

Awarded Article, Annual Meeting of JSA

# Depletion of Thymus-Derived CD4<sup>+</sup>CD25<sup>+</sup> T Cells Abrogates the Suppressive Effects of $\alpha$ -galactosylceramide Treatment on Experimental Allergic Conjunctivitis

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## ABSTRACT

**Background:** We showed previously that  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) treatment elevated splenic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T-cell numbers and suppressed the development of experimental allergic conjunctivitis (EC). Here, we investigated whether CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells mediate the suppressive effects of  $\alpha$ -GalCer treatment on EC.

**Methods:** To deplete CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells, neonatal mice were thymectomized and intraperitoneally injected with anti-CD25 Ab. At 6 weeks of age, these mice were immunized with ragweed (RW) in aluminum hydroxide. Ten days later, the mice were challenged with RW in eye drops and 24 hours later, the conjunctivas and spleens were harvested for histological and flow cytometric analyses, respectively.  $\alpha$ -GalCer or vehicle was injected 2 hours prior to RW challenge. In addition,  $\alpha$ -GalCer was injected into thymus-intact EC-developing mice that had not been treated with anti-CD25 Ab.

**Results:**  $\alpha$ -GalCer treatment significantly suppressed EC in the thymus-intact mice that had not been treated with anti-CD25 Ab. In contrast,  $\alpha$ -GalCer treatment of thymectomized and anti-CD25 Ab-treated mice did not affect the severity of EC or splenic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T-cell numbers. However,  $\alpha$ -GalCer treatment did significantly increase splenic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T-cell numbers in thymectomized mice that had not received anti-CD25 Ab.

**Conclusions:**  $\alpha$ -GalCer treatment during the effector phase of EC increased CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T-cell numbers, which in turn suppressed the development of EC.

## KEY WORDS

allergic conjunctivitis, eosinophils, regulatory T cells, thymus,  $\alpha$ -galactosylceramide

## INTRODUCTION

Allergic conjunctivitis (AC) is a disease that ranges from mild forms such as seasonal AC to severe forms such as vernal keratoconjunctivitis (VKC).<sup>1</sup> More than 80% of AC patients have mild forms of the disease and their major clinical findings are itching and

hyperemia.<sup>2</sup> In contrast, the clinical manifestations of VKC are proliferative conjunctival giant papillary formations with intense itching, strong pain, foreign body sensation, mucous discharge and corneal ulceration, which may lead to vision loss. Cytological and histological studies have shown that many eosinophils infiltrate the conjunctiva of VKC pa-

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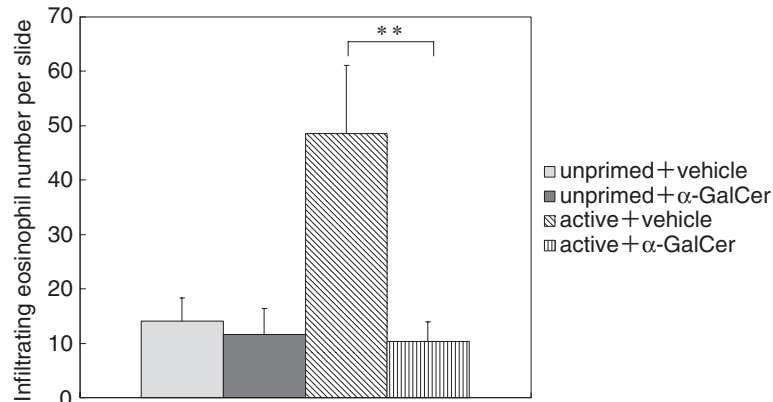
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Received 24 August 2007. Accepted for publication 23 January 2008.

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**Fig. 1** Treatment with  $\alpha$ -GalCer during the effector phase suppressed EC. BALB/c mice were immunized with RW in alum and 10 days later, the mice were challenged with RW in eye drops. Two hours prior to RW challenge,  $\alpha$ -GalCer (active +  $\alpha$ -GalCer) or vehicle (active + vehicle) was injected intraperitoneally ( $n = 3$  per group). Twenty-four hours after RW challenge, the conjunctivas were harvested and the conjunctival eosinophils were counted. Alternatively, conjunctivas were harvested from unprimed mice that had been challenged with RW and treated with either  $\alpha$ -GalCer (unprimed +  $\alpha$ -GalCer) or vehicle (unprimed + vehicle) ( $n = 3$  per group). The data are presented as means  $\pm$  SEM of 3 mice in each group. \*\* $P < 0.01$ .

tients.<sup>3</sup> Furthermore, the numbers of eosinophils in tear fluids increase as the severity of corneal damage increases.<sup>4</sup> Thus, it is believed that eosinophils are the major effector cells in the development of VKC.

