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CD27 and CD70 do not play a critical role in the development of experimental allergic conjunctivitis in mice

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ABSTRACT

CD27, which belongs to the TNF receptor family, is a costimulatory molecule that participates in T-cell activation. Unlike costimulatory molecules such as OX40 and 4-1BB, little is known about the role CD27 plays in the development of experimental diseases. We asked whether CD27 and its ligand CD70 participate in the development of experimental allergic conjunctivitis (EC) in BALB/c mice, which is generated by immunization with ragweed (RW) in alum and challenged 10 days later with RW in eye drops. The roles of CD27 and CD70 were tested by intraperitoneally injecting the mice with anti-CD27, anti-CD70 or a control Ab during the induction or effector phase. Twenty-four hours after challenge, the conjunctiva, blood and spleens were harvested for histological analysis, measuring Ig levels and cytokine analysis, respectively. Regardless of when the mice were treated, anti-CD27 or anti-CD70 Ab treatment did not significantly affect the severity of EC as evaluated by conjunctival eosinophil numbers. However, anti-CD27 or anti-CD70 Ab treatment during the induction phase did significantly modulate systemic humoral and cellular immune responses. *In vitro* treatment of RW-primed splenocytes with anti-CD27 or anti-CD70 Ab did not affect the EC-inducing capability of the splenocytes. Taken together, CD27 and CD70 do not play a critical role in the development of EC.

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1. Introduction

Experimental allergic conjunctivitis (EC) [1] is a model of severe forms of human allergic conjunctivitis (AC) since it is characterized by conjunctival eosinophilic infiltration, a hallmark of severe human AC [2,3]. We have investigated the mechanism by which EC develops and have found that T cells play a prominent role in inducing the conjunctival eosinophilic infiltration [4,5]. For T cells to exert their effects, they must be activated via both the T-cell receptor and co-stimulatory molecules. Therefore, we have also investigated the roles co-stimulatory molecules play in the development of EC [6–10].

Our series of experiments have revealed that TNF receptor family molecules such as 4-1BB [6] and OX40 [7] play a critical role in the development of EC. Moreover, many studies have demonstrated that 4-1BB [11,12] and OX40 [13–16] play a critical role in the development of other experimental allergic diseases such as

airway inflammation. CD27 is also a TNF receptor family molecule [17]. However, little is known about the role it plays in the development of allergic diseases. Here we asked whether CD27 and its ligand CD70 participate in the development of EC.

2. Materials and methods

2.1. Mice

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 6–12-week-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 Polysciences, Inc., Warrington, PA. RW extract was obtained from LSL Co. Ltd.,

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Tokyo, Japan. Aluminum hydroxide (alum) was purchased from Sigma, St. Louis, MO. The anti-mouse CD27 mAb (RM27-3E5, rat IgG2a) was raised against mouse CD27-human IgG1 Fc fusion protein (R&D Systems, Minneapolis, MN). This mAb enhanced anti-CD3-stimulated T-cell proliferation when immobilized *in vitro* and augmented OVA-specific CTL induction when administered *in vivo* (details will be described elsewhere). The neutralizing anti-mouse CD70 mAb (FR70, rat IgG2b) has been characterized previously [18]. These Abs were purified from ascites by using protein G columns. The Ab preparations contained less than 100 pg/ml endotoxin. Normal rat IgG (nrlgG) was obtained from MP Biomedicals Inc., Aurora, OH.

2.3. 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 μ g of RW and 675 μ g of alum) was injected into each site. The mice were injected intraperitoneally with 200 μ g of purified anti-CD27, anti-CD70 Ab or nrlgG ($n = 10$ per group) on days 0, 2, 4, 6 and 8 after immunization (induction phase). In a separate experiment, the actively immunized mice ($n = 10$ per group) were injected intraperitoneally with 200 μ g of Ab only once 2 h before the RW challenge (effector phase). Ten days after active immunization, 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 and blood were harvested for histological analysis and measurement of serum Ig levels, respectively. The spleens were also harvested for cytokine assays and splenocyte transfer.

