

# T-cell Ig and mucin domain-containing protein (Tim)-2 regulates murine allergic conjunctivitis during the effector phase

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## Abstract

T-cell Ig and mucin domain-containing protein (Tim)-2 is associated with Th2-dependent immune responses. Here, we investigated whether administration of anti-Tim-2 Abs affects the development of murine experimental allergic conjunctivitis (EC), a Th2-mediated disease. While treatment with anti-Tim-2 Abs *in vivo* during the induction phase did not affect the severity of EC, treatment during the effector phase augmented EC. The *in vitro* stimulation of ragweed (RW)-primed splenocytes with RW in the presence of anti-Tim-2 Abs induced significantly more IL-5 and IL-13 production but significantly less IFN- $\gamma$  production compared with the control splenocytes treated with normal rat IgG. In addition, the transfer of the anti-Tim-2 Ab-treated splenocytes induced significantly more severe EC than the control splenocytes. Thus, Tim-2 negatively regulates the Th2 differentiation of Ag-primed T-cells and the development of EC during the effector phase.

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## 1. Introduction

The T-cell Ig and mucin domain-containing proteins (Tim) gene family is located on mouse chromosome 11B1.1 and on human chromosome 5q33.2 [1,2] and has been linked to both autoimmune and allergic diseases [3–5]. For example, human chromosome 5q33.2 has been linked to asthma susceptibility [3,4] and further analysis using mice suggested that the Tim gene family could be responsible for this genetic susceptibility [1].

To date, four Tim proteins have been identified. 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 [6–9]. Indeed, we recently reported that treatment with anti-Tim-1 or anti-Tim-3 Ab could suppress both the induction and progression of experimental allergic conjunc-

tivitis (EC) in mice [10]. However, with regard to Tim-2, its roles in the development of experimental allergic diseases have not been investigated extensively [11,12]. Therefore, we here sought to determine whether Tim-2 participates in the development of EC, which is a Th2-mediated disease.

## 2. Materials and methods

### 2.1. Mice

BALB/c mice were purchased from Japan SLC Inc., Hamamatsu, Shizuoka, Japan, and 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.

### 2.2. Reagents

Short ragweed pollen (RW) was purchased from Poly-sciences, Inc. (Warrington, PA). RW extract was obtained from

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LSL Co. Ltd. (Tokyo, Japan). Aluminum hydroxide (alum) was purchased from Sigma (St. Louis, MO). Anti-Tim-2 (RMT2-1 and RMT2-14, both rat IgG2a) monoclonal Abs were generated by immunizing rats with Tim-2-Fc fusion proteins. Normal rat IgG (nrIgG) was purchased from MP Biomedicals Inc. (Aurora, OH).

### 2.3. 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. Fifty microliters of the emulsion (50  $\mu$ g of RW and 675  $\mu$ g of 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 (2 h before RW challenge; effector phase treatment) with 200  $\mu$ g anti-Tim-2 Ab or nrIgG. On day 10, the eyes of the immunized mice were challenged with RW in PBS (2 mg in 10  $\mu$ l 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.

### 2.4. Induction of EC by adoptive transfer of *in vitro*-stimulated and Ab-treated RW-primed splenocytes

Naïve 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 with 5  $\mu$ g/ml RW extract as described previously [13] in the presence of 10  $\mu$ g/ml anti-Tim-2 Abs or nrIgG. After incubation for 72 h at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>,  $2 \times 10^7$  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  $\mu$ l per eye). Twenty-four hours later, the eyes were harvested for histological analysis.

### 2.5. 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 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  $\pm$  S.E.M. of all the mice examined.

