Roles of OX40 in the Development of Murine Experimental Allergic Conjunctivitis: Exacerbation and Attenuation by Stimulation and Blocking of OX40

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PURPOSE. To investigate the roles of interaction between OX40 and OX40 ligand (OX40L) in the development of experimental allergic conjunctivitis (EC) in mice.

METHODS. BALB/c mice actively immunized with short ragweed pollen (RW) were intraperitoneally injected on days 0, 2, 4, 6, and 8 with agonistic anti-OX40 Ab, blocking anti-OX40L Ab, or normal rat (nr)IgG. On day 10, the mice were challenged with RW in eye drops, and 24 hours later their conjunctivae, spleens, and blood were harvested for analyses. For examination of the effects of the Abs during the late induction (or effector) phase, actively immunized mice were treated with the Abs just before or at the same time as the challenge. In addition, splenocytes from RW-primed mice were transferred into syngeneic naïve mice, and the recipients were treated with Abs twice (on days 2 and 4). On day 4, the mice were challenged with RW and evaluated.

RESULTS. When the treatments were performed during the induction phase, anti-OX40 Ab treatment significantly increased clinical EC and eosinophil infiltration into the conjunctiva, whereas anti-OX40L Ab treatment significantly reduced eosinophil infiltration. Compared with splenocytes from nrIgG-treated mice, splenocytes from anti-OX40 Ab-treated mice proliferated vigorously against RW and produced significantly higher amounts of IL-2, -4, and -5 by RW stimulation but a significantly lesser amount of IFN-γ after Con A stimulation. In contrast, splenocytes from anti-OX40L Ab-treated mice produced significantly less IL-5 with RW stimulation and IL-2 and IL-5 with Con A stimulation, whereas significantly more IFN-γ was induced by Con A stimulation. Treatment with anti-OX40 and anti-OX40L Abs during the late induction or effector phase of EC did not affect eosinophil infiltration.

CONCLUSIONS. Blocking of the interaction between OX40 and OX40L in vivo inhibits the development of EC. In contrast, forced stimulation of OX40 in vivo significantly exacerbates EC by activating T cells, especially Th2 cells. These effects were noted only in the induction phase of EC, suggesting that the interaction between OX40 and OX40L is important in the generation of Th2 immune responses in the development of EC.

A llergic conjunctivitis (AC) is one of the most common ocular diseases. It is estimated that it affects more than 20% of the population in the industrialized countries such as the United States.1 Seasonal and perennial AC, which are not sight threatening, are the most common types of AC.2 However, severe types of AC, such as vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC), cause conjunctival tissue and corneal damage and may lead to blindness.3-5 The prominent pathologic changes in these severe types are giant papillae formation that is composed of proliferation of fibroblasts and infiltration of inflammatory cells.2-4 Among the infiltrating cells, eosinophils are considered to be involved in the development of severe AC, based on the fact that severity of AC is in parallel with the number of infiltrating eosinophils in tears.5 Furthermore, inflammatory mediators released from eosinophils are abundantly detected in the damaged conjunctiva and cornea.6-8 Therefore, the idea that eosinophils are the major effector cells in severe AC is generally well accepted.

Ag-specific immune responses initiate the development of AC. However, eosinophils do not have Ag-specific receptors.6 Therefore, certain types of cells that have Ag-specific receptors have been considered to be involved in the initiation of AC. To clarify this question, we established an experimental model of AC in rats9,10 and mice11,12 and designated it experimental immune-mediated blepharoconjunctivitis (EC). From the series of experiments, we confirmed that Ag-specific T cells, especially Th2 type cells, play a pivotal role in the infiltration of inflammatory cells, including eosinophils.