However, AC is an Ag-specific disease and eosinophils do not have Ag-specific receptors. Therefore, T cells or Igs, which have Ag-specific receptors, are believed to play a crucial role in the development of AC. To examine this issue, our group has investigated the mechanism by which VKC develops by using animal models of AC (experimental AC, EC).<sup>5-10</sup> Adoptive transfer experiments demonstrated that Ag-primed T cells, but not Ag-specific IgE, trigger conjunctival eosinophil infiltration.<sup>11</sup> Furthermore, adoptive transfer of Ag-specific Th2 cells, but not Ag-specific Th1 cells or CD8<sup>+</sup> T cells, was able to induce conjunctival eosinophil infiltration.<sup>12</sup> Thus, it appears that Th2 cells play an essential role in the development of EC and possibly VKC as well.

Th2 cells are regulated *in vivo* by many types of cells, including regulatory T cells (Tregs) and NKT cells. The depletion of naturally occurring thymus-selected Tregs augments the severity of EC,<sup>13,14</sup> which demonstrates that Tregs regulate the development of EC. We also found that the forced activation of NKT cells by administration of  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer) during the effector phase of EC attenuated the severity of EC.<sup>15</sup> Interestingly,  $\alpha$ -GalCer treatment during the effector phase significantly increased the numbers of splenic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>

Tregs.<sup>15</sup> Therefore, we speculated that CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs may mediate the attenuation of EC induced by  $\alpha$ -GalCer treatment during the effector phase. Here, to test this notion, Treg-depleted mice were treated with  $\alpha$ -GalCer during the effector phase.

## METHODS

### MICE

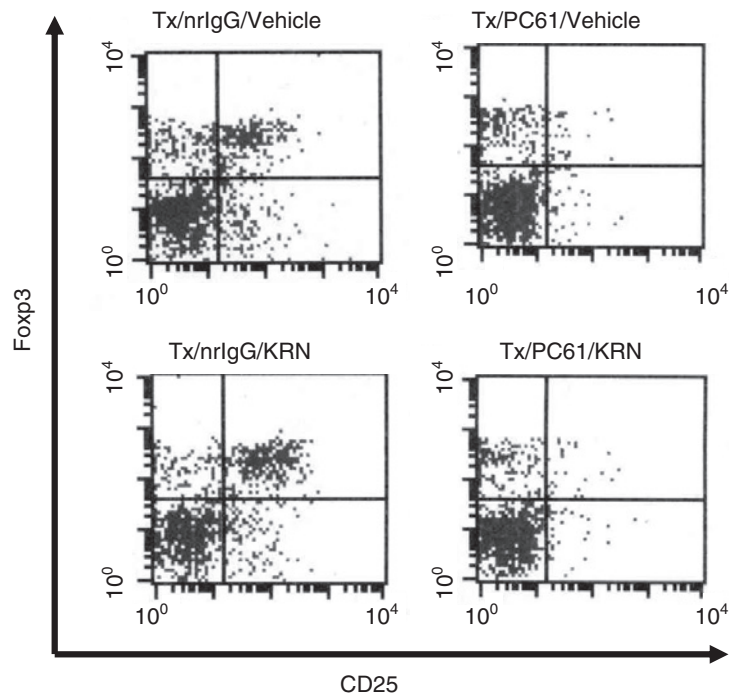
Inbred BALB/c mice were purchased from Japan SLC Inc., Hamamatsu, Shizuoka, Japan, and kept in pathogen-free conditions at the animal facility of the Kochi Medical School. All research adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

### REAGENTS

Short ragweed pollen (RW) was purchased from Polysciences, Inc, Warrington, PA, USA. RW extract was obtained from LSL Co. Ltd., Tokyo, Japan. Aluminum hydroxide (alum) was purchased from Sigma, St. Louis, MO, USA.  $\alpha$ -GalCer was kindly donated by Kirin, Tokyo, Japan.

