

Modulation of murine experimental allergic conjunctivitis by treatment with α -galactosylceramide

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Abstract

When mice are treated with α -galactosylceramide (α -GalCer), NKT cells are activated and suppress the development of experimental airway inflammation. This suppressive effect is believed to be mediated by the upregulation of IFN- γ . Here, we investigated whether α -GalCer treatment can also modulate the development of experimental allergic conjunctivitis (EC). EC was induced in wild-type and IFN- γ -deficient Balb/c mice by active immunization with ragweed (RW) followed by challenge with RW in eye drops. The mice were intraperitoneally injected with α -GalCer or vehicle at the time of immunization or before RW challenge. Twenty-four hours after RW challenge, conjunctivas, spleens and sera were harvested for histological analysis, flow cytometric, proliferation and cytokine assays, and measurement of immunoglobulin levels, respectively. Treatment with α -GalCer at the time of the EC-priming immunization significantly increased Th2 responses and markedly upregulated the severity of the EC. However, treatment with α -GalCer just before the Ag challenge that triggers EC in primed animals significantly suppressed the disease. This was associated with an increased frequency of CD4⁺CD25⁺ cells, which express Foxp3, in the spleen. α -GalCer treatment just prior to Ag challenge also suppressed the development of EC in IFN- γ -deficient mice, and we found apoptosis and anergy are unlikely to play a major role in the mechanism by which pre-challenge α -GalCer treatment suppresses EC. These data suggest that NKT cells can play a downregulatory role in the development of EC and that α -GalCer may be useful for treating allergic conjunctivitis.

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1. Introduction

Allergic conjunctivitis (AC) encompasses a wide variety of disorders [1] that range from the mild type, such as seasonal AC, to the severe type, such as vernal keratoconjunctivitis (VKC), which is characterized by massive eosinophil infiltration [2,3]. AC is mediated by both genetic and environmental factors [4], both of which lead to the polarized Th2 immune responses that are believed to induce eosinophil infiltration [5]. Our group has investigated the roles Th2 cells play in the development of AC by using an experimental AC model in rats [6–8] and mice [9–12] that is termed experimental immune-mediated blepharoconjunctivitis (EC). The essential role Th2 cells play in the development of EC was revealed when it was found that

the transfer of Ag-specific Th2 cells, but not Th1 cells, induced eosinophil infiltration into the conjunctiva [13].

Given that Th2 responses can be suppressed by Th1 responses [14], it has been speculated that the development of EC could be inhibited by treatments that upregulate Th1 responses. This idea was tested by injecting the Th1 cytokines [15] or by using IFN- γ -deficient mice [16,17]. The administration of recombinant IFN- γ indeed suppressed EC in Brown Norway rats [15], while IFN- γ -deficient C57BL/6 mice suffered severe conjunctival inflammation when subjected to the normal EC-inducing regimen [16]. Thus, IFN- γ appears to be able to negatively regulate the development of EC. However, in the Balb/c background, endogenous IFN- γ promotes the development of EC [17]. Furthermore, IL-12-deficient mice showed diminished conjunctival inflammation [16]. These observations together suggest that EC cannot be suppressed by the upregulation of a single Th1 cytokine [18].

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Upon T cell receptor stimulation, NKT cells very rapidly produce both Th1 and Th2 cytokines [19]. These cytokines produced by NKT cells have been postulated to regulate allergic inflammation. With regard to murine allergic airway diseases, analysis of NKT cell-deficient mice has revealed that NKT cells are required for the development of airway inflammation [20–22]. However, three recently published reports have demonstrated that allergic inflammation is inhibited when the animals are treated with α -galactosylceramide (α -GalCer), which is recognized by NKT cells in combination with the CD1d molecule and activates them [23–25]. These studies prompted us to investigate the effect of α -GalCer treatment on the development of EC.

2. Materials and methods

2.1. Mice

Inbred wild-type (WT) Balb/c mice were purchased from Japan SLC Inc., Hamamatsu, Shizuoka, Japan. IFN- γ -deficient Balb/c (GKO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were kept in pathogen-free conditions at the animal facility of the Kochi Medical School and age- and gender-matched mice were used when they were 6- to 12-week-old. All research adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

2.2. Reagents

Short ragweed pollen (RW) was purchased from Poly-sciences, Inc., Warrington, PA. RW extract was obtained from LSL CO. Ltd., Tokyo, Japan. Aluminum hydroxide (alum) was purchased from Sigma, St. Louis, MO. FITC-labeled anti-CD3 (145–2C11) and anti-CD4 (GK1.5), biotin-labeled anti-CD25 (PC61) and biotin-labeled anti-CD8 (53–6.7) were purchased from eBioscience, San Diego, CA. FITC-labeled anti-CD45R/B220 (RA3-6B2), FITC-labeled anti-CD11c (HL3) and streptavidin-PE were obtained from BD Biosciences, Franklin Lakes, NJ, while biotin-labeled anti-F4/80 (A3-1) was from Caltag Laboratories, Burlingame, CA.

