BAL fluids, lung interstitium, and peripheral blood. The lungs were lavaged with five repeat instillations of 1 mL saline *via* the trachea, and lung interstitial cells were extracted using a method described previously [39]. In brief, the whole lavaged lungs were chopped into small pieces, incubated with RPMI 1640 containing 10% FCS and 75 U/mL type I collagenase (Sigma) for 2 h at 37°C and filtered through a nylon mesh to remove the large tissue fragments. Peripheral blood was collected by cardiac puncture, and leukocytes were separated from erythrocytes by differential sedimentation in 6% dextran solution. Cells were counted using a hemocytometer, and a differential cell count was performed by standard light microscopic techniques, based on staining with Diff-Quik (American Scientific Products, McGaw Park, IL).

OVA-specific IgE assay

Serum was obtained from each group and OVA-specific IgE levels were measured by ELISA using microplates coated with OVA. Diluted serum samples or control serum from non-immunized wild-type mice were added to each well, and the bound IgE was detected with biotinylated anti-mouse IgE. The reactions were developed with streptavidin-peroxidase conjugate, using tetramethylbenzidine as substrate. The plates were read at 450 nm. All these materials were purchased from Seikagaku Kogyo (Tokyo, Japan). The results were expressed as titers, representing the highest dilution of samples giving an extinction value higher than that of non-immunized serum.

Histopathology

Mice were sacrificed by exsanguination under deep anesthesia and the lungs were dissected after perfusion with saline *via* the heart to clear the blood. Following fixation, the lungs were embedded in paraffin. The sections were then evaluated using Congo red staining for identification of eosinophils, with counterstaining with hematoxylin.

Measurement of airway responsiveness

At 24 h after the challenge, mice were anesthetized, tracheostomized and connected to a mechanical ventilator (FlexiVent; SCIREQ, Montreal, Canada). Methacholine was intravenously injected at increasing doses (0.04–1 mg kg⁻¹), and the peak value of respiratory resistance for each dose was recorded.

PCR analysis

Total RNA was extracted from the lung tissues, and RT-PCR was performed using the OneStep RT-PCR kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The sequences of the PCR primers were: 5'-ATTGG-TAATGTTGGTGCT-3' and 5'-GTGACCCTTTATGCCTCTGT-3' for collagen type I, and 5'-ACCACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Semi-quantitative analysis was performed using NIH Image software.

ELISA

The supernatant from the first BAL sample was used for cytokine and chemokine assays. We performed a preliminary examination of cytokine concentrations in BAL fluids after administration of α -GalCer, prior to starting the experiments. Since we found that the cytokine concentrations showed a peak from 24 to 72 h after α -GalCer administration, we selected the time point of 48 h for evaluating cytokine levels. The concentrations of IFN- γ , IL-4, IL-5, IL-13, eotaxin, RANTES and MCP-1 were determined by ELISA, according to the manufacturer's instruction (IFN- γ , IL-4, IL-5, RANTES and MCP-1: BioSource International, Inc., Camarillo, CA; IL-13 and eotaxin: R&D Systems, Inc., Minneapolis, MN). The limits of detection were 1 pg/mL for IFN- γ and eotaxin, 5 pg/mL for IL-4 and RANTES, 3 pg/mL for IL-5, 2 pg/mL for IL-13, and 9 pg/mL for MCP-1.

Flow cytometry

The proportion of lung lymphocyte subsets and intracellular production of IFN- γ and IL-4 were evaluated by flow cytometric analysis, using a protocol described elsewhere [40]. Briefly, after lung interstitial cells were isolated, these cells were stimulated with 25 ng/mL PMA (Sigma), 1 µg/mL ionomycin (Sigma) and 10 µg/mL brefeldin A (Epicentre, Madison, WI) for 4 h at 37°C, and thereafter they were incubated with Alexa 488-conjugated α -GalCer-loaded CD1d tetramer, anti-DX5-FITC, and anti-TCR β -allophycocyanin Ab (PharMingen, San Diego, CA). Cells were then fixed,

permeabilized using IntraPrep permeabilization reagent (Immunotech, Marseille, France) and stained with anti-IFN- γ -PE or anti-IL-4-PE Ab (PharMingen). Data were analyzed by FACSCalibur using the CellQuest program (Becton Dickinson, Mountain View, CA).

