
LABORATORY INVESTIGATION

Aggravation of Conjunctival Early-Phase Reaction by *Staphylococcus* Enterotoxin B via Augmentation of IgE Production

Dai Miyazaki¹, Waka Ishida², Takeshi Tominaga¹, Tamaki Sumi²,
and Atsuki Fukushima²

¹ Division of Ophthalmology and Visual Science, Tottori University Faculty of Medicine, Yonago, Japan; ² Department of Ophthalmology and Visual Science, Kochi Medical School, Nankoku, Japan

Abstract

Purpose: To investigate whether *Staphylococcus* enterotoxin B (SEB) affects the early-phase reaction (EPR) in experimental conjunctivitis.

Methods: Nc/Nga mice were sensitized to ragweed (RW) or phosphate-buffered saline (PBS) in alum. The mice were subsequently treated three times a day with eye drops adulterated with SEB or vehicle on postimmunization days 29 to 31. On postimmunization day 32, the mice were administered eye drops adulterated with RW, and the EPR was evaluated. Ninety minutes after the RW challenge, the eyes were harvested for histological evaluation of degranulation of mast cells, and blood was drawn for subsequent measurement of serum antibody levels.

Results: The total EPR score was significantly higher in the RW-sensitized mice than in the PBS-sensitized mice. Among the RW-sensitized mice, the SEB-treated mice had significantly higher EPR scores than did the vehicle-treated mice. Treatment with SEB significantly increased the degranulated mast cells in the eyes of the RW-sensitized mice. Serum levels of RW-specific IgG1 and IgG2a were significantly higher in the RW-sensitized mice than in the control mice. The total IgE level was significantly higher in the RW-sensitized, SEB-treated mice than in the other three groups of mice.

Conclusions: Topical SEB treatment upregulated systemic IgE production, which may augment conjunctival EPR. **Jpn J Ophthalmol** 2010;54:476–480 © Japanese Ophthalmological Society 2010

Keywords: allergic conjunctivitis, early-phase reaction, IgE, *Staphylococcus* enterotoxin B

Introduction

Allergic inflammation consists of two different phases: the early-phase reaction (EPR) and the late-phase reaction (LPR). The results of several studies suggest that the EPR usually involves cells that are normal residents of the epithelium (mast cells) and preformed substances (histamine), whereas cells participating in the LPR are recruited from circulation (eosinophils, basophils, and T cells).¹ Among

these inflammatory cells, eosinophils influence the severity of allergic conjunctivitis (AC).² Most studies of experimental AC predominantly investigated the LPR rather than the EPR because evaluation of conjunctival eosinophilia is easier and more quantitative than that of clinical EPR findings such as edema and hyperemia.³

Staphylococcus aureus has been identified as a trigger factor in atopic dermatitis (AD). Most patients with AD are colonized with *S. aureus* without showing any sign of bacterial infection.^{4–6} On the other hand, only 5%–30% of healthy individuals are carriers of this microorganism.^{4–6} *Staphylococcus aureus* can be isolated from clinically affected and unaffected skin, and bacterial counts on the nonlesional skin of patients with AD are lower than those on lesional skin. Up to 65% of *S. aureus* strains isolated from patients with AD produce exotoxins such as staphylococcal entero-

Received: November 11, 2009 / Accepted: April 13, 2010

Correspondence and reprint requests to: Atsuki Fukushima, Department of Ophthalmology, Kochi Medical School, Kohasu, Oko-cho, Nankoku 783-8505, Japan
e-mail: fukushima@kochi-u.ac.jp

toxins A, B, C, and D and toxic shock syndrome toxin 1,⁷⁻⁹ which play an important role in allergic inflammation.

Staphylococcus enterotoxin B (SEB) reportedly aggravates eosinophilic inflammation in the airway lumen and bronchial tissue.¹⁰ More recently, inoculation with *S. aureus* markedly accelerated conjunctival eosinophilia, which is a sign of AC.¹¹ However, to date, the effects of SEB on the EPR have not been clarified in a model of allergic disease. Therefore, we aimed to investigate how SEB affects the EPR in experimental conjunctivitis (EC), a model of AC.

