

Engagement of 4-1BB Inhibits the Development of Experimental Allergic Conjunctivitis in Mice¹

Atsuki Fukushima,^{2*} Tomoko Yamaguchi,* Waka Ishida,* Kazuyo Fukata,* Robert S. Mittler,[†] Hideo Yagita,[‡] and Hisayuki Ueno*

The 4-1BB receptor acts as a costimulator in CD8⁺ T cell activation. Agonistic stimulation through this molecule by treatment with anti-4-1BB Abs has been demonstrated to inhibit various experimentally induced diseases in animals. However, the effect of anti-4-1BB Abs on experimental allergic diseases has not been reported. We investigated the effect of anti-4-1BB Abs on the development and progression of experimental allergic conjunctivitis in mice. To examine the effects of Abs during the induction or effector phase, actively immunized mice or passively immunized mice by splenocyte transfer were treated with agonistic anti-4-1BB Abs, blocking anti-4-1BB ligand Abs, or normal rat IgG. Eosinophil infiltration into the conjunctiva was significantly reduced in wild-type mice by the anti-4-1BB Ab treatment during either induction or effector phase. Th2 cytokine production by splenocytes and total serum IgE were significantly reduced by the anti-4-1BB Ab treatment, while IFN- γ production was increased. The anti-4-1BB Ab treatment induced a relative increase of CD8-positive cell numbers in the spleens. Moreover, inhibition of eosinophil infiltration by the treatment with anti-4-1BB Abs was also noted in actively immunized IFN- γ knockout mice. Taken altogether, in vivo treatment with agonistic anti-4-1BB Abs in either induction or effector phase inhibits the development of experimental allergic conjunctivitis, and this inhibition is likely to be mediated by suppression of Th2 immune responses rather than up-regulation of IFN- γ . *The Journal of Immunology*, 2005, 175: 4897–4903.

Allergic conjunctivitis (AC)³ is an IgE-mediated ocular disease characterized by the infiltration of eosinophils into the conjunctiva (1). Generally, the more severe the AC, the more eosinophils are detected in the conjunctiva (2). In fact, molecules secreted from eosinophils such as major basic protein have been demonstrated to cause corneal damage (3, 4), which is often noted in severe AC patients. Because eosinophil infiltration is up-regulated by eotaxin and the Th2-type cytokine IL-4 (5), and IgE production is also up-regulated by IL-4 (6), AC is believed to be predominantly mediated by Th2-type immune responses. Supporting this is that Th2-type cytokines are detected in tears of most patients with severe AC (7, 8). To understand how Th2 cells are involved in AC development, our group has established an experimental model of AC in rats (9, 10) and mice (11, 12) denoted as experimental immune-mediated blepharoconjunctivitis (EC). This model involves injecting the animals with an allergen, followed by challenge with allergen-containing eye drops. Alternatively, the animals are passively primed by adoptive transfer of splenocytes or T cells from allergen-primed animals or allergen-specific IgE. In mice, the active sensitization method induces

prominent Th2 immune responses and abundant eosinophilic infiltration into the conjunctiva, especially in Th2-prone BALB/c mice (11). The passive sensitization of naive BALB/c mice by allergen-primed T cells or allergen-specific IgE also induces severe eosinophilic infiltration (13). These results support the notion that Th2-type immune responses are involved in the development of AC in humans.

The 4-1BB (CD137), a T cell costimulatory molecule, is expressed on activated T cells, NK cells, and dendritic cells (14–18). Signals through 4-1BB induce T cells to carry out effector functions such as the CD8⁺ T cell-mediated eradication of established tumors (15, 19). Moreover, 4-1BB-activated CD8⁺ T cells produce IFN- γ and TNF- α (20, 21). In addition to up-regulating these cellular immune responses, 4-1BB is actively involved in humoral immune responses (22). This is shown by the fact that agonistic Abs to 4-1BB inhibit T cell-dependent humoral immune responses and suppress acute disease in systemic lupus erythematosus-prone mice (23, 24).

To date, a number of studies have examined the effects of agonistic anti-4-1BB Ab treatment on various experimentally induced diseases, including autoimmune diseases such as rheumatoid arthritis (25, 26), cancer (15, 19), graft-vs-host disease (27), and infectious disease (28). However, the effect of agonistic anti-4-1BB Abs on the development of experimental allergic diseases has not been investigated. In this study, we sought to investigate the involvement of 4-1BB in the development of experimental AC.

