

Short communication

B7-H3 regulates the development of experimental allergic conjunctivitis in mice

Atsuki Fukushima^{a,*}, Tamaki Sumi^a, Ken Fukuda^b, Naoki Kumagai^b, Teruo Nishida^b,
Tomohide Yamazaki^c, Hisaya Akiba^c, Ko Okumura^c, Hideo Yagita^c, Hisayuki Ueno^a

^a Department of Ophthalmology and Visual Science, Kochi Medical School, Kohasu, Ogo-cho, Nankoku-city 783-8505, Japan

^b Department of Ophthalmology, Yamaguchi University School of Medicine, Japan

^c Department of Immunology, Juntendo University School of Medicine, Japan

Received 14 May 2007; received in revised form 8 July 2007; accepted 19 July 2007

Available online 23 August 2007

Abstract

B7-H3 negatively regulates Th1-mediated immune responses. Here, we aimed to investigate whether B7-H3 is involved in the development of murine experimental allergic conjunctivitis (EC), which is predominantly mediated by Th2 cells. Intraperitoneal injection of anti-B7-H3 Ab during the induction phase of EC significantly augmented the severity of EC evaluated as conjunctival eosinophil numbers and Ag-induced IL-5 production by splenocytes. Injection of anti-B7-H3 Ab during the effector phase of EC did not significantly affect the severity of EC. In addition, transfer of Ag-primed splenocytes treated with anti-B7-H3 Ab *in vitro* did not significantly affect the severity of EC, compared to the splenocytes treated with the control Ab. Thus, regulation of EC by blocking of B7-H3 was observed during the induction phase but not the effector phase. Moreover, this study provides a new notion that B7-H3 regulates not only Th1-mediated but also Th2-mediated immune reactions.

© 2007 Published by Elsevier B.V.

Keywords: Allergic conjunctivitis; Eosinophil; B7-H3; Th1; Th2

1. Introduction

B7 homolog 3 (B7-H3) has been identified both in humans and mice [1,2]. B7-H3 is expressed in various types of organs and is induced by inflammatory cytokines in dendritic cells and monocytes [1]. B7-H3 binds a receptor on activated T cells that is distinct from so far identified CD28 family proteins such as CD28 and inducible costimulator [1]. *In vitro* studies have demonstrated that B7-H3 costimulation increases proliferation of both CD4-positive and CD8-positive T cells, stimulates IFN- γ production and induces cytotoxic T lymphocytes (CTLs) [1]. These results indicate that B7-H3 provides a positive signal to Th1 immune responses and CTL.

With regard to roles of B7-H3 *in vivo*, B7-H3-deficient mice were established to analyze its roles [3]. B7-H3-deficient mice developed more severe airway inflammation compared with wild-type mice in the situation that this inflammation was pre-

dominantly mediated by Th1 cells, but not by Th2 cells. This result is contradictory to a previous report that B7-H3 provides a positive signal to Th1 responses [1]. Similarly, in that report, it was also demonstrated that B7-H3 was not involved in CTL responses in mice [3]. Thus, these results are contradictory to the initial report that B7-H3 plays an activating role in inducing Th1 cells and CTL [1]. The reason why the outcomes are different in these two reports remains to be elucidated; nevertheless, it is clear that B7-H3 plays a role in the regulation of Th1-mediated immune responses.

Here, we have sought to investigate whether B7-H3 plays a role in the development of experimental murine allergic conjunctivitis (EC) that is mediated by Th2 cells [4–7].

2. Materials and methods

2.1. Mice

BALB/c mice were purchased from Japan SLC Inc., Hamamatsu, Shizuoka, Japan, and kept in pathogen-free conditions at the animal facility of Kochi Medical School. Age- and

* Corresponding author. Tel.: +81 88 880 2391; fax: +81 88 880 2392.
E-mail address: fukushima@kochi-u.ac.jp (A. Fukushima).

gender-matched mice were used when they were 6- to 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. (Tokyo, Japan). Aluminum hydroxide (alum) was purchased from Sigma (St. Louis, MO). The anti-mouse B7-H3 monoclonal Ab (MJ18, rat IgG1) was generated by immunizing rats with mouse B7-H3 fusion proteins. This monoclonal Ab selectively reacted with mouse B7-H3-transfected P815 cells but not with untransfected P815 cells as estimated by flow cytometry. This Ab blocked the rejection of mouse B7-H3-transfected P815 tumor cells in DBA/2 mice, indicating an antagonistic effect *in vivo* (details will be described elsewhere). Normal rat IgG (nrIgG) was purchased from MP Biomedicals Inc. (Aurora, OH).

