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INFLAMMATORY DISORDERS

B and T lymphocyte attenuator regulates the development of antigen-induced experimental conjunctivitis

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Abstract
Purpose To investigate the roles that B and T lymphocyte attenuator (BTLA) and herpesvirus entry mediator (HVEM) play in the development of antigen-induced experimental conjunctivitis (EC).
Methods BALB/c mice were immunized with ragweed (RW) in alum. Ten days later, the mice were challenged with RW in eye drops. After 24 hours, the conjunctivas, blood and spleens were collected for histological analysis, measurement of serum immunoglobulin (Ig) levels, and both flow cytometric analysis and cytokine assays, respectively. The mice were injected intraperitoneally with anti-BTLA antibody, anti-HVEM antibody or control antibody during either induction phase or effector phase.
Results Induction-phase treatment with anti-BTLA antibody but not anti-HVEM antibody significantly increased conjunctival eosinophil infiltration. Treatment with either antibody during the effector phase did not affect conjunctival eosinophil infiltration. Anti-BTLA antibody treatment during the induction phase reduced the B cell compartment and increased the CD11b-positive cell compartment in splenocytes. Additionally, anti-BTLA treatment upregulated IL-4 and IL-10 production of splenocytes stimulated by RW.
Conclusions BTLA regulated the development of EC possibly by downregulating Th2 cytokine production and adjusting the compartments of immunocompetent cells. The regulation of EC by BTLA may be mediated by BTLA ligands other than HVEM.

Keywords Antigen-induced experimental conjunctivitis · B and T lymphocyte attenuator · Herpesvirus entry mediator · Th2

Introduction

Allergic conjunctival diseases (ACDs) encompass a variety of pathological conditions, ranging from a mild form such as seasonal allergic conjunctivitis to severe forms such as vernal keratoconjunctivitis (VKC) and atopic keratoconjunctivitis (AKC) [1]. In the mild form, IgE-mediated type I allergic reactions are predominant [2]. In the severe forms, unlike allergic conjunctivitis, T cells play an important role in the development, in addition to type I allergic reaction [3]. This has been confirmed by the finding that treatment with T-cell-specific immunosuppressant eye drops such as cyclosporine A [4] and tacrolimus [5] effectively suppressed the severity of VKC in patients.

To investigate the developing mechanism of severe types of ACD, experimental conjunctivitis (EC) was established in mice by using ragweed (RW) as the antigen [6, 7]. Histopathological features of EC include conjunctival eosinophilia [6, 7], which is a marker for the severity of ACDs like AKC and VKC [8, 9]. By assessing the degree of conjunctival eosinophilia, the roles of costimulatory molecules in the development of EC were evaluated by treating mice with antibodies directed against these mole-

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cules, because signals through costimulatory molecules are crucial for T-cell activation. We found that OX40 [10] and 4-1BB [11] upregulated and downregulated respectively the severity of EC.

In addition to 4-1BB, cytotoxic T lymphocyte-associated Ag-4 (CTLA-4), programmed death receptor-1 (PD-1), and B and T lymphocyte attenuator (BTLA) transduce signals that inhibit T-cell activation [12–14]. CTLA-4 [15] and programmed death-ligand 2 (PD-L2) [16] are involved in the development of EC during the induction and effector phase respectively. A few reports have demonstrated that BTLA regulates the development of antigen-induced airway inflammation by using BTLA-deficient mice [17, 18]. However, it is not known how BTLA is involved in the development of antigen-induced conjunctivitis, an experimental model of ACD. Here, the role of BTLA in EC was examined by treating mice with antibodies against BTLA and its ligand herpesvirus entry mediator (HVEM).