2.3. Induction of EC by active immunization and treatment with α -GalCer

RW adsorbed on alum was injected into the left hind-footpad and the tail base. Fifty microliters of the emulsion (50 μ g of RW and 2 mg of alum) was injected into each site. The mice were challenged with RW in PBS (2 mg in 10 μ l per eye) 10 days after the immunization. Some of the mice were injected intraperitoneally with 2 μ g of α -GalCer (Kirin, Tokyo, Japan) at the time of challenge ($n = 10$ per group) or on day 10, 2 h before RW challenge (pre-challenge group, $n = 13$ per group). In a separate experiment, 2 μ g of α -GalCer was topically administered in eye drops 2 h before RW challenge ($n = 10$ per group). As a control, the vehicle (polysorbate-20, 0.5%) was used. Twenty-four hours

after the challenge with RW, the eyes, sera and spleens were harvested for histological analysis, measurement of Ig levels, and T cell culture for transfer, cytokine or proliferation assays, respectively.

2.4. EC induction by adoptive transfer of splenocytes from actively immunized and α -GalCer-treated mice

Splenocytes harvested from the mice intraperitoneally injected with α -GalCer or vehicle before RW challenge described above were cultured with RW extract at final concentrations of 5 μ g/ml in 75-cm² flasks at a concentration of 10^7 cells per ml in a final volume of 20 ml RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, ICN Biomedical Japan Co., Tokyo, Japan), 2-mercaptoethanol (2-ME, 5×10^{-5} M), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μ g/ml) either in the absence or presence of recombinant human IL-2 (100 JRU/ml, Takeda, Tokyo, Japan). After incubation for 72 h at 37 °C in a humidified atmosphere with 5% CO₂, 2×10^7 splenocytes/mouse were intraperitoneally injected into naïve Balb/c mice ($n = 10$ mice per group). Four days after the transfer, the eyes of the recipient mice were challenged with RW in PBS (2 mg in 10 μ l per eye). Twenty-four hours later, the eyes and spleens were harvested for histological analysis.

2.5. Histological analysis

The eyes including the conjunctivas were harvested and fixed in 10% buffered formalin. Vertical 2 μ m-thick sections were cut and stained with Giemsa. Infiltrating eosinophils in the lamina propria mucosae of the tarsal and bulbar conjunctivas throughout each section were counted by two masked observers. The sections counted were those of the central portion of the eye, which included the pupil and optic nerve head. The data are presented as an average $\pm$ S.E.M. per slide of all the mice examined.

2.6. Flow cytometric analysis

Splenocytes from actively immunized α -GalCer- or vehicle-treated mice were incubated for 30 min on ice with FITC- or biotin-labeled Abs at optimal concentrations. After the incubation, the cells were washed with cold 2% FCS-PBS. In the case of the biotin-labeled Abs, the cells were then incubated with streptavidin-PE for 10 min and then washed again. To detect annexin V-positive cells, whole splenocytes were used directly. Alternatively, the splenocytes were initially stained with biotin-labeled anti-CD4 Ab followed by streptavidin-PE staining. Thereafter, these cells were stained with annexin V according to the manufacturer's recommendations. Dead cells were excluded by 7-amino-actinomycin D (7-AAD). To examine Foxp3 expression, mouse regulatory T cell staining kit (eBioscience) was used following the manufacturer's recommendation. The cells were then analyzed on a FACScan (Becton Dickinson, Mountain View, CA) and the data were acquired and analyzed by using CellQuest software.

2.7. Cellular proliferation assay

RBC-depleted splenocytes (3×10^5 cells/well) were cultured in 96-well flat-bottom plates in a final volume of 0.2 ml RPMI 1640 medium supplemented with 5% FCS and 2-ME. The cells were stimulated with RW at a final concentration of 25 μ g/ml or with concanavalin A (Con A) at 5 μ g/ml. After an 72 h incubation at 37 °C in a humidified atmosphere containing 5% CO₂, the cultures were pulsed for 16 h with 0.5 μ Ci/well [³H]thymidine (Japan Atomic Energy Research Institute, Tokai, Japan). The cultures were then harvested and the incorporated radioactivity was measured by standard techniques. The data were expressed as delta counts per minute [cpm] (mean cpm of stimulated cultures-mean cpm of unstimulated control cultures).

2.8. Measurement of IgE, IgG1 and IgG2a in serum

Twenty-four hours after RW challenge, the blood of actively immunized mice was collected and serum was prepared. RW-specific and total IgE, IgG1 and IgG2a levels in the sera were measured by ELISA detailed in elsewhere [26]. The data are expressed as actual concentrations except for RW-specific IgG1 and IgG2a, which are expressed units (U)/ml relative to the pooled hyperimmune serum.

2.9. Measurement of cytokines in the culture supernatants

RBC-depleted splenocytes (10^7 cells/ml) were cultured for 48 h with RW (25 μ g/ml) in 96-well flat-bottom plates in a final volume of 0.2 ml RPMI 1640 medium supplemented with 10% FCS and 2-ME. The levels of IL-2, IL-4, IL-5, IL-10, IL-13 and IFN- γ produced were measured by using the Bioplex system (Bio-Rad, Hercules, CA) according to the manufacturer's recommendation.

2.10. Measurement of cytokines and chemokines in the conjunctiva

The levels of multiple cytokines and chemokines, including 17 cytokines (IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, G-CSF, GM-CSF, IFN- γ , and TNF- α) and six chemokines (eotaxin, KC, MCP-1, MIP-1 α , MIP-1 β , and RANTES), in the conjunctiva were measured by using the Bioplex system. Thus, conjunctivas from α -GalCer- or vehicle-treated mice that were developing EC ($n = 4$ per group) were weighed, cut into small pieces, homogenized in 100 μ l of lysis buffer and centrifuged at 6000 rpm for 5 min. The levels of 23 different cytokines and chemokines were measured in 50 μ l of the resulting conjunctiva supernatants. The data are expressed as pg (cytokine)/mg (conjunctiva).