### THYMECTOMY, TREATMENT WITH ABS AND $\alpha$ -GALCER AND INDUCTION OF EC

Neonates were thymectomized 3 days after birth as described previously<sup>13</sup> and then injected intraperitoneally on days 4, 21 and 38 after birth with 150  $\mu$ g of rat mAbs against CD25 (PC61, rat IgG1,  $n = 17$ ) or



**Fig. 2** Flow cytometric analysis of CD25 and Foxp3 expression in CD4<sup>+</sup> T-cell compartment. Thymectomized (Tx) mice were intraperitoneally injected with PC61 or nrlgG. These mice were immunized with RW and 10 days later, the mice were challenged with RW in eye drops. Two hours before RW challenge, the mice were injected intraperitoneally with either  $\alpha$ -GalCer or vehicle. Twenty-four hours after RW challenge, spleens were harvested for flow cytometric analysis to determine the levels of Foxp3 expression. Expression levels of CD25 and Foxp3 in CD4<sup>+</sup> T-cell compartment are presented as dot plot. Representative data of each group summarized in Table 1 are shown.

control rat IgG (MP Biomedicals Inc., Aurora, OH, USA,  $n = 19$ ). Six weeks after birth, RW (Polysciences, Inc, Warrington, PA, USA) adsorbed on alum (Sigma, St. Louis, MO, USA) was injected into the left hind-footpad and the tail base; each site received 50  $\mu$ l emulsion, which contained 50  $\mu$ g of RW and 675  $\mu$ g of alum. Ten days later, the eyes of the immunized mice were challenged with RW in PBS (2 mg in 10  $\mu$ l per eye). Two  $\mu$ g of  $\alpha$ -GalCer was intraperitoneally injected 2 hours before RW challenge ( $n = 9$  in nrlgG-treated group and  $n = 8$  in PC61-treated group). As a control, the vehicle (polysorbate-20, 0.5%) was used. Twenty-four hours later, the eyes and spleens were harvested for histological and flow cytometric analyses, respectively. In addition, EC was induced and  $\alpha$ -GalCer or vehicle was injected in unthymectomized and unprimed naïve mice for histological analysis ( $n = 3$  per group), as described above. Furthermore, to evaluate the effect of thymectomy alone on EC, EC was induced in thymectomized and unthymectomized immunocompetent mice without

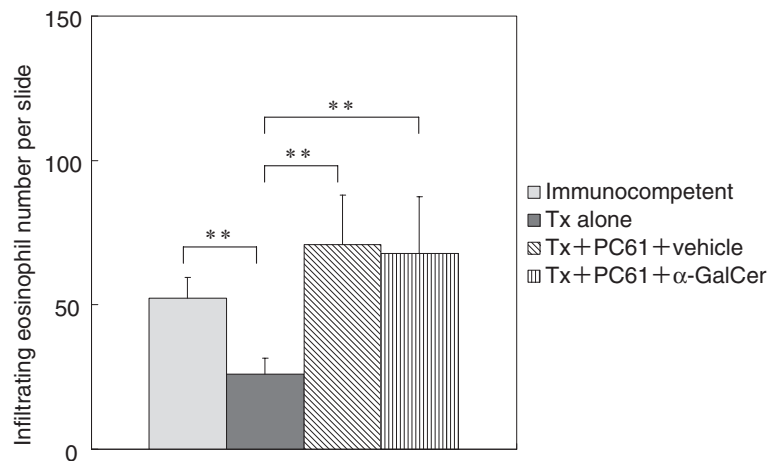
treatment of any Abs and  $\alpha$ -GalCer or vehicle ( $n = 17$  per group).

### HISTOLOGICAL ANALYSIS

The eyes including the conjunctivas were harvested and fixed in 10% buffered formalin. Vertical 2  $\mu$ 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 averages  $\pm$  SEM per slide of all the mice examined.

