

Roles of Galectin-9 in the Development of Experimental Allergic Conjunctivitis in Mice

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Key Words

Allergic conjunctivitis · Eosinophil · Galectin-9 · Th1 · Th2

Abstract

Background: Galectin-9 was recently identified as a ligand for Tim-3, which negatively regulates Th1 cells. Here, we investigated whether administration of anti-galectin-9 antibodies (Abs) affects the development of murine experimental allergic conjunctivitis (EC), a Th2-mediated disease.

Methods: EC was induced in BALB/c mice by active immunization with ragweed (RW) followed by RW challenge in eye drops. Normal rat IgG (nrlgG) or anti-galectin-9 Ab was injected intraperitoneally into the mice either during the induction phase treatment or effector phase treatment. Alternatively, Abs were injected into the subconjunctival space during the effector phase. To evaluate in vitro effects of anti-galectin-9 Abs, splenocytes from RW-sensitized mice were cultured in vitro with RW in the presence of nrlgG or anti-galectin-9 Abs. To induce EC, these splenocytes were transferred into naïve BALB/c mice followed by RW challenge. **Results:** Treatment with anti-galectin-9 Abs in vivo during either the induction or effector phase did not significantly affect the severity of EC. However, when the splenocytes from RW-primed mice treated with anti-galectin-9 Abs during the induction phase were stimulated in vitro and adoptively transferred into naïve recipients, they induced significantly

cantly severer EC. In contrast, when RW-primed splenocytes were restimulated in vitro with RW in the presence of anti-galectin-9 Abs and then adoptively transferred, they induced less severe EC and produced significantly less IL-5 and IL-13 and more IFN- γ compared to nrlgG-treated control splenocytes. **Conclusions:** These observations may reflect the fact that galectin-9 not only regulates T-cell function, it is also involved in many other biological functions.

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Introduction

The T-cell immunoglobulin and mucin domain-containing proteins (Tims) were recently found to be important costimulatory molecules in T-cell activation [1]. Four different Tim proteins, namely Tim-1–4, have been identified [2]. With regard to Tim-1 and Tim-3, many reports have shown that Tim-1 and Tim-3 regulate immune responses and the development of experimental allergic diseases [3–7]. Indeed, we recently reported that treatment with anti-Tim-1 or anti-Tim-3 Abs can suppress both the induction and progression of experimental allergic conjunctivitis (EC) in mice [8]. With regard to Tim-2, it was found to play a role in airway hyperresponsiveness [9, 10] as Tim-2-Fc fusion protein treatment of mice just before their nebulization with the allergen increased

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eosinophil counts in the bronchoalveolar lavage fluid and Th2 cytokine production of bronchial lymph node cells [9]. Furthermore, we also demonstrated that Tim-2 negatively regulates the development of EC during the effector phase [11]. Thus, it appears that Tim proteins play an important role in the development of allergic conjunctivitis.

The signals from costimulatory molecules that are received by T cells are generated when these molecules bind to their ligands. It was recently reported that the Tim-3 ligand is galectin-9 [12]. The roles galectin-9 plays in vivo have been investigated by injecting mice that were developing experimental autoimmune encephalomyelitis (EAE) with recombinant galectin-9 or galectin-9 siRNA (which up- and downregulate the Tim-3 signals, respectively) [12]. It was found that the interaction between Tim-3 and galectin-9 downregulated the development of EAE as it eliminated IFN- γ -producing Th1 cells [12]. Whether the Tim-3/galectin-9 interactions are also involved in experimental allergic diseases has not been investigated yet. However, it is possible that it does play a role in such diseases since it has been observed that galectin-9 is a T cell-produced chemoattractant for eosinophils [13], which are major effector cells in allergic diseases.

Here, we sought to determine the involvement of galectin-9 in the development of EC [14–17], which is a Th2-mediated disease that is characterized by eosinophilic infiltration.

Materials and Methods

Mice

Wild-type (WT) BALB/c mice were purchased from Japan SLC, Hamamatsu, Japan. IFN- γ -deficient (GKO) BALB/c mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). These mice were kept in pathogen-free conditions at the animal facility of Kochi Medical School. Age- and gender-matched mice were used when they were 6–12 weeks old. 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 (Warrington, Pa., USA). RW extract was obtained from LSL (Tokyo, Japan). Aluminum hydroxide (alum) was purchased from Sigma (St. Louis, Mo., USA). Anti-galectin-9 monoclonal antibodies (Abs; RG9-1, rat IgG2b and RG9-35, rat IgG2a) were generated by immunizing rats with recombinant mouse galectin-9 kindly provided by Dr. J. Wada (Okayama University) [18]. These Abs were purified from ascites using a protein G column, more than 95% pure as estimated by SDS-PAGE, and contained <100

pg/ml endotoxin in 1 mg/ml antibody solution as estimated by the Limulus test. With regard to the characteristics of RG9-1 and RG9-35 [Okumura et al., in preparation], these Abs block Tim-3-Fc binding to galectin-9 and inhibit galectin-9-induced Th1 cell death in vitro. In addition, both RG9-1 and RG9-35 bind with galectin-9 but not with either galectin-1 or galectin-3. RG9-1 and RG9-35 did not compete the binding with galectin-9, suggesting that both Abs are likely to recognize different epitopes. Both Abs equally inhibit the binding between galectin-9 and Tim-3-Ig. Normal rat IgG (nrIgG) was purchased from MP Biomedicals (Aurora, Ohio, USA). For flow-cytometric analysis, both FITC- and biotin-labeled anti-CD4 (GK1.5) were purchased from eBioscience, San Diego, Calif., USA. Streptavidin-PE, FITC-labeled anti-mouse T1ST2 (DJ8), and PE-labeled anti-mouse CXCR3 (220803) were purchased from BD Biosciences, Franklin Lakes, N.J., USA, MD Biosciences, Zurich, Switzerland, and R & D systems, Minneapolis, Minn., USA, respectively.