Materials and Methods

Mice

Inbred wild-type (WT) BALB/c mice were purchased from Japan SLC. IFN- γ -deficient BALB/c (IFN- γ knockout (GKO)) mice were purchased from The Jackson Laboratory. 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- to 12-wk old. All research adhered to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

*Department of Ophthalmology, Kochi Medical School, Nankoku-city, Japan; [†]Department of Surgery and Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA 30329; and [‡]Department of Immunology, School of Medicine, Juntendo University, Tokyo, Japan

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² Address correspondence and reprint requests to Dr. Atsuki Fukushima, Department of Ophthalmology, Kochi Medical School, Kohasu, Oko-cho, Nankoku-city 783-8505, Japan. E-mail address: fukushima@med.kochi-u.ac.jp

³ Abbreviations used in this paper: AC, allergic conjunctivitis; 4-1BBL, 4-1BB ligand; alum, aluminum hydroxide; EC, experimental immune-mediated blepharoconjunctivitis; GKO, IFN- γ knockout; nrlgG, normal rat IgG; RW, ragweed; WT, wild type.

Reagents

Short ragweed pollen (RW) was purchased from Polysciences. RW extract was obtained from LSL. Aluminum hydroxide (alum) was purchased from Sigma-Aldrich. A hybridoma producing an Ab to 4-1BB (3H3) was established, as described previously (20), and a hybridoma producing an Ab to 4-1BB ligand (4-1BBL, TKS-1) was established, as described previously (18). These Abs for *in vivo* treatments were purified from ascites using protein G column and contained <100 pg/ml endotoxin. The following Abs were purchased: normal rat IgG (nrIgG) (MP Biomedicals); FITC-labeled anti-CD3 (145-2C11) and anti-CD4 (GK1.5) and biotin-labeled CD8 (53-6.7) (eBioscience); FITC-labeled anti-CD45R/B220 (RA3-6B2), FITC-labeled anti-CD11c (HL3), and streptavidin-PE (BD Biosciences); and biotin-labeled anti-F4/80 (A3-1) (Caltag Laboratories).

EC induction by active immunization and treatment with Abs

RW adsorbed on alum was injected into the left hind footpad and the tail base. A total of 50 μ l of the emulsion (50 μ g of RW and 2 mg of alum) was injected into each site. The mice were injected i.p. with 200 μ g of purified anti-4-1BB ($n = 20$ in WT and $n = 9$ in GKO), anti-4-1BBL ($n = 19$ in WT), or control rat IgG ($n = 20$ in WT and $n = 9$ in GKO) on days 0, 2, 4, 6, and 8 after immunization. Ten days later, 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, sera, and spleens were harvested for histological analysis, measurement of IgE levels, and T cell culture for transfer, cytokine, or proliferation assays after measuring the weight of spleens, respectively.

EC induction by adoptive transfer of splenocytes from actively immunized and Ab-treated mice

Splenocytes harvested from the mice described above were cultured with RW extract at final concentrations of 5 μ g/ml in 75-cm² flasks at a concentration of 10^7 cells/ml in a final volume of 20 ml of RPMI 1640 medium supplemented with 10% FCS (ICN Biomedical), 2-ME (5×10^{-5} M), L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μ g/ml). After incubation for 72 h at 37°C in a humidified atmosphere with 5% CO₂, 2×10^7 splenocytes were i.p. injected into naive BALB/c mice ($n = 10$ mice per group). Four days after the transfer, the eyes of the recipient mice were challenged with RW in PBS (2 mg in 10 μ l per eye). Twenty-four hours later, the eyes and spleens were harvested for histological analysis.

EC induction by adoptive transfer of *in vitro*-stimulated RW-primed splenocytes and treatment with Abs

Naive 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, splenocytes were prepared and cultured with RW, as detailed in the previous section. After incubation for 72 h at 37°C in a humidified atmosphere with 5% CO₂, 2×10^7 splenocytes were i.p. injected into syngeneic naive BALB/c mice. After the splenocyte transfer, the mice were injected i.p. with 200 μ g of anti-4-1BB ($n = 10$), anti-4-1BBL ($n = 10$), or nrIgG ($n = 10$) either once (on day 4) or twice (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 μ l 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. Horizontal 2- μ m-thick sections were cut and stained with Giemsa. Infiltrating eosinophils in the lamina propria mucosae of the tarsal and bulbar conjunctivas throughout each section were counted by two blinded observers. The sections counted were those of the central portion of the eye, which included the pupil and optic nerve head. Because the counts vary depending on the severity of inflammation (when inflammation is severe, the thickness of lamina propria mucosae increases), the cell count data are expressed as infiltrating eosinophil numbers divided by area (mm²), as measured by Scion Image (Scion). The data are presented as an average $\pm$ SEM of all the mice examined.

Flow cytometric analysis

Splenocytes were incubated for 30 min on ice with FITC- or biotin-labeled Abs at optimal concentrations. After the incubation, the cells were washed with cold 2% FCS-PBS. In the case of biotin-labeled Abs, the cells were then incubated with streptavidin-PE for 10 min and then washed again. These cells were analyzed on a FACScan (BD Biosciences), and acquisition and analysis were performed using CellQuest software. The data are presented as an average of Ab-stained cell number $\pm$ SEM per spleen.