2.11. Statistical analysis of data

Differences between the α -GalCer- and vehicle-treated mice in terms of their serum Ig levels, flow cytometric analysis of splenocytes, splenocyte proliferation and cytokine production, and infiltrating eosinophil numbers were tested for significance

by Student's *t*-test. *P* values less than 0.05 were considered significant. The number of mice for each experiment (the sum of the number of mice used in individual experiment) is shown as above. Each experiment was repeated for 2–4 times except for measurement of cytokines and chemokines in the conjunctiva.

3. Results

3.1. Systemic α -GalCer treatment at the time of the priming immunization exacerbates EC, while treatment just before the challenge attenuates EC

It is known that α -GalCer activates NKT cells and induces them to produce a variety of cytokines *in vivo* that suppresses experimental airway inflammation [23–25]. Consequently, we sought to determine whether α -GalCer-induced NKT-cell activation also affects the development of EC. To this aim, we injected actively immunized mice intraperitoneally with α -GalCer either at the time of immunization or just before the challenge. Compared to the vehicle-treated control mice, treatment with α -GalCer at the time of immunization significantly increased the eosinophil infiltration into the conjunctiva that was triggered by RW challenge (Fig. 1A and C). In contrast, treatment with α -GalCer just before the challenge significantly decreased eosinophil infiltration (Fig. 1B and D).

3.2. Systemic α -GalCer treatment affects immune responses

To examine whether these disparate modulating effects of α -GalCer are mediated by altered systemic immune responses, we harvested the spleens from the two groups of mice described above 1 day after the challenge. The splenocytes were analyzed by flow cytometric analysis to examine the frequencies of various cell types. As summarized in Table 1, relative to the vehicle-treated control animals, animals treated with α -GalCer at the time of immunization showed significantly lower CD4⁺ T cell frequencies. In contrast, treating the mice just before challenge significantly elevated the frequencies of the CD25⁺, CD4⁺CD25⁺ and B220⁺ cell compartments. Analysis of Foxp3 expression in CD4⁺CD25⁺ T cell compartment confirmed that the number of Foxp3⁺ cells and the expression levels of Foxp3 were significantly higher in α -GalCer-treated group (Table 2). The splenocytes of the animals described above were also cultured with RW for 88 h to determine the effect of α -GalCer on their recall responses. The proliferative response to RW was significantly upregulated by α -GalCer treatment at the time of immunization (Fig. 2A). In contrast, treatment just prior to challenge had no effect on the proliferative responses (Fig. 2A). The splenocyte proliferation induced by Con A was equivalent for the two groups (Fig. 2A). The cytokines produced during the *in vitro* recall response to RW by the splenocytes were analyzed 48 h after culture. This revealed that treatment with α -GalCer at the time of immunization generally increased RW-induced cytokine production, particularly with regard to IL-5 and IL-13 (Fig. 2B). In contrast, treatment with α -GalCer before the challenge significantly decreased IL-2 production (Fig. 2B). IL-4 was below detectable levels in both groups regardless of the