### 2.6. Immunohistochemistry

The eyes including the conjunctivas from EC-developing mice treated with Abs during the effector phase were harvested and sections for immunohistochemistry were prepared following the method as previously described [14]. Briefly, the eyes were immediately frozen in 3% carboxymethyl cellulose (CMC) gel. Four-micrometer thick sections were prepared and fixed in methanol. Endogenous peroxidase activity was

inhibited with 0.1% NaN<sub>3</sub> and 0.3% H<sub>2</sub>O<sub>2</sub> in distilled water for 10 min at room temperature. Samples were exposed to first antibodies for 30 min and another 30 min to appropriate biotinylated secondary antibodies. All slides were revealed using an Avidin–Biotin–Complex kit (Vector laboratories Inc., USA), then developed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma). The antibodies used were rat anti-mouse CD3 (CT-CD3, Caltag, Burlingame, CA, USA), and rabbit anti-mouse major basic protein (MBP, provided by Dr. Lee, Mayo Clinic).

### 2.7. Measurement of RW-specific Igs in serum

Twenty-four hours after RW challenge of actively immunized mice, blood was collected and serum was prepared. RW-specific Ig levels in the serum were assessed by ELISA as described elsewhere in detail [15]. Quantified RW-specific IgE (kindly provided by Prof. Fu-Tong Liu) and pooled hyperimmune serum were used as controls. IgG1 concentrations are expressed as units

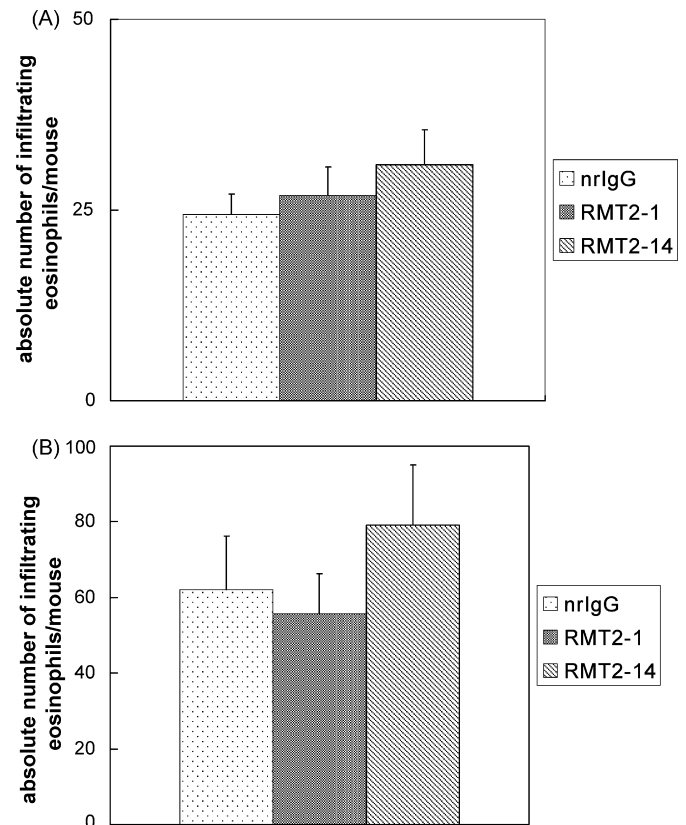


Fig. 1. Treatment with anti-Tim-2 Abs during the induction phase did not affect the severity of EC. BALB/c mice were actively immunized with RW in alum, injected intraperitoneally five times every other day starting on the day of immunization with 200  $\mu$ g of nrIgG or anti-Tim-2 Abs (RMT2-1, RMT2-14), and challenged on day 10 with RW in eye drops to induce EC. Twenty-four hours later, the conjunctivas and spleens were harvested for histological analysis (A, 10 mice per group) and splenocyte transfer (B), respectively. The harvested splenocytes were cultured *in vitro* with RW extract for 3 days and then  $2 \times 10^7$  cells were intraperitoneally injected into naïve BALB/c mice (8 mice per group). Four days later, the recipient mice were challenged with RW in eye drops and 24 h later, the conjunctivas were harvested for histological analysis. Absolute numbers of infiltrating eosinophils/mouse are shown as means  $\pm$  S.E.M.

(U)/ml relative to the pooled hyperimmune serum, whereas IgE concentrations are expressed as ng/ml.