2.3. Induction of EC by active immunization and treatment with Abs

RW adsorbed on alum was injected into the left hind-footpad and at the base of the tail. Fifty microliters of the emulsion (50 μ g of RW and 675 μ g of alum) was injected into each site. The mice were also injected intraperitoneally on days 0, 2, 4, 6 and 8 after RW immunization (induction phase treatment) or on day 10 only (2 h before RW challenge; effector phase treatment) with 200 μ g anti-B7-H3 Ab or nrIgG. On day 10, 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 spleens were harvested for histological analysis and cytokine assays, respectively.

2.4. Induction of EC by adoptive transfer of RW-primed splenocytes and treatment with Abs

Naïve BALB/c mice were immunized with RW in both their left hind-footpad and tail base and were not treated with any Abs. Ten days later, their splenocytes were prepared and cultured with 5 μ g/ml RW extract as described previously [4,6,7]. After incubation for 72 h at 37 °C in a humidified atmosphere with 5% CO₂, 2×10^7 splenocytes were intraperitoneally injected into naïve mice. Anti-B7-H3 Ab or nrIgG (200 μ g each) was intraperitoneally injected into recipient mice twice for 2 and 4 days after splenocyte transfer. Two hours after the last Ab injection, 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 *in vitro* with Abs

Naïve BALB/c mice were immunized with RW in both their left hind-footpad and tail base and were not treated with any Abs. Ten days later, their splenocytes were prepared and cultured

with 5 μ g/ml RW extract as described previously [4,6,7] in the presence of 10 μ g/ml anti-B7-H3 Ab or nrIgG. After incubation for 72 h at 37 °C in a humidified atmosphere with 5% CO₂, 2×10^7 splenocytes were intraperitoneally injected into naïve mice. Four days after the splenocyte transfer, the eyes of the recipient mice were challenged with RW in PBS (2 mg in 10 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. Vertically 2 μ m thick sections were cut and stained with Giemsa. Infiltrating eosinophils in the lamina propria mucosae of the tarsal and bulbar conjunctivas in the entire section were counted by two observers in given blinded samples. The sections counted were those from the central portion of the eye, which included the pupil and optic nerve head. The data are presented as means $\pm$ S.E.M. of all the mice examined.

2.7. Immunohistochemistry

The eyes including the conjunctivas from EC-developing mice treated with Abs during the induction phase were harvested and sections for immunohistochemistry were prepared following the method as previously described [8]. Briefly, the eyes were immediately frozen in 3% carboxymethyl cellulose (CMC) gel. Four μ m thick sections were prepared and fixed in methanol. Endogenous peroxidase activity was inhibited with 0.1% NaN₃ and 0.3% H₂O₂ in distilled water for 10 min at room temperature. Samples were exposed to first antibodies for 30 min and another 30 min to appropriate biotinylated secondary antibodies. All slides were revealed using an avidin–biotin–complex kit (Vector Laboratories Inc., USA), then developed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma St. Louis, MO). The antibodies used were rat anti-mouse CD3 (CT-CD3, Caltag, Burlingame, CA, USA), rabbit anti-mouse major basic protein (MBP, provided by Dr. Lee, Mayo Clinic) and anti-F4/80 (A3-1) (Caltag Laboratories, Burlingame, CA).

2.8. Cytokine assay

Splenocytes harvested from mice actively immunized with RW and treated with Abs were cultured *in vitro* for 48 h with 25 μ g/ml RW extract. The IL-5, IL-13 and IFN- γ levels in the culture supernatants were measured by the Bioplex system (Bio-rad, Hercules, CA) according to the manufacturer's recommendations.