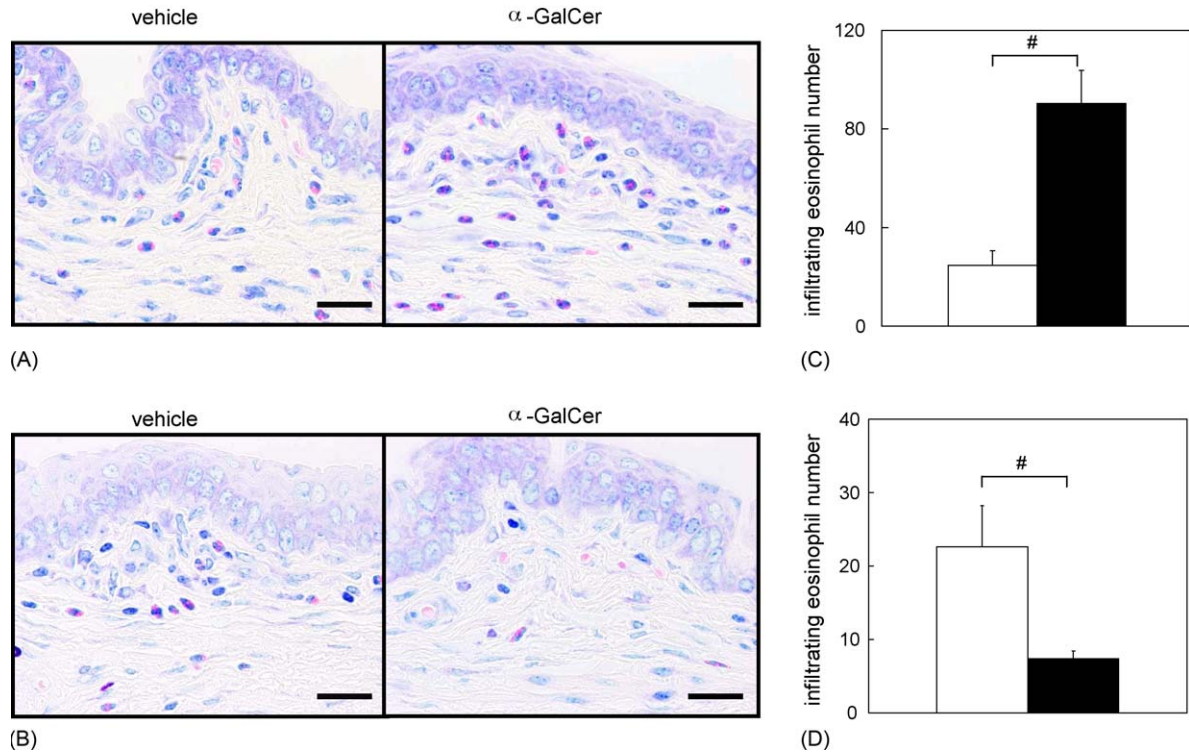


Fig. 1. Effect of systemic α -GalCer treatment on EC induced by active immunization. EC was induced in Balb/c WT mice by active immunization with RW followed by RW challenge in eye drops. These mice were treated with α -GalCer or vehicle at the time of immunization (A and C) or just before RW challenge (B and D). Twenty-four hours after RW challenge, the conjunctivas were harvested for histological evaluation. (A and C) microphotographs. (B and D) infiltrating eosinophil number per slide. Bar = 20 micrometer. White bar, vehicle-treated mice; black bar: α -GalCer-treated mice, $\#P < 0.01$.

Table 1
Flow cytometric analysis of splenocytes from actively immunized mice

	CD3	CD4	CD8	CD25	CD4/CD25	B220	TCR β /DX5
Vehicle day 0 i.p.	41.45 $\pm$ 1.23	25.13 $\pm$ 0.49*	14.96 $\pm$ 0.62	3.19 $\pm$ 0.11	2.63 $\pm$ 0.13	53.06 $\pm$ 1.27	0.75 $\pm$ 0.04
α -GalCer day 0 i.p.	37.40 $\pm$ 1.62	20.32 $\pm$ 0.75*	13.68 $\pm$ 1.84	3.20 $\pm$ 0.19	2.45 $\pm$ 0.15	55.67 $\pm$ 1.56	0.83 $\pm$ 0.10
Vehicle day 10 i.p.	46.38 $\pm$ 1.58	2947 $\pm$ 1.75	14.16 $\pm$ 0.25	1.64 $\pm$ 0.28*	1.16 $\pm$ 0.29*	46.91 $\pm$ 1.20*	0.82 $\pm$ 0.09
α -GalCer day 10 i.p.	42.12 $\pm$ 2.79	28.70 $\pm$ 1.34	12.48 $\pm$ 1.06	3.77 $\pm$ 0.70*	2.98 $\pm$ 0.64*	56.15 $\pm$ 2.37*	0.73 $\pm$ 0.03

Data are presented as percentage of cells in the total splenocyte population that are positive for the indicated cell surface marker ($\pm$ S.E.M.). "Day 0" indicates that the treatment was conducted at the time of immunization, while "Day 10" indicates that the treatment was conducted before RW challenge. * $P < 0.05$, $n = 6$ mice per group.

timing of treatment (data not shown). The serum harvested from the mice 1 day after challenge was assessed for RW-specific (Fig. 2C) or total (Fig. 2D) IgE, IgG1 and IgG2a levels. No differences between the sera from vehicle- and α -GalCer-treated mice were observed except that the RW-specific IgG2a levels were significantly upregulated by α -GalCer treatment at the time of immunization (Fig. 2C).

3.3. Transfer of splenocytes from the EC-developing mice treated with α -GalCer before the challenge induced significantly less eosinophil infiltration

That α -GalCer delivered just prior to the triggering challenge can block the response to this challenge suggests that this agent could be therapeutic value in AC diseases. Consequently, we investigated further why pre-challenge treatment with α -GalCer attenuated eosinophil infiltration into the conjunctiva. To this aim, we induced EC in naïve WT mice by

transferring RW-primed splenocytes from actively immunized mice that were treated with α -GalCer or vehicle before the challenge. The transfer of splenocytes from α -GalCer-treated mice induced significantly less eosinophilic infiltration than the transfer of cells from vehicle-treated mice (Fig. 3). This is consistent with the lower eosinophilic response by the day 10-treated

Table 2
Foxp3 expression in CD4 $^{+}$ /CD25 $^{+}$ compartment

	% Positive	MFI
Vehicle	0.03 $\pm$ 0.01*	20.6 $\pm$ 10.7*
α -GalCer	0.33 $\pm$ 0.06*	290.7 $\pm$ 52.6*

Freshly isolated splenocytes from EC-developing mice treated either with vehicle or α -GalCer just before RW challenge were subjected to flow cytometric analysis to determine Foxp3 expression. % Positive (Foxp3 $^{+}$ CD25 $^{+}$) was calculated in CD4 $^{+}$ T cell compartment. MFI (mean fluorescence intensity) of Foxp3 was measured in Foxp3 $^{+}$ CD25 $^{+}$ CD4 $^{+}$ T cell compartment. Data are presented as mean $\pm$ S.E.M. * $P < 0.001$, $n = 3$ mice per group.