Materials and methods

Mice

Inbred wild-type (WT) BALB/c mice were purchased from Japan SLC Inc., Hamamatsu, Shizuoka, Japan, and were kept in pathogen-free conditions at the animal facility of Kochi Medical School. Age- and gender-matched mice were used when they were 6 to 12 weeks of age. 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 RW pollen was purchased from Polysciences, Inc, Warrington, PA, USA. RW extract was obtained from LSL CO. LTD., Tokyo, Japan. Aluminum hydroxide (alum) was purchased from Sigma, St. Louis, MO, USA. Hybridomas producing monoclonal antibody specific for BTLA (HMBT-6B2) and HVEM (HMHV-1B1B) was established by immunizing Armenian hamsters with mouse BTLA/Fc (R&D Systems, Minneapolis, MN, USA) and mouse HVEM/Fc (R&D Systems) fusion protein respectively, and fusing the immune splenocytes with P3U1 myeloma. The antibodies for in vivo treatments were purified from ascites fluid using a protein G column, and contained less than 100 pg/ml of endotoxin. The following antibodies were purchased: normal hamster immunoglobulin G (nhIgG) (MP Biomedicals Inc., Aurora, OH, USA), FITC-labeled anti-CD3 (145-2C11), FITC-labeled anti-CD45R/B220 (RA3-6B2), FITC-labeled anti-mouse CD11b (BioLegend, San Diego, CA, USA), and FITC-labeled anti-CD11c (BD Biosciences, Franklin Lakes, NJ, USA).

EC induction and treatment with antibodies

RW adsorbed on alum was injected into the left hind-footpad and the tail base. Fifty μl of the emulsion (50 μg of RW and 675 μg of alum) was injected into each site. The mice were injected five times (every other day from the day of immunization) intraperitoneally with 200 μg of purified anti-BTLA antibody, anti-HVEM antibody or control hamster IgG (n=10 per group) . Alternatively, the mice were injected intraperitoneally with 200 μg of these antibodies (n=5) once, 2 hours prior to RW challenge. Ten days after immunization, the eyes of the immunized mice were challenged with RW in PBS (2 mg in 10 μl per eye). Twenty-four hours later, the eyes, blood and spleens were harvested for histological analysis, measurement of serum Ig levels, and both flow cytometric analysis and cytokine assays respectively.

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 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 presented are an average ± SEM of all the mice examined.

Flow cytometric analysis

Splenocytes were incubated for 30 minutes on ice with FITC-labeled antibodies at optimal concentrations. After the incubation, the cells were washed with cold 2% fetal calf serum (FCS)-phosphate buffered saline (PBS). Dead cells were excluded by 7-amino-actinomycin D (7-AAD). Cells were analyzed using a FACScan (Becton Dickinson, Mountain View, CA, USA), and data acquisition and analysis were performed using CellQuest software. The data presented are the average percentage of antibody-stained cells ± SEM per spleen.

Measurement of serum Ig levels

Twenty-four hours after RW challenge, blood was collected and serum was prepared. Both total and RW-specific Ig levels in sera were assessed by ELISA, as described before [16, 19]. Data presented are ng/ml (total IgE, RW-specific