2.9. Flow cytometric analysis

Naïve BALB/c mice were actively immunized with RW and were not treated with any Abs. Ten days later, the mice were challenged with RW in eye drops and 24 h later, spleens were harvested. As a control, naïve mice were challenged with RW and 24 h later, spleens were harvested. Splenocytes were

incubated for 30 min on ice with FITC-(anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD45R/B220 (RA3-6B2)), PE-(anti-F4/80 (A3-1)) and biotin-labeled (anti-B7-H3 (MJ18)) Abs at optimal concentrations. After the incubation, the cells were washed with cold 2% FCS-PBS. In the case of biotin-labeled anti-B7-H3 Ab, the cells were then incubated with streptavidin-PE or streptavidin-FITC for 10 min and then washed again. These cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA) to assess B7-H3 expression in whole splenocytes or CD3-, CD4-, CD8-, B220- and F4/80-positive cell compartments. Acquisition and analysis were performed using CellQuest software. The data are presented as positive % of B7-H3 expressing cells out of a particular cell compartment in splenocytes (CD3⁺, CD4⁺, B220⁺, F4/80⁺) $\pm$ S.E.M. per spleen.

2.10. Statistical analysis

Significant differences between anti-B7-H3 Ab- and nrIgG-treated groups with regard to the infiltrating eosinophil numbers and splenocyte cytokine production were evaluated by Student's *t*-test. *P* values less than 0.05 were considered significant.

3. Results

3.1. Effects of anti-B7-H3 Ab treatment during the induction phase on conjunctival eosinophil infiltration

Anti-B7-H3 Ab was injected into RW-immunized mice for five times, every other day from the day of immunization (day 0). These mice were challenged with RW in eye drops on day 10 and 24 h after the challenge, the conjunctivas were harvested for histological analysis to determine conjunctival eosinophil numbers. Compared with the control that had been treated with nrIgG, significantly more eosinophils were counted in the conjunctiva of mice treated with anti-B7-H3 Ab (Fig. 1A). To determine the phenotypes of infiltrating inflammatory cells into the conjunctiva, the harvested conjunctivas were subjected to immunohistochemical analysis. More MBP-positive eosinophils were detected in the anti-B7-H3 Ab-treated group, whereas infiltration of CD3-positive T cells and F4/80-positive macrophages was not different between the two groups (Fig. 1B). To examine why more eosinophils were detected after the treatment with anti-B7-H3 Ab, splenocytes were cultured *in vitro* with RW extract and cytokine production was evaluated. More IFN- γ , IL-5 and IL-13 were detected in the culture supernatant of splenocytes from mice treated with anti-B7-H3 Ab, although a significant difference was noted only in IL-5.

3.2. Effects of anti-B7-H3 Ab treatment during the effector phase on conjunctival eosinophil infiltration

To investigate whether B7-H3 is involved in the development of EC during the effector phase, anti-B7-H3 Ab was injected into actively RW-immunized mice once on day 10, 2 h prior to RW challenge (Fig. 2A). Alternatively, anti-B7-H3 Ab was

injected twice (2 days after splenocyte transfer and 2 h prior to RW challenge) into the mice that received RW-primed splenocytes (Fig. 2B). Then, these mice were challenged with RW in eye drops and 24 h after the challenge, the conjunctivas were harvested for histological analysis to determine conjunctival eosinophil numbers. Regardless of the sensitization methods, slightly more eosinophils were detected in the conjunctiva from the mice that were treated with anti-B7-H3 Ab, although significant difference was not observed (Fig. 2A and B).

