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Antibodies to T-cell Ig and mucin domain-containing proteins (Tim)-1 and -3 suppress the induction and progression of murine allergic conjunctivitis

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Abstract

The T cell Ig and mucin domain-containing proteins (Tim) regulate Th1- and Th2-mediated immune responses. We investigated the ability of Abs blocking Tim-1 or Tim-3 ligand-binding activity to prevent and treat murine experimental allergic conjunctivitis (EC), a Th2-mediated disease. Treatment with either Ab during the induction phase of EC in actively immunized wild-type mice suppressed EC and upregulated Th1 and Th2 immune responses. In contrast, both Abs exacerbated EC in actively immunized IFN-γ-knockout mice. Thus, both anti-Tim Abs suppress the pathogenic immune responses generated in the induction phase by upregulating systemic IFN-γ production. Treatment of actively immunized mice and passively immunized mice with either anti-Tim Ab just prior to RW challenge also suppressed EC. Thus, treatment with anti-Tim-1 or anti-Tim-3 Ab can suppress both the induction and progression of EC, which could indicate potential preventive and/or therapeutic approaches for allergic diseases such as allergic conjunctivitis.

Keywords: Allergic conjunctivitis; Eosinophil; Tim-1; Tim-3; Th2

Allergic conjunctivitis (AC) is one of the most commonly occurring ocular diseases. While most AC patients have mild disease [1] (such as seasonal and perennial AC which causes itching and hyperemia), some suffer from severe forms of AC such as vernal keratoconjunctivitis (VKC), that induce not only conjunctival inflammation but also corneal ulceration that can lead to vision loss [2]. Accumulating evidence suggests that the corneal damage in VKC is predominantly mediated by eosinophils that infiltrate the conjunctiva [3,4]. Consequently, the presence of eosinophil infiltration into the conjunctiva is highly indicative of severe AC [5].

Our recent studies of experimental AC (EC) have revealed that IgE-mediated mast cell activation in the conjunctiva on its own cannot induce massive eosinophil infiltration [6]; rather, conjunctival eosinophil infiltration is driven by Ag-primed T cells [6]. Since T-cell activation requires signals from both the TcR and costimulatory molecules [7], we have been investigating how various costimulatory molecules contribute to the development of EC. Depending on the stage of disease, a number of costimulatory molecules have been found to play important roles [8–11]. For example, during the induction phase of EC, agonistic stimulation of 4-1BB, a TNF receptor family molecule, suppresses EC [8]. In contrast, agonistic stimulation during the induction phase of another TNF receptor family molecule, OX40, exacerbates EC [9]. Such observations not only enhance our understanding of the immunological

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mechanisms that drive AC, they also reveal therapeutic opportunities, as up or downregulating various costimulatory signals may ameliorate AC.

Recently, a new family of T cell costimulatory molecules was discovered, namely, T cell Ig and mucin domain-containing (Tim) protein family [12,13]. Tim-3 was first found during a search for molecules that would distinguish Th1 from Th2 cells and was then shown to be preferentially expressed on Th1 cells and to negatively regulate Th1 cells [13–15]. Supporting this is that blockade of Tim-3 during the development of experimental autoimmune encephalomyelitis (EAE) [13] and diabetes in non-obese diabetic (NOD) mice [14] exacerbates diseases, which indicates that Tim-3 negatively regulates the development of Th1-mediated diseases. Tim-1, which was initially identified as the hepatitis A virus receptor [16], was subsequently found to be expressed at higher levels in Th2 cells than Th1 cells [17,18]. Blocking of Tim-1 decreases Th2 immune responses and airway inflammation [19] as well as abrogating the induction of high-dose tolerance and restoring airway hyperresponsiveness in mice [18]. Thus, Tim-1 appears to be involved in upregulating Th2 cells, while Tim-3 downregulates Th1 cells.

In this study, we investigated the involvement of Tim-1 and Tim-3 in the development of EC, which is mediated by Ag-specific Th2 cells. In addition, we investigated the therapeutic potential of blocking Tim-1 and Tim-3 in ongoing EC.