## 2.8. Cytokine assay

BALB/c mice were actively immunized with RW in alum and 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-Tim-2 Ab. 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.

## 2.9. Statistical analysis

Significant differences between anti-Tim-2 Ab-treated and nrIgG-treated groups with regard to the infiltrating eosinophil

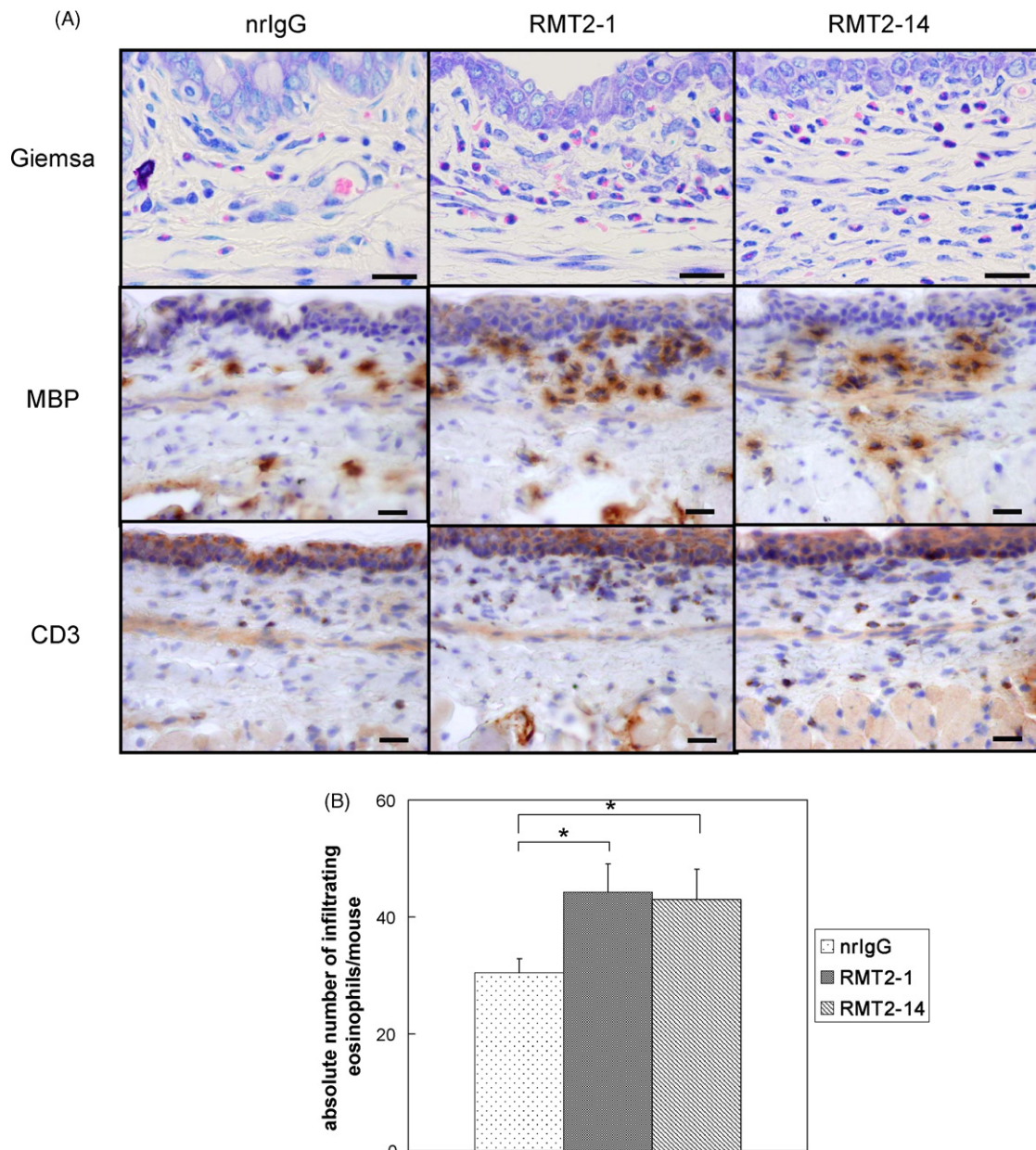


Fig. 2. Treatment with anti-Tim-2 Abs during the effector phase augmented EC. BALB/c mice were actively immunized with RW in alum and injected intraperitoneally once with 200 µg of nrIgG or anti-Tim-2 Abs (RMT2-1 and RMT2-14) 2 h prior to RW challenge on day 10. Twenty-four hours later, the conjunctivas, sera and spleens were harvested for histological (A, B, 15 mice per group) and immunohistochemical (A, 5 mice per group) analyses, measurement of RW-specific Ig levels in serum (C, 15 mice per group) and splenocyte transfer (D), respectively. (A) Microphotograph. Eosinophils were evaluated either by Giemsa staining or anti-MBP staining. T-cells were evaluated by anti-CD3 staining. Bar = 20 µm. (B) Absolute numbers of infiltrating eosinophils/mouse evaluated by Giemsa staining are shown as means ± S.E.M. \*  $P < 0.05$ . (C) RW-specific IgE and IgG1 levels. Data are presented as ng/ml (IgE) or U/ml (IgG1). (D) The harvested splenocytes were cultured *in vitro* with RW extract for 3 days and then  $2 \times 10^7$  cells were intraperitoneally injected into naïve BALB/c mice (8 mice per group). Four days later, the recipient mice were challenged with RW in eye drops and 24 h later, the conjunctivas were harvested for histological analysis. Absolute numbers of infiltrating eosinophils/mouse are shown as means ± S.E.M. \*\*  $P < 0.01$ .