Acquiring effector function in T cells requires activation. Optimal activation of naïve T cells requires two signals: one from the T cell receptor and the other from costimulatory molecules.13 Among costimulatory molecules, CD28, which is constitutively expressed in T cells, has been characterized most extensively.14,15 In addition to CD28, molecules recently classified within the TNF receptor superfamily have been identified as essential in T-cell activation.16 In contrast to CD28, these molecules are inducible in T cells by activation. An example is OX40 (CD134),17 which is expressed predominantly in CD4+ T cells. By using agonistic or antagonistic Abs and gene-deficient mice, OX40 has been identified to be involved in memory formation of CD4+ T cells.18-21 In addition, signals from OX40 facilitate Th2 development in vitro22-24 and in vivo, by the fact that blockade of OX40 ligand (OX40L) in Leishmania-infected BALB/c mice inhibits Th2-associated Ab production and induces inflammatory CD4 Th1 responses.25 Furthermore, in experimental airway inflammation in mice, blockade of OX40L attenuates airway hypersensitivity reaction and airway eosinophilia.26-28 These reports strongly suggest that OX40 is positively involved in the generation of Th2-type immune responses. However, in experimental autoimmune disease29 and graft-versus-host disease,30,31 in which Ag is
continuously present, the OX40 signal induces proliferation of Th1 cells. Therefore, it is still controversial whether OX40 is involved in Th1 and Th2 differentiation.

Although the roles of OX40 in the development of murine experimental asthma have been reported, the involvement of OX40 in AC has not been reported. In the present study, we sought to investigate the roles of OX40 in the development of murine AC (EC), which is predominantly mediated by Th2 cells, by using both agonistic and antagonistic Abs.

**MATERIALS AND METHODS**

**Mice**

Inbred wild-type (WT) BALB/c mice were purchased from Japan SLC Inc., (Hamamatsu, Shizuoka, Japan). The mice were kept in specific 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 weeks old. All research adhered to the ARVO 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 from LSL Co. Ltd. (Tokyo, Japan); a hybridoma producing an Ab to OX40 (OX80)32 from European Cell Culture Collection (Porton Down, UK). A hybridoma producing an Ab to OX40 L (RM134L)33 was established as described previously. These Abs for in vivo treatments were purified from ascites by using a protein G column and contained less than 100 pg/mL endotoxin. The following Abs were purchased: normal rat IgG (nrIgG; MP Biomedicals Inc., Aurora, OH), FITC-labeled anti-CD5 (145-2C11), and anti-CD4 (GK1.5) and biotin-labeled anti-CD8 (53-6.7; ebioscience, San Diego, CA), FITC-labeled anti-CD45RB/B220 (RA3-6B2), streptavidin-PE (BD Biosciences, Franklin Lakes, NJ), biotin-labeled anti-CD4+44 (IM7), and biotin-labeled anti-CD62L (MElli; BioLegend, San Diego, CA), and biotin-labeled anti-F4/80 (A5;1; Caltag Laboratories, Burlingame, CA).

**EC Induction by Active Immunization and Treatment with Abs**

RW adsorbed on alum was injected into the left hind footpad and the tail base. Fifty microliters of the emulsion (50 μg of RW and 2 mg of alum) was injected into each site. The mice were injected intraperitoneally with 200 μg of purified anti-OX40, anti-OX40L, or control rat IgG (n = 15 per group) on days 0, 2, 4, 6, and 8 after immunization. In a separate experiment, the actively immunized mice (n = 10 per group) were injected intraperitoneally with 200 μg of each Ab only once 2 hours before or at the same time as the RW challenge. Ten days after the active immunization, the eyes of the immunized mice were challenged with RW in PBS (2 μg in 10 μL per eye). Twenty-four hours later, photographs were obtained of the anterior segments of the eye, the eyes, sera, and spleens were harvested for histologic analysis, measurement of IgE levels, and T-cell culture for transfer, cytokine, and proliferation assays, respectively (Fig. 1A). As comparative control groups for the evaluation of eosinophil infiltration into the conjunctiva, naïve mice, Ab-untreated mice actively immunized with RW in alum that were challenged with PBS, and PBS mice were immunized with alum alone that were challenged with RW were used (n = 10 per group).

**EC Induction by Adoptive Transfer of Splenocytes from Actively Immunized and Ab-Treated Mice**

Splenocytes harvested from the actively immunized and 1 mg of the Ab-treated mice were cultured with RW extract at final concentrations of 5 μg/mL in 75-cm² flasks at a concentration of 10⁷ cells/mL in a final volume of 20 mL RPMI 1640 medium supplemented with 10% fetal calf serum (FCS; ICN Biomedical Japan Co., Tokyo, Japan), 2-mercaptoethanol (2-ME: 5 × 10⁻⁵ M), L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL). After incubation for 72 hours at 37°C in a humidified atmosphere with 5% CO₂ 2 × 10⁷ splenocytes were intraperitoneally injected into naïve BALB/c mice (n = 10; nrlgG; n = 11, anti-OX40; and n = 9, anti-OX40L). Four days after the 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 histologic analysis.