Cellular proliferation assay

RBC-depleted splenocytes (3×10^5 cells/well) were cultured in 96-well flat-bottom plates in a final volume of 0.2 ml of RPMI 1640 medium supplemented with 5% FCS and 2-ME. The cells were stimulated with RW at final concentrations of 0.2, 1, 5, and 25 μ g/ml or with Con A at 5 μ g/ml. After an 80-h incubation at 37°C in a humidified atmosphere containing 5% CO₂, the cultures were pulsed for 16 h with 0.5 μ Ci/well [³H]thymidine (Japan Atomic Energy Research Institute). The cultures were then harvested, and the incorporated radioactivity was measured by standard techniques. The data were expressed as δ cpm (mean cpm of stimulated cultures – mean cpm of unstimulated control cultures) or as stimulation indices (mean cpm of stimulated cultures/mean cpm of unstimulated control cultures).

Measurement of total IgE in serum

Twenty-four hours after RW challenge of actively immunized mice, the blood was collected and serum was prepared. Total IgE in the sera was measured by ELISA. Briefly, affinity-purified anti-mouse IgE (2 μ g/ml; eBioscience) was coated in a 96-well enzyme immunoassay plate (Costar) overnight at 4°C. The plates were then washed and incubated with blocking buffer (1% BSA in PBS) for 3 h at room temperature. The plates were washed again, and the samples or IgE standards were applied to each well. After 2-h incubation at room temperature, the plates were washed and biotin-conjugated rat anti-mouse IgE (BD Biosciences) was added to each well for 1 h at room temperature. After washing, avidin-alkaline phosphatase (Sigma-Aldrich) was added to each well for 1 h. After washing, the substrate *p*-nitrophenyl phosphate (*p*-nitrophenyl phosphate liquid substrate system; Sigma-Aldrich) was added to each well. Fifteen minutes later, absorbance was measured at 405 nm. The concentration of IgE was standardized by reference to the known concentrations of the IgE standards (BD Biosciences).

Measurement of cytokines in the culture supernatants

RBC-depleted splenocytes (10^7 cells/ml) were cultured for 48 h with Con A (5 μ g/ml) or RW (25 μ g/ml) in 96-well flat-bottom plates in a final volume of 0.2 ml of RPMI 1640 medium supplemented with 10% FCS and 2-ME. The levels of IL-4, IL-5, IL-13, and IFN- γ produced were measured by using commercially available ELISA kits (DuoSet; R&D Systems), according to the manufacturer's recommendations.

Statistical analysis

Differences between the Ab-treated and nrIgG-treated mice in terms of their serum IgE levels, splenocyte proliferation, cytokine production, and infiltrating eosinophil numbers were tested for significance by Student's *t* test. Values of $p < 0.05$ were considered significant.

Results

Treatment with agonistic anti-4-1BB Abs during the induction phase inhibits the infiltration of eosinophils into the conjunctiva

To investigate the role that the 4-1BB molecule plays in the development of EC, we induced EC in WT BALB/c mice by active immunization and treated the mice with i.p. injections of either an agonistic anti-4-1BB Ab or a blocking anti-4-1BBL Ab. As a control, the mice were treated with nrIgG. The untreated and nrIgG-treated actively immunized mice showed equivalent eosinophilic infiltration (Fig. 1, A and D, and data not shown). However, relative to the nrIgG-treated mice, the agonistic anti-4-1BB Ab-treated mice showed a marked inhibition in eosinophilic infiltration (Fig. 1, B and E). In contrast, the eosinophil infiltration was not apparently affected by treatment with the blocking anti-4-1BBL Ab (Fig. 1, C and F). When the eosinophils were counted, the reduced eosinophil infiltration seen after the agonistic Ab treatment compared with that in the nrIgG treatments was found to be significant (Fig. 1G, $p < 0.001$). Difference between nrIgG-treated and anti-4-1BBL Ab-treated mice was not statistically significant.