Materials and methods

Mice. Inbred wild-type (WT) Balb/c mice were purchased from Japan SLC Inc., Hamamatsu, Shizuoka, Japan. IFN-γ-deficient (GKO) Balb/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were kept in pathogen-free conditions at the animal facility of Kochi Medical School and 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 Inc (Warrington, PA). RW extract was obtained from LSL Co. Ltd (Tokyo, Japan). Aluminium hydroxide (alum) was purchased from Sigma (St. Louis, MO). Anti-Tim-1 (RMT1-10, rat IgG2a) and anti-Tim-3 (RMT3-23, rat IgG2a) monoclonal Abs were generated by immunizing rats with Tim-1-Fc and Tim-3-Fc fusion proteins, respectively [20]. Flow cytometric analysis revealed that RMT1-10 binds to Tim-1-transfectants but not to either Tim-2 or Tim-3 transfectants and blocks the binding of Tim-1-Fc to a Tim-1 ligand (possibly Tim-4) [17]; similarly, RMT3-23 binds to Tim-3 transfectants but not to either Tim-1 or Tim-2 transfectants, and blocks the binding of Tim-3-Fc to a Tim-3 ligand (galectin-9) [15] (H. Akiba, K. Okumura and H. Yagita, details will be described elsewhere). Normal rat IgG (nrIgG) was purchased from MP Biomedicals Inc (Aurora, OH).

EC induction 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. Fifty microliters of the emulsion (50 μg RW and 675 μg alum) was 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 (Two hours before RW challenge; effector phase treatment) with 200 μg anti-Tim-1 Ab, anti-Tim-3 Ab, or nrIgG. On day 10, the eyes of the immunized mice were challenged with RW in PBS (2 mg in 10 μl per eye). Twenty-four hours later, the eyes, spleens, and sera were harvested for histological analysis, proliferation and cytokine assays, and measurement of Ig levels, respectively.

EC induction by adoptive transfer of in vitro-stimulated RW-primed splenocytes and treatment with Abs. Naïve Balb/c mice were immunized with RW in both left hind-footpad and tail base and were not treated with any Abs. Ten days later, their splenocytes were prepared and cultured with RW extract at 5 μg/ml as described before [8–11]. After incubation for 72 h at 37 °C in a humidified atmosphere with 5% CO_2, 2×10^7 splenocytes were intraperitoneally injected into naïve Balb/c mice. After the splenocyte transfer, the mice were injected intraperitoneally with 200 μg anti-Tim-1 Ab, anti-Tim-3 Ab or nrIgG on days 2 and 4. Soon after the injection of Abs on day 4, 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 averages ± SEM of all the mice examined.

Proliferation and cytokine assays. As described before [8–11], splenocyte proliferation and cytokine assays were performed by stimulation with RW extract (25 μg/ml) or concanavalin A (Con A, 5 μg/ml). The data were expressed as the change in counts per minute (cpm) (Δcpm = mean cpm of stimulated cultures − mean cpm of unstimulated control cultures). With regard to the cytokine assay, the IL-4, IL-5, IL-10, IL-13, and IFN-γ levels in the culture supernatants were measured by the Bioplex system (Bio-Rad, Hercules, CA) according to the manufacturer's recommendations.

Measurement of Igs in serum. Twenty-four hours after RW challenge of actively immunized mice, blood was collected and serum was prepared. Serum Ig levels were assessed by ELISA as described before in detail [10,11]. The absorbance was measured at 405 nm and the Ig concentrations were calculated by using standard curves generated with purified mouse IgG_1, IgG_{2a}, and IgE.

Statistical analysis. Significant differences between the three groups (nrIgG, anti-Tim-1Ab, and anti-Tim-3Ab) 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 less than 0.05 were considered significant.

Results

Treatment with anti-Tim-1 and anti-Tim-3 Abs during the induction phase of EC suppresses conjunctival eosinophil infiltration

To investigate the roles Tim-1 and Tim-3 play in the development of EC during the induction phase, actively immunized mice were intraperitoneally injected with anti-Tim-1 Ab, anti-Tim-3 Ab or nrIgG five times every other day starting on the day of immunization. Treatment with anti-Tim-1 Ab suppressed eosinophil infiltration into the conjunctiva (Fig. 1A). Anti-Tim-3 Ab also inhibited eosinophil infiltration by 34%, although this did not achieve statistical significance ($P = 0.08$, Fig. 1).