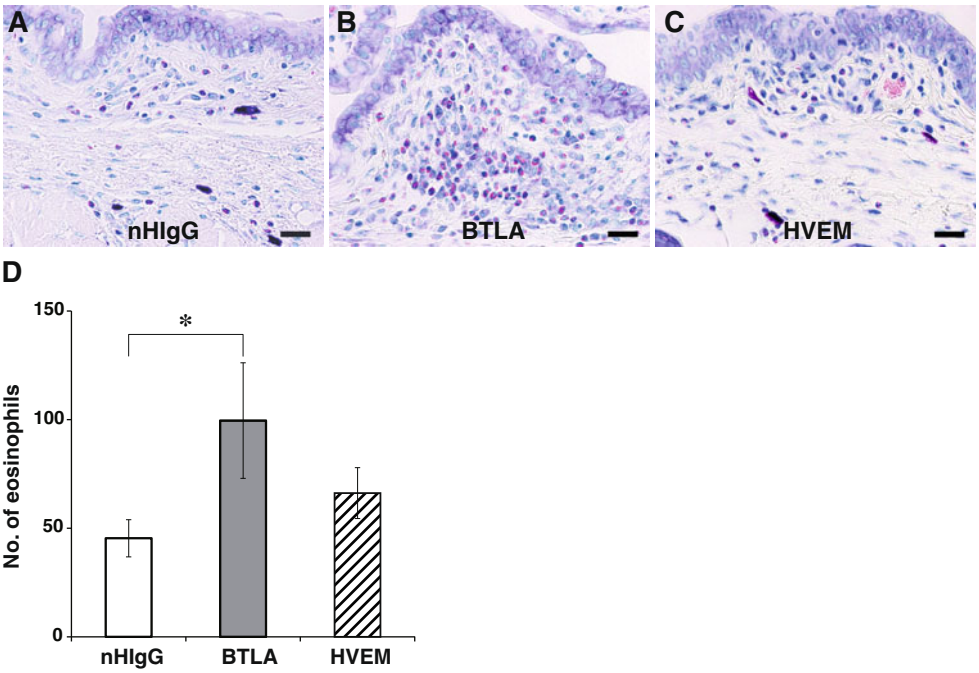

Fig. 1 Anti-BTLA antibody treatment during the induction phase augments conjunctival eosniophilia in EC. EC was induced in BALB/c mice by immunization with RW in alum and followed by RW challenge. The mice were intraperitoneally injected with anti-BTLA antibody, anti-HVEM antibody or control nhIgG during the induction phase. **a–c** Microphotographs of the conjunctiva. *Bar* = 20 μm. **d** Eosinophil counts per conjunctiva. Average ± SEM is shown. **P*<0.05

IgE and total IgG2a), μg/ml (total IgG1) or units/ml (RW-specific IgG1 and IgG2a).

Measurement of cytokines in the culture supernatants

Red blood cell (RBC)-depleted splenocytes (10^7 cells/ml) from anti-BTLA antibody and nhIgG-treated mice were cultured for 48 hours with RW extract (25 μg/ml) in 96-well flat-bottom plates in 0.2 ml RPMI 1640 medium supplemented with 10% FCS and 2-ME. Levels of IL-2, IL-4, IL-5, IL-10, IL-12 and IFN-γ were measured using the Bioplex system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Data shown are the average ± SEM (pg/ml) of four individual experiments.

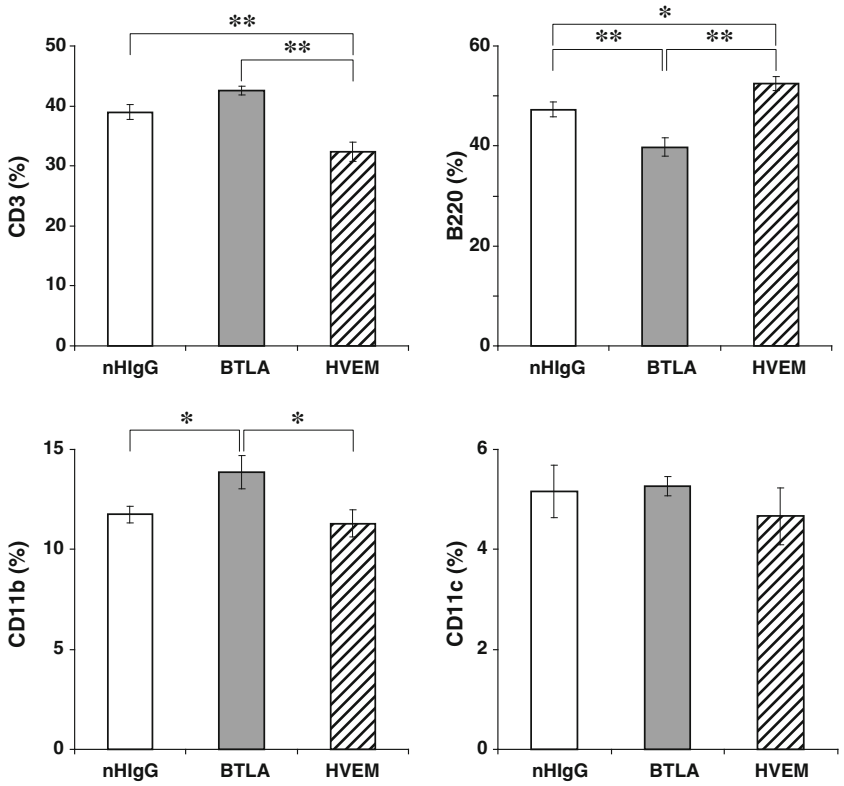

Fig. 2 Anti-BTLA antibody treatment during the induction phase decreases the B-cell compartment and increases the CD11b-positive cell compartment. EC was induced in BALB/c mice, and the mice were treated with antibodies as described in the legend to Fig. 1. Spleens were collected at the time of sacrifice, and splenocytes were subjected to flow cytometric analysis. Data are shown as the average % ± SEM out of whole splenocytes. **P*<0.05

Statistical analysis

Differences between the two groups in terms of the numbers of infiltrating eosinophils and serum Ig levels were analyzed by Fisher's PLSD test. To compare cytokine production between anti-BTLA antibody-treated and nhIgG-treated groups, Student's *t*-test was used. *P* values less than 0.05 were considered statistically significant.