### FLOW CYTOMETRIC ANALYSIS

Splenocytes were prepared as described above. To examine Foxp3 expression in these splenocytes, a mouse regulatory T cell staining kit (eBioscience, San Diego, CA, USA) was used according to the manufacturer's instructions. The cells were then ana-



**Fig. 3** Suppressive effect of  $\alpha$ -GalCer on EC was abrogated by depletion of CD4<sup>+</sup> CD25<sup>+</sup> Foxp3<sup>+</sup> T cells. Thymectomized and unthymectomized immunocompetent BALB/c mice without treatment of any Abs and  $\alpha$ -GalCer or vehicle were immunized with RW in alum and 10 days later, they were challenged with RW in eye drops ( $n = 17$  per group). In another set of experiments, thymectomized BALB/c mice were injected intraperitoneally 3 times with PC61 or nrlgG. Six weeks after birth, the mice were immunized with RW in alum and 10 days later, they were challenged with RW in eye drops. Two hours prior to RW challenge,  $\alpha$ -GalCer (Tx + PC61 +  $\alpha$ -GalCer,  $n=8$  mice) or vehicle (Tx + PC61 + vehicle,  $n=9$  mice) was injected intraperitoneally. Twenty-four hours after RW challenge, the conjunctivas were harvested and the conjunctival eosinophils were counted. The data are presented as means  $\pm$  SEM in each group. \*\* $P < 0.01$ .

**Table 1** Percentage of Foxp3 expressing in CD4<sup>+</sup> CD25<sup>+</sup> splenic T cells

	% of Foxp3 <sup>+</sup> in CD4 <sup>+</sup> CD25 <sup>+</sup> T cells	
Tx/nrlgG/Vehicle	16.36 $\pm$ 0.23	] * ] * ] * ] NS ] * ] *
Tx/nrlgG/ $\alpha$ -GalCer	23.41 $\pm$ 0.10	
Tx/PC61/Vehicle	2.23 $\pm$ 0.02	
Tx/PC61/ $\alpha$ -GalCer	1.90 $\pm$ 0.09	

Thymectomized (Tx) mice were intraperitoneally injected with PC61 or nrlgG. These mice were immunized with RW and 10 days later, the mice were challenged with RW in eye drops. Two hours before RW challenge, the mice were injected intraperitoneally with either  $\alpha$ -GalCer or vehicle. Twenty-four hours after RW challenge, spleens were harvested for flow cytometric analysis to determine the levels of Foxp3 expression.

Data are presented as % of CD4<sup>+</sup> CD25<sup>+</sup> splenic T cells that are Foxp3<sup>+</sup>.  $n = 8-10$  mice per group.

\* $P < 0.001$ , NS: not significantly different.

lyzed on a FACScan (Becton Dickinson, Mountain View, CA, USA) and the data were acquired and analyzed by using CellQuest software.

### STATISTICAL ANALYSIS OF DATA

Differences between the  $\alpha$ -GalCer- and vehicle-treated mice in terms of flow cytometric data and infiltrating eosinophil numbers were tested for significance by Student's *t*-test. *P* values less than 0.05 were considered to indicate significant differences.

## RESULTS AND DISCUSSION

To confirm the suppressive effects of  $\alpha$ -GalCer on EC, RW-immunized mice were injected intraperitoneally with  $\alpha$ -GalCer or vehicle 2 hours prior to RW challenge. Conjunctivas harvested 24 hours after RW challenge were subjected to histological analysis to determine the numbers of conjunctival eosinophils. Identical to our previous report,<sup>15</sup> treatment with  $\alpha$ -GalCer significantly suppressed conjunctival eosinophil numbers (Fig. 1). In addition,  $\alpha$ -GalCer treat-

ment did not affect conjunctival eosinophil numbers in naïve mice (Fig. 1), which suggests that the activation of NKT cells by  $\alpha$ -GalCer treatment specifically affected RW-sensitized immunocompetent cells.

To determine whether CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells mediate the suppressive effect of  $\alpha$ -GalCer treatment on EC, we depleted CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells from murine neonates by thymectomy plus PC61 treatment, as previously described.<sup>13</sup> At the age of 6 weeks, these mice were then immunized with RW, treated with  $\alpha$ -GalCer or vehicle, and challenged with RW to induce EC. Twenty-four hours after RW challenge, the conjunctivas and spleens were harvested for histological and flow cytometric analyses, respectively. Flow cytometric analysis demonstrated that thymectomy plus PC61 treatment significantly depleted Foxp3-expressing CD4<sup>+</sup>CD25<sup>+</sup> cells relative to the numbers observed in thymectomized animals treated with nrIgG (Tx/nrIgG/vehicle vs Tx/PC61/vehicle and Tx/nrIgG/ $\alpha$ -GalCer vs Tx/PC61/ $\alpha$ -GalCer, Fig. 2, Table 1). Similar to our previous report,<sup>15</sup>  $\alpha$ -GalCer treatment significantly increased the numbers of Foxp3-expressing CD4<sup>+</sup>CD25<sup>+</sup> cells in the nrIgG-treated group (Fig. 2, Table 1). However,  $\alpha$ -GalCer treatment did not affect the Foxp3-expressing CD4<sup>+</sup>CD25<sup>+</sup> cell numbers in the PC61-treated group (Fig. 2, Table 1). These data show that  $\alpha$ -GalCer treatment cannot increase CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T-cell numbers in the absence of thymus-derived CD4<sup>+</sup>CD25<sup>+</sup> T cells.