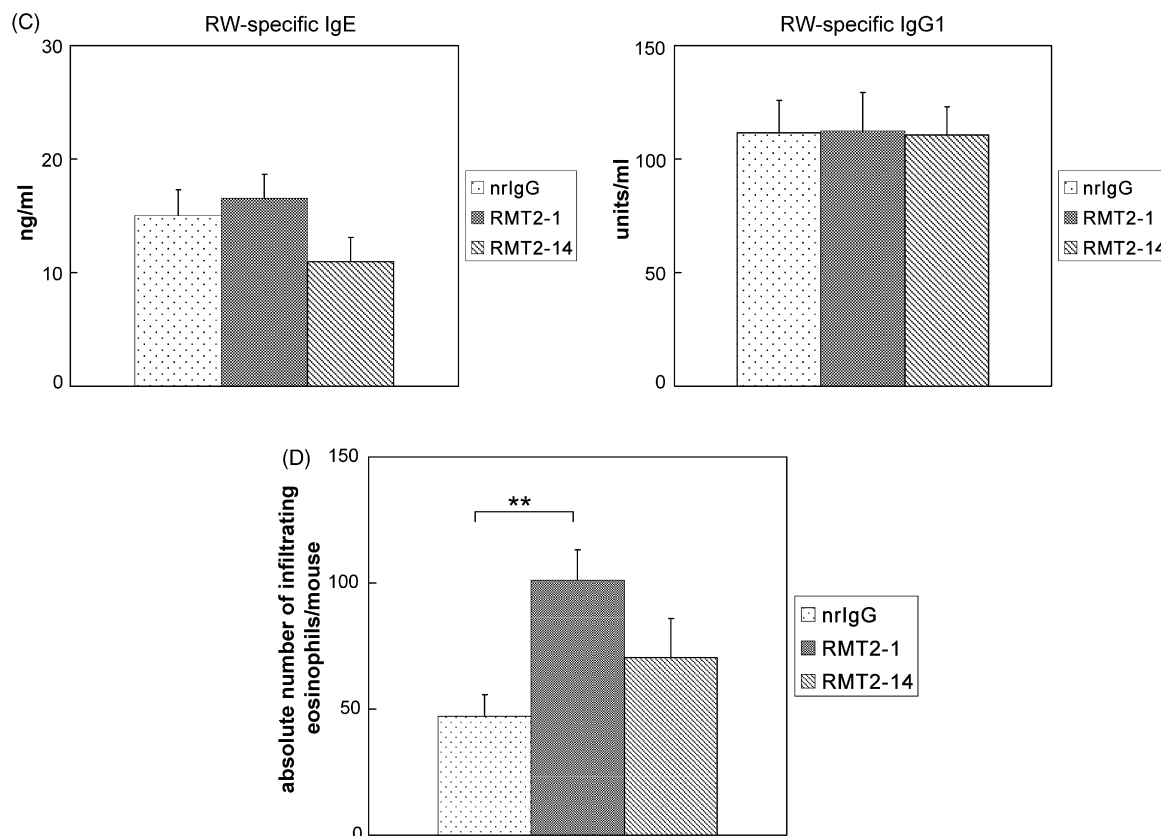


Fig. 2. (Continued).

numbers and splenocyte cytokine production were evaluated by Student's *t*-test. *P* values less than 0.05 were considered significant.

### 3. Results

#### 3.1. Treatment with anti-Tim-2 Abs during the induction phase did not affect EC induced by active immunization

To investigate whether Tim-2 is involved in the induction phase of EC, we intraperitoneally injected groups of 10 RW-sensitized mice with anti-Tim-2 Abs RMT2-1 or RMT2-14 (or, as a control, nrIgG) five times every other day starting on the day of immunization. Two days after the last injection, the mice were challenged with RW in eye drops and 24 h later, the conjunctivas were harvested for histological analysis to determine the infiltrating eosinophil numbers. Treatment with anti-Tim-2 Abs, regardless of the Ab clone used, did not significantly affect the severity of EC compared to nrIgG (Fig. 1A). In addition, the transfer into naïve mice of splenocytes from mice that had been treated with anti-Tim-2 Abs while they were developing EC, induced EC that was as severe as that generated by splenocytes from nrIgG-treated mice (8 mice per group, Fig. 1B). Thus, Tim-2 is unlikely to play a role in the development of EC during the induction phase.

#### 3.2. Treatment with anti-Tim-2 Abs during the effector phase augmented the severity of EC induced by active immunization

Next, to investigate whether Tim-2 is involved in the development of EC during the effector phase, groups of 15 RW-sensitized mice were injected intraperitoneally once with anti-Tim-2 Abs 2 h prior to RW challenge. Harvested conjunctivas were subjected to histological (Giemsa staining) and immunohistochemical analysis (anti-MBP and anti-CD3 staining) to evaluate the infiltration of eosinophils and T-cells. As shown in Fig. 2A, eosinophils and T-cells infiltrated into the conjunctiva and it was likely that more eosinophils and T-cells infiltrated into the conjunctiva from EC-developing mice treated with anti-Tim-2 Abs compared to those with nrIgG. Counting of eosinophils in the conjunctiva confirmed that the anti-Tim-2 Abs significantly augmented the severity of EC, regardless of the Ab clone used (Fig. 2B). Effects of Ab treatment on immune responses were evaluated by RW-specific IgE and IgG1 levels in serum; however, significant difference was not observed between nrIgG- and anti-Tim-2 Ab-treated groups (Fig. 2C). Similar to the data of EC-developing mice treated with anti-Tim-2 Abs during the effector phase (Fig. 2B), the transfer into naïve mice of splenocytes from the anti-Tim-2 Ab-treated mice induced more severe EC than the splenocytes from the nrIgG-treated mice, although this was statistically significant only for RMT2-1 (8 mice per group, Fig. 2D). Thus, it appears

that Tim-2 regulates the development of EC during the effector phase.