Results

Treatment with anti-BTLA antibody during the induction phase exacerbates EC

BALB/c mice were immunized with RW in alum, and 10 days later they were challenged with RW in eye drops. Twenty-four hours later, conjunctivas, blood and spleens were collected. The mice were treated with 200 μg of anti-BTLA antibody, anti-HVEM antibody or control nhIgG five times (every other day from the day of immunization). Conjunctival eosinophil infiltration was upregulated by treatment with anti-BTLA antibody and was not affected by anti-HVEM antibody, as compared to nhIgG (Fig. 1a–c). Cell counting confirmed that significantly more eosinophils infiltrated into the conjunctiva of mice treated with anti-BTLA antibody compared to nhIgG-treated group (Fig. 1d).

Treatment with anti-BTLA antibody during the induction phase decreases B-cell population and upregulates Th2 cytokine production

To investigate the mechanism whereby anti-BTLA antibody exacerbates EC, surface marker expression and cytokine

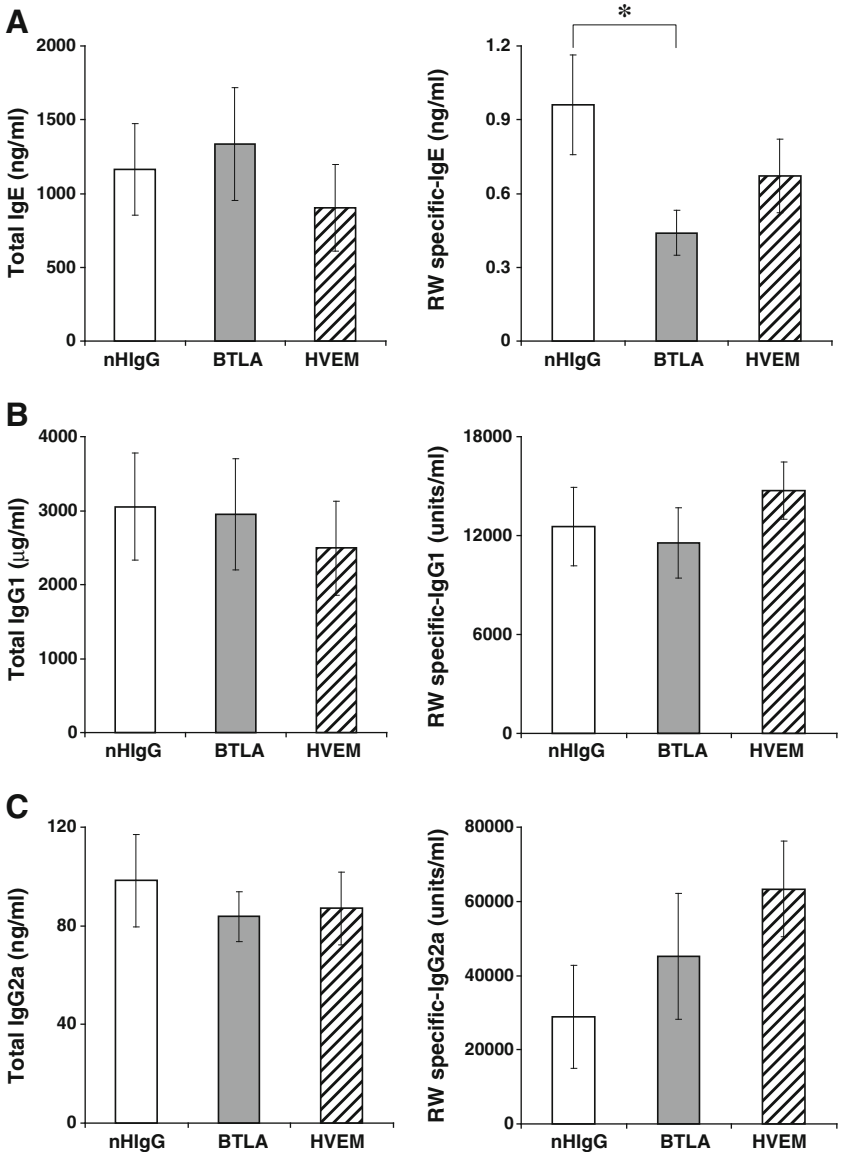

Fig. 3 Systemic humoral immune responses affected by anti-BTLA antibody treatment. When collecting the conjunctivas, blood was collected for the measurement of serum IgE (**a**) and IgG1 (**b**) and IgG2a levels (**c**). Data presented are ng/ml (total IgE, RW-specific IgE and total IgG2a), μg/ml (total IgG1) or units/ml (RW-specific IgG1 and IgG2a). *$P<0.05$