3.3. Effects of anti-B7-H3 Ab on the culture of RW-primed splenocytes *in vitro*

The above findings indicate that B7-H3 regulates priming of RW-specific splenocytes, but B7-H3 does not play a significant role once the mice are primed with RW. To further investigate the roles B7-H3 plays in RW-primed splenocytes, anti-B7-H3 Ab was added to the culture of RW-primed splenocytes secondarily stimulated with RW *in vitro*. Three days later, these splenocytes were transferred into mice and 4 days after splenocyte transfer, the recipient mice were challenged with RW in eye drops. Conjunctivas were harvested for 24 h after the challenge to determine conjunctival eosinophil numbers. Slightly more, but not significantly more, eosinophils were detected in the conjunctivas of mice that received the anti-B7-H3 Ab-treated splenocytes as compared to the nrIgG-treated splenocytes (Fig. 3).

3.4. Expression of B7-H3 in splenocytes

To investigate whether RW sensitization affect B7-H3 expression in splenocytes, B7-H3 expression levels in the different cell compartments of splenocytes were analyzed by flow cytometry. We compared the B7-H3 expression levels in splenocytes between from naïve mice challenged with RW and from RW-immunized mice challenged with RW. B7-H3 expression levels in CD3⁺, CD4⁺, B220⁺ and F4/80⁺ cell compartments were not different between naïve mice and actively immunized mice (Table 1).

4. Discussion

To date, several reports have been examined that the roles of B7-H3 plays in immune responses *in vivo*. As described in Section 1, B7-H3 negatively regulated the airway inflammation when this inflammation was predominantly mediated by Th1 cells [3]. In accord with this report, it was demonstrated that experimental autoimmune encephalomyelitis in mice was accelerated when the mice were treated with anti-B7-H3 Ab as compared with the control Ab [9]. Taken together, B7-H3 appears to negatively regulate Th1 immune responses *in vivo*.

With regard to *in vivo* roles of B7-H3 in Th2-mediated immune responses, similar numbers of inflammatory cells including eosinophils were detected in the bronchoalveolar lavage fluid when airway inflammation was polarized to Th2 conditions [3]. On the contrary to this report, we found that treatment with anti-B7-H3 Ab during the induction phase significantly augmented conjunctival eosinophil infiltration. In