Materials and Methods

Isolation of Connective Tissue-Type Mast Cells and β -Hexosaminidase Release Assay

We evaluated skin-derived connective tissue-type mast cells because the NC/Nga mouse is a murine model of AD. Mast cells were isolated from mouse skin using an enzymatic digestion protocol described earlier.¹² Briefly, the skin from 8- to 12-week old NC/Nga mice was enzymatically digested with 1.5 mg/ml collagenase (Nitta Gelatin, Osaka, Japan), 0.5 mg/ml hyaluronidase (Sigma, St. Louis, MO, USA), and 0.5 mg/ml DNase I (Sigma) for 2 h at 37°C. The dispersed cells were layered onto isotonic Percoll density medium (density, 1.041; Amersham Pharmacia Biotech, Piscataway, NJ, USA) and centrifuged at 800g for 20 min. The cell pellet was collected and cultured in RPMI1640 with murine recombinant interleukin (IL)-3 (10 ng/ml; Peprotech, Rocky Hill, NJ, USA), recombinant stem cell factor (SCF; 10 ng/ml; Peprotech), and 5% serum for 6 days. Lineage marker-positive cells and plasmacytoid dendritic cells were depleted after labeling with rat anti-CD4, rat anti-CD8a, rat anti-CD11b, rat anti-B220 (all from eBiosciences, San Diego, CA, USA), and rat anti-PDCA-1 (Miltenyi Biotec, Auburn, CA, USA) using anti-rat immunoglobulin κ chain antibody-conjugated microbeads (BD Biosciences, Franklin Lakes, NJ, USA). Isolated mast cells were sensitized by incubation with anti-dinitrophenol (DNP) IgE (SPE7; Sigma) in RPMI1640 containing 10 ng/ml of murine recombinant IL-3, 10 ng/ml of recombinant SCF, and 5% murine serum, and cultured with SEB for 2 days. The cells at a concentration of $(2-8) \times 10^4$ cells/100 μ l were stimulated with DNP-albumin (Sigma) or compound 48/80 (Sigma). After 45 min, the collected supernatant was measured for β -hexosaminidase activity using a published protocol.¹² The relative release of β -hexosaminidase was defined as the activity in the supernatant of the tested cells divided by the activity in the positive control cell supernatant multiplied by 100. Compound 48/80 stimulus was used for the assay control.

Induction of EC and SEB Treatment

Four-week-old female NC/Nga mice (Charles River Laboratories Japan, Yokohama, Japan) were immunized by intraperitoneal injection of 50 μ g of ragweed (RW) (Polysciences,

Warrington, PA, USA) in 1 mg of alum (Sigma). As the control, additional mice received phosphate-buffered saline (PBS) in 1 mg of alum instead of RW in alum. All mice were maintained under specific pathogen-free conditions at the Tottori University animal facility. All procedures were carried out in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Mice were treated with eye drops adulterated with SEB (250 ng/2.5 μ l; Sigma) or vehicle (2.5 μ l PBS) three times daily on postimmunization days 29 to 31.

Evaluation of EPR and Immune Responses

On postimmunization day 32, the mice received eye drops adulterated with RW, and the EPR was evaluated. After 90 min, the eyes, blood, and lymph nodes were harvested for histological evaluation of degranulation of mast cells and measurement of serum antibody levels and RW-induced cytokine production, respectively. The EPR was recorded within the first 30 min, and the responses were graded using criteria described elsewhere.¹³ Degranulation of mast cells was evaluated as previously reported¹⁴ (described in the next section), and the data were presented as the percentage of degranulated mast cells in the total number of mast cells. The antibody levels were measured by enzyme-linked immunosorbent assay, as detailed previously.¹⁵

Histological Evaluation

For histological evaluation of the immediate hypersensitivity reactions, the mice were killed and their tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Serial sagittal sections (3 μ m in thickness) were stained with Giemsa. Three consecutive sections from each eye were examined, and the number of degranulated mast cells was counted in a 200 $\times$ microscopic field in a masked fashion by an independent investigator.

Statistical Analysis

For evaluation of clinical grading, degranulation of mast cells, and antibody titers, one-way analysis of variance followed by Tukey-Kramer post hoc tests were used. The *t* test was used for the β -hexosaminidase assay.