Western blot analysis

The lungs were removed and homogenized in PBS containing a cocktail of protease inhibitors (Boehringer Mannheim, GmbH, Germany). The homogenates were then centrifuged at 100 000 g for 1 h at 4°C. The supernatants were collected, and their protein concentrations were determined. Each sample containing 30 μ g of protein was subjected to SDS-PAGE and subsequent Western blot analysis, using an anti-VCAM-1 mAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Relative protein levels were quantified using the NIH Image software.

Neutralization of IFN- γ

For neutralization of IFN- γ , anti-IFN- γ mAb (XMG1.2, 500 μ g/body; PharMingen) or an appropriate amount of the isotype control (R3-34; PharMingen) was injected intraperitoneally into wild-type mice just before and 24 h after the challenge with OVA.

Statistical analysis

Data are represented as mean $\pm$ SEM. Airway responsiveness to methacholine was analyzed by repeated-measures analysis of variance (ANOVA), using StatView 5.0 (Abacus Concepts, Inc., Cary, NC). All other parameters were compared by one-way ANOVA with the *post hoc* Bonferroni–Dunn procedure. Differences were considered to be statistically significant at $p < 0.05$.

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References

- Bradley, B. L., Azzawi, M., Jacobson, M., Assoufi, B., Collins, J. V., Irani, A. M., Schwartz, L. B. *et al.* Eosinophils, T-lymphocytes, mast cells, neutrophils, and macrophages in bronchial biopsy specimens from atopic subjects with asthma: comparison with biopsy specimens from atopic subjects without asthma and normal control subjects and relationship to bronchial hyperresponsiveness. *J. Allergy Clin. Immunol.* 1991. **88**: 661–674.
- Bousquet, J., Chanez, P., Lacoste, J. Y., Barnéon, G., Ghavanian, N., Enander, I., Venge, P. *et al.* Eosinophilic inflammation in asthma. *N. Engl. J. Med.* 1990. **323**: 1033–1039.
- Motojima, S., Frigas, E., Loegering, D. A. and Gleich, G. J., Toxicity of eosinophil cationic proteins for guinea pig tracheal epithelium *in vitro*. *Am. Rev. Respir. Dis.* 1989. **139**: 801–805.
- Flood-Page, P., Menzies-Gow, A., Phipps, S., Ying, S., Wangoo, A., Ludwig, M. S., Barnes, N., Robinson, D. and Kay, A. B., Anti-IL-5 treatment reduces deposition of ECM proteins in the bronchial subepithelial basement membrane of mild atopic asthmatics. *J. Clin. Invest.* 2003. **112**: 1029–1036.
- Cho, J. Y., Miller, M., Baek, K. J., Han, J. W., Nayar, J., Lee, S. Y., McElwain, K. *et al.* Inhibition of airway remodeling in IL-5-deficient mice. *J. Clin. Invest.* 2004. **113**: 551–560.
- Green, R. H., Brightling, C. E., McKenna, S., Hargadon, B., Parker, D., Bradding, P., Wardlaw, A. J. and Pavord, I. D., Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* 2002. **360**: 1715–1721.
- Brossay, L., Chioda, M., Burdin, N., Koezuka, Y., Casorati, G., Dellabona, P. and Kronenberg, M., CD1d-mediated recognition of an alpha-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J. Exp. Med.* 1998. **188**: 1521–1528.
- Yoshimoto, T. and Paul, W. E., CD4^{pos}, NK1.1^{pos} T cells promptly produce interleukin 4 in response to *in vivo* challenge with anti-CD3. *J. Exp. Med.* 1994. **179**: 1285–1295.