Induction of EC by Active Immunization and Treatment with Abs

RW adsorbed on alum was injected into the left hind footpad and at the base of the tail of WT mice. Fifty microliters of the emulsion (50 μ g of RW and 675 μ g of alum) were injected into each site. The mice were also injected intraperitoneally on days 0, 2, 4, 6 and 8 after RW immunization (induction phase treatment) or on day 10 only (2 h before RW challenge; effector phase treatment) with 200 μ g anti-galectin-9 Ab or nrIgG. In a separate experiment, 2 μ g of nrIgG or anti-galectin-9 Ab in a volume of 2 μ l were injected into the subconjunctival space on day 10, 2 h prior to RW challenge. On day 10, the eyes of the immunized mice were challenged with RW in PBS (2 mg in 10 μ l per eye). Twenty-four hours later, the eyes, spleens and blood were collected for immunohistochemical or histological analysis, splenocyte transfer, cytokine assays or flow-cytometric analysis, and counting of white blood cells using a hemocytometer, respectively.

Adoptive Transfer of RW-Primed Splenocytes from Mice Treated with Abs in vivo

The spleens harvested from the mice described above were stimulated in vitro with 5 μ g/ml RW extract as described previously [14, 15, 17]. After incubation for 72 h at 37°C in a humidified atmosphere with 5% CO₂, 2×10^7 splenocytes were intraperitoneally injected into naïve WT mice. Four days after the splenocyte 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 were harvested for histological analysis.

Adoptive Transfer of RW-Primed Splenocytes Followed by Treatment with Abs

Naïve WT BALB/c mice were immunized with RW in both their left hind footpad and tail base and were not treated with any Abs. Ten days later, their splenocytes were prepared and cultured as above. Three days later, 2×10^7 splenocytes were intraperitoneally injected into naïve mice. These recipient mice were intraperitoneally injected with 200 μ g anti-galectin-9 Ab or nrIgG twice (2 days after transfer and 2 h prior to RW challenge). Four days after the splenocyte 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 were harvested for histological analysis.

Adoptive Transfer of in vitro Ab-Treated RW-Primed Splenocytes

RW-primed splenocytes prepared as described in the preceding section were cultured in vitro with RW in the presence of 10 µg/ml anti-galectin-9 Abs or nrIgG. Three days later, 2×10^7 splenocytes were intraperitoneally injected into naïve mice. Four days after the splenocyte transfer, the eyes of the recipient mice were challenged with RW in PBS (2 mg in 10 µl per eye). Twenty-four hours later, the eyes were harvested for histological analysis.

Histological Analysis

The eyes including the conjunctivas were harvested and fixed in 10% buffered formalin. Vertical 2-µm-thick sections were cut and stained with Giemsa. Infiltrating eosinophils in the lamina propria mucosae of the tarsal and bulbar conjunctivas in the entire section were counted by two observers given blinded samples. The sections counted were those from the central portion of the eye, which included the pupil and optic nerve head. The data are presented as means $\pm$ SEM of all the mice examined.

Immunohistochemistry

The eyes including the conjunctivas from EC-developing mice treated with Abs during the induction phase were harvested and sections for immunohistochemistry were prepared following the method described previously [15]. Briefly, the eyes were immediately frozen in 3% carboxymethyl cellulose gel. Four-micrometer-thick sections were prepared and fixed in methanol. Endogenous peroxidase activity was inhibited with 0.1% NaN₃ and 0.3% H₂O₂ in distilled water for 10 min at room temperature. Samples were exposed to first Abs for 30 min and another 30 min to appropriate biotinylated secondary Abs. All slides were revealed using an avidin-biotin-complex kit (Vector Laboratories, Burlingame, Calif., USA), then developed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma). The Abs used were rat anti-mouse CD3 (CT-CD3; Caltag Laboratories, Burlingame, Calif., USA), rat anti-mouse CD45R/B220 (RA3-6B2; BD Biosciences), rat anti-mouse F4/80 (A3-1; Caltag Laboratories) and rabbit anti-mouse major basic protein (provided by Dr. Lee, Mayo Clinic).