Effect of Ab treatment during the induction phase on systemic immune responses

To investigate the effect of Ab treatment during the induction phase on systemic immune responses, we

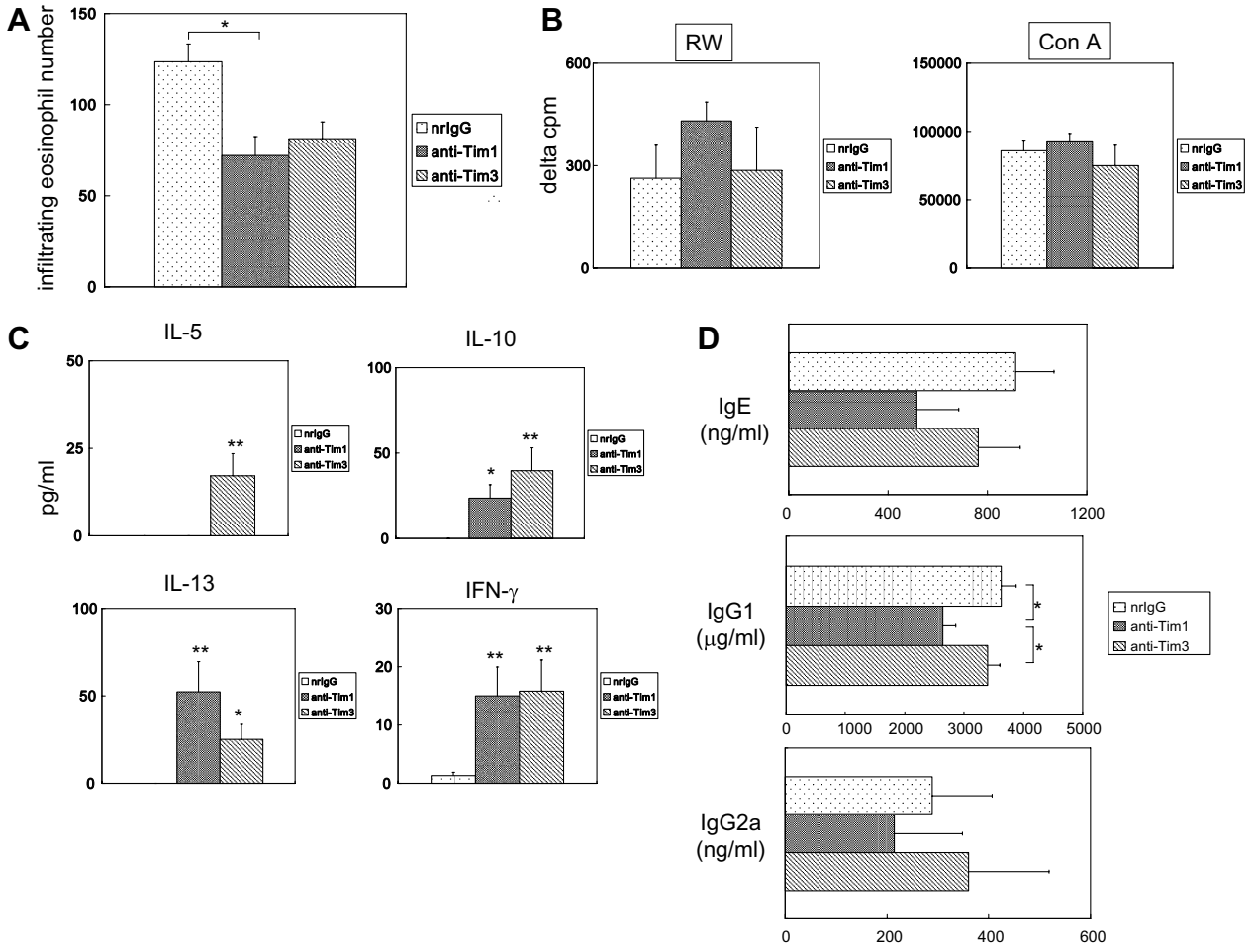

Fig. 1. Suppression of EC by treatment with anti-Tim-1 or anti-Tim-3 Ab during the induction phase. Balb/c WT mice were actively immunized with RW in alum and 10 days later, the mice were challenged with RW in eye drops to induce EC. The mice were intraperitoneally injected with 200 μg nrIgG, anti-Tim-1 Ab or anti-Tim-3 Ab five times every other day starting on the day of immunization. Twenty-four hours after RW challenge, the conjunctivas, spleens, and blood were harvested for histological analysis (A), splenocyte responses (B,C), and serum Ig levels (D), respectively. (A) Conjunctival eosinophil numbers are shown as means ± SEM of 13 mice in each group. $^{*}P < 0.05$. (B) Proliferative responses of splenocytes against RW and Con A. A representative data of five different samples in each group is shown as Δcpms ± SEM. Background cpms ± SEM were 639 ± 39, 681 ± 48, and 551 ± 34 for the nrIgG-, anti-Tim-1 Ab-, and anti-Tim-3-Ab-treated groups, respectively. (C) Cytokine production by RW-stimulated splenocytes. The data are presented as means ± SEM (pg/ml) of five different samples in each group. Note that both anti-Tim Abs upregulated Th1 and Th2 cytokine production. $^{*}P < 0.05$ and $^{**}P < 0.01$. (D) Total Ig levels in serum. Data are shown as means ± SEM of 13 mice in each group. $^{*}P < 0.05$.