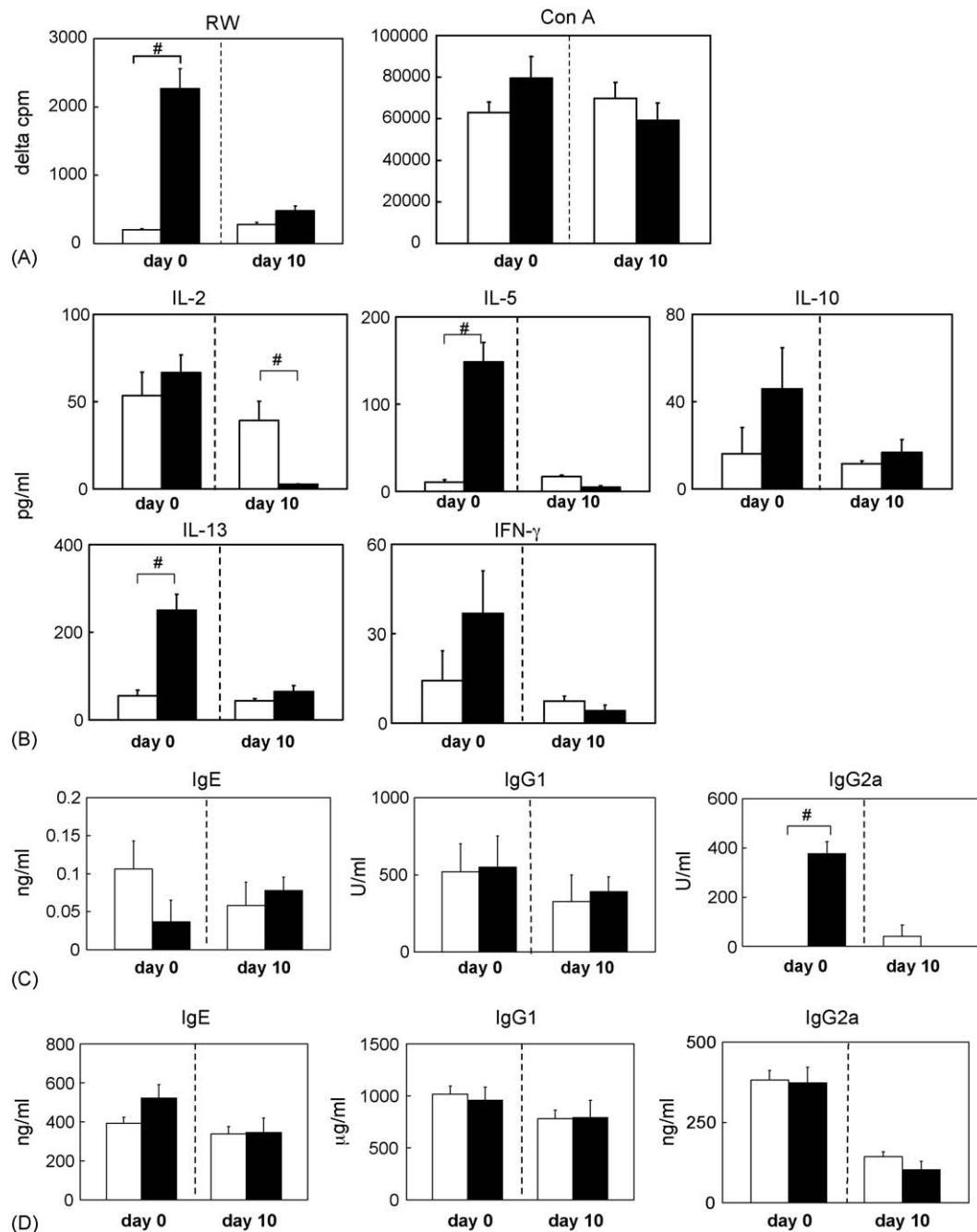


Fig. 2. Effect of systemic α -GalCer treatment on immune responses. Spleens and sera were harvested from the mice described in the legend of Fig. 1, 24 h after RW challenge. "Day 0" and "Day 10" indicate that the animals were treated at the time of immunization or just before RW challenge, respectively. Splenocytes were prepared and cultured with RW or Con A. Their proliferative (A) and cytokine (B) responses were determined after 88 and 48 h of culture, respectively. The background cpm $\pm$ S.E.M.s for the day 0 vehicle-treated, day 0 α -GalCer-treated, day 10 vehicle-treated, and day 10 α -GalCer-treated groups were 744 ± 36 , 970 ± 46 , 745 ± 48 and 1648 ± 98 . The sera were examined for the levels of RW-specific (C) or total (D) Ig. White bar: vehicle-treated mice; black bar: α -GalCer-treated mice, [#] $P < 0.01$.

actively immunized mice (Fig. 1B and D). Thus, α -GalCer treatment just before challenge suppresses EC by attenuating the EC-inducing capability of splenocytes.

3.4. IFN- γ is unlikely to be involved in the suppression of EC that is induced by pre-challenge α -GalCer treatment

Recent reports have demonstrated that α -GalCer treatment inhibits allergic airway inflammation in an IFN- γ -dependent

manner [23–25]. To investigate whether IFN- γ is involved in the mechanism by which pre-challenge treatment with α -GalCer suppresses the development of EC upon challenge, GKO mice were actively immunized with RW and treated with α -GalCer 2 h prior to RW challenge. As with the WT mice, treatment of GKO mice with α -GalCer just before RW challenge inhibited their development of EC (Fig. 4). Thus, IFN- γ is unlikely to be involved in the mechanism by which pre-challenge α -GalCer treatment suppresses EC.