Cytokine Assays

Splenocytes were harvested from WT BALB/c mice actively immunized with RW in alum treated with the Abs for 5 times and challenged with RW. These splenocytes were cultured in vitro for 48 h with 25 µg/ml RW extract, and the culture supernatants were collected for assays. Additionally, either WT or GKO BALB/c mice were actively immunized with RW in alum and were not treated with any Abs. Ten days later, the mice were sacrificed and their spleens were harvested. The splenocytes were cultured in vitro for 48 h with 25 µg/ml RW extract in the presence of 10 µg/ml nrIgG or anti-galectin-9 Abs. The IL-4, IL-5, IL-13 and IFN-γ levels in the culture supernatants were measured by the Bioplex system (Bio-Rad, Hercules, Calif., USA) according to the manufacturer's recommendations.

Flow-Cytometric Analysis

Splenocytes from EC-developing mice treated with Abs during the induction phase were incubated for 30 min on ice with FITC- or biotin-labeled anti-CD4 Ab at optimal concentrations and washed with cold 2% FCS-PBS. In the case of biotin-labeled Abs, the cells were then incubated with streptavidin-PE for 10

min and washed again. These cells were then incubated with anti-CXCR3 Ab or anti-T1ST2 Ab. After washing, these cells were analyzed on a FACScan (Becton Dickinson, Mountain View, Calif., USA), and data acquisition and analysis were performed using CellQuest software. The data are presented as average percentages of CD4+/CXCR3+ or CD4+/T1ST2+ cells per whole splenocytes $\pm$ SEM per spleen.

Statistical Analysis

Significant differences between anti-galectin-9 Ab-treated and nrIgG-treated groups with regard to the infiltrating eosinophil numbers and splenocyte cytokine production were evaluated by ANOVA and then by Fisher's protected least significant difference method. *p* values <0.05 were considered significant.

Results

Effects of Anti-Galectin-9 Ab Treatment during the Induction Phase of EC

To investigate whether galectin-9 plays a role in the induction phase of EC, mice were immunized with RW in alum and then intraperitoneally injected 5 times every other day with anti-galectin-9 Abs starting on the day of immunization. As a control, mice were injected with nrIgG instead. Ten days after the immunization, the mice were challenged with RW in eye drops and 24 h later, the conjunctivas were harvested for immunohistochemical and histological analyses. Infiltration of CD3+ T cells, major basic protein-positive eosinophils and F4/80+ macrophages was not different between nrIgG-treated and anti-galectin-9 Ab-treated groups (fig. 1a). Neutro-

Fig. 1. Treatment with anti-galectin-9 Abs during the induction phase of EC. BALB/c mice were actively immunized with RW in alum and injected intraperitoneally 5 times every other day starting on the day of immunization with 200 µg of nrIgG or anti-galectin-9 Abs (RG9-1 or RG9-35). On day 10, the mice were challenged with RW in eye drops to induce EC, and 24 h later the conjunctivas and spleens were harvested for immunohistochemical (a, 3 mice per group) or histological analysis (b, 10 mice per group) and splenocyte transfer (c, 8 mice per group) or cytokine assays (d-f, 4 samples per group), respectively. With regard to splenocyte transfer (c), the harvested splenocytes were cultured in vitro with RW extract for 3 days and then 2×10^7 cells were intraperitoneally injected into naïve BALB/c 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. **a** One representative microphotograph is shown. Bar = 20 µm. **b, c** Conjunctival eosinophil numbers are shown as means $\pm$ SEM. With regard to cytokine assays (d-f), the harvested splenocytes were cultured in vitro with 25 µg/ml RW extract; 48 h later, the culture supernatants were collected and used for cytokine assays. * *p* < 0.05 vs. the nrIgG-treated group. MBP = Major basic protein.