evaluated the splenocyte responses and the Ig levels in serum. Neither anti-Tim Ab significantly affected RW-specific or Con A-induced splenocyte proliferation (Fig. 1B). However, anti-Tim-3 Ab treatment significantly increased RW-induced IL-5 production, while both anti-Tim Abs significantly increased RW-induced IL-10, IL-13, and IFN-γ production (Fig. 1C). IL-4 was below detectable levels for all the tested groups. Measurement of serum Ig levels revealed that anti-Tim-1 Ab significantly decreased total IgG1 levels, whereas total IgE and IgG2a levels were not affected by the treatment with either anti-Tim Ab (Fig. 1D).

Effects of anti-Tim-1 and anti-Tim-3 Ab treatments in IFN-γ-deficient (GKO) mice

That treatment with either anti-Tim Ab during the induction phase upregulated RW-specific splenocyte production of IFN-γ led us to investigate the role IFN-γ plays in the Ab-mediated suppression of EC. For this, GKO mice were actively immunized with RW and treated with anti-Tim Abs during the induction phase. Unlike the WT mice, the anti-Tim-1 Ab exacerbated conjunctival eosinophil infiltration (Fig. 2A). Anti-Tim-3 Ab also tended to increase the severity of EC, although this did not reach statistical significance ($P = 0.210$, Fig. 2A). However, like the WT mice, the splenocytes from the three groups of mice did not differ significantly in their RW- or Con A-induced proliferation (data not shown). With regard to cytokine production, while treatment with either anti-Tim Ab tended to elevate IL-5 production of splenocytes and anti-Tim-3 Ab tended to increase IL-13 production, none of these differences attained statistical significance (Fig. 2B). The anti-Tim Abs also did not significantly affect the serum Ig levels (data not shown).

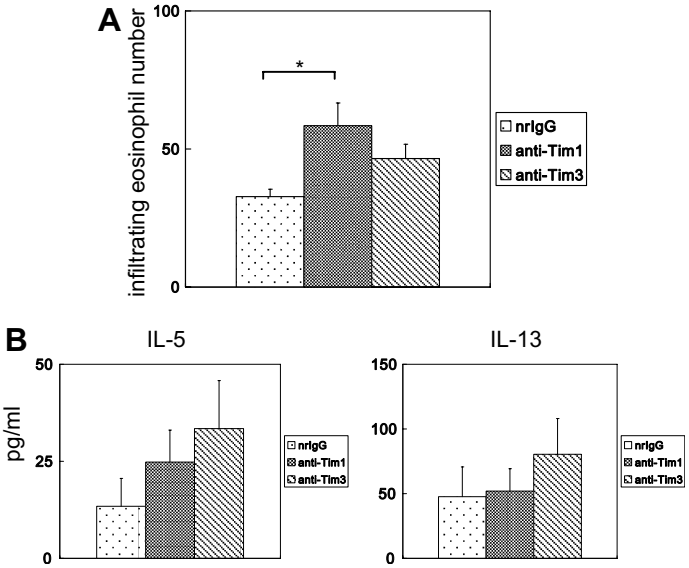

Fig. 2. Exacerbation of EC in GKO mice by treatment with anti-Tim-1 and anti-Tim-3 Abs during the induction phase. GKO mice actively immunized with RW were intraperitoneally injected five times with nrIgG, anti-Tim-1 or anti-Tim-3 Ab and then challenged with RW to induce EC, as described in the legend of Fig. 1. Twenty-four hours after RW challenge, the conjunctivas and spleens were harvested for histological analysis (A) and cytokine production (B), respectively. (A) Conjunctival eosinophil numbers are presented as means ± SEM ($n = 11$ in nrIgG-treated group, $n = 12$ in anti-Tim-1 Ab-treated group and anti-Tim-3 Ab-treated groups). $^{*}P < 0.05$. (B) Both anti-Tim Abs tended to elevate IL-5 production, while anti-Tim-3 Ab tended to increase IL-13 secretion, although these differences did not attain statistical significance compared to nrIgG treatment.