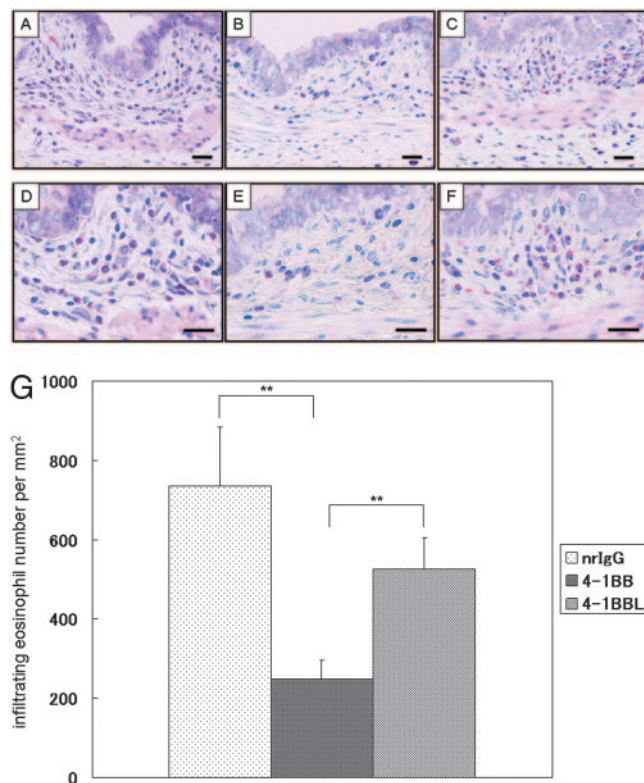


FIGURE 1. Treatment with agonistic anti-4-1BB Abs during the induction phase of EC inhibits eosinophilic infiltration into the conjunctiva of WT mice. BALB/c WT mice were immunized with RW in alum and then i.p. injected with 200 μ g of nrIgG (A and D; $n = 20$), anti-4-1BB Abs (B and E; $n = 20$), or anti-4-1BBL Abs (C and F; $n = 19$) on days 0, 2, 4, 6, and 8. On day 10, the mice were challenged with RW in eye drops, and their conjunctivas were harvested 24 h later for histologic analysis. Bar = 20 μ m. The infiltrating eosinophils were counted, and the data are expressed as eosinophils per mm² (G). **, $p < 0.01$.

Transfer of splenocytes from actively immunized mice treated with the agonistic anti-4-1BB Ab induces less eosinophil infiltration

EC can be transferred by Ag-primed splenocytes. Consequently, we next examined the severity of eosinophilic infiltration into the conjunctiva of naive BALB/c mice upon adoptive transfer of the splenocytes from the actively immunized mice after the treatment with anti-4-1BB Abs, anti-4-1BBL Abs, or nrIgG. The splenocytes from anti-4-1BB Ab-treated mice induced significantly less eosinophilic infiltration compared with those from nrIgG-treated or anti-4-1BBL Ab-treated mice (Fig. 2, $p < 0.001$). Difference between nrIgG-treated and anti-4-1BBL Ab-treated mice was not statistically significant.

Effect of agonistic anti-4-1BB Ab treatment on immune responses

The above findings indicate that the agonistic anti-4-1BB Ab inhibits EC, possibly by modifying the function of splenocytes. To examine this, we evaluated the effect of Ab treatment on immune responses of the splenocytes from actively immunized mice by in vitro assays. In addition, the humoral immune responses of these mice were evaluated by measuring total serum IgE levels. The spleens in the agonistic anti-4-1BB Ab-treated group were significantly larger and contained significantly more splenocytes than those of the other two groups (Fig. 3A). Moreover, flow cytometric analysis showed the spleens of the agonistic anti-4-1BB Ab-treated

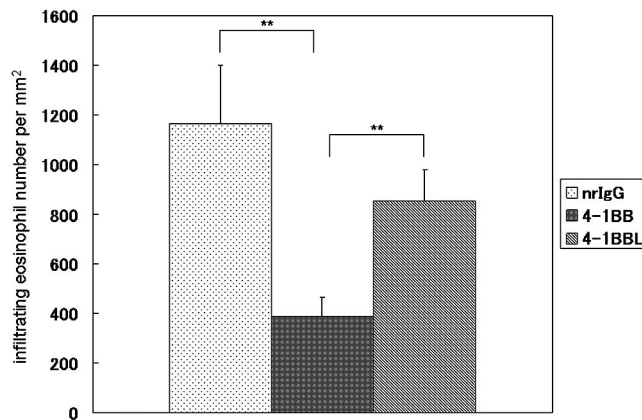


FIGURE 2. Reduced eosinophilic infiltration is observed upon adoptive transfer of splenocytes from the mice treated with agonistic anti-4-1BB Abs. Spleens were harvested from the mice described in the legend to Fig. 1 at the same time the conjunctiva were removed. Splenocytes were prepared and cultured in vitro with RW and then transferred into naive BALB/c WT mice. Four days later, the recipient mice were challenged with RW in eye drops, and 24 h later, their conjunctivas were subjected to histologic analysis. The infiltrating eosinophils were counted, and the data are expressed as eosinophils per mm². **, $p < 0.01$.

group contained a higher proportion of CD3⁺ cells, especially CD8⁺ cells (Tables I and II). In accordance with the previous report (26), the number and proportion of CD8⁺CD11c⁺ positive cells were significantly higher in the agonistic anti-4-1BB Ab-treated group. In addition, these splenocytes proliferated against RW significantly less vigorously (Fig. 3B). Compared with splenocytes from the nrIgG-treated mice, splenocytes from the blocking anti-4-1BBL Ab-treated mice also proliferated less vigorously (Fig. 3B). In contrast, Con A-induced proliferation was similar among the three groups (Fig. 3C). Analyses of the cytokine production by the cultured splenocytes revealed that the Con A-stimulated splenocytes from the agonistic anti-4-1BB Ab-treated mice produced more IFN- γ and less IL-4, IL-5, and IL-13, compared with the nrIgG-treated group (Fig. 3D). In addition, RW-stimulated splenocytes from the agonistic anti-4-1BB Ab-treated mice produced less IL-4 and IL-13 than the nrIgG-treated group (Fig. 3D). Finally, the agonistic anti-4-1BB Ab-treated mice had significantly lower total serum IgE levels, compared with the other two groups (Fig. 3E). Compared with the nrIgG-treated group, total serum IgE levels were significantly lower in the blocking anti-4-1BBL Ab-treated group (Fig. 3E).