Finally, we investigated whether EC induced in thymectomized and PC61-treated mice can be suppressed by treatment with  $\alpha$ -GalCer. To investigate whether thymectomy alone affects the severity of EC, we compared the conjunctival eosinophil numbers between EC-developing thymectomized and unthymectomized immunocompetent mice without treatment of any Abs and  $\alpha$ -GalCer or vehicle. Significantly less eosinophils were detected in thymectomized mice (Fig. 3). In accord with conjunctival eosinophil numbers, the percentage of T cells in splenocytes was significantly lower in thymectomized mice (10.9  $\pm$  2.1) than in unthymectomized immunocompetent mice (39.0  $\pm$  0.5). Thus, thymectomy alone effectively decreased T cell compartment and the reduction of T cell population, especially RW-specific effector T cells, may have attenuated EC development in thymectomized mice. On the contrary, significantly more conjunctival eosinophils were counted in thymectomized mice treated with PC61 than in thymectomized mice without any treatment (Fig. 3), similar to our previous report.<sup>13</sup> Strikingly, conjunctival eosinophil numbers were almost the same between the vehicle-treated and  $\alpha$ -GalCer-treated groups (Fig. 3). These data clearly demonstrate that thymectomy together with PC61 treatment abrogate the suppressive effects of  $\alpha$ -GalCer on EC. We previously reported that transfer of CD4<sup>+</sup>CD25<sup>+</sup>

Foxp3<sup>+</sup> T cells suppressed EC.<sup>13</sup> Together with the findings summarized in Table 1, it appears that the suppressive effects of  $\alpha$ -GalCer on EC are, at least in part, mediated by CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells.

It has been reported that administration of  $\alpha$ -GalCer expands CD4<sup>+</sup>CD25<sup>+</sup> T cells, which in turn suppresses the development of experimental autoimmune myasthenia gravis (EAMG).<sup>16</sup> In that report, administration of  $\alpha$ -GalCer in thymectomized mice increased CD4<sup>+</sup>CD25<sup>+</sup> T-cell numbers. This is in accordance with our observation that  $\alpha$ -GalCer treatment increased CD4<sup>+</sup>CD25<sup>+</sup> T-cell numbers in nrIgG-treated thymectomized mice (Fig. 2, Table 1). In the EAMG report, to deplete CD4<sup>+</sup>CD25<sup>+</sup> T cells, anti-CD25 Ab was injected 15 times starting from when EAMG was induced. It is certainly conceivable that this experimental procedure affects not only CD25<sup>+</sup> Treg but also CD25<sup>+</sup> effector T cells, although the authors stated that this protocol did not affect EAMG when  $\alpha$ -GalCer was not administered. Furthermore, data confirming the depletion of CD4<sup>+</sup>CD25<sup>+</sup> T cells were not shown in the EAMG report. Thus, how anti-CD25 Ab treatment affects the effects of  $\alpha$ -GalCer was unclear in the EAMG report. Here, in contrast, we showed the suppressive effects of  $\alpha$ -GalCer treatment on EC are abrogated by depletion of CD4<sup>+</sup>CD25<sup>+</sup> T cells.

Basically,  $\alpha$ -GalCer exerts its effects by its nature as a NKT cell ligand. In a previous report of experimental asthma, it has been pointed out that  $\alpha$ -GalCer inhibits asthma through both NKT cell- and IFN- $\gamma$  dependent pathway.<sup>17</sup> It was also reported that production of IFN- $\gamma$  by NKT cells in response to  $\alpha$ -GalCer requires IL-12 produced by dendritic cells (DCs).<sup>18</sup> Thus, it is possible that these mechanisms other than increase in CD4<sup>+</sup>CD25<sup>+</sup> T cells play a role in the regulation of EC by treatment with  $\alpha$ -GalCer.

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