

The interaction between ICOS and B7RP-1 is not required for the development of experimental murine allergic conjunctivitis

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Abstract

It is still unclear whether the interaction between inducible costimulator (ICOS) and its ligand, B7 related protein (B7RP)-1, is important for the development of allergic diseases. We investigated whether blocking the ICOS/B7RP-1 interaction affects the development of murine experimental allergic conjunctivitis (EC). EC was induced in Balb/c mice either by active immunization of ragweed (RW) or by transferring RW-primed splenocytes, followed by challenge with RW-containing eye drops. The mice were treated with anti-B7RP-1 antibody (Ab) or normal rat immunoglobulin G (IgG) during either the induction or effector phase. Regardless of the induction method or when the animals were treated, eosinophil infiltration into the conjunctiva was not affected by the anti-B7RP-1 Ab treatment. Splenocyte responses were not largely affected by this treatment. However, serum Ig levels were significantly reduced. These data suggest that blocking the ICOS/B7RP-1 in allergic diseases may not always be therapeutic.

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Vernal keratoconjunctivitis (VKC), the most severe type of allergic conjunctivitis (AC), causes tissue damage not only in the conjunctiva but also in the cornea, and therefore is a vision-threatening disease [1]. Fibroblast proliferation and massive infiltration of inflammatory cells are the typical pathological features of VKC [2]. While the predominant effector cells in VKC are believed to be eosinophils and mast cells, many CD4⁺ T cells also infiltrate into the conjunctiva [3,4]. Although the IgE-mediated type I allergic reaction is apparently involved in the pathogenesis of VKC [1–4], we have demonstrated previously that this reaction alone is not sufficient to induce the severe infiltration of inflammatory cells into the conjunctiva in murine experimental AC (experimental immune-mediated blepharoconjunctivitis, EC) [5].

Furthermore, the transfer of Ag-specific T cells into naïve animals followed by Ag challenge in the conjunctiva induces severe infiltration of eosinophils into the conjunctiva [6–8]. Thus, CD4⁺ T cells are likely to play a central role in the infiltration of inflammatory cells into the conjunctiva. A further detailed analysis using signal transducer and activator of transcription (STAT)-6-deficient, suppressor of cytokine signaling (SOCS)-3-heterozygous and SOCS-5-transgenic mice confirmed that Th2 type CD4⁺ T cells play an essential role in the development of EC [9]. These experimental animal data together with the finding that Th2 cytokines are abundantly detected in the tear fluids and conjunctiva of VKC patients [10–12] strongly support the notion that Th2 cells are critically involved in the development of severe types of AC.

To acquire effector function, T cells must be activated. Activation of naïve T cells requires both signal 1 from

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the T cell receptor and signal 2 from costimulatory molecules [13]. One of these costimulatory molecules is CD28 which appears to provide a critical signal for IL-2 production and cell-cycle progression in naïve T cells [14]. Recently, inducible costimulator (ICOS), a new member of the CD28 family, was identified [15]. Like CD28, ICOS augments T-cell differentiation and cytokine production and provides critical signals for the production of immunoglobulins (Igs) [16]. However, in contrast to CD28, ICOS costimulation has not been shown to upregulate IL-2 production [17]. Furthermore, unlike CD28, ICOS expression is inducible and Th2 cells continue to express ICOS after activation [18,19]. Several studies have demonstrated that ICOS costimulation induces Th2 differentiation and blockade of ICOS suppresses Th2-mediated airway inflammation [20–23]. However, another study showed that a deficiency of B7 related protein-1 (B7RP-1), the ICOS ligand, rather augments Th2-mediated inflammation in the airway [24]. Thus, it remains unclear whether ICOS blockade can be used to inhibit Th2-mediated allergic diseases. Here, we investigated whether blocking the interaction between ICOS and B7RP-1 suppresses the development of EC, which is predominantly mediated by Th2 cells.