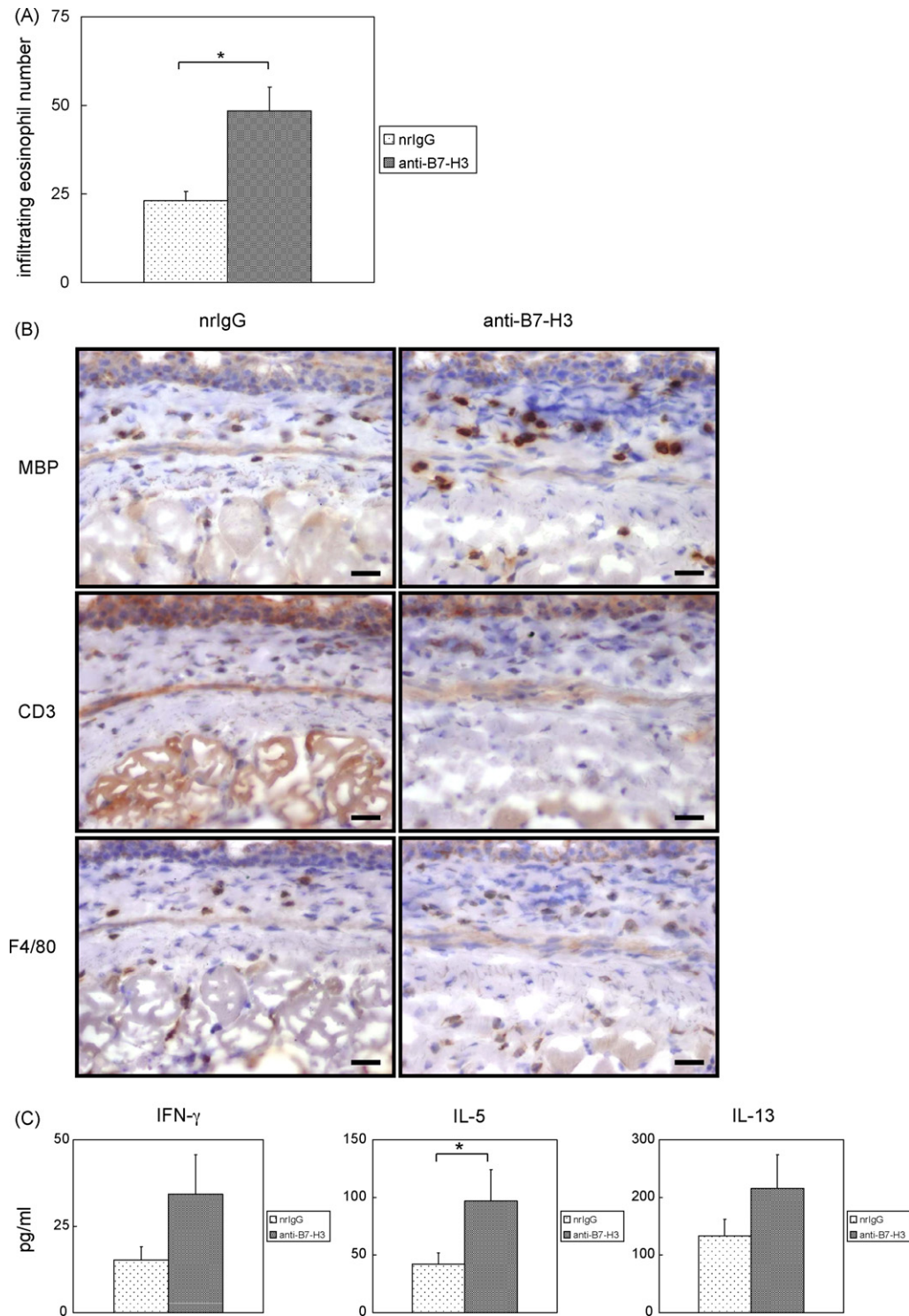


Fig. 1. Treatment with anti-B7-H3 Ab during the induction phase augmented the severity of EC. BALB/c mice were actively immunized with RW in alum, injected intraperitoneally five times for every other day starting on the day of immunization with 200 μ g of nrlgG or anti-B7-H3 Ab, and challenged on day 10 with RW in eye drops to induce EC. Twenty-four hours later, the conjunctivas and spleens were harvested for histological (A, 10 mice per group) and immunohistochemical (B, 3 mice per group) analyses and cytokine assays (C, 3 samples per group), respectively. (A) Absolute numbers of infiltrating eosinophils/mouse evaluated by Giemsa staining are shown as means $\pm$ S.E.M. (B) One representative microphotograph is shown. Bar = 20 μ m. (C) Data are expressed as mean $\pm$ S.E.M. (pg/ml). * P < 0.05.

addition, IL-5 production by splenocytes in response to *in vitro* re-stimulation with RW was significantly higher in the anti-B7-H3 Ab-treated group. These results demonstrate that blocking of B7-H3 during the induction phase of EC upregulated Th2

immune responses, thus leading to more severe conjunctival eosinophil infiltration. The different results between the previous report [3] and ours may be due to that B7-H3-deficient mice were used in the previous report while anti-B7-H3 Abs