2.4. 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 per 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 μ g/ml). After incubation for 72 h at 37 °C in a humidified atmosphere with 5% CO₂, 2×10^7 splenocytes were injected intraperitoneally into naïve BALB/c mice ($n = 8$ 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 were harvested for histological analysis.

2.5. Induction of EC by adoptive transfer of RW-primed splenocytes treated with Abs *in vitro*

Naïve BALB/c mice were immunized with RW in 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 μ g/ml RW extract in the presence of 10 μ g/ml anti-CD27, anti-CD70 or nrlgG ($n = 8$ per group). After incubation for 72 h at 37 °C in a humidified atmosphere with 5% CO₂, 2×10^7 splenocytes were injected intraperitoneally 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 μ l per eye). Twenty-four hours later, the eyes were harvested for histological analysis.

2.6. Histological analysis

The eyes including the conjunctivas were harvested and fixed in 10% buffered formalin. Vertical 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. The data are presented as averages $\pm$ S.E.M. of all the mice examined.

2.7. Measurement of Igs in serum

Twenty-four hours after RW challenge of actively immunized mice, blood was collected and serum was prepared. Serum total Ig levels were assessed by ELISA, as detailed elsewhere [8], and the data are presented as concentrations of Igs.

2.8. Measurement of cytokines in the culture supernatants

RBC-depleted splenocytes (10^7 cells/ml) were cultured for 48 h with RW extract (25 μ g/ml) in 96-well flat-bottom plates in a final volume of 0.2 ml RPMI 1640 medium supplemented with 10% FCS and 2-ME. The levels of IL-4, IL-5, IL-10, IFN- γ and TNF- α in the culture supernatants were measured by using Luminex100 (Luminex corporation, Austin, TX) and the Bio-Plex Cytokine Assay Panel (Bio-Rad Laboratories Inc., Hercules, CA) according to the manufacturers' instructions.

2.9. Statistical analysis

Differences between the Ab-treated and control IgG-treated mice in terms of their cytokine production, serum Ig levels and infiltrating eosinophil numbers were tested for significance by ANOVA and Scheffe's *F*-test. *P* values less than 0.05 were considered significant.

3. Results

3.1. Treatment with Abs against CD27 or CD70 during the induction phase did not affect the severity of EC

To investigate whether CD27 and CD70 participate in the development of EC during the induction phase, anti-CD27 or anti-CD70 Ab was injected intraperitoneally into actively immunized mice 5 times every other day from the day of immunization through to day 8. As a control, nrlgG was injected. The three groups did not differ significantly in terms of EC severity, as evaluated by conjunctival eosinophil numbers (Fig. 1A). To ascertain the effects of the Abs on systemic immune responses, we subjected the splenocytes of these mice, which were harvested 24 h after RW challenge, to cytokine production assay and splenocyte transfer. We also measured the serum Ig levels. Analysis of the splenocyte cytokine production revealed that compared to nrlgG treatment, anti-CD27 or anti-CD70 Ab treatment significantly decreased IL-5 production (Fig. 1B). Moreover, anti-CD27 Ab treatment significantly suppressed IFN- γ production compared to the effects of nrlgG or anti-CD70 Ab treatment (Fig. 1B). It also significantly increased TNF- α production compared to the nrlgG-treated group (Fig. 1B). Anti-CD27 or anti-CD70 Ab treatment significantly suppressed total serum IgE levels relative to the effect of nrlgG treatment, while anti-CD27 Ab treatment significantly increased total serum IgG1 and IgG2a levels compared to the effect of the other two Abs (Fig. 1C). Interestingly, the adoptive transfer of splenocytes from anti-CD70 Ab-treated mice induced significantly more severe conjunctival eosinophil infiltration than the adoptive transfer of splenocytes from nrlgG-treated or anti-CD27 Ab-treated mice (Fig. 1D). Thus, it appears that anti-CD27 or anti-CD70 Ab treatment during the induction phase significantly affected the sys-