Materials and methods

Mice. Inbred wild-type (WT) Balb/c mice were purchased from Japan SLC (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 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 Poly-sciences, (Warrington, PA). RW extract was obtained from LSL (Tokyo, Japan). Aluminum hydroxide (alum) was purchased from Sigma (St. Louis, MO). Anti-B7RP-1 mAb (HK5.3, rat IgG2a) was prepared as described previously [25] and was purified from ascites fluid using a Protein G column. It contained less than 100 pg/ml endotoxin. Normal rat IgG (nrIgG) was purchased from MP Biomedicals (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 of RW and 2 mg of alum) was injected into each site. The mice were injected intraperitoneally with 200 µg of purified anti-B7RP-1 or control rat IgG ($n = 10$ per group) on days 0, 2, 4, 6, and 8 after RW immunization. On day 10, the eyes of the immunized mice were challenged with RW in PBS (2 mg in 10 µl per eye). In a separate experiment, the actively immunized mice ($n = 10$ per group) were injected intraperitoneally with 200 µg of either Ab on day 10 only, 2 h before RW challenge. Twenty-four hours after the RW challenge, the eyes, sera, and spleens were harvested for histological analysis, measurement of Ig levels, and flow cytometric analysis, cytokine production or proliferation assays, respectively.

EC induction by adoptive transfer of in vitro-stimulated RW-primed splenocytes and treatment with Abs. Naïve Balb/c mice were immunized by injection of RW both in their left hind-footpad and at the base of the tail. Ten days later, splenocytes were prepared and cultured with RW extract at final concentrations of 5 µg/ml in 75-cm² flasks at a concentration of 10^7 cells per ml in a final volume of 20 ml RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, ICN Biomedical Japan, 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 h at 37 °C in a humidified atmosphere with 5% CO₂, 2×10^7 splenocytes were intraperitoneally injected into syngeneic naïve Balb/c mice. After the splenocyte transfer, the mice were injected intraperitoneally with 200 µg anti-B7RP-1 or nrIgG ($n = 10$ per group) 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). The eyes were harvested for histological analysis 24 h after the challenge.

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 blind samples. The sections counted were those from the central portion of the eye, which included the pupil and optic nerve head. Since the counts vary depending on the counting area, the cell count data are expressed as infiltrating eosinophil numbers divided by area (mm²) as measured by Scion Image (Scion Corporation, Frederick, MD). The data are presented as averages $\pm$ SEM of all the mice examined.

Cellular proliferation assay. RBC-depleted splenocytes (3×10^5 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 a 72 h incubation at 37 °C in a humidified atmosphere containing 5% CO₂, the cultures were pulsed for 16 h 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 the change in counts per minute [cpm] (delta cpm: mean cpm of stimulated cultures – mean cpm of unstimulated control cultures).

Measurement of cytokines in the culture supernatants. RBC-depleted splenocytes (10^7 cells/ml) were cultured for 48 h 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. As the control, splenocytes were cultured in the same manner without any stimulants. 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, Minneapolis, MN) 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. Briefly, EIA plates (Costar, Corning, NY) were coated with goat anti-mouse IgG (2 µg/ml, Sigma) or affinity-purified anti-mouse IgE (2 µg/ml, eBioscience, San Diego, CA). After blocking with 1% bovine serum albumin in PBS, serum samples that had been diluted from 1/100 to 1/10,000 in PBS were added in duplicate and incubated for 2 h at room temperature or overnight at 4 °C. The plates were washed with PBS plus 0.05% Tween 20 (PBS/T) (Wako, Osaka, Japan) and incubated for 2 h at room temperature with alkaline phosphatase (ALP)-conjugated goat antibodies specific for IgG, IgG₁, and IgG_{2a} (Zymed, San Francisco, CA). In the case of IgE, biotin-conjugated rat anti-mouse IgE (BD Biosciences, Franklin Lakes, NJ) was added to each well for 2 h at room temperature. After washing, avidin-ALP (Sigma) was added to each well for 1 h. After washing with PBS/T, *p*-nitrophenyl phosphate (*p*-nitrophenyl phosphate liquid substrate system, Sigma) was added to each well and allowed to develop for 15 min. Absorbance was measured at 405 nm. Ig concentrations were calculated by using the linear ranges of the dilution and standard curves generated with purified mouse IgG₁ (BioLegend, San Diego, CA), mouse IgG_{2a} (eBioscience), and mouse IgE (BD Biosciences).