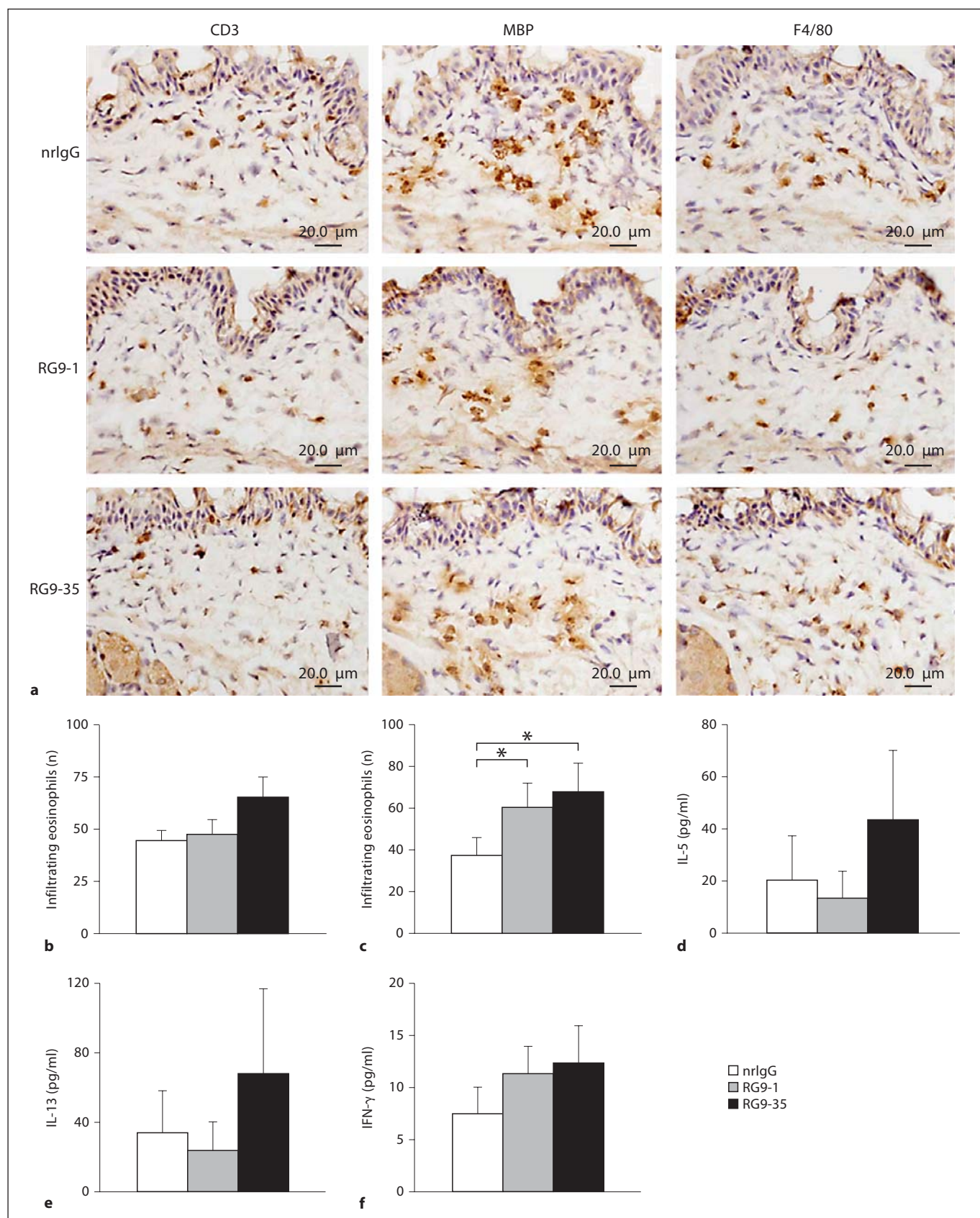


Table 1. Expression levels of CXCR3 or T1ST2 in CD4+ splenic T cells

	nrIgG	RG9-1	RG9-35
CD4+/CXCR3+	2.53 ± 0.52	2.87 ± 0.41	2.29 ± 0.08
CD4+/T1ST2+	0.47 ± 0.14	0.48 ± 0.03	0.54 ± 0.22

Splenocytes from EC-developing mice treated with Abs during the induction phase were harvested to examine the expression levels of CXCR3 or T1ST2 in CD4+ splenic T cells using flow cytometry. The data are presented as average percentages of CD4+/T1ST2+ or CD4+/CXCR3+ cells per whole splenocytes ± SEM per spleen. A significant difference was not observed between nrIgG-treated and anti-galectin-9 Ab-treated groups.

phils and B cells were barely detectable (data not shown). Counting of conjunctival eosinophils revealed that the anti-galectin-9 Ab treatment did not significantly affect conjunctival eosinophil infiltration, although a slight, but insignificant, increase was observed in the RG9-35-treated group (fig. 1b). Conjunctival eosinophil numbers were similar between nrIgG-treated and PBS-treated groups (data not shown), indicating that nrIgG treatment did not significantly affect the development of EC.

To investigate whether the *in vivo* treatment with anti-galectin-9 Abs affected the disease-inducing properties of RW-primed splenocytes, the splenocytes from the RW-primed and anti-galectin-9 Ab-treated mice described above were cultured *in vitro* with RW extract and then transferred into naïve syngeneic mice. Four days later, the recipient mice were challenged with RW and 24 h later, the conjunctivas were harvested to determine the conjunctival eosinophil numbers. The splenocytes from the anti-galectin-9 Ab-treated mice induced significantly more severe eosinophil infiltration compared to those from the nrIgG-treated mice (fig. 1c). This was true for both anti-galectin-9 Abs. Thus, although treatment with anti-galectin-9 Abs during the induction phase of EC had no discernible effects on disease severity, splenocytes showed enhanced EC-inducing capacity upon their adoptive transfer. To further examine the effects of treatment with anti-galectin-9 Abs on splenocyte responses, cytokine production of splenocytes was evaluated following RW stimulation. Although slight increases in IL-5, IL-13 and IFN- γ production were noted when mice were treated with RG9-35, the differences did not reach statistical significance (fig. 1d–f). To further examine the effects of anti-galectin-9 Abs on Th1/Th2 balance, flow-cytometric analysis was performed to evaluate expression of

CXCR3 or T1ST2 in CD4+ splenic T cells. Similarly, a significant difference was not observed between nrIgG-treated and anti-galectin-9 Ab-treated groups (table 1). In addition, the number of peripheral white blood cells did not significantly differ ($3,857 \pm 1,028$ per μl in nrIgG-treated group, $3,200 \pm 150$ per μl in the RG9-1-treated group and $3,540 \pm 1,390$ per μl in the RG9-35-treated group).