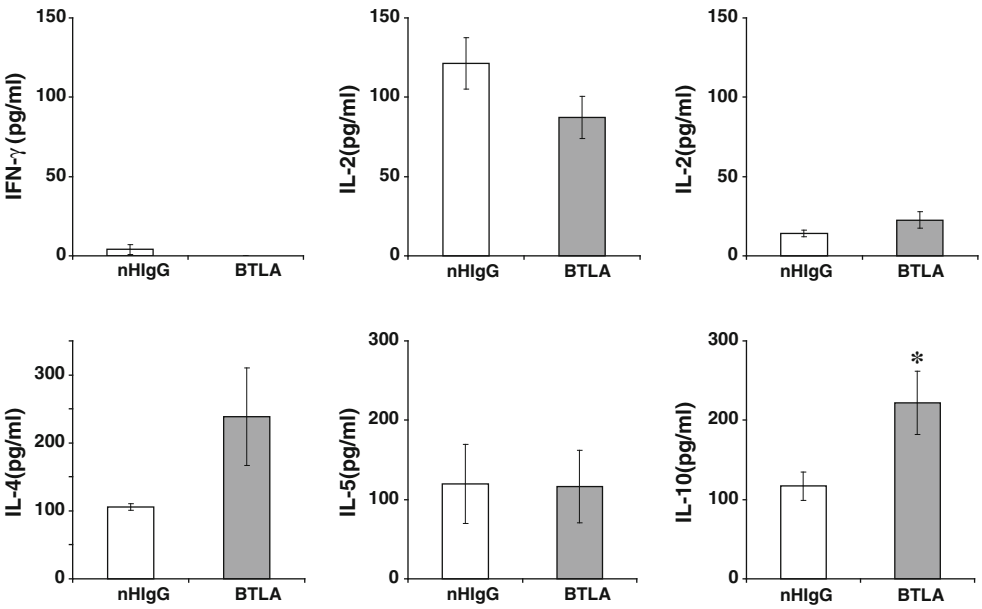

Fig. 4 Systemic cellular immune responses affected by anti-BTLA antibody treatment. When collecting the conjunctivas, spleens were collected for evaluation of RW-specific cytokine production. Data presented are pg/ml in the culture supernatants. *$P<0.05$

production of splenocytes were examined by flow cytometry and multiplex assay respectively. The total number of splenocytes did not differ among nhIgG-treated, anti-BTLA antibody-treated and anti-HVEM antibody-treated groups (data not shown). Treatment with anti-BTLA antibody decreased the B-cell compartment (Fig. 2, *upper right*) and significantly increased the CD11b-positive cell compartments compared to the other two groups (Fig. 2, *lower left*). In contrast, anti-HVEM antibody decreased the T-cell compartment (Fig. 2, *upper left*) and increased the B cell compartment compared to the other two groups (Fig. 2, *upper right*). The levels of total serum IgE did not differ among the three groups, but RW-specific IgE was reduced by treatment with anti-BTLA antibody compared to the nhIgG-treated group (Fig. 3a). Both total and RW-specific IgG levels were not significantly different among the three groups (Fig. 3b, c). Compared to the nhIgG-treated group, RW-recall cytokine production revealed that IL-4 and IL-10 were upregulated by anti-BTLA antibody treatment, although the difference regarding IL-4 did not reach significance (Fig. 4). The levels of other tested cytokines were not different between the two groups (Fig. 4).

Treatment with anti-BTLA antibody during the effector phase does not affect EC

To determine whether BTLA and HVEM participates in the development of EC during the effector phase, 200 μg of anti-BTLA or anti-HVEM antibody was intraperitoneally injected once, 2 hours prior to RW challenge. Conjunctival eosinophil numbers were not different among the three groups (Fig. 5).

Discussion

BTLA regulated the development of EC in the induction phase because treatment with anti-BTLA antibody during the induction phase increased the conjunctival eosinophil numbers. However, treatment with anti-BTLA antibody during the effector phase did not affect EC. In contrast, treatment with an antibody against HVEM, which is a ligand for BTLA, did not affect the development of EC in either the induction or the effector phase.

BTLA is constitutively expressed in murine splenic B cells and is expressed much less in splenic T cells [20].