Statistical analysis. Significant differences in serum Ig levels, splenocyte proliferation, cytokine production, and infiltrating eosinophil numbers between the anti-B7RP-1-treated and nrIgG-treated groups were determined by Student's *t* test. *P* values less than 0.05 were considered significant.

Results

The interaction between ICOS and B7RP-1 is not required for EC development during the induction phase

A previous report demonstrated that blocking ICOS every other day starting the day before immunization and continuing until two days before sacrifice reduced *Schistosoma mansoni*-induced airway inflammation in mice [21]. We therefore investigated whether the interaction between ICOS and B7RP-1 is also important for the development of EC during the induction phase. Thus, we induced EC by active immunization and treated the mice with a blocking anti-B7RP-1 Ab every other day starting on the day of immunization and continuing until day 8. The numbers of eosinophils infiltrating the conjunctiva did not significantly differ between the anti-B7RP-1-treated group and the nrIgG-treated group (Fig. 1). This result suggests that the ICOS/B7RP-1 interaction is not required for the induction phase of EC.

The interaction between ICOS and B7RP-1 is not required for EC development during the effector phase

A previous report suggested that ICOS is not critical for Ag priming and Th differentiation but regulates Th2 effector function; consequently, ICOS blockade just prior to Ag challenge reduced airway inflammation [20]. Furthermore, it was shown that ICOS blockade reduced the airway inflammation and airway hyper-reactivity induced by transferring highly polarized Th2 cells into naïve mice [18]. Therefore, we next examined whether blocking B7RP-1 during the effector phase inhibits eosinophil infiltration into the conjunctiva. Thus, actively immunized mice were treated with anti-B7RP-1 Ab only once on day 10 just before RW challenge. However, compared to nrIgG

treatment, this did not influence eosinophil infiltration into the conjunctiva (Fig. 2A). Furthermore, when passively immunized mice were treated with anti-B7RP-1 Ab twice (two days before and on the day of RW challenge), no effects on the infiltration of eosinophils were observed compared with nrIgG treatment (Fig. 2B). These results suggest that the interaction between ICOS and B7RP-1 is not required for the effector phase of EC.

B7RP-1 blockade does not affect splenocyte responses

That the ICOS/B7RP-1 interaction is unlikely to be involved in the infiltration of eosinophils in EC suggests that the treatment with anti-B7RP-1 Ab did not significantly affect the relevant immune responses in this experimental system. To examine this issue, we evaluated splenocyte responses as a marker for cellular immune responses. Flow cytometric analysis showed that the splenocytes from the two groups expressed CD3, CD4, CD8, CD25, F4/80, T1/ST2, and ICOS at equivalent frequencies and levels (data not shown). The splenocytes from the anti-B7RP-1 Ab-treated mice proliferated better against the lower concentrations of RW than the splenocytes from the nrIgG-treated mice; however, equivalent proliferation was observed when higher concentrations of RW were used (Fig. 3A). Con A-induced proliferation was similar between the two groups (Fig. 3B). The splenocytes from the two groups did not differ in the production of cytokines, except for IL-5 (RW stimulation), and IL-13 and IFN- γ (Con A stimulation) (Fig. 3C). These results suggest that the blockade of B7RP-1 did not profoundly affect cellular immune responses.

B7RP-1 blockade suppresses Ig levels in the serum

Finally, we investigated whether B7RP-1 blockade affected serum Ig levels, namely, humoral immune responses. Treatment with anti-B7RP-1 Ab significantly suppressed the total IgE levels in the serum (Fig. 4A) as well as the total IgG (Fig. 4B), IgG1 (Fig. 4C), and IgG2a (Fig. 4D) levels. Thus, the anti-B7RP-1 Ab treatment attenuated systemic humoral immune responses. However, this did not affect the development of EC.

Discussion

Most previous reports examining the effects of ICOS blockade on experimental airway inflammation demonstrated that it attenuated the development of this disease [20–23]. Contradicting this, however, was the report that airway inflammation was more severe in B7RP-1-deficient mice compared with WT littermates [24]. This controversy led us to investigate whether blocking the interaction between ICOS and B7RP-1 affects EC, an experimental AC.