Effects of Anti-Galectin-9 Ab Treatment during the Effector Phase of EC

Next, to investigate whether galectin-9 plays a role in the effector phase of EC, mice immunized with RW in alum were intraperitoneally injected once with anti-galectin-9 Abs 2 h prior to RW challenge. Treatment with anti-galectin-9 Abs did increase eosinophil infiltration into the conjunctiva, but this difference did not achieve statistical significance (fig. 2a).

To further investigate the effects of anti-galectin-9 Abs delivered during the effector phase on the severity of the disease, RW-primed splenocytes from RW-sensitized mice that had not been treated with any Abs were cultured and then injected into naïve recipients. These mice were then injected with anti-galectin-9 Abs twice (2 and 4 days after transfer). Two hours after the second Ab injection, the mice were challenged with RW and 24 h later, the conjunctivas were harvested and the eosinophils were counted. Similar to the actively immunized mice (fig. 2a), treatment with anti-galectin-9 Abs did not significantly affect conjunctival eosinophil infiltration (fig. 2b). Moreover, anti-galectin-9 Abs were injected into the subconjunctival space in the actively immunized mice, 2 h prior to RW challenge. Conjunctival eosinophil numbers were not significantly different between nrIgG-treated and anti-galectin-9 Ab-treated groups (fig. 2c). Thus, anti-galectin-9 Abs did not affect EC when delivered during the effector phase.

Effect of in vitro Treatment with Anti-Galectin-9 Abs on the Ability of RW-Primed Splenocytes to Induce EC upon Adoptive Transfer

Finally, we investigated whether RW-primed splenocytes from mice that had not been treated with any Abs showed altered EC-inducing ability if they were treated with anti-galectin-9 Abs *in vitro* prior to their adoptive transfer. Thus, these *in vitro* stimulated splenocytes were transferred into syngeneic recipient mice, and 4 days after the transfer these mice were challenged with RW in eye drops. Twenty-four hours later, conjunctivas were harvested to count conjunctival eosinophil numbers.

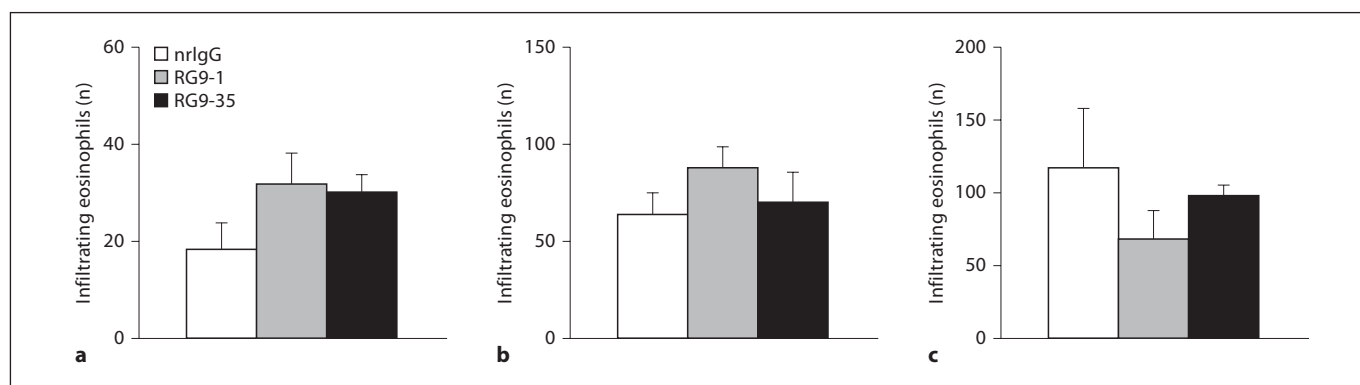


Fig. 2. Treatment with anti-galectin-9 Abs during the effector phase of EC. **a** Two hours prior to RW challenge, actively immunized BALB/c mice were intraperitoneally injected once with 200 μ g of nrlgG or anti-galectin-9 Abs (RG9-1 or RG9-35, $n = 10$ per group); 24 h after RW challenge, the conjunctivas were harvested for histological analysis and the conjunctival eosinophils were counted. The data are presented as means $\pm$ SEM of 10 mice in each group. **b** Splenocytes from BALB/c mice that had been immunized with RW in alum and not treated with any Ab were transferred into naïve recipients. Two and four days later, the mice were intraperitoneally injected with nrlgG or anti-galectin-9 Abs (RG9-1 or RG9-35, $n = 10$ per group). Two hours after the second

injection, the mice were challenged with RW in eye drops. Twenty-four hours after the challenge, the conjunctivas were harvested for histological analysis and the conjunctival eosinophils were counted. The data are presented as means $\pm$ SEM of 10 mice in each group. **c** Two hours prior to RW challenge, actively immunized BALB/c mice were injected with 2 μ g of nrlgG or anti-galectin-9 Abs into the subconjunctival space (RG9-1 or RG9-35, $n = 4$ per group). Twenty-four hours after RW challenge, the conjunctivas were harvested for histological analysis and the conjunctival eosinophils were counted. The data are presented as means $\pm$ SEM.

Compared to mice receiving the nrlgG-treated splenocytes, significantly fewer conjunctival eosinophils were detected in mice that had received the splenocytes treated with anti-galectin-9 Abs in vitro (fig. 3a).