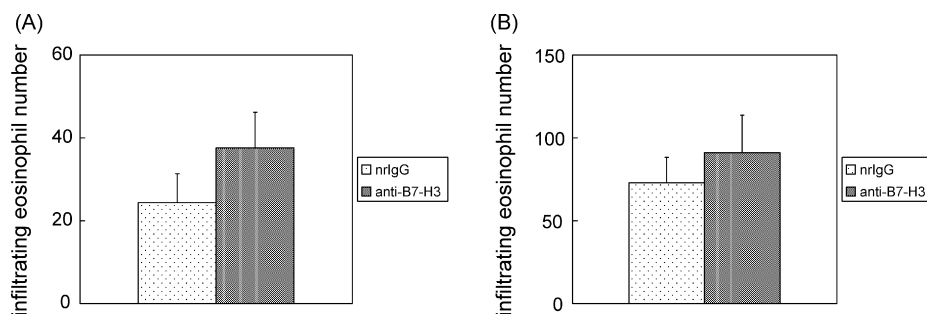


Fig. 2. Treatment with anti-B7-H3 Ab during the effector phase did not significantly affect the severity of EC. (A) BALB/c mice were actively immunized with RW in alum and injected intraperitoneally once with 200 μ g of nrIgG or anti-B7-H3 Ab for 2 h prior to RW challenge on day 10. Twenty-four hours later, the conjunctivas, sera and spleens were harvested for histological analysis (10 mice per group). (B) BALB/c mice were actively immunized with RW in alum without treatment with Abs. Ten days later, spleens were harvested and the splenocytes were cultured *in vitro* with RW extract for 3 days. These *in vitro* stimulated splenocytes (2×10^7 cells) were transferred into naïve BALB/c mice. Four days after splenocyte transfer, the recipient mice were challenged with RW in eye drops. The recipient mice were injected intraperitoneally twice with 200 μ g of nrIgG or anti-B7-H3 Ab, 2 days after the transfer and 2 h prior to RW challenge. Twenty-four hours later, the conjunctivas were harvested for histological analysis (10 mice per group). Absolute numbers of infiltrating eosinophils/mouse evaluated by Giemsa staining are shown as means $\pm$ S.E.M.

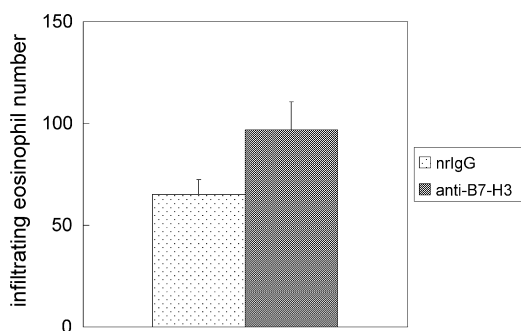


Fig. 3. *In vitro* treatment of RW-primed splenocytes with anti-B7-H3 Ab did not significantly affect the severity of EC. BALB/c mice were actively immunized with RW in alum and not treated with Abs. Ten days later, the mice were sacrificed and their spleens were harvested. The splenocytes were cultured *in vitro* with RW extract in the presence of 10 μ g/ml nrIgG or anti-B7-H3 Ab for 3 days and then 2×10^7 cells were injected intraperitoneally into naïve BALB/c mice (10 mice per group). Four days after the transfer, the recipient mice were challenged with RW in eye drops and 24 h later, the conjunctivas were harvested for histological analysis. Absolute numbers of infiltrating eosinophils/mouse evaluated by Giemsa staining are shown as means $\pm$ S.E.M.

were used in our study. It may be also possible that different target organs and different Ags participate in different results between the two studies. It is of note that IFN- γ production was also upregulated, although not statistically significant. Thus, it appears that B7-H3 negatively regulates Th2 immune responses as well as Th1 immune responses [1,3,9].

Table 1
Expression of B7-H3 in splenocytes

	CD3 ⁺	CD4 ⁺	B220 ⁺	F4/80 ⁺
Naïve	0.58 $\pm$ 0.26	0.36 $\pm$ 0.17	1.49 $\pm$ 0.33	0.51 $\pm$ 0.18
Actively immunized	0.73 $\pm$ 0.31	0.34 $\pm$ 0.14	1.84 $\pm$ 0.76	0.53 $\pm$ 0.02

Naïve mice were actively immunized with RW and were not treated with any Abs. Ten days later, the mice were challenged with RW in eye drops and 24 h later, spleens were harvested for flow cytometric analysis ($n=4$). As a control, splenocytes from naïve mice challenged with RW were used ($n=4$). Data are presented as positive % of both B7-H3 and a particular cell marker (CD3, CD4, B220 and F4/80) expressing cells $\pm$ S.E.M. per spleen.