Results

Effect of SEB Treatment on Connective Tissue-Type Mast Cell Degranulation

To investigate the effects of SEB on mast cell degranulation, connective tissue-type mast cells were sensitized with DNP-

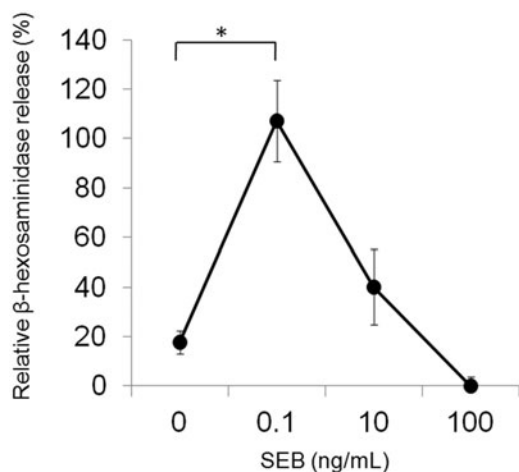


Figure 1. Effect of *Staphylococcus enterotoxin B* (SEB) treatment on mast cell degranulation of connective tissue-type mast cells. Dinitrophenol-specific IgE-sensitized mast cells were cultured with SEB and assessed for allergen-induced mast cell degranulation using a β-hexosaminidase release assay. SEB treatment significantly enhanced β-hexosaminidase at 0.1 ng/ml ($n = 6$, $P < 0.05$).

specific IgE, cultured with SEB, and assessed for allergen-induced mast cell degranulation using a β-hexosaminidase release assay. Treatment with SEB at a dose of 0.1 ng/ml significantly enhanced β-hexosaminidase ($P < 0.05$; Fig. 1).

Effects of SEB Treatment on EC

On the basis of the above finding and a previous report,¹⁶ we decided to treat mice with SEB at a dose of 250 ng/2.5 μl in eye drops. The total EPR score was significantly higher in the RW-sensitized group of mice than in the control group that had not been sensitized with RW ($P < 0.01$; Fig. 2A). Treatment with SEB did not affect the scores of the control mice. In contrast, SEB treatment significantly increased the scores of the RW-sensitized mice ($P < 0.05$; Fig. 2A).

The histological analysis revealed that mast cell degranulation was exacerbated in the SEB-treated sensitized mice (upper panel, Fig. 2B) as compared to the vehicle-treated sensitized mice (lower panel, Fig. 2B). Counting of degranulated mast cells demonstrated that the percentage of degranulated mast cells was significantly higher in the RW-

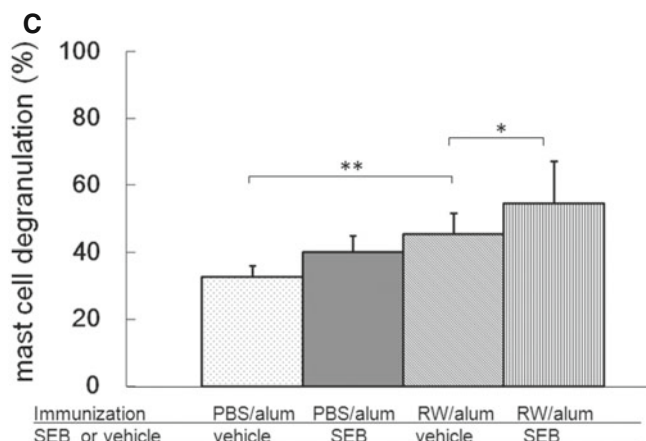
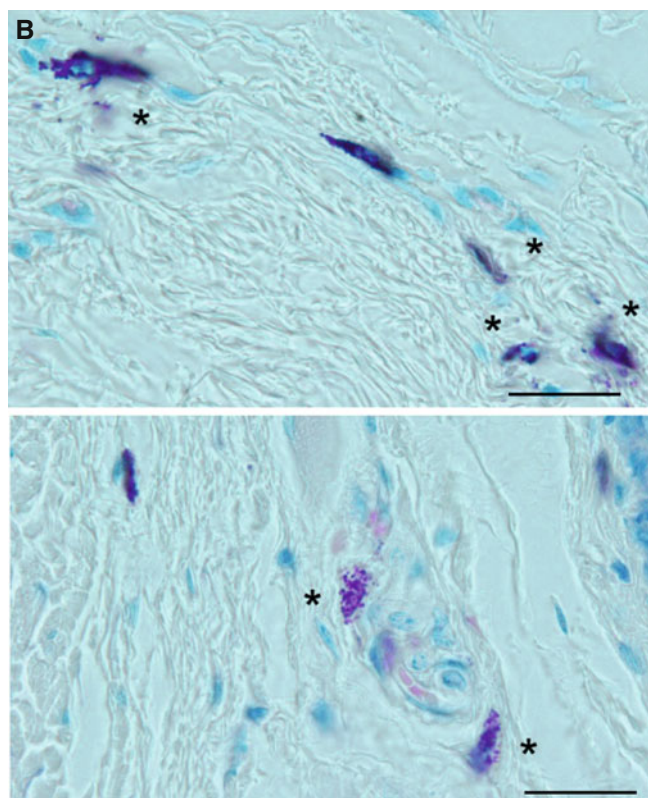
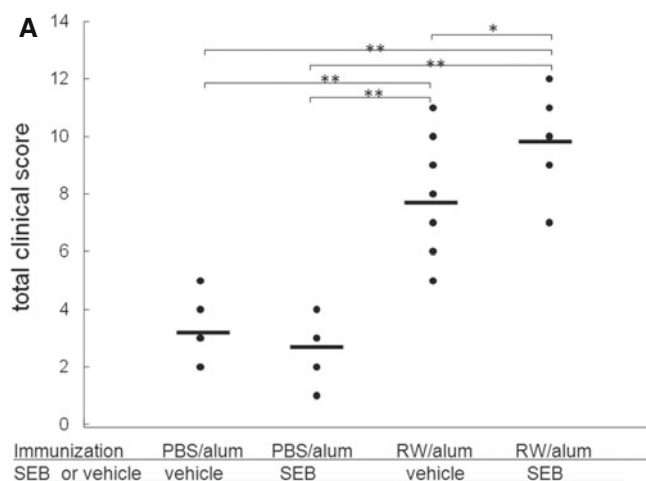