We found that blocking the interaction between ICOS and B7RP-1 during either the induction or effector phase did not affect the development of EC. This suggests that

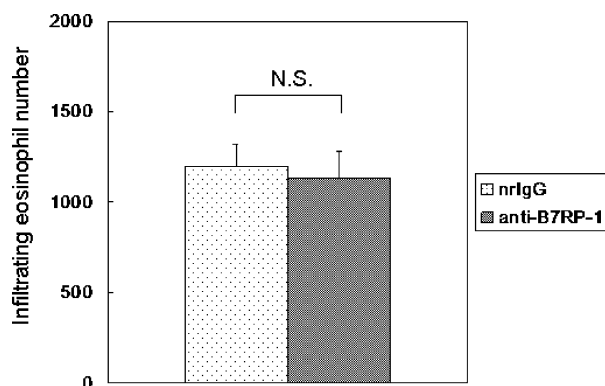


Fig. 1. Treatment with anti-B7RP-1 Ab during the induction phase of EC does not affect eosinophil infiltration into the conjunctiva. Balb/c mice were immunized with RW in alum and then intraperitoneally injected with 200 μ g nrIgG or anti-B7RP-1 Ab on days 0, 2, 4, 6, and 8. On day 10, the mice were challenged with RW in eye drops and their conjunctivas were harvested 24 h later for histological analysis by Giemsa staining ($n = 10$ mice per group). The infiltrating eosinophils were counted and the data are expressed as eosinophils per mm². N.S., not significantly different.

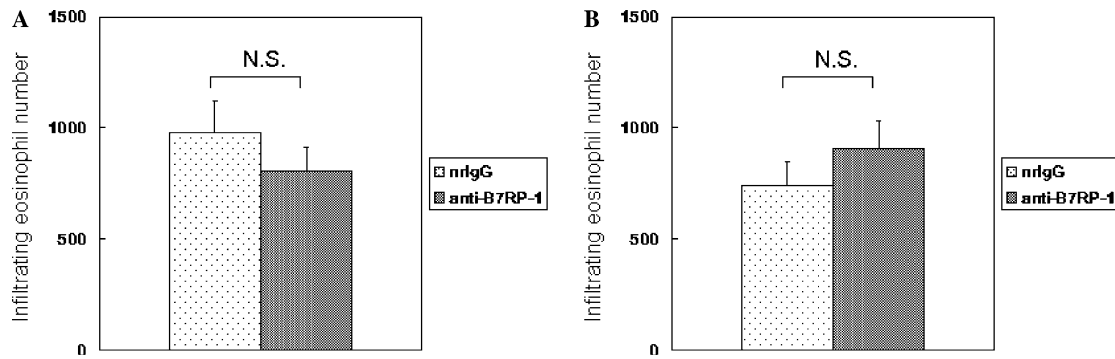


Fig. 2. Treatment with anti-B7RP-1 Ab during the effector phase of EC does not affect eosinophil infiltration into the conjunctiva. (A) Balb/c mice were immunized with RW in alum and then intraperitoneally injected with 200 μ g nrIgG or anti-B7RP-1 Ab once on day 10. Two hours later, the mice were challenged with RW in eye drops. Their conjunctivas were harvested 24 h later and subjected to histological analysis so that the infiltrating eosinophils would be counted ($n = 10$ mice per group). (B) RW-primed splenocytes were stimulated in vitro with RW and then transferred into naïve Balb/c mice. The mice were intraperitoneally injected with 200 μ g nrIgG or anti-B7RP-1 Ab twice on day 2 and 2 h prior to RW challenge on day 4. The conjunctivas of the mice were harvested 24 h later and subjected to histological analysis so that the infiltrating eosinophils could be counted ($n = 10$ mice per group). The data are expressed as eosinophils per mm^2 . N.S., not significantly different.