Treatment with agonistic anti-4-1BB Abs during the induction phase inhibits the infiltration of eosinophils into the conjunctiva of GKO mice

Next, we induced EC in GKO mice and treated the mice with nrIgG or anti-4-1BB Ab to investigate whether the suppressive effects of the agonistic anti-4-1BB Ab are mediated by IFN- γ . Similar to the data in WT BALB/c mice, the agonistic anti-4-1BB Ab treatment significantly suppressed the infiltration of eosinophils into the conjunctiva (Fig. 4).

Treatment with the agonistic anti-4-1BB Ab during the effector phase inhibits the infiltration of eosinophils into the conjunctiva

Finally, we investigated whether the treatment with agonistic anti-4-1BB Abs can affect the effector phase of EC. For this purpose, we transferred in vitro stimulated RW-primed splenocytes from Ab-untreated mice and treated the recipients with the agonistic anti-4-1BB Ab. The anti-4-1BB Ab treatment suppressed the infiltration of eosinophils into the conjunctiva as compared with the

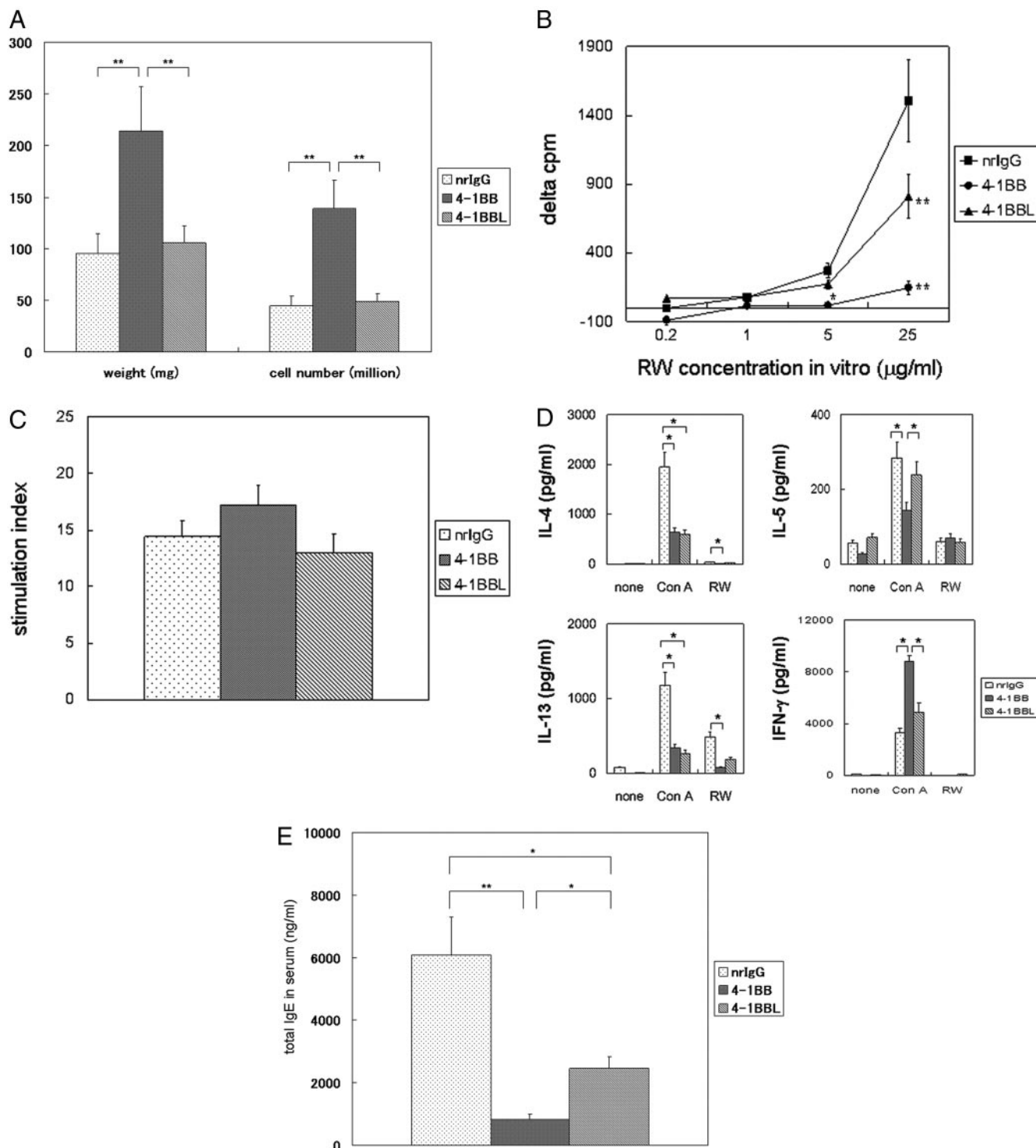


FIGURE 3. Effect of agonistic anti-4-1BB Ab treatment of actively immunized mice on splenocyte immune responses. Spleens and sera were harvested from the mice described in the legend to Fig. 1 at the same time the conjunctivas were collected. The spleens were weighed and then dissociated, and the recovered splenocyte numbers were counted. Data are presented as weight and cell number per spleen in each group (A; **, $p < 0.01$). The resulting splenocytes were stimulated in vitro with RW (B; *, $p < 0.05$ and **, $p < 0.01$ compared with nrIgG) or Con A (C). Splenocyte proliferation was evaluated by [^{3} H]thymidine incorporation. Background cpm $\pm$ SEM was 2376 ± 87 , 1393 ± 41 , and 1426 ± 68 for the nrIgG-treated, anti-4-1BB Ab-treated, and anti-4-1BBL Ab-treated groups, respectively. The supernatants of splenocytes cultured with RW and Con A, or without any stimulants, were harvested for measuring the concentrations of IL-4, IL-5, IL-13, and IFN- γ by ELISA (D; *, $p < 0.05$). Total serum IgE was measured by ELISA (E; *, $p < 0.05$ and **, $p < 0.01$).