Figure 2A–C. Effect of SEB treatment on the conjunctival early-phase reaction (EPR). Experimental conjunctivitis was induced in NC/Nga mice, and the EPR was evaluated. **A** Total EPR score. ●, data of one individual mouse; —, median values. The total EPR score was significantly higher in the ragweed (RW)-sensitized group of mice. SEB treatment significantly increased the scores of the RW-sensitized mice. PBS, phosphate-buffered saline. **B** Mast cell degranulation was exacerbated in the SEB-treated sensitized mice (upper panel). The lower panel shows vehicle-treated sensitized mice. *, degranulated mast cells. Bar = 25 μm. **C** The percentage of degranulated mast cells relative to total mast cells. SEB treatment significantly increased the percentage of degranulated mast cells in RW-sensitized mice. * $P < 0.05$, ** $P < 0.01$.

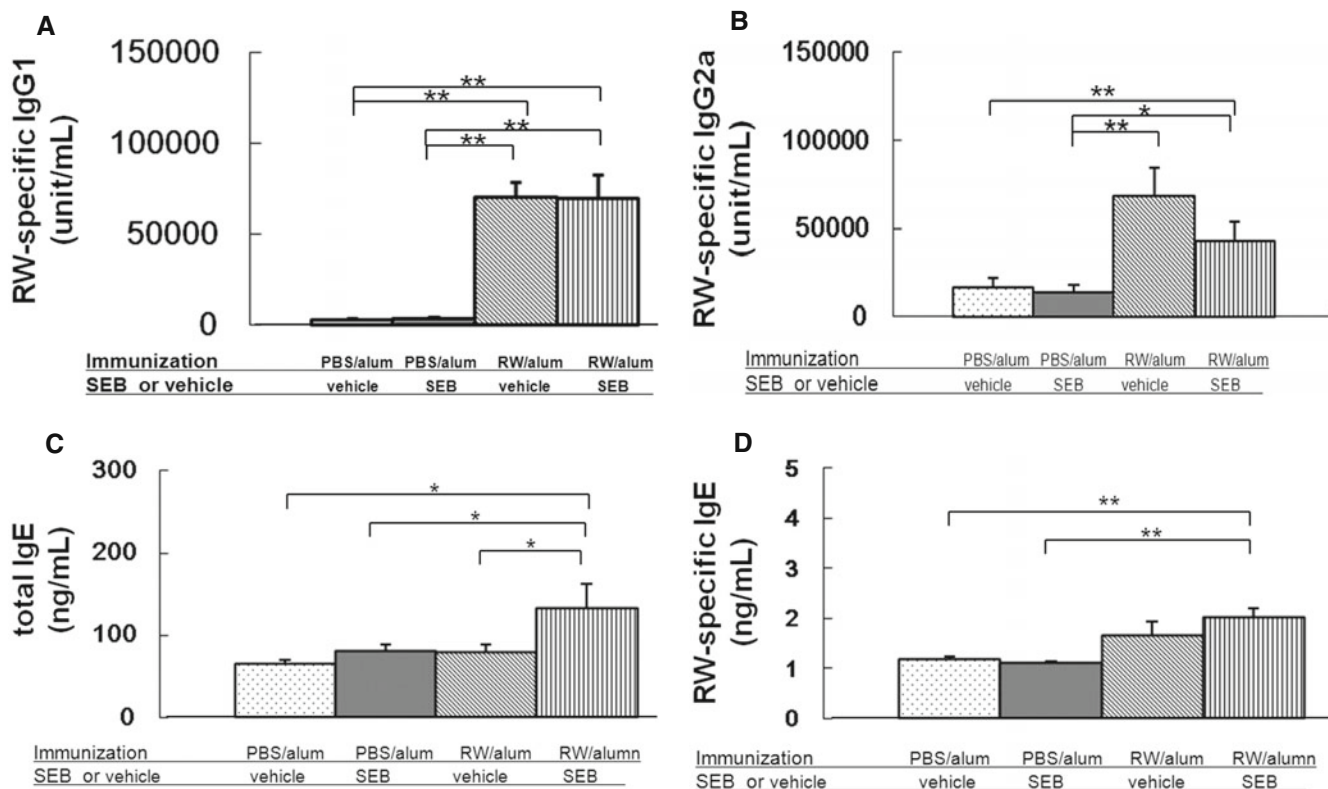


Figure 3A–D. Effect of SEB on antibody levels in serum. After the mice were killed, blood was collected and serum was prepared for enzyme-linked immunosorbent assay. Total and RW-specific IgG1, IgG2a, and IgE were evaluated. **A** RW-specific IgG1 and **B** IgG2a levels. The levels of RW-specific IgG1 and IgG2a were significantly higher in the blood serum of RW-sensitized mice than in the PBS-sensitized mice. **C** Total and **D** RW-specific IgE levels. The serum level of total and RW-specific IgE was significantly higher in the RW-sensitized, SEB-treated mice than in the PBS-sensitized groups of mice. * $P < 0.05$, ** $P < 0.01$.

sensitized, vehicle-treated mice than in the PBS-sensitized, vehicle-treated mice ($P < 0.01$; Fig. 2C). Importantly, a significant difference in the percentage of degranulated mast cells was observed between the RW-sensitized mice treated with SEB and those treated with vehicle ($P < 0.05$; Fig. 2C).

Evaluation of Antibody Titers