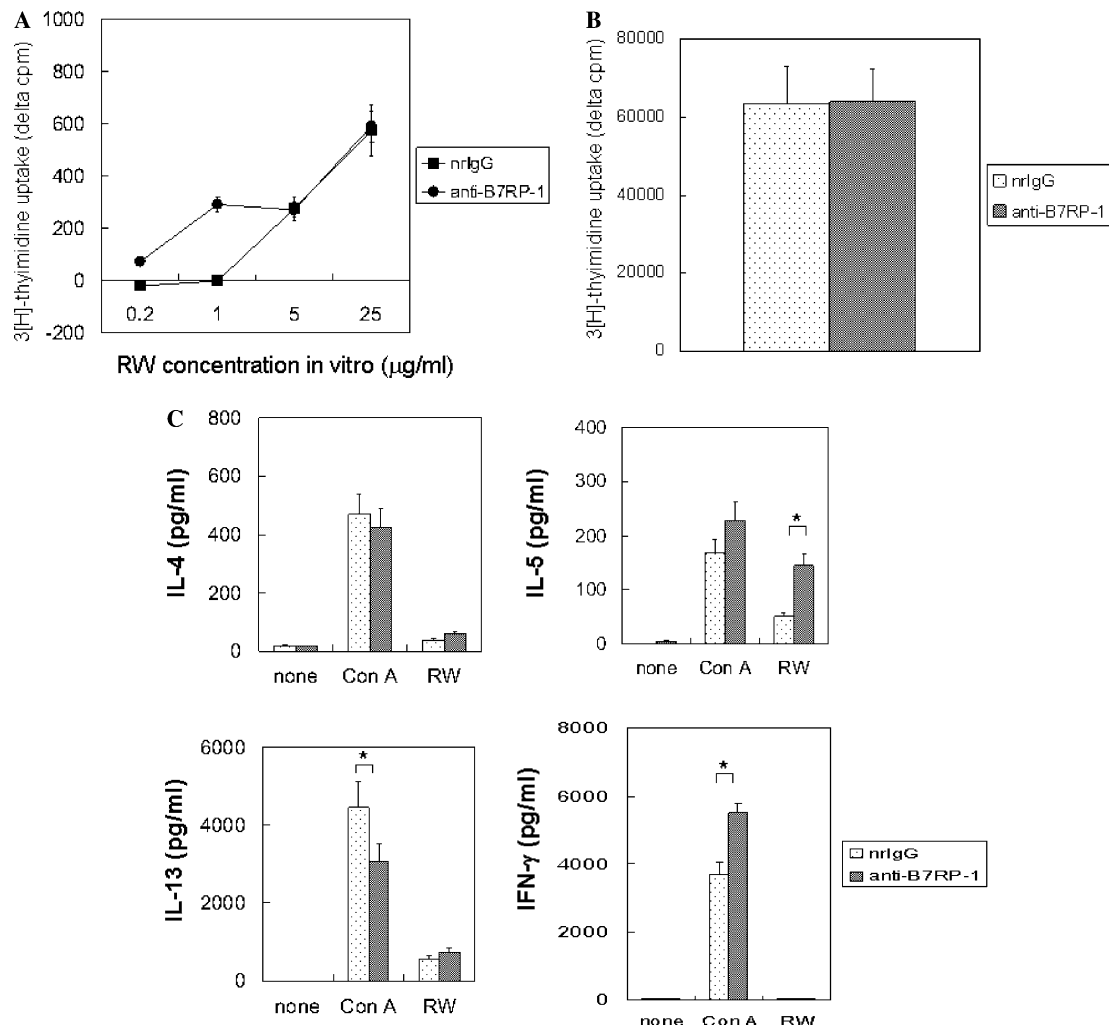


Fig. 3. Splenocyte responses. Splens were collected at the time of harvesting the conjunctivas from actively immunized mice treated 5 times with nrIgG or anti-B7RP-1 Ab as described in the legend to Fig. 1. The proliferative responses of the splenocytes against RW (A) or Con A (B) were evaluated by ^3H thymidine uptake and the data are expressed as delta cpm. Background cpms were 774 ± 22 in nrIgG-treated group and 753 ± 29 in anti-B7RP-1-treated group. The IL-4, IL-5, IL-13, and IFN- γ levels in the culture supernatants of splenocytes stimulated with RW or Con A were measured by ELISA (C). Culture supernatants of unstimulated splenocytes (none) served as the controls. * $P < 0.05$.

