

Topical Administration of β -1,3-Glucan to Modulate Allergic Conjunctivitis in a Murine Model

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PURPOSE. Recent studies on β -1,3-glucan (BG), a cell wall component of a variety of fungi, yeasts, and bacteria, demonstrated that it affects the balance of Th1/Th2 immune responses. We therefore determined whether topical application of BG modulates ocular allergy in a murine model.

METHODS. We sensitized 7- to 8-week-old BALB/c mice once with ovalbumin (OVA) and aluminum hydroxide via intraperitoneal injection. Mice were rested for 2 weeks and then challenged by instillation of OVA eye drops once daily for 13 days. We administered BG eye drops 5 minutes after OVA challenge once daily. Clinical signs were measured, the infiltration of eosinophils and mast cells into conjunctiva was assessed with flow cytometry, and the serum levels of OVA-specific IgE production and Th2 cytokines after in vitro stimulation of T cells in draining lymph nodes (LN) were determined.

RESULTS. Mice treated with BG showed attenuated allergic conjunctivitis, as indicated by clinical signs and decreased production of serum OVA-specific IgE. In addition, BG treatment led to decreased infiltration of CD45+ immune cells, eosinophils, and mast cells into the conjunctiva, compared with the mice treated with vehicle alone (control mice). Administration of BG suppressed Th2 cytokine production in in vitro T-cell assays partially through the induction of interleukin (IL)-10-producing CD4 T cells in draining LNs.

CONCLUSIONS. Taken together, these results suggest that BG is capable of stimulating IL-10-producing CD4+ T cells and suppressing both the Th2 response in draining LNs and conjunctival eosinophil infiltration. We therefore demonstrated the therapeutic potential of topical BG administration for allergic conjunctivitis.

Keywords: allergic conjunctivitis, β -1,3-glucan, IL-10, immune modulation, treg

The conjunctiva is the most common site of allergic inflammation because its mucosal surfaces are highly accessible to airborne allergens. The prevalence of allergic diseases is rapidly increasing worldwide, and allergic conjunctivitis (AC) is one of the most common diseases encountered in eye clinics. Many types of topical and systemic drugs are used to manage its symptoms, which include ocular itching, eye discharges, and redness.^{1,2} Currently, the drugs used for treatment of AC include mast cell stabilizers, antihistamines, nonsteroidal anti-inflammatory drugs, steroids, and immunosuppressants such as cyclosporine and tacrolimus. In addition to pharmacotherapy, allergen avoidance and immunotherapy, for example, allergen-specific immunotherapy (SIT), are also recommended for patients. However, environmental control is very difficult especially against airborne allergens, and SIT has some risks such as anaphylaxis and immunologic adverse effects. In addition, many antiallergic treatments have variable responses and significant complications, such as irritation, cataract, or glaucoma. Thus, a new generation of therapeutics to modulate allergic responses is needed to overcome the problems associated with medical treatment of allergic disease.^{1,3,4}

Recently β -glucans, extracted from yeast, fungi, bacteria, and mushrooms, are emerging as possible safe and effective

immunotherapeutic drugs to modulate the immunologic response and to reduce susceptibility to infection and cancer.^{5,6} Previous studies have shown that systemic application of β -1,3-glucan (BG) could suppress IgE-mediated allergy in a murine model and human clinical studies.^{6,7} The authors suggested that BGs alleviate Th2-mediated-allergic reactions due to their Th1-skewing ability, which is achieved through immune modulatory action on macrophages or dendritic cells.^{6,7} However, the therapeutic potential of topical BG for AC and the underlying mechanisms of its actions remain still unknown.

The present study was designed to evaluate the efficacy of topically administering BG to suppress the development of ovalbumin (OVA)-sensitized AC in a murine model.

MATERIALS AND METHODS

Animals and Anesthesia

Eight-week-old BALB/c male mice were purchased from Charles River Laboratories (Strain code: 028, Orient Co., Sungnam, Korea). Mice were cared for in a specific pathogen-free environment at the animal facility in accordance with the Catholic Institutional Animal Care and Use Committee and the ARVO Statement for the Use of Animals in Ophthalmic and



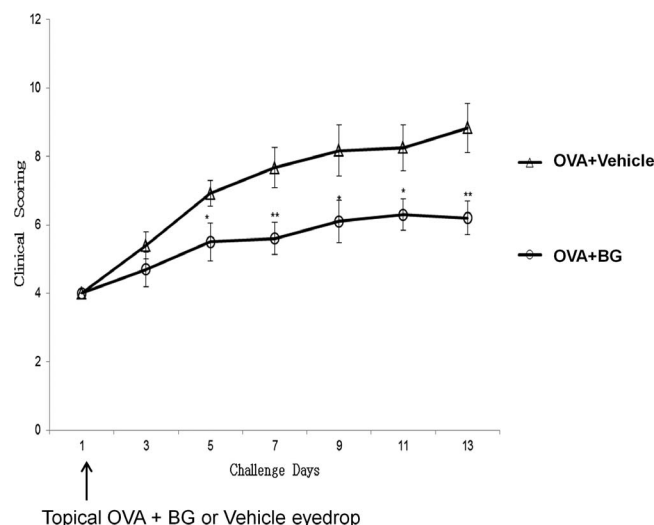


FIGURE 1. β -1,3-glucan inhibits development of AC. Mice were injected intraperitoneally with 100 μ g OVA/Al and the others were naïve as a control. After 2 weeks, BG or vehicle eye drops were applied 5 minutes posttopical OVA challenge in the right eye of each mouse once a day for 13 days. Clinical scorings (i.e., lid edema, conjunctival hyperemia, chemosis, and tearing/discharge) were evaluated in a masked fashion, 20 minutes posttopical OVA challenge. Graphs are representative of three independent experiments and data are presented as mean and standard deviation ($n = 6/\text{group}$). * $P < 0.05$, ** $P < 0.01$.