nrIgG treatment (Fig. 5), although significant inhibition was noted only when the treatment was conducted twice (Fig. 5B, $p < 0.05$). In contrast, treatment with the blocking anti-4-1BBL Ab did not affect the infiltration of eosinophils (Fig. 5).

Discussion

Agonistic anti-4-1BB Abs have been reported to augment activation of T cells, especially CD8 $^+$ subset, and the production of IFN- γ (15, 19–21). These Abs have also been observed to ameliorate several

Table I. Flow cytometric analysis of splenocytes from actively immunized mice: cell number per spleen^a

	CD3 ⁺	CD4 ⁺	CD8 ⁺	B220 ⁺	F4/80 ⁺	CD8 ⁺ CD11c ⁺
nrlgG	17.8 ± 2.4	11.6 ± 1.9	6.2 ± 1.1	22.9 ± 5.4	2.1 ± 0.3	0.5 ± 0.2
4-1BB	77.8 ± 16.0**	45.3 ± 8.8**	31.2 ± 6.5**	51.8 ± 7.6**	8.7 ± 1.6**	6.0 ± 0.4**
4-1BBL	20.6 ± 1.4	13.6 ± 0.7	6.2 ± 0.4	26.6 ± 5.4	1.7 ± 0.3	0.3 ± 0.1

^a Mice were actively immunized with RW and i.p. injected with nrlgG, anti-4-1BB, or anti-4-1BBL, as detailed in *Materials and Methods*. Harvested splenocytes were analyzed by flow cytometric analysis and data are presented as mean cell number ± SEM ($\times 10^6$).

**, $p < 0.01$ compared to nrlgG and 4-1BBL groups.

experimentally induced diseases, including autoimmune diseases such as rheumatoid arthritis (25, 26), cancer (15, 19), graft-vs-host disease (27), and infectious diseases (28). However, it has not yet been reported whether these Abs also affect experimental allergic diseases. EC, an experimental AC, is mediated by Th2 cells and has been demonstrated to be negatively regulated by IFN- γ (29, 30). Therefore, in this study, we have sought to determine whether the agonistic anti-4-1BB Ab treatment can suppress EC and whether this may be mediated by the up-regulation of IFN- γ .