We also assessed the effect of in vitro treatment with anti-galectin-9 Abs on the cytokine production of RW-primed splenocytes. The anti-galectin-9 Ab treatment significantly reduced IL-5 and IL-13 production while significantly elevating IFN- γ production (fig. 3b).

To investigate whether the reduction in IL-5 and IL-13 production by treatment with anti-galectin-9 Abs was due to the elevated IFN- γ production, RW-primed splenocytes from GKO mice were stimulated in vitro with RW extract in the presence of anti-galectin-9 Abs, and culture supernatants were collected for cytokine assays. IFN- γ was below the detectable level in the three groups (data not shown). Production of IL-5 was not different between the nrlgG-treated and anti-galectin-9 Ab-treated groups (fig. 3c). Although IL-13 production was significantly reduced in the anti-galectin-9 Ab-treated groups (fig. 3c), the difference between anti-galectin-9 Ab-treated and nrlgG-treated groups was less in GKO splenocytes (fig. 3c) than WT splenocytes (fig. 3b). Thus, it could be considered that upregulated IFN- γ production by anti-galectin-9 Ab treatment inhibited IL-5 and IL-13 production in

WT splenocytes (fig. 3b). It may be possible that anti-galectin-9 Abs exerted their effects directly on IL-13-producing cells.

Discussion

We show here that in vivo treatment with anti-galectin-9 Abs did not affect the severity of EC either during the induction or the effector phase. However, when the RW-primed splenocytes from these Ab-treated mice were adoptively transferred into naïve recipients, they induced significantly severer EC than the cells from nrlgG-treated mice. In another set of experiments using RW-primed splenocytes from mice that had not been treated with Abs, their EC-inducing capability was attenuated along with their Th2 cytokine response to RW extract when these cells were treated with anti-galectin-9 Abs in vitro. They also showed upregulated IFN- γ production in response to RW. Thus, the anti-galectin-9 Abs altered the pathogenic properties of the EC-inducing immune cells but did so in disparate directions depending on the environmental context in which they had been exposed to the Abs. Caution should be paid to the dose of Abs used, since the dose of Ab may not be sufficient to completely block galectin-9