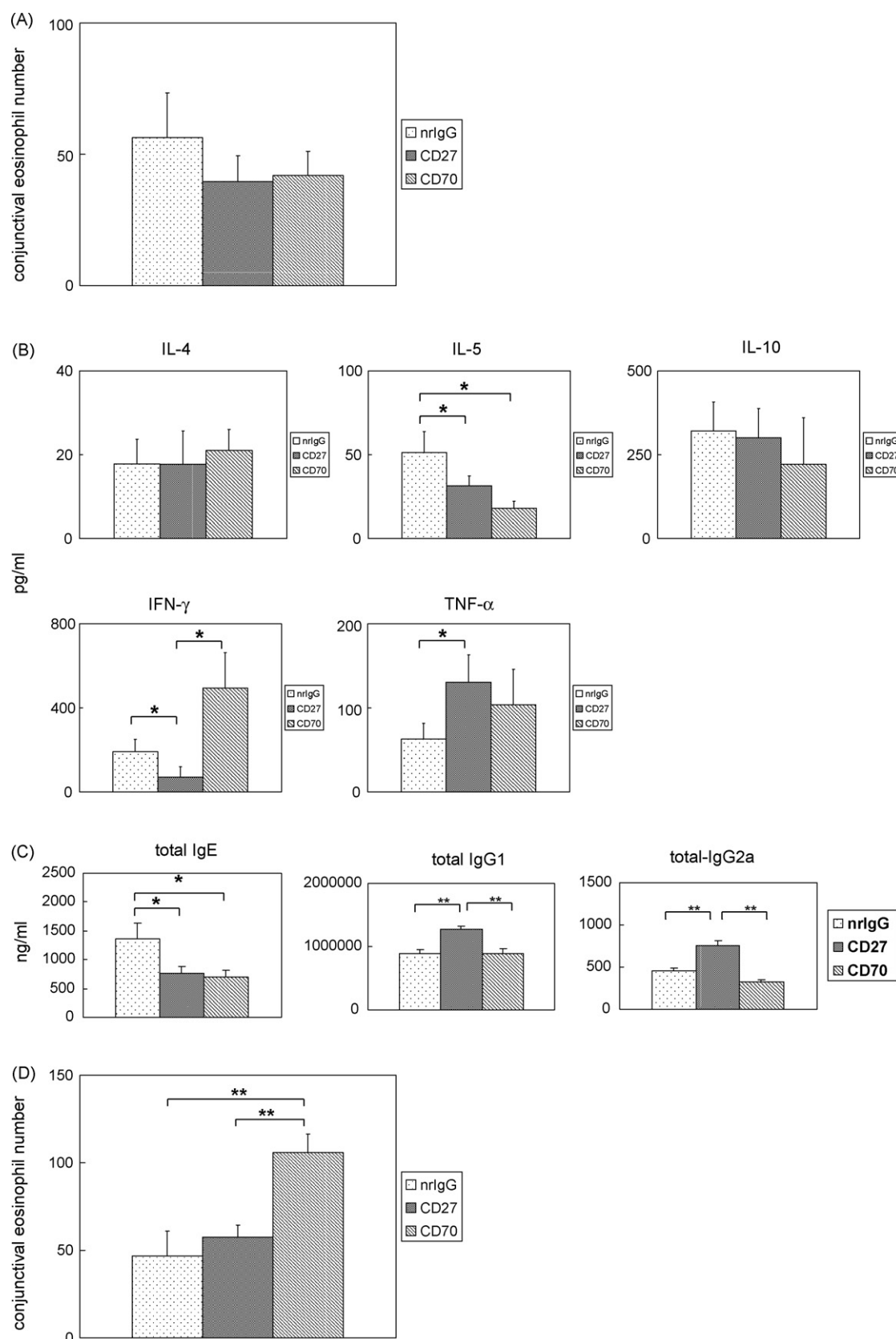


Fig. 1. Effects of Ab treatment during the induction phase of EC. BALB/c mice were immunized with RW in alum and challenged 10 days later with RW in eye drops. The mice were injected intraperitoneally five times every other day starting on the day of immunization with anti-CD27 Ab, anti-CD70 Ab or nrlgG. Twenty-four hours after RW challenge, the conjunctivas and blood were harvested for histological analysis and measurement of serum Ig levels, respectively. The spleens were also harvested for cytokine production analysis and splenocyte transfer. (A) Conjunctival eosinophil numbers in actively immunized and Ab-treated mice. (B) Cytokine assay. (C) Serum Ig levels. (D) Conjunctival eosinophil numbers in naïve mice that had received the RW-primed splenocytes from actively immunized and Ab-treated mice and were challenged four days later. * $P < 0.05$, ** $P < 0.01$.