**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 the 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 hours at 37°C in a humidified atmosphere with 5% CO₂ 2 × 10⁷ splenocytes were intraperitoneally injected into syngeneic naïve BALB/c mice. Four days after the splenocyte transfer, the mice were injected intraperitoneally with 200 μg anti-OX40 (n = 8), anti-OX40L (n = 8), or nrIgG (n = 8) twice (on days 2 and 4). Two hours 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 histologic analysis (Fig. 1B).

**Histologic Analysis**

The eyes, including the conjunctivae, were harvested and fixed in 10% buffered formalin. Horizontal 2-μm-thick sections were cut and stained with Giemsa. Infiltrating eosinophils in the lamina propria mucosae of the tarsal and bulbar conjunctivae 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. The data are presented as the average ± SEM of all the mice examined.
Cell-Proliferation Assay
Red blood cell (RBC)-depleted splenocytes (3 x 10^7 cells/well) were cultured in 96-well flat-bottomed plates in a final volume of 0.2 mL 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 µg/mL or with concanavalin A (Con A) at 5 µg/mL. After an 80-hour incubation at 37°C in a humidified atmosphere containing 5% CO2, the cultures were pulsed for 16 hours with 0.5 µCi/well [3H]thymidine (Japan Atomic Energy Research Institute, Tokai, Japan). The cultures were then harvested, and the incorporated radioactivity was measured by standard techniques. The data were expressed as Δ counts per minute (mean cpm of stimulated cultures – mean cpm of unstimulated control cultures).

Measurement of Total and RW-Specific IgE in Serum
Twenty-four hours after RW challenge of actively immunized mice, the blood was collected, and serum was prepared. Either total or RW-specific IgE in the sera was measured by ELISA. Briefly, either affinity-purified anti-mouse IgE (2 µg/mL; eBioscience) or RW (2 µg/mL) was used to coat a 96-well EIA plate (Corning-Costar, Corning, NY) overnight at 4°C. The plates were then washed and incubated with blocking buffer (1% bovine serum albumin in PBS) for 3 hours at room temperature. The plates were washed again and the samples or IgE standards (either TNP-KLH-specific IgE [BD Biosciences] or quantified RW-specific IgE; kindly provided by Fu-Tong Liu, University of California, Davis) were applied to each well. After 2 hours' incubation at room temperature, the plates were washed, and biotin-conjugated rat anti-mouse IgE (BD Biosciences) was added to each well for 1 hour at room temperature. After the plates were washed, avidin-alkaline phosphatase (Sigma-Aldrich) was added to each well for 1 hour. After another washing, the substrate p-nitrophenyl phosphatase (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.

Measurement of Cytokines in the Culture Supernatants
RBC-depleted splenocytes (10^7 cells/mL) were cultured for 48 hours with Con A (5 µg/mL) or RW (25 µg/mL) in 96-well flat-bottomed plates in a final volume of 0.2 mL RPMI 1640 medium supplemented with 10% FCS and 2-ME. The levels of IL-2, IL-4, IL-5, and IFN-γ produced were measured by using commercially available ELISA kits (Duoset; R&D Systems, Minneapolis, MN), 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, and cytokine production, and the number of infiltrating eosinophils were tested for significance by Student’s t-test. P < 0.05 was considered significant.

RESULTS
Effect of Treatment with Anti-OX40 or Anti-OX40L Ab during the Induction Phase of EC
To examine whether the interaction between OX40 and OX40L is essential for the development of EC, we treated actively immunized, EC-developing mice with an agonistic anti-OX40 Ab and a blocking anti-OX40L Ab during the induction phase. Late-phase clinical findings (24 hours after RW challenge) demonstrated that all the mice treated with anti-OX40 Ab exhibited severe lid swelling and discharge (Fig. 2B), whereas nrIgG- (Fig. 2A) and anti-OX40L-treated mice (Fig. 2C) showed mild conjunctival hyperemia. In accord with clinical findings, massive infiltration of eosinophils into the conjunctiva was demonstrated in anti-OX40-treated mice (Fig. 3B) compared with the nrIgG-treated mice (Fig. 3A). Fewer eosinophils were observed in anti-OX40L-treated mice (Fig. 3C) than in nrIgG-treated mice. Counting of infiltrating eosinophils confirmed that there was significantly more eosinophil infiltration in anti-OX40-treated mice and significantly less in anti-OX40L-treated mice than in nrIgG-treated mice (Fig. 3D). Compared with the control groups (naive, RW/alum immunized and PBS challenged, and alum immunized and RW challenged), infiltration of eosinophils was significantly more in RW/alum immunized and RW challenged mice that were treated with Abs (Fig. 3D), indicating that the mice were properly sensitized with RW.