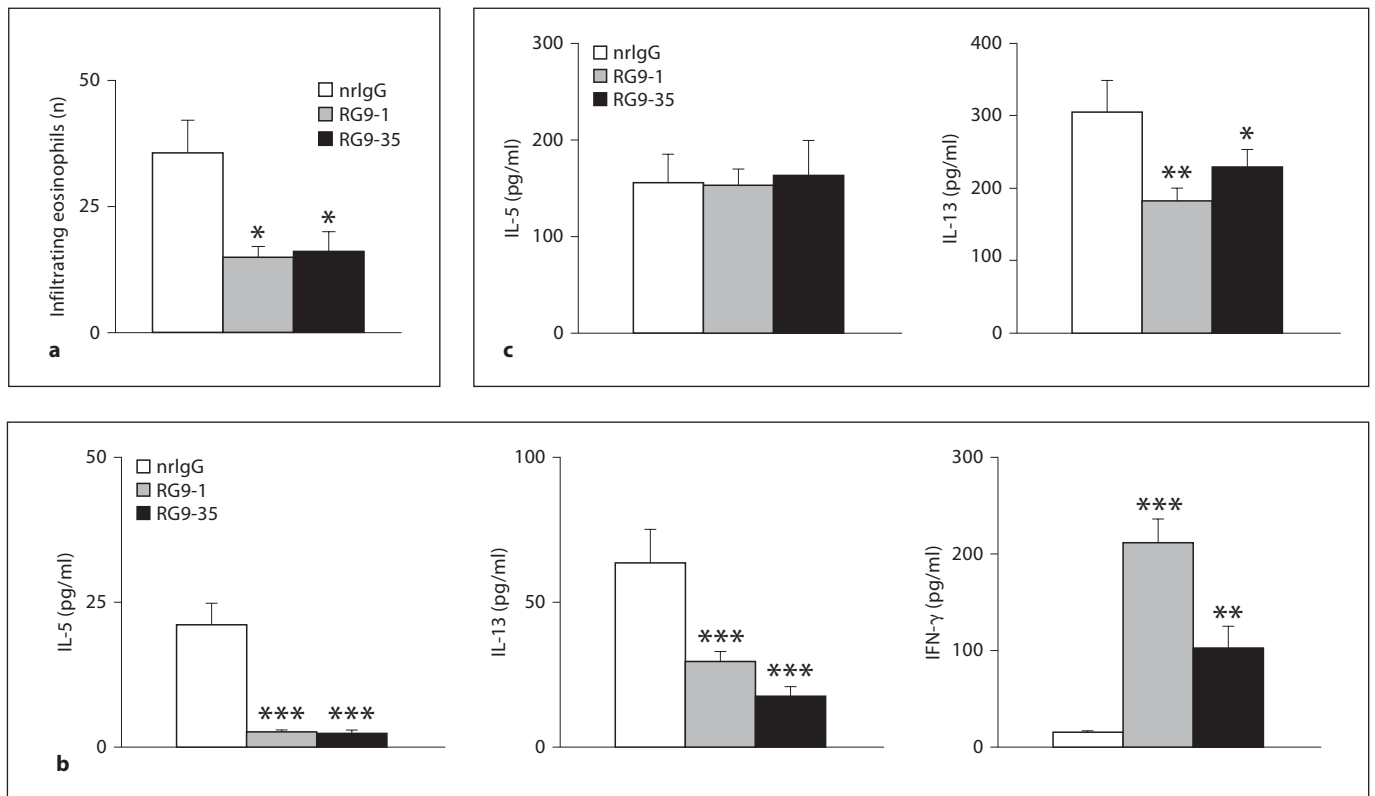


Fig. 3. Effect of in vitro treatment with anti-galectin-9 Abs on the EC-inducing capacity of RW-primed splenocytes. BALB/c mice were actively immunized with RW in alum and not treated with Abs. Ten days later, the mice were sacrificed and their spleens were harvested. **a** Splenocytes were cultured in vitro with RW extract (5 μ g/ml) in the presence of 10 μ g/ml nrlgG or anti-galectin-9 Abs (RG9-1 or RG9-35) for 3 days, after which 2×10^7 cells were injected intraperitoneally into naïve BALB/c mice (10 mice per group). Four days after the transfer, the recipient mice were challenged with RW in eye drops and 24 h later, the conjunctivas were

harvested for histological analysis. Conjunctival eosinophil numbers are shown as means $\pm$ SEM. * $p < 0.05$ vs. nrlgG-treated splenocytes. **b, c** Cytokine assays of WT (**b**) and GKO splenocytes (**c**). The RW-primed WT (**b**) and GKO splenocytes (**c**) were cultured in vitro with 25 μ g/ml RW extract in the presence of 10 μ g/ml nrlgG or anti-galectin-9 Abs (RG9-1 or RG9-35). Forty-eight hours later, the culture supernatants were collected and used for cytokine assays. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. the nrlgG-treated group.

in vivo and in vitro, although the same dose of anti-Tim Abs used in vivo significantly affected the severity of EC [8, 11].

We have previously demonstrated that the in vivo treatment with anti-Tim-3 Abs inhibited the development of EC. Therefore, we initially hypothesized that treatment with anti-galectin-9 Abs blocks the interaction between galectin-9 and Tim-3, thus leading to upregulation of Th1 responses and attenuation of EC. However, the in vivo treatment with the anti-galectin-9 Abs failed to affect the severity of EC. Recently, it was reported that intravenous administration of galectin-9 inhibited allergic asthma in mice [19]. In that report, it was demonstrated that galectin-9 bound with CD44 and this binding inhibited the

interaction between CD44 and its ligand hyaluronan, and therefore, migration of eosinophils and lymphocytes into the airway was attenuated [19]. Thus, galectin-9 not only affects the Th1/Th2 balance mediated by Tim-3, it is also involved in leukocyte migration into the inflammatory site. Contrary to the injection of galectin-9 [19], anti-galectin-9 Ab treatment may increase the interaction between CD44 and hyaluronan in our experimental settings. This may be the reason why anti-galectin-9 Ab treatment increased, rather than decreased, conjunctival eosinophil infiltration either during the induction or effector phase.

In addition to the two different functions described above, galectin-9 has a wide variety of biological func-

tions, including roles in cell aggregation, proliferation, and death and the modulation of inflammation [20]. Indeed, galectin-9 is known to be a chemoattractant for eosinophils [21]. Thus, treatment with anti-galectin-9 Abs would affect not only T cells but also eosinophils and conjunctival residential cells. Therefore, in vivo treatment with anti-galectin-9 Abs may have blocked pleiotropic galectin-9 functions. This observation may also help answer our question, namely, why did EC-inducing immune cells induce severer EC when they were exposed to anti-galectin-9 Ab treatment in vivo and less severe EC when they were exposed to these Abs in vitro? In the lat-

ter experiment, the Abs have a direct effect on immune cells, reflecting only the T cell-regulatory functions of galectin-9. In the former experiment, the Abs also affect other cell types that may influence T-cell function differently. Further studies are needed to address these issues.