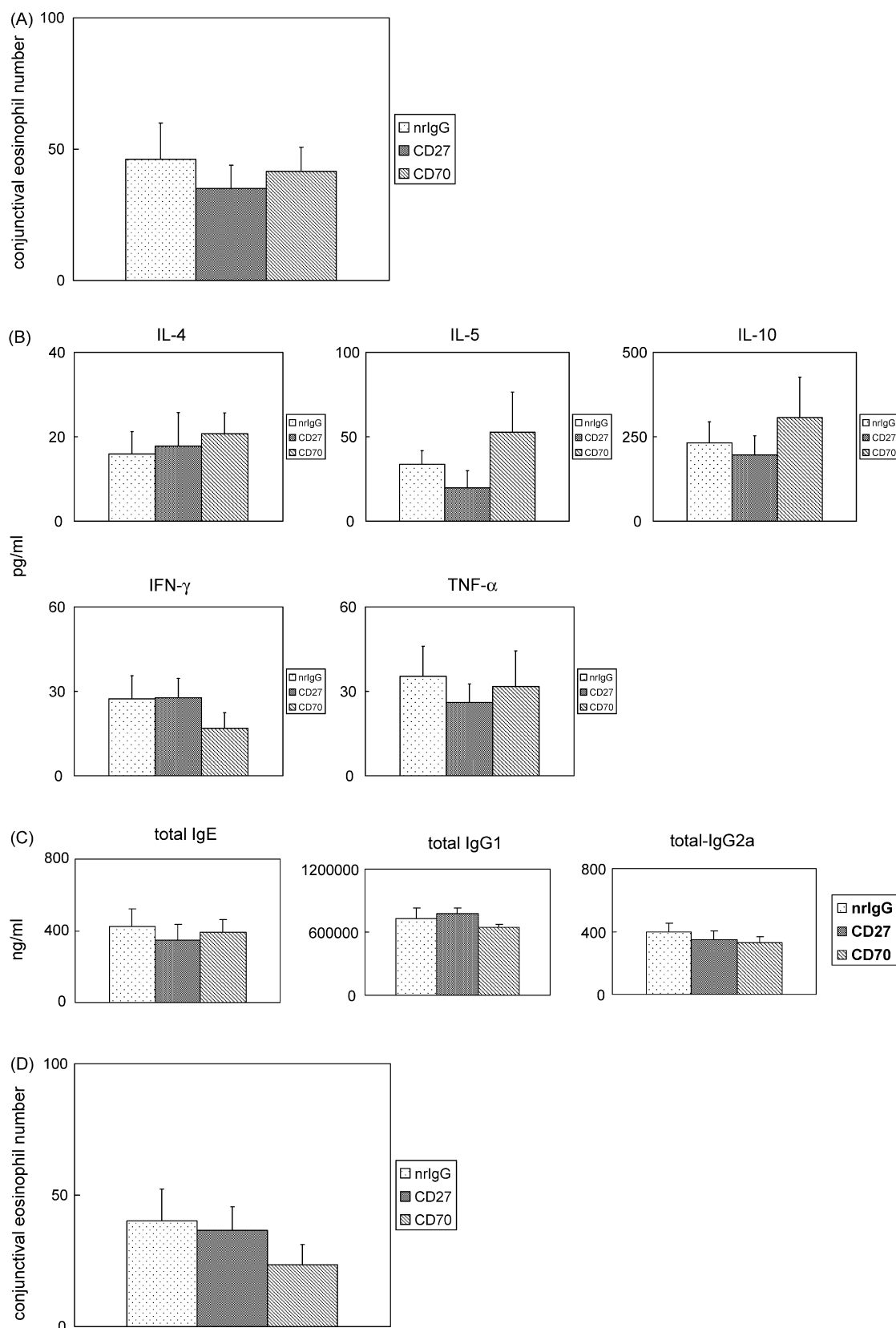


Fig. 2. Effects of Ab treatment during the effector phase of EC. BALB/c mice were immunized with RW in alum and challenged 10 days later with RW in eye drops. The mice were injected intraperitoneally once 2 h prior to RW challenge with anti-CD27 Ab, anti-CD70 Ab or nrlgG. Twenty-four hours after RW challenge, the conjunctivas and blood were harvested for histological analysis and measurement of serum Ig levels, respectively. The spleens were also harvested for cytokine production analysis and splenocyte transfer. (A) Conjunctival eosinophil numbers in actively immunized and Ab-treated mice. (B) Cytokine assay. (C) Serum Ig levels. (D) Conjunctival eosinophil numbers in naïve mice that received the RW-primed splenocytes from the actively immunized and Ab-treated mice and were challenged 4 days later.

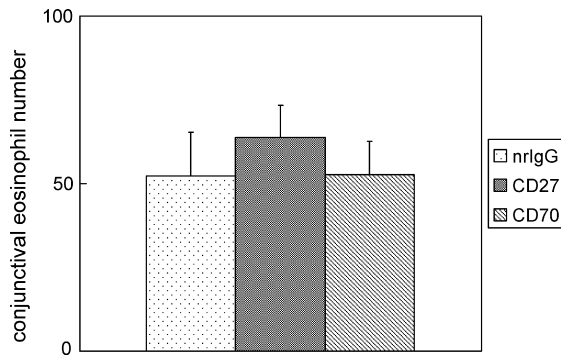


Fig. 3. Effect of Ab treatment *in vitro* on the EC-inducing capability of RW-primed splenocytes. BALB/c mice were immunized with RW in alum and not treated with any Abs. Ten days later, their splenocytes were prepared and cultured with 5 μ g/ml RW extract in the presence of 10 μ g/ml anti-CD27 Ab, anti-CD70 Ab or nrlgG for 72 h. These splenocytes were transferred into naïve syngeneic mice, which were challenged with RW in eye drops 4 days after the transfer. Twenty-four hours later, the conjunctivas were harvested to evaluate the conjunctival eosinophil numbers.

temic immune responses, although these changes did not affect the severity of EC.

3.2. Treatment with Abs against CD27 or CD70 during the effector phase did not affect the severity of EC