### 3.3. Effect of anti-Tim-2 Abs on the cytokine production of RW-stimulated splenocytes

Splenocytes were obtained from RW-sensitized mice that had not been treated with Abs and were cultured *in vitro* with RW in the presence of anti-Tim-2 Abs or nrIgG. The splenocytes produced significantly more IL-5 and IL-13 and significantly less IL-10 and IFN- $\gamma$  in the presence of anti-Tim-2 Abs than in the presence of nrIgG (four samples per group, Fig. 3A). In addition, when groups of 10 naïve mice were given these RW-primed splenocytes, they exhibited more severe EC when the

splenocytes had been treated with anti-Tim-2 Abs *in vitro* as compared to nrIgG treatment (Fig. 3B). Thus, it appears that the anti-Tim-2 Abs used in this study promoted Th2 deviation while suppressing Th1 deviation of RW-primed splenocytes upon the secondary stimulation with RW *in vitro*.

## 4. Discussion

Here, we demonstrate that treatment with anti-Tim-2 Abs during the induction phase of EC did not affect the severity of EC. In addition, the transfer of splenocytes from mice treated with anti-Tim-2 Abs during the induction phase, induced EC that was as severe as that generated by splenocytes from nrIgG-treated mice. Thus, Tim-2 is unlikely to be involved in the initial steps