- Chen, H. and Paul, W. E., Cultured NK1.1⁺ CD4⁺ T cells produce large amounts of IL-4 and IFN-gamma upon activation by anti-CD3 or CD1. *J. Immunol.* 1997. **159**: 2240–2249.
- Kawano, T., Cui, J., Koezuka, Y., Taura, I., Kaneko, Y., Motoki, K., Ueno, H. *et al.* CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* 1997. **278**: 1626–1629.
- Hameg, A., Apostolou, I., Leite De Moraes, M., Gombert, J. M., Garcia, C., Koezuka, Y., Bach, J. F. and Herbelin, A., A subset of NKT cells that lacks the NK1.1 marker, expresses CD1d molecules, and autophenotypes the alpha-galactosylceramide antigen. *J. Immunol.* 2000. **165**: 4917–4926.
- Schmiege, J., Yang, G., Franck, R. W. and Tsuji, M., Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand alpha-galactosylceramide. *J. Exp. Med.* 2003. **198**: 1631–1641.
- Kawakami, K., Kinjo, Y., Yara, S., Koguchi, Y., Uezu, K., Nakayama, T., Taniguchi, M. and Saito, A., Activation of Valpha14(+) natural killer T cells by alpha-galactosylceramide results in development of Th1 response and local host resistance in mice infected with *Cryptococcus neoformans*. *Infect. Immun.* 2001. **69**: 213–220.
- Kimura, T., Ishii, Y., Morishima, Y., Shibuya, A., Shibuya, K., Taniguchi, M., Mochizuki, M. *et al.* Treatment with alpha-galactosylceramide attenuates the development of bleomycin-induced pulmonary fibrosis. *J. Immunol.* 2004. **172**: 5782–5789.
- Seder, R. A., Gazzinelli, R., Sher, A. and Paul, W. E., Interleukin 12 acts directly on CD4⁺ T cells to enhance priming for interferon gamma production and diminishes interleukin 4 inhibition of such priming. *Proc. Natl. Acad. Sci. USA* 1993. **90**: 10188–10192.
- Wenner, C. A., Güler, M. L., Macatonia, S. E., O'Garra, A. and Murphy, K. M., Roles of IFN-gamma and IFN-alpha in IL-12-induced T helper cell-1 development. *J. Immunol.* 1996. **156**: 1442–1447.
- Yoshida, M., Leigh, R., Matsumoto, K., Wattie, J., Ellis, R., O'Byrne, P. M. and Inman, M. D., Effect of interferon-gamma on allergic airway responses in interferon-gamma-deficient mice. *Am. J. Respir. Crit. Care Med.* 2002. **166**: 451–456.
- Kawano, T., Cui, J., Koezuka, Y., Taura, I., Kaneko, Y., Sato, H., Kondo, E. *et al.* Natural killer-like nonspecific tumor cell lysis mediated by specific ligand-activated Valpha14 NKT cells. *Proc. Natl. Acad. Sci. USA* 1998. **95**: 5690–5693.
- Smyth, M. J., Thia, K. Y., Street, S. E., Cretney, E., Trapani, J. A., Taniguchi, M., Kawano, T. *et al.* Differential tumor surveillance by natural killer (NK) and NKT cells. *J. Exp. Med.* 2000. **191**: 661–668.
- Wilson, S. B., Kent, S. C., Patton, K. T., Orban, T., Jackson, R. A., Exley, M., Porcelli, S. *et al.* Extreme Th1 bias of invariant Valpha24JalphaQ T cells in type 1 diabetes. *Nature* 1998. **391**: 177–181.
- Miyamoto, K., Miyake, S. and Yamamura, T., A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature* 2001. **413**: 531–534.
- Akbari, O., Stock, P., Meyer, E., Kronenberg, M., Sidobre, S., Nakayama, T., Taniguchi, M. *et al.* 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.