Next, to investigate whether CD27 and CD70 participate in the development of EC during the effector phase, anti-CD27 or anti-CD70 Ab was injected intraperitoneally into actively immunized mice once 2 h prior to RW challenge in eye drops. As a control, nrlgG was injected. Similar to the induction phase treatment, the three groups did not differ in EC severity, as evaluated by conjunctival eosinophil numbers (Fig. 2A). Analysis of the cytokine production of the splenocytes revealed that three groups did not differ significantly with regard to IL-4, IL-5, IL-10, IFN- γ or TNF- α production (Fig. 2B). Anti-CD27 or anti-CD70 Ab treatment also did not affect the total serum IgE, IgG1 and IgG2a levels (Fig. 2C). Finally, the adoptive transfer of the splenocytes from the three groups induced equivalent levels of conjunctival eosinophil infiltration (Fig. 2D). Thus, it appears that anti-CD27 or anti-CD70 Ab treatment during the effector phase did not significantly affect systemic immune responses or the severity of EC.

3.3. Treatment of RW-primed splenocytes with Abs against CD27 or CD70 *in vitro* did not affect their EC-inducing capability

Finally, to investigate whether *in vitro* treatment of RW-primed splenocytes with anti-CD27 or anti-CD70 Ab would affect their ability to induce conjunctivitis upon adoptive transfer. RW-primed splenocytes were stimulated *in vitro* with RW extract in the presence of anti-CD27, anti-CD70 Ab or nrlgG and transferred into naïve syngeneic mice. Four days after the transfer, the recipient mice were challenged with RW in eye drops to induce EC. Twenty-four hours later, the conjunctivas were harvested to evaluate conjunctival eosinophilia. As with Ab treatment *in vivo*, the three groups did not differ in conjunctival eosinophil numbers (Fig. 3).