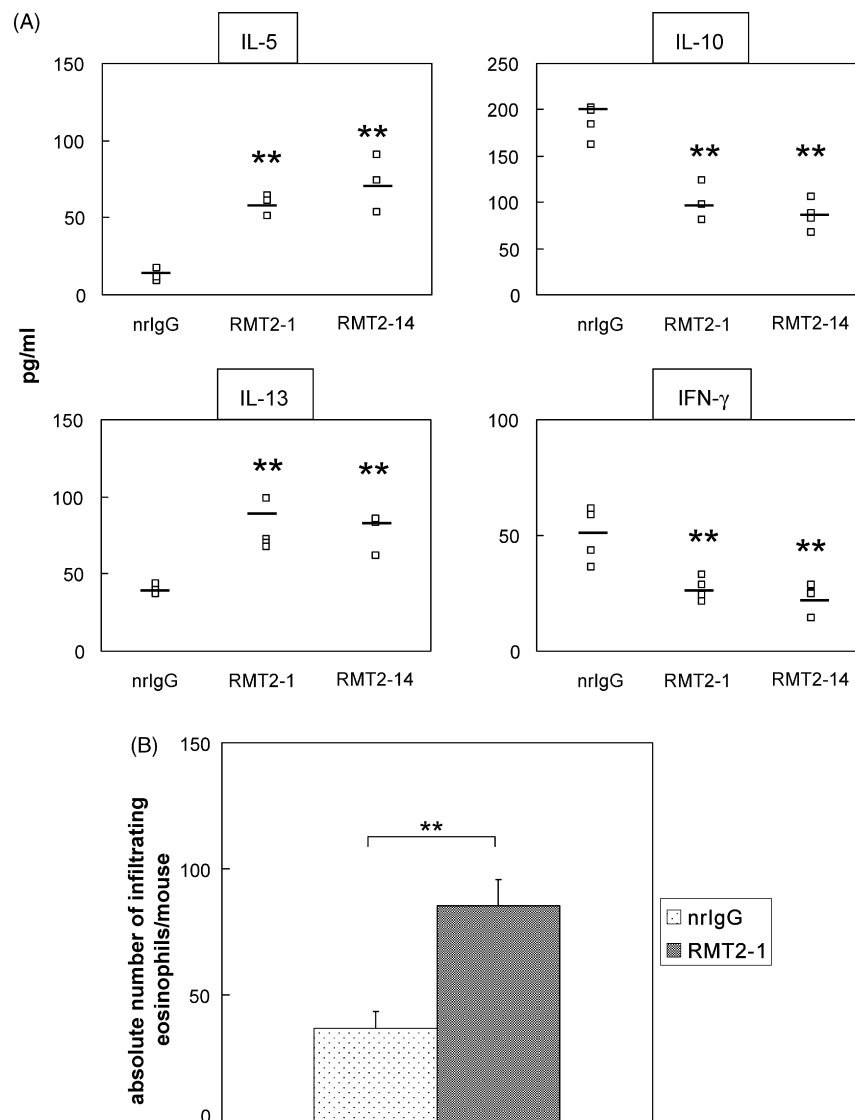


Fig. 3. *In vitro* treatment of RW-primed splenocytes with anti-Tim-2 Abs upregulated Th2 cytokine production and increased the severity of EC. BALB/c mice were actively immunized with RW in alum and not treated with Abs. Ten days later, the mice were sacrificed and their spleens were harvested. (A) Cytokine assay: the splenocytes were cultured *in vitro* with 25  $\mu$ g/ml RW extract in the presence of 10  $\mu$ g/ml nrIgG or anti-Tim-2 Abs. Forty-eight hours later, the culture supernatants were collected and used for cytokine assays. \*\* $P < 0.01$  as compared to the nrIgG-treated group. (B) Splenocyte transfer: the splenocytes were cultured *in vitro* with RW extract (5  $\mu$ g/ml) in the presence of 10  $\mu$ g/ml nrIgG or the RMT2-1 anti-Tim-2 Ab for 3 days and then  $2 \times 10^7$  cells were injected intraperitoneally into naïve BALB/c mice (8 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. Absolute numbers of infiltrating eosinophils/mouse are shown as means  $\pm$  S.E.M. \*\* $P < 0.01$ .

that generate Ag-specific Th2 cells. In contrast, treatment with anti-Tim-2 Abs during the effector phase augmented the severity of EC. Moreover, the splenocytes from these mice induced more severe EC than the control splenocytes. These data suggest that Tim-2 appears to regulate Th2 differentiation once the T-cells have been primed with an Ag. These observations are in good agreement with a previous report showing that TIM-2-Fc fusion protein treatment of mice just before the nebulization period increased the eosinophil counts in the bronchoalveolar lavage fluid and the Th2 cytokine production of bronchial lymph node cells [12].

It has not been clarified whether the Abs used in this study (RMT2-1 and RMT2-14) exert agonistic or antagonistic activity. Recently, it was reported that expression of Tim-2 in T-cells significantly impairs the induction of NFAT and AP-1 activity [16]. Additionally, Tim-2 gene-deficient mouse exhibits dysregulated expression of Th2 cytokines [12]. Together with the data that treatment with RMT2-1 and RMT2-14 either *in vivo* (Fig. 2) or *in vitro* (Fig. 3) augmented Th2 responses, it could be considered that the Abs used in this study exert antagonistic activity rather than agonistic activity.