Treatment of WT mice with anti-Tim Abs during the effector phase of EC suppresses conjunctival eosinophil infiltration

Next, to investigate whether the anti-Tim Abs delivered during the effector phase of EC could also suppress eosinophil infiltration, actively immunized WT mice were intraperitoneally injected with anti-Tim-1 Ab, anti-Tim-3 Ab or nrIgG once on day 10, two hours prior to RW challenge. Anti-Tim-3 Ab significantly suppressed conjunctival eosinophil infiltration (Fig. 3A). Anti-Tim-1 Ab also tended to suppress eosinophil infiltration, although this did not attain statistical significance ($P = 0.134$, Fig. 3A). The Abs did not affect splenocyte responses or the serum Ig levels (data not shown).

To confirm the observation that treatment with anti-Tim-1 and anti-Tim-3 Abs during the effector phase suppress EC, naïve WT mice were injected with RW-primed splenocytes, treated with Abs, and challenged with RW. Treatment of these passively immunized mice with either anti-Tim Ab significantly suppressed conjunctival eosinophil infiltration (Fig. 3B).

Discussion

In this study, we found that when WT mice were treated with anti-Tim-1 and anti-Tim-3 Abs during either the induction or the effector phase of EC, the severity of EC was suppressed. This indicates that anti-Tim Ab treatment can both inhibit the induction of EC and suppress this disease once it has been initiated. Notably, both anti-Tim Ab treatments in the induction phase elevated WT splenocyte IFN-γ production, and anti-Tim Ab-treated GKO mice showed exacerbated disease. Thus, it appears that both of the anti-Tim Abs suppressed the pathogenic immune responses in the induction phase by upregulating IFN-γ.

Previous reports have demonstrated that Tim-1 is expressed on naïve T cells upon activation and then preferentially on Th2 cells but not on Th1 cells [17,19], while Th1 cells express Tim-3 [13]. Moreover, as described in the Introduction, Tim-1 expression appears to regulate Th2 cells, while Tim-3 downregulates Th1 cells. Since the anti-Tim-1 (RMT1-10) and anti-Tim-3 (RMT3-23) Abs used in this study block Tim-1-Fc binding to a Tim-1 ligand (possibly Tim-4) [17] and Tim-3-Fc binding to a Tim-3 ligand (galectin-9) [15], respectively, and EC is a Th2-mediated disease [21], we hypothesized that both Abs would act as antagonistic and suppress EC when delivered during the induction phase: anti-Tim-1 by blocking the activation of Th2 cells and anti-Tim-3 Ab by augmenting the Th1 response (which would in turn downregulate the Th2 response). Indeed, both Abs suppressed EC in WT mice (although the effect of anti-Tim-3 Ab treatment did not reach statistical significance). However, when we assessed the systemic immune responses of the treated mice to understand how the anti-Tim Abs delivered in the induction phase modulated EC severity, we found that anti-Tim-3 Ab treatment increased the production of both Th1 (IFN-γ) and Th2 (IL-5, IL-10, and IL-13) cytokines. Therefore, it appears that anti-Tim-3 augments not just RW-specific Th1 responses but also Th2 responses. Moreover, although we expected that anti-Tim-1 Ab treatment would suppress Th2 cytokine production (since Tim-1