4. Discussion

Only a single report has examined the direct contribution of CD27/CD70 to experimental diseases [19]. It revealed that anti-CD70 Ab treatment prevented experimental autoimmune encephalomyelitis in mice, presumably because it inhibited TNF production [19]. Thus, it was not yet known whether CD27/CD70 participates in the development of experimental allergic diseases.

Therefore, we asked whether CD27/CD70 plays a role in the development of EC, which is an ocular allergy disease model.

Anti-CD27 or anti-CD70 Ab treatment *in vivo* did not significantly affect the severity of EC regardless of when in the disease process the treatment was instituted. When delivered during the induction phase, both the anti-CD27 Ab and the anti-CD70 Ab significantly affected splenocyte cytokine production and serum Ig levels. However, these changes were not consistent with a deviation toward Th1- or Th2-dominance. Since EC in BALB/c mice can be induced by Ag-specific Th2 cells, but not by Th1 cells [4], this observation may explain, at least in part, why anti-CD27 or anti-CD70 Ab treatment did not affect EC severity. This possibility is further supported by our previous finding that treatment with anti-4-1BB [6] or anti-OX40 Ab [7] significantly affected EC severity along with markedly changing the Th1/Th2 balance into a direction that either exacerbated or attenuated the severity of EC.

An interesting observation is that anti-CD27 Ab treatment and anti-CD70 Ab treatment during the induction phase promoted opposite effects on the IFN- γ production by splenocytes. The reason for this difference remains unclear; however, one possible explanation is that the mode of action is different between anti-CD27 and anti-CD70 Abs. Anti-CD70 Ab is an antagonistic Ab [18], while anti-CD27 Ab is an agonistic Ab, because it enhanced anti-CD3-stimulated T-cell proliferation when immobilized *in vitro*, as described in Materials and Methods. In contrast to IFN- γ , productions of IL-4, IL-5, IL-10 and TNF- α were basically similar between the two groups. From these data, it may be speculated that CD27 signaling may be preferentially involved in IFN- γ production. Indeed, it was reported that the number of IFN- γ -producing T cells and the expression levels of IFN- γ per T cell increased in CD70 transgenic mice, in which CD27 and CD70 are constitutively interacted [20].

Another interesting observation is that adoptive transfer of splenocytes from the mice treated with anti-CD70 Ab during the induction phase induced significantly more severe EC. In contrast, treatment with anti-CD70 Ab during the induction phase did not affect the severity of EC induced by active immunization. The former finding indicates that RW-primed T cells in anti-CD70 Ab-treated mice have more EC-inducing capability. EC induced by active immunization is mediated by both IgE and Th2 cells [4,5]. Serum IgE levels were significantly suppressed by anti-CD70 Ab treatment during the induction phase. Therefore, it could be considered that the modulation of these two parameters (up-regulation of EC-inducing capability of T cells and down-regulation of IgE) may be the reason why anti-CD70 Ab treatment did not affect the severity of EC induced by active immunization.

It has been reported that CD27 signaling maintains T-cell survival and enhances T-cell accumulation in the peripheral tissues [21,22]. CD27 appears to promote T-cell expansion by allowing T cells to survive through successive divisions but it does not actually influence the rate of cell division, which is promoted by CD28 [23]. Thus, CD27 exerts its effects after CD28 engagement and is likely to influence secondary responses more than primary responses. In our study, Ab treatment was conducted during primary immune responses. This may be another reason why anti-CD27 or anti-CD70 Ab treatment during the induction phase of EC did not affect the severity of EC. In any case it seems that the CD27/CD70 costimulatory system does not appear to participate in the EC-inducing pathogenic process. This is further supported by the observation that *in vitro*-treatment with anti-CD27 or anti-CD70 Ab did not affect the EC-inducing capability of RW-primed splenocytes.

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