Effect of Transfer of Splenocytes from Actively Immunized Mice Treated with Anti-OX40 or Anti-OX40L Ab on Eosinophil Infiltration
EC can be transferred by Ag-primed splenocytes. Consequently, we next examined the severity of eosinophil infiltration into the conjunctiva of naïve BALB/c mice upon adoptive transfer of the splenocytes from the actively immunized mice after the treatment with anti-OX40 Abs, anti-OX40L Abs, or nrIgG. The splenocytes from anti-OX40-treated mice induced significantly more eosinophil infiltration than those from nrIgG-treated mice. In contrast, the splenocytes from anti-OX40L-treated mice induced significantly less eosinophil infiltration (Fig. 4).

Effect of Treatment with Anti-OX40 and Anti-OX40L Abs on Immune Responses
The findings thus far indicated that the agonistic anti-OX40 Ab exacerbated and blocking anti-OX40L Ab inhibited EC, possibly...
by modifying the function of splenocytes. To examine this further, we evaluated the effect of Ab treatment on immune responses of the splenocytes from actively immunized mice by in vitro assays. In addition, total and RW-specific serum IgE levels were evaluated. The number of recovered splenocytes was similar among the three groups, and flow cytometric analysis showed that the expression levels of CD3, CD4, CD8, CD25, CD44, CD45R, CD62L, and F4/80 were similar (data not shown). The splenocytes from anti-OX40-treated mice proliferated significantly more vigorously against RW (Fig. 5A) and Con A (Fig. 5B). In contrast, splenocytes from anti-OX40L-treated mice did not show significant proliferation against RW (Fig. 5A). Analyses of the cytokine production by the cultured splenocytes revealed that the RW-stimulated splenocytes from the agonistic anti-OX40 Ab-treated mice produced more IL-2, -4, and -5 than did the other two groups (Fig. 5C). In addition, Con A-stimulated splenocytes from the agonistic anti-OX40-treated mice produced more IL-4 and -5 and less IFN-γ than did the other two groups (Fig. 5C). When nrgG-treated and anti-OX40L-treated groups were compared, Con A stimulation induced significantly less IL-2 and -5 but more IFN-γ in anti-OX40L-treated mice (Fig. 5C). Finally, both anti-OX40 Ab-treated mice and anti-OX40L Ab-treated mice had significantly lower total serum IgE levels than did nrgG-treated mice (Fig. 5D), whereas RW-specific IgE levels were significantly lower in anti-OX40 Ab-treated mice than in the other two groups (Fig. 5E).

**Effect of Treatment with Anti-OX40 or Anti-OX40L Ab on EC during the Late Induction or Effector Phase**

Next, we sought to examine the effects of anti-OX40 and anti-OX40L Abs during the effector phase of EC. To this end, we treated the actively immunized mice with these Abs just before (Fig. 6A) or at the same time as RW challenge (Fig. 6B) and treated the passively immunized mice by splenocyte transfer twice (Fig. 7). By either method, both anti-OX40 and anti-OX40L Abs neither increased nor decreased eosinophil infiltration into the conjunctiva (Figs. 6, 7).

**DISCUSSION**

In this study, stimulation of OX40 in vivo exacerbated clinical and histologic EC, whereas blocking of the interaction be-
tween OX40 and OX40L in vivo attenuated histologic EC. The latter data are in good agreement with previous reports in experimental asthma in mice in which used anti-OX40L Ab and OX40L-deficient mice were used.26–28 In accord with these previous reports,26–28 Th2 immune responses including total serum IgE levels and IL-5 production in the culture supernatants were lower in anti-OX40L Ab-treated, anti-OX40 Ab-treated, and anti-OX40L Ab-treated groups, respectively. *P < 0.001 compared with nrIgG and anti-OX40L. The supernatants of splenocytes cultured with RW and Con A, or without any stimulants, were harvested for measuring the concentrations of IL-2, IL-4, IL-5, and IFN-γ by ELISA (C, *P < 0.01, **P < 0.05). Total IgE (D) or RW-specific IgE (E) in serum was measured by ELISA (*P < 0.01, **P < 0.05).