To further investigate how Tim-2 promotes Th2 differentiation once T-cells are Ag-primed, RW-primed splenocytes were stimulated *in vitro* with RW in the presence of anti-Tim-2 Abs or nrIgG. Cytokine analysis demonstrated that blocking Tim-2 *in vitro* upregulated the production of Th2 cytokines, such as IL-5 and IL-13 while significantly suppressing the production of IFN- $\gamma$ . In addition, the transfer of RW-primed splenocytes treated *in vitro* with anti-Tim-2 Abs induced more severe EC than the control splenocytes. These data suggest that Tim-2 negatively regulates Th2 differentiation but augments Th1 differentiation. It has been reported previously that Tim-2 is preferentially upregulated during Th2 differentiation [11]. Furthermore, it was reported that administration of the Tim-2-Ig fusion protein during the initiation and early development of immune responses resulted in the increased production of Th2 cytokines [11]. Taken together, the anti-Tim-2 treatment in our study may have inhibited the negative signal to Th2 cells from the Tim-2 molecules, thereby increasing the Th2 responses and conjunctival eosinophil infiltration. However, unlike the previous studies, the treatment with anti-Tim-2 Abs during the induction phase did not affect Th2 development in this study. This may be due to the different experimental models (experimental autoimmune encephalomyelitis and allergic conjunctivitis) and blocking agents (Tim-2-Ig fusion protein and anti-Tim-2 Ab) that were used. Nevertheless, our observations provide additional information, namely, that Tim-2 negatively regulates Th2 differentiation and the development of allergic disease.

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