- 23 Lisbonne, M., Diem, S., de Castro Keller, A., Lefort, J., Araujo, L. M., Hachem, P., Fourneau, J. M. *et al.* Cutting Edge: Invariant V alpha 14 NKT cells are required for allergen-induced airway inflammation and hyper-reactivity in an experimental asthma model. *J. Immunol.* 2003. **171**: 1637–1641.
- 24 Brown, D. R., Fowell, D. J., Corry, D. B., Wynn, T. A., Moskowitz, N. H., Cheever, A. W., Locksley, R. M. and Reiner, S. L., Beta 2-microglobulin-dependent NK1.1⁺ T cells are not essential for T helper cell 2 immune responses. *J. Exp. Med.* 1996. **184**: 1295–1304.
- 25 Korsgren, M., Persson, C. G., Sundler, F., Bjerke, T., Hansson, T., Chambers, B. J., Hong, S. *et al.* Natural killer cells determine development of allergen-induced eosinophilic airway inflammation in mice. *J. Exp. Med.* 1999. **189**: 553–562.
- 26 Singh, N., Hong, S., Scherer, D. C., Serizawa, I., Burdin, N., Kronenberg, M., Koezuka, Y. and Van Kaer, L., Cutting Edge: Activation of NK T cells by CD1d and alpha-galactosylceramide directs conventional T cells to the acquisition of a Th2 phenotype. *J. Immunol.* 1999. **163**: 2373–2377.
- 27 Bilenki, L., Yang, J., Fan, Y., Wang, S. and Yang, X., Natural killer T cells contribute to airway eosinophilic inflammation induced by ragweed through enhanced IL-4 and eotaxin production. *Eur. J. Immunol.* 2004. **34**: 345–354.
- 28 Cui, J., Watanabe, N., Kawano, T., Yamashita, M., Kamata, T., Shimizu, C., Kimura, M. *et al.* Inhibition of T helper cell type 2 cell differentiation and immunoglobulin E response by ligand-activated Valpha14 natural killer T cells. *J. Exp. Med.* 1999. **190**: 783–792.
- 29 Burdin, N., Brossay, L. and Kronenberg, M., Immunization with α -galactosylceramide polarizes CD1-reactive NKT cells towards Th2 cytokine synthesis. *Eur. J. Immunol.* 1999. **29**: 2014–2025.
- 30 Li, X. M., Chopra, R. K., Chou, T. Y., Schofield, B. H., Wills-Karp, M. and Huang, S. K., Mucosal IFN-gamma gene transfer inhibits pulmonary allergic responses in mice. *J. Immunol.* 1996. **157**: 3216–3219.
- 31 Cohn, L., Homer, R. J., Niu, N. and Bottomly, K., T helper 1 cells and interferon gamma regulate allergic airway inflammation and mucus production. *J. Exp. Med.* 1999. **190**: 1309–1318.
- 32 Nakajima, H., Iwamoto, I. and Yoshida, S., Aerosolized recombinant interferon-gamma prevents antigen-induced eosinophil recruitment in mouse trachea. *Am. Rev. Respir. Dis.* 1993. **148**: 1102–1104.
- 33 Ford, J. G., Rennick, D., Donaldson, D. D., Venkayya, R., McArthur, C., Hansell, E., Kurup, V. P., Warnock, M. and Grünig, G., IL-13 and IFN-gamma: interactions in lung inflammation. *J. Immunol.* 2001. **167**: 1769–1777.
- 34 Matsuda, J. L., Naidenko, O.V., Gapin, L., Nakayama, T., Taniguchi, M., Wang, C. R., Koezuka, Y. and Kronenberg, M., Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J. Exp. Med.* 2000. **192**: 741–754.
- 35 Gosset, P., Tillie-Leblond, I., Janin, A., Marquette, C. H., Copin, M. C., Wallaert, B. and Tonnel, A. B., Increased expression of ELAM-1, ICAM-1, and VCAM-1 on bronchial biopsies from allergic asthmatic patients. *Ann. N. Y. Acad. Sci.* 1994. **725**: 163–172.
- 36 Nakajima, H., Sano, H., Nishimura, T., Yoshida, S. and Iwamoto, I., Role of vascular cell adhesion molecule 1/very late activation antigen 4 and intercellular adhesion molecule 1/lymphocyte function-associated antigen 1 interactions in antigen-induced eosinophil and T cell recruitment into the tissue. *J. Exp. Med.* 1994. **179**: 1145–1154.
- 37 Fukuda, T., Fukushima, Y., Numao, T., Ando, N., Arima, M., Nakajima, H., Sagara, H. *et al.* Role of interleukin-4 and vascular cell adhesion molecule-1 in selective eosinophil migration into the airways in allergic asthma. *Am. J. Respir. Cell Mol. Biol.* 1996. **14**: 84–94.
- 38 Bochner, B. S., Klunk, D. A., Sterbinsky, S. A., Coffman, R. L. and Schleimer, R. P., IL-13 selectively induces vascular cell adhesion molecule-1 expression in human endothelial cells. *J. Immunol.* 1995. **154**: 799–803.
- 39 Holt, P. G., Robinson, B. W., Reid, M., Kees, U. R., Warton, A., Dawson, V. H., Rose, A. *et al.* Extraction of immune and inflammatory cells from human lung parenchyma: Evaluation of an enzymatic digestion procedure. *Clin. Exp. Immunol.* 1986. **66**: 188–200.
- 40 Murphy, E., Shibuya, K., Hosken, N., Openshaw, P., Maino, V., Davis, K., Murphy, K. and O'Garra, A., Reversibility of T helper 1 and 2 populations is lost after long-term stimulation. *J. Exp. Med.* 1996. **183**: 901–913.