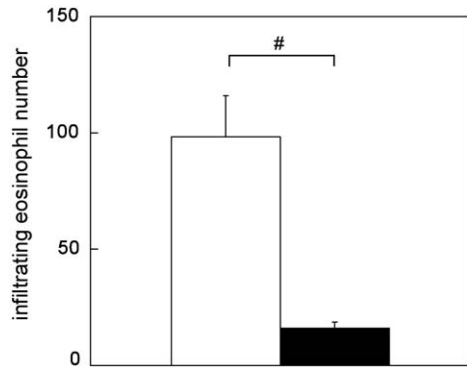


Fig. 3. Conjunctival infiltration of eosinophils induced by the transfer of splenocytes from mice treated with α -GalCer. Splenocytes prepared from the mice treated with either vehicle or α -GalCer as described in Fig. 2 were cultured *in vitro* with RW for 72 h, and transferred into naïve syngeneic WT mice. Four days later, the recipient mice were challenged with RW in eye drops and 24 h later, the conjunctivas were harvested for histological analysis to evaluate eosinophil infiltration. The data are presented as infiltrating eosinophil numbers per slide. White bar: vehicle-treated mice; black bar: α -GalCer-treated mice, $^{\#}P < 0.01$.

3.5. Pre-challenge treatment with α -GalCer is unlikely to induce splenic $CD4^{+}$ T cell apoptosis or anergy

The data in Fig. 3 suggest that pre-challenge treatment with α -GalCer impairs the EC-inducing capability of primed splenocytes. Moreover, while the splenocytes from pre-challenge-treated and -untreated animals exhibited few obvious differences in terms of cytokine production upon *in vitro* culture with RW, the treated animals did produce less IL-2 (Fig. 2B). This may be significant as reduced IL-2 expression can indicate a state of T cell anergy [27]. Consequently, we next examined whether pre-challenge treatment with α -GalCer causes the splenocytes to become apoptotic or anergic. However, while the whole splenocyte population of the α -GalCer-treated group did show slightly higher apoptosis, this was not statistically significant (Table 3). The same was true for the $CD4^{+}$ T cells in the splenocytes (Table 3). Thus, it is unlikely that pre-challenge treatment

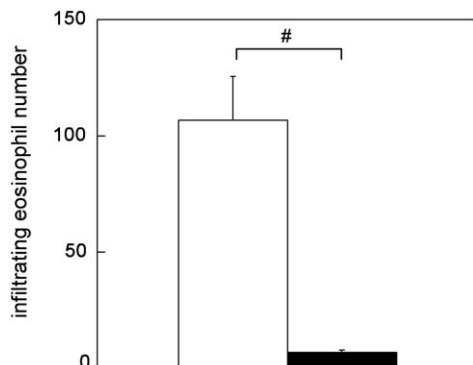


Fig. 4. Effect of systemic α -GalCer treatment on EC induced by active immunization in GKO mice. EC was induced in Balb/c GKO mice by active immunization with RW followed by RW challenge in eye drops. These mice were treated with α -GalCer or vehicle just before RW challenge. Twenty-four hours after RW challenge, the conjunctivas were harvested for histological analysis to evaluate eosinophil infiltration. The data are presented as infiltrating eosinophil number per slide. White bar: vehicle-treated mice; black bar: α -GalCer-treated mice, $^{\#}P < 0.01$.

Table 3
Percentage of apoptotic cells

	Whole splenocytes	$CD4^{+}$ compartment
Vehicle	5.84 ± 0.75	1.56 ± 0.82
α -GalCer	7.06 ± 3.29	2.57 ± 0.49

Freshly isolated splenocytes were subjected to flow cytometric analysis to determine the percentage of apoptotic cells by staining with annexin V and 7-AAD. Data are presented as mean $\pm$ S.E.M. Significant differences were not observed between vehicle-treated and alpha-GalCer-treated groups.

with α -GalCer suppresses the EC-inducing capacity of splenocytes by causing their apoptosis. Next, to examine whether splenocytes become anergic upon pre-challenge treatment with α -GalCer, we cultured the splenocytes from pre-challenge α -GalCer-treated mice with RW in the presence or absence of exogenous IL-2. These splenocytes were then transferred into naïve WT mice and the recipients mice were challenged with RW. However, culture with exogenous IL-2 did not elevate the ability of the splenocytes from pre-challenge α -GalCer-treated mice to induce EC (Fig. 5), nor did it alter the severity of the EC that was generated (Fig. 5).

3.6. Pre-challenge treatment with α -GalCer in eye drops does not affect the development of EC

It has been reported that intranasal α -GalCer treatment inhibits the airway inflammation [23]. Consequently, we examined whether treatment of primed mice with α -GalCer in eye drops just prior to their challenge with RW can affect the development of EC. However, the treatment had no effect on the eosinophilic infiltration (Fig. 6), although it did significantly increase the levels of IL-12p40, TNF- α and MCP-1 in the conjunctiva while significantly decreasing the IL-6 levels (Fig. 7).