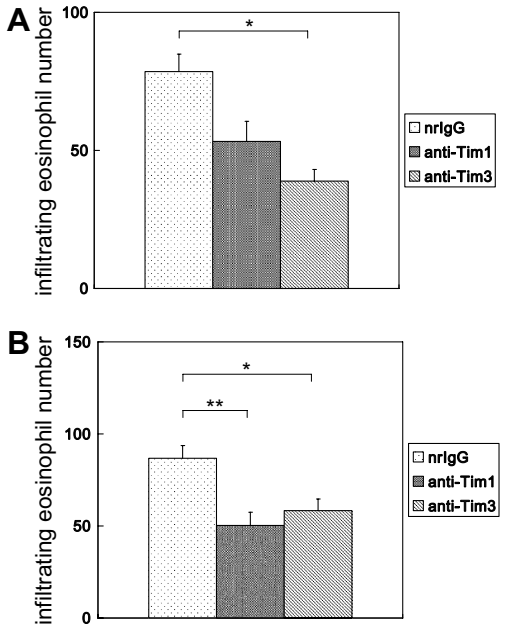

Fig. 3. Suppression of EC by treatment with anti-Tim-1 and anti-Tim-3 Abs during the effector phase. (A) Two hours prior to RW challenge, the actively immunized WT mice were intraperitoneally injected once with 200 μg nrIgG, anti-Tim-1 or anti-Tim-3 Ab ($n = 13$ per group). Twenty-four hours after RW challenge, the conjunctivas were harvested for histological analysis and the conjunctival eosinophils were counted. The data are presented as means ± SEM of 13 mice in each group. $^{*}P < 0.05$. (B) RW-primed *in vitro*-stimulated splenocytes were transferred into naïve WT mice. Two and four days later, the mice were intraperitoneally injected with nrIgG, anti-Tim-1 or anti-Tim-3 Abs ($n = 8$ 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 were presented as means ± SEM of 8 mice in each group. $^{*}P < 0.05$, $^{**}P < 0.01$.

appears to positively regulate Th2 responses), this Ab upregulated both Th1 (IFN-γ) and Th2 (IL-10 and IL-13) cytokine production. This suggests that RMT1-10 may have agonistic effects *in vivo* that augment the priming of RW-reactive T cells in general. Further studies are needed to address these possibilities.

The effect of anti-Tim-1 Ab on allergic disease induction has been investigated only once before, namely, by Encinas et al. who showed that anti-Tim-1 Ab treatment suppressed experimental allergic airway disease [19]. This effect was associated with reduced Th2 cytokine production of lung draining lymph node cells and decreased inflammatory cell infiltration, goblet cell hyperplasia, and mucus overproduction in the lung. This contrasts with our observation that EC induction phase treatment with anti-Tim-1 Ab upregulated Th2 cytokine production. This discrepancy may be due to the different anti-Tim-1 Abs being used; as suggested above, our RMT1-10 Ab may act agonistically while that of Encinas et al. may act antagonistically.

Both Abs delivered during the induction phase of EC significantly upregulated IFN-γ production. Since EC can be induced by transfer of Th2, but not Th1, cells [21] and IFN-γ suppresses the function of Th2 cells and the development of EC [22,23], we used GKO mice to investigate whether Ab-induced upregulation of IFN-γ helps suppress EC. In contrast to WT mice, treatment with either anti-Tim Ab exacerbated EC in GKO mice (although treatment with anti-Tim-3 Ab did not reach statistical significance). Thus, it seems that when anti-Tim Abs were delivered during the induction phase of EC, they suppress the severity of this disease by upregulating IFN-γ production.

Ours is the first study to assess the therapeutic potential of anti-Tim Abs, namely, the ability of these Abs to block the progression of EC once the disease has entered the effector phase. This is a more clinically relevant experiment since patients with allergic diseases are already Ag-sensitized when they are treated. We found that treatment of actively sensitized WT mice with either anti-Tim Ab just prior to Ag challenge suppressed EC (although the effect of the anti-Tim-1 Ab treatment did not reach statistical significance). This observation was confirmed by injecting the Abs into mice that had been passively sensitized by transfer of Ag-primed splenocytes (both Abs had statistically significant effects). Thus, Tim-1 and Tim-3 may be suitable therapeutic targets for allergic diseases. Other possible targets may be the Tim-1 and Tim-3 ligands, such as galectin-9, which is an eosinophil chemoattractant [24]. Although galectin-9 has been reported to be a Tim-3 ligand [15], we have found that Tim-1-Fc binds to galectin-9 as well as Tim-3-Fc (H. Akiba, unpublished observation). Thus, the interactions between galectin-9 and eosinophil-expressed Tim-1 and/or Tim-3 may play a role in conjunctival eosinophil infiltration and may be suitable therapeutic targets. Further studies are needed to address this possibility.

In conclusion, the data in this study suggest that it may be possible to treat allergic inflammatory diseases by administrating anti-Tim-1 and -Tim-3 Abs. While we have shown that anti-Tim-1 and -Tim-3 Abs suppresses EC by upregulating IFN-γ, additional studies will be needed to further delineate the mechanism involved. Further research into the effects of the anti-Tim Abs on EC may also improve our understanding of the Tim molecules, about which we still know relatively little.

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