We observed that treatment with the agonistic anti-4-1BB Ab during the induction phase of EC suppressed the infiltration of eosinophils into the conjunctiva. In contrast, the blocking anti-4-1BBL Ab had little, if any, effect on eosinophil infiltration. However, the treatment with anti-4-1BBL Ab did significantly suppress the proliferation of splenocytes against RW and the total serum IgE levels. The reason for this discrepancy between eosinophil infiltration and immune responses in anti-4-1BBL Ab-treated mice remains unclear. Because suppression of immune responses in anti-4-1BBL Ab-treated mice was less than that in anti-4-1BB Ab-treated mice, the immune responses in anti-4-1BBL Ab-treated mice might be above the threshold for the development of EC. In contrast, we found the forced stimulation of 4-1BB actively suppressed the sensitization. This effect on sensitization could be transferred to naive mice by the splenocytes, because the transfer of anti-4-1BB-treated splenocytes induced significantly less infiltration of eosinophils into the conjunctiva. Therefore, to investigate the mechanism that is affected by anti-4-1BB Abs, we examined the splenocytes. The splenocytes from anti-4-1BB Ab-treated mice showed markedly impaired proliferation and produced lower amounts of IL-4 and IL-13 upon RW stimulation. Moreover, the spleens of anti-4-1BB Ab-treated mice were larger and contained a higher ratio of CD8⁺ T cells, especially CD8⁺CD11c⁺ cells, and the splenocytes produced less IL-4, IL-5, and IL-13 and more IFN- γ upon Con A stimulation. These alterations in splenic phenotype are in accordance with previous reports demonstrating the suppressive effect of anti-4-1BB Abs on collagen-induced arthritis (25, 26). In addition, similar to the previous report demonstrating that anti-4-1BB Abs abrogated T cell-dependent humoral immune responses in vivo (22), we found total serum IgE levels (whose production is supported by Th2 cells) were significantly suppressed by the treatment with anti-4-1BB Abs. Thus, the treatment

with anti-4-1BB Abs during the induction phase generally suppressed Th2 responses in vivo.

It was demonstrated that transfer of Ag-primed CD8⁺ T cells has a potent suppressive effect on late allergic airway responses in Brown Norway rats (31). In addition, it also has been demonstrated recently that adoptively transferred in vitro cultured CD8⁺ T cells are capable of suppressing allergic airway eosinophilia in an Ag-independent, but IFN- γ -dependent manner in mice (32). Thus, CD8⁺ T cells are likely to have suppressive potential for the development of allergic diseases. Furthermore, transfer of CD8⁺CD11c⁺ cells from anti-4-1BB Ab-treated mice suppressed collagen-induced arthritis (26). Together with our finding that CD8⁺ cells, especially CD8⁺CD11c⁺ cells, increased in the spleen of anti-4-1BB Ab-treated mice, it could be considered that splenic CD8⁺ cells may have the suppressive capability in the development of EC. To examine this possibility, we adoptively transferred 5×10^7 splenocytes from nrlgG-treated and anti-4-1BB Ab-treated mice into naive mice and then induced EC by active immunization. The average infiltrating eosinophil numbers were not significantly different (353 in nrlgG-treated mice and 391 in anti-4-1BB Ab-treated mice). Although purified CD8⁺ cells were not transferred in our study, the splenocytes from anti-4-1BB Ab-treated mice are less likely to contain suppressive regulatory cells. Detailed analysis as to how CD8⁺ T cells are involved in the development of EC will be required.

In a previous report (33), IFN- γ was essential for the generation of tumor-specific immune responses by the treatment with anti-4-1BB Abs. To investigate whether the suppressive effect of agonistic anti-4-1BB Abs on the development of EC was mediated by IFN- γ , we induced EC in GKO mice and treated the mice with nrlgG or anti-4-1BB Abs. The suppressive effect of anti-4-1BB Abs was not impaired in GKO mice (Fig. 4), as compared with that in WT mice (Fig. 1). This indicates that the suppressive effect of agonistic anti-4-1BB Abs was not mediated by up-regulation of IFN- γ , but most likely mediated by suppression of Th2 responses. The mechanism by which the anti-4-1BB Ab treatment suppressed the Th2 responses independently of IFN- γ remains to be determined. It was recently reported that 4-1BB expressed on CD4⁺ T cells may play a negative regulatory role (34). Moreover, it also has been reported recently that the agonistic anti-4-1BB Abs induced activation-induced cell death of pathogenic CD4⁺ T cells in

Table II. Flow cytometric analysis of splenocytes from actively immunized mice: percent positive^a

	CD3 ⁺	CD4 ⁺	CD8 ⁺	B220 ⁺	F4/80 ⁺	CD8 ⁺ CD11c ⁺
nrlgG	40.1 ± 3.0	26.2 ± 1.6	13.9 ± 0.9	50.9 ± 1.3	4.6 ± 0.3	1.2 ± 0.2
4-1BB	56.8 ± 2.1**	32.5 ± 1.8**	24.0 ± 1.0**	37.3 ± 2.1**	6.2 ± 0.4*	4.4 ± 1.0**
4-1BBL	42.6 ± 3.2	28.2 ± 2.5	12.8 ± 0.9	54.2 ± 3.1	3.5 ± 0.1	0.6 ± 0.2

^a Mice were actively immunized with RW and i.p. injected with nrlgG, anti-4-1BB or anti-4-1BBL, as detailed in *Materials and Methods*. Harvested splenocytes were analyzed by flow cytometric analysis and data are presented as percent positive ± SEM.

*, $p < 0.05$. **, $p < 0.01$ compared to nrlgG and 4-1BBL groups.