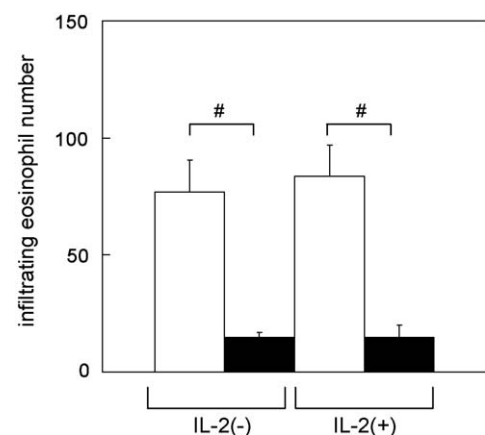


Fig. 5. Effect on eosinophilic infiltration of culturing primed splenocytes from α -GalCer-treated mice with IL-2 prior to their adoptive transfer. Splenocytes were prepared and cultured *in vitro* with RW for 72 h as described in the legend of Fig. 3 with (+) or without (–) exogenous IL-2. These cells were then transferred into syngeneic naïve WT mice. Four days after the transfer, the recipient mice were challenged with RW in eye drops and 24 h later, their conjunctivas were harvested for histological analysis to evaluate eosinophil infiltration. The data are presented as infiltrating eosinophil numbers per slide. White bar: vehicle-treated mice; black bar: α -GalCer-treated mice, $^{\#}P < 0.01$.

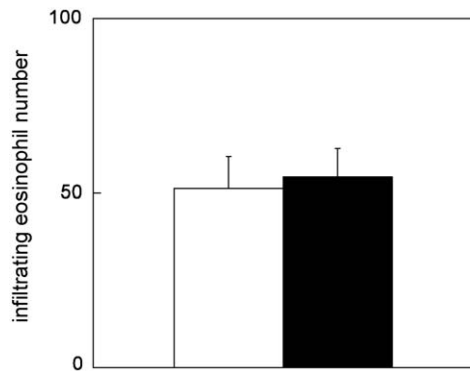


Fig. 6. Effects of pre-challenge topical α -GalCer treatment on EC induced by active immunization. EC was induced in Balb/c WT mice by active immunization with RW followed by RW challenge in eye drops. These mice were topically treated with α -GalCer or vehicle in eye drops 2 h before RW challenge. Twenty-four hours after RW challenge, the conjunctivas were harvested for histological analysis to evaluate eosinophil infiltration. The data are presented as infiltrating eosinophil numbers per slide. White bar: vehicle-treated mice; black bar: α -GalCer-treated mice.

4. Discussion

In this study, we found that the activation of NKT cells by intraperitoneal injection of α -GalCer at the time of sensitization augmented EC, while treatment just before the challenge suppressed EC. The observations are in good agreement with previous reports of the effects of α -GalCer on experimental airway inflammation. However, we found that topical administration of α -GalCer in eye drops just prior to challenge did not suppress EC. In contrast, it has been shown that intranasal administration of α -GalCer inhibited airway inflammation [23].

We found that the intraperitoneal injection of α -GalCer at the time of sensitization significantly increased the RW-specific proliferation of splenocytes and their elaboration of Th2 cytokines such as IL-5 and IL-13. However, little effect on the humoral immune responses was observed. This is consistent with our previous observations that Ag-specific cellular immunity plays a more important role in the development of EC than Ag-specific humoral immune responses [6,9,28]. Furthermore, it has been shown that the upregulation of Ag-specific cellular immunity and Th2 cytokine production induces more severe conjunctival inflammation [29]. Thus, it may be that α -GalCer delivered at the time of priming upregulates cellular immune responses, especially Th2 responses, and that these then generate significantly more severe EC.

Three publications have shown that α -GalCer treatment just prior to the challenge suppresses experimental airway inflammation [23–25]. Although these papers differ somewhat with regard to the suppressive mechanism that appears to be involved, all three suggest that α -GalCer upregulates the production of IFN- γ , which then negatively regulates Th2 immune responses and attenuates airway inflammation [23–25]. To investigate whether the same mechanism is responsible for the suppression of EC by pre-challenge α -GalCer treatment, we induced EC in GKO mice and treated them with α -GalCer just before the challenge. However, the inability of these mice to produce IFN- γ did not alter the EC-suppressive effects of pre-challenge α -GalCer. Therefore, IFN- γ is unlikely to participate in the mechanism by which pre-challenge-administered α -GalCer suppresses the development of EC.

How, then, does pre-challenge α -GalCer attenuate the development of EC? Analysis of the cytokines elaborated by the splenocytes from actively immunized mice treated with α -