The levels of RW-specific IgG1 and IgG2a were significantly higher in the blood serum of RW-sensitized mice than in the PBS-sensitized mice (Fig. 3A, B). However, the total IgG1 and IgG2a levels did not differ between the mice of these two groups (data not shown). The total IgE level was significantly higher in the RW-sensitized, SEB-treated mice than in the mice of the other three experimental groups ($P < 0.05$; Fig. 3C). The serum level of RW-specific IgE was also significantly higher in the RW-sensitized, SEB-treated mice than in the PBS-sensitized groups of mice ($P < 0.01$; Fig. 3D).

Stimulation with RW increased lymph node production of IL-4 slightly more in the RW-sensitized mice than in the PBS-sensitized mice, whereas interferon γ production did not differ among the four groups of mice (data not shown).

Discussion

In this study, we demonstrated that topical SEB treatment upregulated systemic IgE production, which may augment conjunctival EPR. Importantly, these data are in good agreement with a previous report demonstrating that epicutaneous sensitization of mice with SEB increased the IgE titers in serum and elicited allergic skin inflammation.¹⁶

Several questions remain as to how SEB augments IgE production. The actions of SEB may directly involve B-cell activation,¹⁷ although we have not investigated the levels of SEB in serum. Alternatively, SEB may activate T cells by serving as a superantigen, leading to antibody production.¹⁸ These potential mechanisms need to be investigated further. *Staphylococcus aureus* infections that produce SEB are significantly more frequent in patients with atopic dermatitis than in healthy control patients.¹⁹ Furthermore, some evidence suggests that *S. aureus* colonization may play a role in the pathogenesis of chronic AC.²⁰ Together, these data suggest that SEB participates in the EPR in AC and that control over *S. aureus* infections may help suppress AC.

We have demonstrated that antigen-specific T cells play a crucial role in the development of conjunctival eosinophilia in EC.²¹ Since SEB appears to stimulate T cells as a

superantigen,¹⁸ it is possible that SEB treatment affects T cell-mediated conjunctival eosinophilia in EC. Future studies will focus on whether and how SEB affects conjunctival eosinophilia.