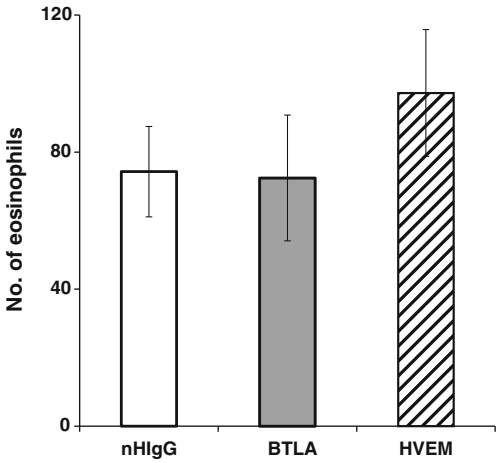

Fig. 5 Anti-BTLA or anti-HVEM antibody treatment during the effector phase does not affect conjunctival eosniophilia in EC. EC was induced in BALB/c mice by immunization with RW in alum following RW challenge. The mice were intraperitoneally injected with anti-BTLA antibody, anti-HVEM antibody or control nhIgG once before RW challenge. Data are presented as eosinophil counts per conjunctiva

BTLA is detected in thymic T cells during positive selection, and is less abundantly expressed in CD11c-positive dendritic cells (DCs) and naive T cells [21, 22]. Studies using expression cloning identified HVEM as the BTLA ligand [23], and the crosslinking of BTLA by HVEM-Ig inhibits T-cell proliferation and cytokine production [23, 24]. In contrast, HVEM is widely expressed in many organs and in various types of cells, such as T cells, B cells and DCs, and all other types of cells within the lymphoid tissue can express HVEM [25]. Furthermore, HVEM binds not only with BTLA but also with LIGHT (lymphotoxin-like, inducible expression, competes with herpes simplex virus glycoprotein D for HVEM, a ligand expressed by activated T cells), lymphotoxin α (LTα), CD160 and glycoprotein D (gD) [23, 26–28] . Thus, cellular distribution and ligand specificity are different between BTLA and HVEM and therefore, the effects of anti-BTLA antibody treatment in EC differ from those of anti-HVEM antibody treatment.

Although IL-4 was not significantly upregulated, anti-BTLA antibody treatment upregulated both RW-specific IL-4 and IL-10 production, which indicates the upregulation of Th2 immune responses. Together with the fact that EC can be induced by the transfer of Th2 cells but not Th1 cells [6], the upregulation of Th2 cytokines by anti-BTLA antibody treatment may account for the increased number of eosinophils in the conjunctiva of mice treated with anti-BTLA antibody. Although BTLA was originally identified as a murine Th1-specific gene owing to the finding that BTLA mRNA expression in Th1 cells was much higher than in Th2 cells by Northern blot analysis [20], it was also reported that BTLA is equally expressed on Th1 and Th2 cells, and its expression is lost in Th1/Th2 clones [29]. Because IFN-γ production was not affected by anti-BTLA antibody treatment, it may be possible that BTLA participates in Th2 deviation in vivo. In addition to Th1 and Th2 cells, follicular Th (Tfh) cells have been identified to be a subset of effector T cells that helps the development of Ag-specific B-cell responses in the germinal centers [30]. With regard to the relationship between BTLA and Tfh cells, adoptive transfer of $BTLA^{-/-}$ CD4-positive T cells to wild-type (WT) mice induced more Ag-specific IgG2a and IgG2b production than the transfer of WT cells, when transferring cells were cultured under Tfh cell-inducing conditions [31]. The involvement of Tfh cells in EC is uncertain, but it is possible that Tfh cells modulated by anti-BTLA antibody affect the development of EC.

Recently, it has been demonstrated that systemic administration of recombinant plasmid DNA encoding BTLA led to a decreased infiltration of $CD4^+$ T cells into herpes simplex virus type 1(HSV-1)-infected corneas, and resulted in a diminished incidence and severity of HSV-1 stromal keratitis compared to the control group [32]. Together with our finding that BTLA inhibits antigen-induced conjunctival eosinophilia in BALB/c mice, BTLA may regulate ocular surface inflammatory diseases mediated by either Th1 or Th2 cells. Therefore, BTLA may be prophylactic and therapeutic targets to treat a variety of ocular surface inflammatory diseases, including HSV-1 stromal keratitis and severe ACDs.

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