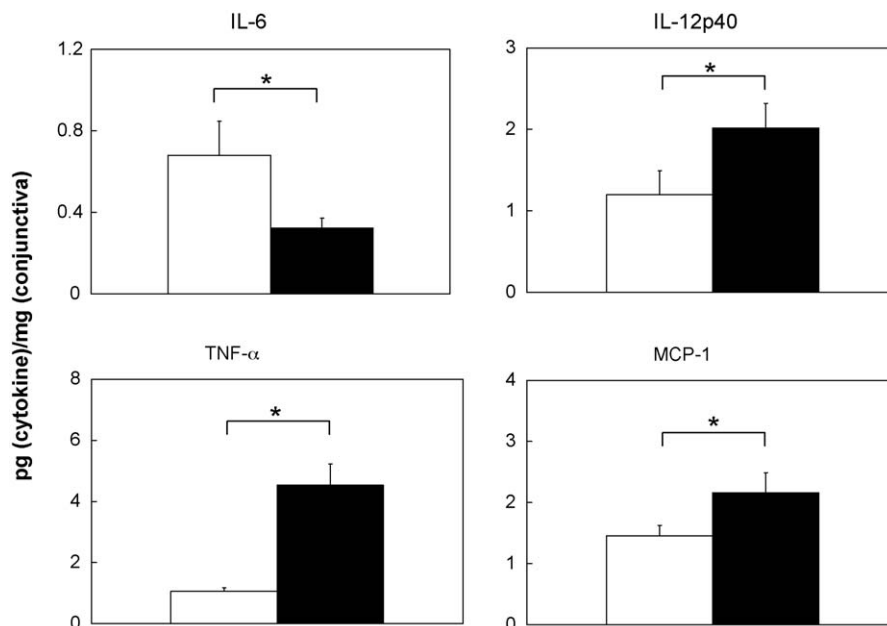


Fig. 7. Effect of pre-challenge topical α -GalCer treatment on cytokine expression in the conjunctiva of EC-developing mice. The conjunctivas prepared as described in the legend of Fig. 6 were analyzed for the levels of 23 cytokines/chemokines by using the Bioplex system. Of the 17 cytokines and six chemokines, only IL-6, IL-12p40, TNF- α and MCP-1 showed detectable levels. The data are expressed as pg (cytokine)/mg (conjunctiva). White bar: vehicle-treated mice; black bar: α -GalCer-treated mice, * P < 0.05.

GalCer just before challenge demonstrated that this treatment almost completely suppressed RW-stimulated production of IL-2 by splenocytes. The number of splenocyte recovered from each spleen did not differ between the vehicle-treated and α -GalCer-treated mice (data not shown). Moreover, flow cytometric analysis failed to detect differences in the frequencies of CD4⁺ and CD8⁺ T cells in these spleens. Therefore, next, we examined whether T cells from α -GalCer-treated mice become either apoptotic or anergic. Flow cytometric analysis confirmed that apoptosis is unlikely to play a role in the mechanism by which pre-challenge α -GalCer treatment attenuates eosinophil infiltration into the conjunctiva. Anergy is also unlikely to play a role, as cultivation with IL-2 failed to improve the EC-inducing potential of adoptively transferred splenocytes from mice treated with α -GalCer just before RW challenge.

We did note that primed mice treated with α -GalCer just prior to challenge had a higher frequency of CD4⁺CD25⁺ cells in their spleens than the vehicle-treated control group. Furthermore, Foxp3 expression in CD4⁺CD25⁺ cells was significantly higher in α -GalCer-treated group. Naturally occurring thymus-selected CD4⁺CD25⁺ cells, which express Foxp3, have been termed regulatory T cells as they have been found to play a suppressive role in a variety of immune responses [30]. It was recently reported that α -GalCer treatment activates CD4⁺CD25⁺ regulatory T cells and in turn inhibits experimental autoimmune myasthenia gravis [31]. With regard to allergic inflammation, it was recently demonstrated that CD4⁺CD25⁺ regulatory T cells play a suppressive role in the development of murine experimental airway inflammation [32,33]. In addition, we found that CD4⁺CD25⁺ cells regulate the development of EC during both the induction and effector phases (submitted for publication). Moreover, transfer of CD4⁺CD25⁺ cells to RW sensitized mice suppressed the severity of EC (manuscript in preparation). These observations together suggest that α -GalCer administered just prior to challenge may stimulate CD4⁺CD25⁺ regulatory T cells that suppress EC.

Although it has been reported that the intranasal administration of α -GalCer inhibits experimental airway inflammation [23] and we showed here that systemic administration of α -GalCer just before challenge suppresses EC, but topical administration of α -GalCer at the same time point did not affect the development of EC. The splenocytes from the topically treated mice did not differ in cytokine production profile relative to those from mice topically treated with vehicle (data not shown). Thus, topical administration of α -GalCer in eye drops does not affect systemic immune responses. However, analysis of the conjunctival expression of a variety of cytokines and chemokines showed that IL-6, IL-12p40, TNF- α and MCP-1 expression was modulated by topical treatment with α -GalCer. These data suggest that NKT cells may be in the conjunctiva. However, these four molecules are unlikely to play a direct role in the infiltration of eosinophils [34]. Thus, unlike the case of experimental airway inflammation, treatment with α -GalCer in eye drops is unlikely to be a suitable therapeutic strategy for AC. However, in severe forms of AC such as VKC, treatment with topical medication by eye drops is not always adequate to suppress conjunctival inflammation and systemic administration of corticosteroid is

sometimes necessary [1]. Therefore, the data here provide, at least some, therapeutic insight to treat severe forms of AC.

In conclusion, systemic treatment with α -GalCer before Ag challenge inhibits eosinophil infiltration into the conjunctiva. While the mechanism behind this suppressive effect remains elusive, our observation suggests that it is unlikely to involve the upregulation of IFN- γ or the induction of apoptosis or anergy. Instead, it is possible that α -GalCer increases the levels of CD4⁺CD25⁺ regulatory T cells, and that these somehow inhibit the immune responses that would normally drive the infiltration of eosinophils in the conjunctiva. To further clarify the roles of NKT cells in the development of EC, it is important to compare the characteristics and severity of EC between wild-type and NKT cell-deficient mice.

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