Next, we have investigated whether B7-H3 also regulates Th2-mediated immune responses during the effector phase of inflammation. For this end, anti-B7-H3 Ab was administered only during the effector phase of EC. Conjunctival eosinophil numbers increased slightly, but not significantly, by the treatment with anti-B7-H3 Ab. To further investigate whether B7-H3 participates in the regulation of Ag-primed immune responses, anti-B7-H3 Ab was added to the culture of RW-primed splenocytes. Transfer of RW-primed splenocytes treated with anti-B7-H3 Ab *in vitro* induced slightly more conjunctival eosinophil infiltration compared to nrIgG-treated splenocytes, although not significantly different. These results suggest that B7-H3 does not play a significant role in Th2 immune responses once Th2 cells are Ag-primed; rather, it provides a negative signal during the establishment of Ag-primed Th2 immune responses.

Finally, to investigate the effects of anti-B7-H3 Ab treatment on the expression levels of B7-H3 in immunocompetent cells, flow cytometric analysis was performed to evaluate the expression of B7-H3 in splenocytes. On the contrary to the report that nearly all splenic B cells and dendritic cells express B7-H3 [9], expression levels of B7-H3 in splenic CD3⁺, CD4⁺, B220⁺ and F4/80⁺ cells were very low in our study. In accord with our results, it was reported that B7-H3 expression was hardly detectable in splenic B cells from wild-type mice [3]. Furthermore, only a slight increase of B7-H3 expression was noted even when the cells were stimulated with LPS [3]. Thus, the difference of B7-H3 expression levels in splenocytes between the Ref. [9] and our data (as well as the Ref. [3]) may depend on the different Abs used for the assay. In our study, it is less likely that B7-H3 molecules were completely blocked by anti-B7-H3 Ab treatment, since B7-H3 expression levels in splenocytes were not affected (data not shown). Yet, cytokine production was significantly affected by this treatment. In contrast, in B7-H3-deficient mice, B7-H3 molecules were completely absent [3]. Taken together, it may be possible that B7-H3 may exerts its regulatory effects on Th2 cells when B7-H3 molecules are partially, but not completely, blocked. Further studies are necessary to elucidate how B7-H3 plays a regulatory role in Th2 immune responses.

References

- [1] Chapoval AI, Ni J, Lau JS, Wilcox RA, Flies DB, Liu D, et al. B7-H3: a costimulatory molecule for T cell activation and IFN- γ production. *Nat Immunol* 2001;2:269–74.
- [2] Sun M, Richards S, Prasad DV, Mai XM, Rudensky A, Dong C. Characterization of mouse and human B7-H3 genes. *J Immunol* 2002;168: 6294–7.
- [3] Suh WK, Gajewska BU, Okada H, Gronski MA, Bertram EM, Dawicki W, et al. The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses. *Nat Immunol* 2003;4: 899–906.
- [4] Fukushima A, Ozaki A, Jian Z, Ishida W, Fukata K, Ueno H, et al. Dissection of antigen-specific humoral and cellular immune responses for the development of experimental immune-mediated blepharoconjunctivitis in C57BL/6 mice. *Curr Eye Res* 2005;30:241–8.
- [5] Ozaki A, Seki Y, Fukushima A, Kubo M. The control of allergic conjunctivitis by suppressor of cytokine signaling (SOCS)3 and SOCS5 in a murine model. *J Immunol* 2005;175:5489–97.
- [6] Fukushima A, Yamaguchi T, Ishida W, Fukata K, Mittler RS, Yagita H, et al. Engagement of 4-1BB inhibits the development of experimental allergic conjunctivitis in mice. *J Immunol* 2005;175:4897–903.
- [7] Fukushima A, Yamaguchi T, Ishida W, Fukata K, Yagita H, Ueno H. Roles of OX40 in the development of murine experimental allergic conjunctivitis: exacerbation and attenuation by stimulation and blocking of OX40. *Invest Ophthalmol Vis Sci* 2006;47:657–63.
- [8] Fukushima A, Ozaki A, Fukata K, Ishida W, Ueno H. Ag-specific recognition, activation, and effector function of T cells in the conjunctiva with experimental immune-mediated blepharoconjunctivitis. *Invest Ophthalmol Vis Sci* 2003;44:4366–74.
- [9] Prasad DV, Nguyen T, Li Z, Yang Y, Duong J, Wang Y, et al. Murine B7-H3 is a negative regulator of T cells. *J Immunol* 2004;173:2500–6.