A more striking result is that anti-OX40 Ab treatment significantly upregulated eosinophil infiltration. This increase may be explained by the data that RW-stimulated splenocytes proliferated extremely vigorously against RW and produced substantially increased amounts of IL-2, -4, and -5. These data indicate that anti-OX40 Ab treatment induced expansion of RW-specific T cells and upregulation of Th2 immune responses. A contradictory finding was that total and RW-specific IgE levels in serum were significantly lower than in the other two groups, and the reason is still unclear. However, together
with the data that transfer of splenocytes from the mice treated with anti-OX40 Ab induced significantly severe eosinophil infiltration, it is acceptable that severe infiltration of eosinophils in anti-OX40 Ab-treated mice is due to highly Th2-polarized splenocytes. Previous reports demonstrated that triggering OX40 signaling exacerbates experimental autoimmune encephalomyelitis,\textsuperscript{34,35} which is a Th1-mediated autoimmune disease and is different from Th2-mediated EC. Taken together, it could be considered that OX40 contributes to upregulation of ongoing Th1 and Th2 immune responses. There have been reports that the interaction between OX40 and OX40L plays a role in T-cell migration. OX40-deficient mice exhibit two- to threefold decreases in accumulation of CD4\textsuperscript{+} T cells in the bronchoalveolar lavage after intranasal infection with influenza virus.\textsuperscript{36} In addition, blockade of OX40L with OX40-Ig decreases CXCR5 expression by CD4\textsuperscript{+} T cells and ameliorates chronic colitis.\textsuperscript{37} These results suggest that the interaction between OX40 and OX40L is involved in the recruitment of T cells into the inflammatory site. However, eosinophil infiltration was not affected by treatment with either anti-OX40 or anti-OX40L Ab, when the Abs were administered on day 10 (2 hours before or at the same time of RW challenge) in actively induced EC. Furthermore, Ab treatment on days 2 and 4 in mice after splenocyte transfer did not affect eosinophil infiltration. Eosinophil infiltration in EC by splenocyte transfer is predominantly mediated by CD4\textsuperscript{+} T cells in BALB/c mice.\textsuperscript{12,27} Therefore, in EC, the interaction between OX40 and OX40L is less likely to be involved in T-cell recruitment. Rather, from the data that treatment with the Abs affected EC during the induction phase, this interaction seems to be essential for the generation of Th2 immune responses in the development of EC.

It has been reported that treatment of C57BL/6 mice with anti-OX40L Ab effectively regulates memory T-cell reactivation and therefore inhibits lung eosinophilia and airway hyperreactivity.\textsuperscript{28} On the contrary, it was reported that treatment of BALB/c mice with anti-OX40L Ab during the sensitization period abolished the induction of asthmatic responses, whereas treatment during the challenge period did not.\textsuperscript{27} The latter data are in good agreement with our results that were obtained in BALB/c mice. Thus, the fact that effects of OX40L blockade are different between C57BL/6 and BALB/c mice may indicate that the efficiency of OX40–OX40L interaction depends on the genetic background of the mice.

Our recent studies focusing on 4-1BB, another TNF receptor superfamily, demonstrated that agonistic stimulation of 4-1BB during the induction phase of EC suppresses eosinophil infiltration into the conjunctiva and increases CD8\textsuperscript{+} T cell compartment in splenocytes.\textsuperscript{39} From the data in the present report, it can be concluded that agonistic stimulation of OX40 during the induction phase of EC upregulated eosinophil infiltration, but blocking of the interaction between OX40 and OX40L suppressed eosinophil infiltration. The distinctively different function of 4-1BB and OX40, both of which are classified within the same TNF receptor superfamily, shows the biological complexity of T-cell-related costimulatory molecules in the development of AC. Analyses of these important costimulatory molecules one by one will provide a better understanding of the pathophysiological roles of T cells in the development of AC.

**References**