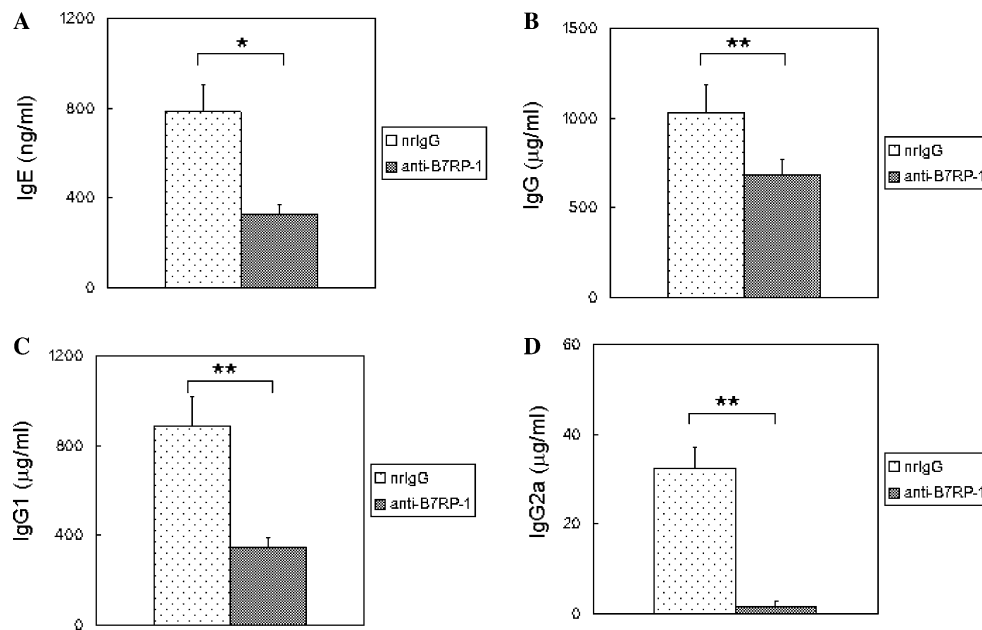


Fig. 4. Serum Ig levels. Sera were prepared from blood collected at the time of harvesting the conjunctivas from actively immunized mice treated five times with nrlgG or anti-B7RP-1 Ab as described in the legend to Fig. 1. Total IgE (A), IgG (B), IgG1 (C), and IgG2a (D) levels were evaluated by ELISA. * $P < 0.05$, ** $P < 0.01$.

the interaction between ICOS and B7RP-1 is not required for the development of EC. The differential effects of ICOS/B7RP-1 blockade with regard to experimental airway inflammation and EC may be due in part to the different experimental protocols involved: the experimental airway inflammation protocol requires repeated immunization and repeated challenge [20–24], whereas EC is generated by a single immunization and a single challenge. Other factors may also participate, such as the nature of the organs involved: unlike the eye, the lung contains more immunocompetent cells, lymphoid tissues, and vasculature. Nevertheless, the data reported here indicate that the ICOS/B7RP-1 interaction is not always critically involved in the development of allergic inflammation.

One possible explanation for the lack of effect of anti-B7RP-1 Ab on EC development is that this treatment did not significantly affect the relevant immune responses and therefore failed to modulate eosinophil infiltration. To examine this issue, we evaluated both the cellular and humoral immune responses of actively immunized animals treated during the induction phase of EC. The flow cytometric profile of the splenocytes and their proliferative and cytokine production responses were largely not affected by treatment with anti-B7RP-1 Ab. In contrast, the serum Ig levels were significantly reduced by anti-B7RP-1 Ab treatment. This confirms that treatment with anti-B7RP-1 Ab affects systemic immune responses, predominantly the humoral immune responses. Clearly, however, the altered humoral immune responses failed to influence the development of EC. This is consistent with the fact that while EC is mediated by both cellular ($CD4^+$ T cells) and humoral (IgE) immune responses [5,8], the cellular immune

responses are predominantly responsible for the development of EC, in terms of eosinophil infiltration [6–9].

In conclusion, blocking the interaction between ICOS and B7RP-1 did not suppress eosinophil infiltration in experimental murine AC. This observation suggests that targeting the interaction between ICOS and B7RP-1 may not always be therapeutic for allergic diseases.

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