References

- Picado C. Early and late-phase asthmatic reactions: a hypothesis. *Allergy* 1992;47:331–333.
- Fukagawa K, Nakajima T, Tsubota K, Shimmura S, Saito H, Hirai K. Presence of eotaxin in tears of patients with atopic keratoconjunctivitis with severe corneal damage. *J Allergy Clin Immunol* 1999;103:1220–1221.
- Niederlorn JY. Immune regulatory mechanisms in allergic conjunctivitis: insights from mouse models. *Curr Opin Allergy Clin Immunol* 2008;8:472–476.
- Breuer K, Kapp A, Werfel T. Bacterial infections and atopic dermatitis. *Allergy* 2001;56:1034–1041.
- Breuer K, Häussler S, Kapp A, Werfel T. *Staphylococcus aureus*: colonizing features and influence of an antibacterial treatment in adults with atopic dermatitis. *Br J Dermatol* 2002;147:55–61.
- Leyden JE, Marples RR, Kligmann AM. *Staphylococcus aureus* in the lesions of atopic dermatitis. *Br J Dermatol* 1974;90:525–530.
- Akiyama H, Toi Y, Kanzaki H, Tada J, Arata J. Prevalence of producers of enterotoxins and the toxic shock syndrome toxin-1 among *Staphylococcus aureus* strains isolated from atopic dermatitis lesions. *Arch Dermatol Res* 1996;288:418–420.
- Bunikowski R, Mielke M, Skarabis H, et al. Evidence for a disease promoting effect of *Staphylococcus aureus*-derived exotoxins in atopic dermatitis. *J Allergy Clin Immunol* 2000;105:814–819.
- McFadden JP, Noble WC, Camp RDR. Superantigenic exotoxin-secreting potential of staphylococci isolated from atopic eczematous skin. *Br J Dermatol* 2000;128:631–632.
- Hellings PW, Hens G, Meyts I, et al. Aggravation of bronchial eosinophilia in mice by nasal and bronchial exposure to *Staphylococcus aureus* enterotoxin B. *Clin Exp Allergy* 2006;36:1063–1071.
- Chung S-H, Namb K-H, Kweon M-N. *Staphylococcus aureus* accelerates an experimental allergic conjunctivitis by Toll-like receptor 2-dependent manner. *Clin Immunol* 2009;131:170–177.
- Tominaga T, Miyazaki D, Sasaki S, et al. Blocking mast cell-mediated type I hypersensitivity in experimental allergic conjunctivitis by monocyte chemoattractant protein-1/CCR2. *Invest Ophthalmol Vis Sci* 2009;50:5181–5188.
- Nakamura T, Toda M, Ohbayashi M, Ono SJ. Detailed criteria for the assessment of clinical symptoms in a new murine model of severe allergic conjunctivitis. *Cornea* 2003;22:S13.
- Miyazaki D, Nakamura T, Toda M, Cheung-Chau KW, Richardson RM, Ono SJ. Macrophage inflammatory protein-1 α as a costimulatory signal for mast cell-mediated immediate hypersensitivity reactions. *J Clin Invest* 2005;115:434–442.
- Fukushima A, Yamaguchi T, Azuma M, Yagita H, Ueno H. Involvement of programmed death-ligand 2 (PD-L2) in the development of experimental allergic conjunctivitis in mice. *Br J Ophthalmol* 2006;90:1040–1045.
- Laouini D, Kawamoto S, Yalcindag A, et al. Epicutaneous sensitization with superantigen induces allergic skin inflammation. *J Allergy Clin Immunol* 2003;112:981–987.
- Franz A, Bryant A, Farrant J. Staphylococcal enterotoxin B up-regulates interleukin-2 receptor beta chain expression on tonsillar B cells. *Eur J Immunol* 1993;23:2696–2699.
- Torres BA, Perrin GQ, Mujtaba MG, Subramaniam PS, Anderson AK, Johnson HM. Superantigen enhancement of specific immunity: antibody production and signaling pathways. *J Immunol* 2002;169:2907–2914.
- Breuer K, Kapp A, Werfel T. Bacterial infections and atopic dermatitis. *Allergy* 2001;56:1034–1041.
- Tuft SJ, Ramakrishnan M, Seal DV, Kemeny DM, Buckley RJ. Role of *Staphylococcus aureus* in chronic allergic conjunctivitis. *Ophthalmology* 1992;99:180–184.
- 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;44:4366–4374.