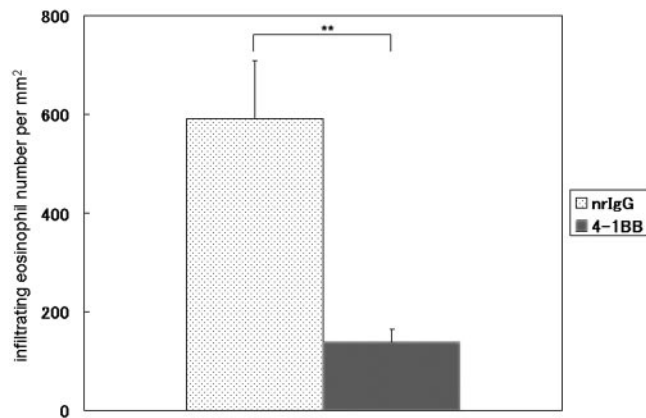


FIGURE 4. Treatment with agonistic anti-4-1BB Abs during the induction phase of EC inhibits eosinophilic infiltration into the conjunctiva of GKO mice. BALB/c GKO mice were immunized with RW in alum and then i.p. injected with 200 μ g of nrIgG ($n = 9$) or anti-4-1BB Abs ($n = 9$) on days 0, 2, 4, 6, and 8. On day 10, the mice were challenged with RW in eye drops, and their conjunctivas were harvested 24 h later for histologic analysis. The infiltrating eosinophils were counted, and the data are expressed as eosinophils per mm². **, $p < 0.01$.

a chronic graft-vs-host disease model (35). Therefore, the anti-4-1BB Abs might act on RW-specific CD4⁺ T cells to prevent their expansion. Alternatively, the agonistic anti-4-1BB Ab treatment might induce CD8⁺ T cells exerting TGF- β -mediated suppression of CD4⁺ T cell responses, as previously reported (36). Further studies are needed to address these possibilities.

Finally, we investigated the inhibitory effects of anti-4-1BB Abs on the effector phase of EC. Although the inhibitory effect was milder than that in the induction phase, the Ab still inhibited eosinophilic infiltration. This inhibition was dose dependent, because significant suppression was noted when the treatment was conducted twice, but not once. Although the suppression was statistically significant, percentage of inhibition of eosinophil infiltration was <30%. Therefore, to confirm the suppressive effect of anti-4-1BB Abs during the effector phase, it is necessary to optimize the treatment protocol by examining the dose of Abs and the timing of treatment.

Disclosures

The authors have no financial conflict of interest.

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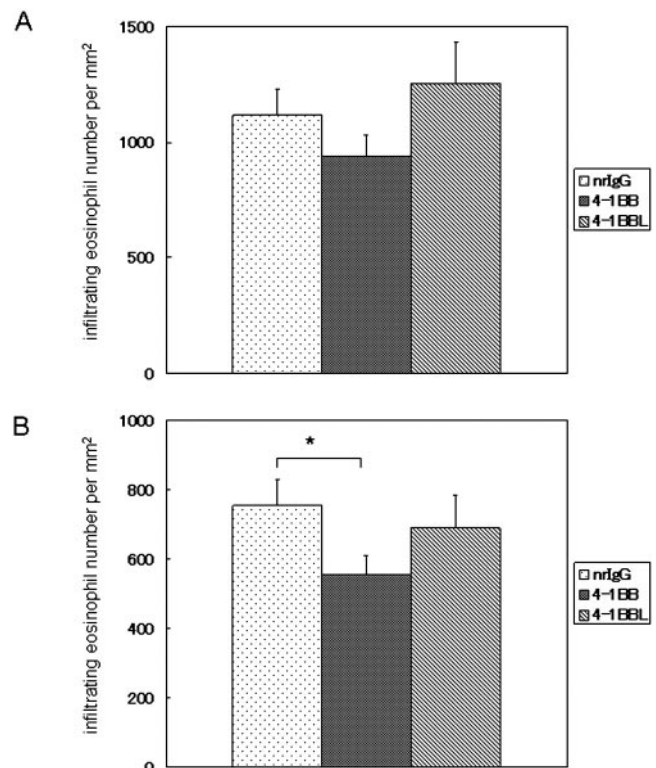


FIGURE 5. Treatment with agonistic anti-4-1BB Abs during the effector phase of EC inhibits the infiltration of eosinophils into the conjunctiva. BALB/c WT mice were immunized with RW in alum and not treated with any Abs. Ten days later, their spleens were harvested and the splenocytes were stimulated in vitro with RW and then transferred into naive BALB/c WT mice. The mice were i.p. injected with 200 μ g of nrIgG ($n = 10$), anti-4-1BB Abs ($n = 10$), or anti-4-1BBL Abs ($n = 10$) for once (A, on day 4) or twice (B, on days 2 and 4). Just after the injection of Abs on day 4, the mice were challenged with RW in eye drops. Twenty-four hours later, conjunctivas were harvested for histologic analysis. The infiltrating eosinophils were counted and the data are expressed as eosinophils per mm². *, $p < 0.05$.

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