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References

- McIntire JJ, Umetsu SE, Akbari O, Potter M, Kuchroo VK, Barsh GS, Freeman GJ, Umetsu DT, DeKruyff RH: Identification of Tapr (an airway hyperreactivity regulatory locus) and the linked Tim gene family. *Nat Immunol* 2001;2:1109–1116.
- Meyers JH, Sabatos CA, Chakravarti S, Kuchroo VK: The TIM gene family regulates autoimmune and allergic diseases. *Trends Mol Med* 2005;11:362–369.
- Monney L, Sabatos CA, Gaglia JL, Ryu A, Waldner H, Chernova T, Manning S, Greenfield EA, Coyle AJ, Sobel RA, Freeman GJ, Kuchroo VK: Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature* 2005;435:536–541.
- Sanchez-Fueyo A, Tian J, Picarella D, Domenig C, Zheng XX, Sabatos CA, Manlongat N, Bender O, Kamradt T, Kuchroo VK, Gutierrez-Ramos JC, Coyle AJ, Strom TB: Tim-3 inhibits T helper type 1-mediated auto- and alloimmune responses and promotes immunological tolerance. *Nat Immunol* 2003;4:1093–1101.
- Meyers JH, Chakravarti S, Schlesinger D, Illes Z, Waldner H, Umetsu SE, Kenny J, Zheng XX, Umetsu DT, DeKruyff RH, Strom TB, Kuchroo VK: TIM-4 is the ligand for TIM-1, and the TIM-1-TIM-4 interaction regulates T cell proliferation. *Nat Immunol* 2005;6:455–464.
- Umetsu SE, Lee WL, McIntire JJ, Downey L, Sanjanwala B, Akbari O, Berry GJ, Nagumo H, Freeman GJ, Umetsu DT, DeKruyff RH: TIM-1 induces T cell activation and inhibits the development of peripheral tolerance. *Nat Immunol* 2005;6:447–454.
- Encinas JA, Janssen EM, Weiner DB, Calarota SA, Nieto D, Moll T, Carlo DJ, Moss RB: Anti-T-cell Ig and mucin domain-containing protein 1 antibody decreases TH2 airway inflammation in a mouse model of asthma. *J Allergy Clin Immunol* 2005;116:1343–1349.
- Fukushima A, Sumi T, Fukuda K, Kumagai N, Nishida T, Akiba H, Okumura K, Yagita H, Ueno H: Antibodies to T-cell Ig and mucin domain-containing proteins (Tim)-1 and -3 suppress the induction and progression of murine allergic conjunctivitis. *Biochem Biophys Res Commun* 2007;353:211–216.
- Chakravarti S, Sabatos CA, Xiao S, Illes Z, Cha EK, Sobel RA, Zheng XX, Strom TB, Kuchroo VK: Tim-2 regulates T helper type 2 responses and autoimmunity. *J Exp Med* 2005;202:437–444.
- Rennert PD, Ichimura T, Sizing ID, Bailly V, Li Z, Rennard R, McCoon P, Pablo L, Miklasz S, Tarilonte L, Bonventre JV: T cell, Ig domain, mucin domain-2 gene-deficient mice reveal a novel mechanism for the regulation of Th2 immune responses and airway inflammation. *J Immunol* 2006;177:4311–4321.
- Fukushima A, Sumi T, Fukuda K, Kumagai N, Nishida T, Okumura K, Akiba H, Yagita H, Ueno H: T-cell Ig and mucin domain-containing protein (Tim)-2 regulates murine allergic conjunctivitis during the effector phase. *Immunol Lett* 2007;110:133–138.
- Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, Zheng XX, Strom TB, Kuchroo VK: The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol* 2005;6:1245–1252.
- Matsumoto R, Matsumoto H, Seki M, Hata M, Asano Y, Kanegasaki S, Stevens RL, Hirashima M: Human ecalectin, a variant of human galectin-9, is a novel eosinophil chemoattractant produced by T lymphocytes. *J Biol Chem* 1998;273:16976–16984.
- Fukushima A, Ozaki A, Jian Z, Ishida W, Fukata K, Ueno H, Liu F-T: Dissection of antigen-specific humoral and cellular immune responses for the development of experimental immune-mediated blepharoconjunctivitis in C57BL/6 mice. *Curr Eye Res* 2005;30:241–248.
- Fukushima A, Yamaguchi T, Ishida W, Fukata K, Mittler RS, Yagita H, Ueno H: Engagement of 4-1BB inhibits the development of experimental allergic conjunctivitis in mice. *J Immunol* 2005;175:4897–4903.
- Ozaki A, Seki Y, Fukushima A, Kubo M: The control of allergic conjunctivitis by suppressor of cytokine signaling (SOCS)3 and SOCS5 in a murine model. *J Immunol* 2005;175:5489–5497.
- Fukushima A, Yamaguchi T, Ishida W, Fukata K, Yagita H, Ueno H: Roles of OX40 in the development of murine experimental allergic conjunctivitis: exacerbation and attenuation by stimulation and blocking of OX40. *Invest Ophthalmol Vis Sci* 2006;47:657–663.
- Tsuyahama Y, Wada J, Zhang H, Morita Y, Hiragushi K, Hida K, Shikata K, Yamamura M, Kanwar YS, Makino H: Efficacy of galectins in the amelioration of nephrotoxic serum nephritis in Wistar Kyoto rats. *Kidney Int* 2000;58:1941–1952.
- Katoh S, Ishii N, Nobumoto A, Takeshita K, Dai SY, Shinonaga R, Niki T, Nishi N, Tomimaga A, Yamauchi A, Hirashima M: Galectin-9 inhibits CD44-hyaluronan interaction and suppresses a murine model of allergic asthma. *Am J Respir Crit Care Med* 2007;176:27–35.
- Hirashima M, Kashio Y, Nishi N, Yamauchi A, Imaizumi TA, Kageshita T, Saita N, Nakamura T: Galectin-9 in physiological and pathological conditions. *Glycoconj J* 2004;19:593–600.
- Asakura H, Kashio Y, Nakamura K, Seki M, Dai S, Shirato Y, Abedin MJ, Yoshida N, Nishi N, Imaizumi T, Saita N, Toyama Y, Takashima H, Nakamura T, Ohkawa M, Hirashima M: Selective eosinophil adhesion to fibroblast via IFN-gamma-induced galectin-9. *J Immunol* 2002;169:5912–5918.