Vision Research. Anesthesia was used for all surgical procedures with intraperitoneally administered ketamine/xylazine suspensions (120 and 20 mg/kg, respectively).

Induction of AC

This procedure was performed using a previously described model.⁸ Briefly, BALB/c mice were immunized with one intraperitoneal injection of 100 μ g OVA in 100 μ L PBS that included 1 mg aluminum hydroxide and 300 ng of pertussis toxin, all purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Mice were sensitized for 2 weeks and then challenged by topical OVA eye drops (250 μ g/5 μ L) once daily for at least 13 days.

Clinical evaluation of AC, which involved checking for immediate hypersensitivity responses 20 minutes after topical OVA challenge, was performed in a masked fashion by two independent observers. Clinical scoring consisted of four parameters: lid swelling, conjunctival chemosis, conjunctival redness, and tearing. Each parameter was scored on a scale from 0 (absence of signs) to 3 (maximal), and the scores for these four parameters were summed together for the total scoring.

Topical Administration of BG

β -1,3-glucan, extracted from *Euglena gracilis*, was purchased from Sigma-Aldrich Corp. (cat. #89862) and diluted to 1 mg/mL in 1% dimethyl sulfoxide and PBS. We administered BG eye drops (5 μ L) 5 minutes after topical OVA eye drops (500 μ g/mL) once daily to prevent dilution of OVA and BG. To analyze whether the interleukin (IL)-10 are involved in the immunomodulation of BG, mice were injected at tail vein intravenously with 200 μ g of anti-IL-10R mAb (clone 1B1.3a; BioLegend, San Diego, CA, USA) or isotype control rat IgG1 κ antibody (clone GL113; BioLegend) every other day up to 9 days, 5 minutes after daily topical OVA with BG or vehicle challenge.

ELISA for OVA-Specific IgE in Serum

Following topical challenges for 13 days, blood was collected and pooled from freshly euthanized mice by cardiac puncture. Sera were isolated via coagulation and centrifugation. Samples were analyzed using an ELISA kit for OVA-specific IgE according to the manufacturer's instructions (AbD Serotec, Raleigh, NC, USA).

Quantitation of Conjunctival Mast Cells and Eosinophils

This procedure has been described previously⁹; 20 minutes after the topical challenge, the mice were euthanized and ipsilateral bulbar and palpebral conjunctivae were collected by vannas scissors. Conjunctival tissues were digested with 2 mg/mL collagenase (Hoffmann-La Roche, Basel, Switzerland) and 0.05 mg/mL DNase I (Hoffmann-La Roche) for 2 hours at 37°C. Suspensions were filtered through a 70- μ m cell strainer and washed carefully. Cells were resuspended in 0.5% bovine serum albumin buffer, and treated with anti-FcR (CD16/CD32; BioLegend) antibodies to block Fc-mediated reactions, as per manufacturer's instructions. Cells were subsequently stained with an AlexaFluor 488-conjugated anti-CD45 antibody (clone 30-F11; BioLegend), a phycoerythrin-cyanin dye 7 (PE-Cy7)-conjugated anti c-kit antibody (clone 2B8; BioLegend), and a PE-conjugated sialic acid binding immunoglobulin-type lectin-F (Siglec-F; clone E50-2440, BD Biosciences, East Rutherford, NJ, USA) antibody. Isotype control was stained with appropriate-matched antibodies (BioLegend). All samples were analyzed using a BD LSRFortessa flow cytometer (BD Biosciences).

Preparation of Bone Marrow Dendritic Cells (BMDCs)

This procedure has been previously described.⁹ Briefly, the femurs and tibia were removed from naïve BALB/c mice and these bones were irrigated with a syringe and Roswell Park Institution Medium (RPMI) 1640 to collect bone marrow cells. Cells were washed and plated ($1 \times 10^5/\text{mL}$) in RPMI 1640 medium that included 10% fetal bovine serum, 20 ng/mL mouse granulocyte-macrophage colony-stimulating factor (BioLegend), and 1% penicillin/streptomycin. Media were changed every other day and loosely adherent cells were harvested on day 7.

In Vitro Stimulation of T Cells

This procedure was previously described.⁹ Briefly, ipsilateral cervical and submandibular lymph nodes (LNs) were collected following topical challenge. T cells were enriched via magnetic-activated cell sorting using anti-CD90.2 antibodies according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Enriched T cells were counted via a trypan blue exclusion assay and plated in round-bottom 96-well plates at a concentration of $1.25 \times 10^6/\text{mL}$. Ovalbumin-pulsed immature BMDCs prepared as described above were plated with T cells at a concentration of $0.625 \times 10^6/\text{mL}$. In some cultures, LN cells were left unfractionated and stimulated with 1 mg/mL OVA (without addition of BMDC). All cultures were plated in triplicate wells for up to 72 hours. In addition, we simulated the inhibitory function of IL-10-secreting CD4 cells. We enriched CD4⁺ cells ($1.0 \times 10^6/\text{mL}$) from draining LNs of both groups via magnetic-activated cell sorting and cocultured with OVA-pulsed BMDCs ($0.5 \times 10^6/\text{mL}$) for 72 hours. Anti-IL-10R blocking antibody (10 μ g/mL, clone 1B1.3a; BioLegend) or isotype control rat IgG1 κ antibody (clone

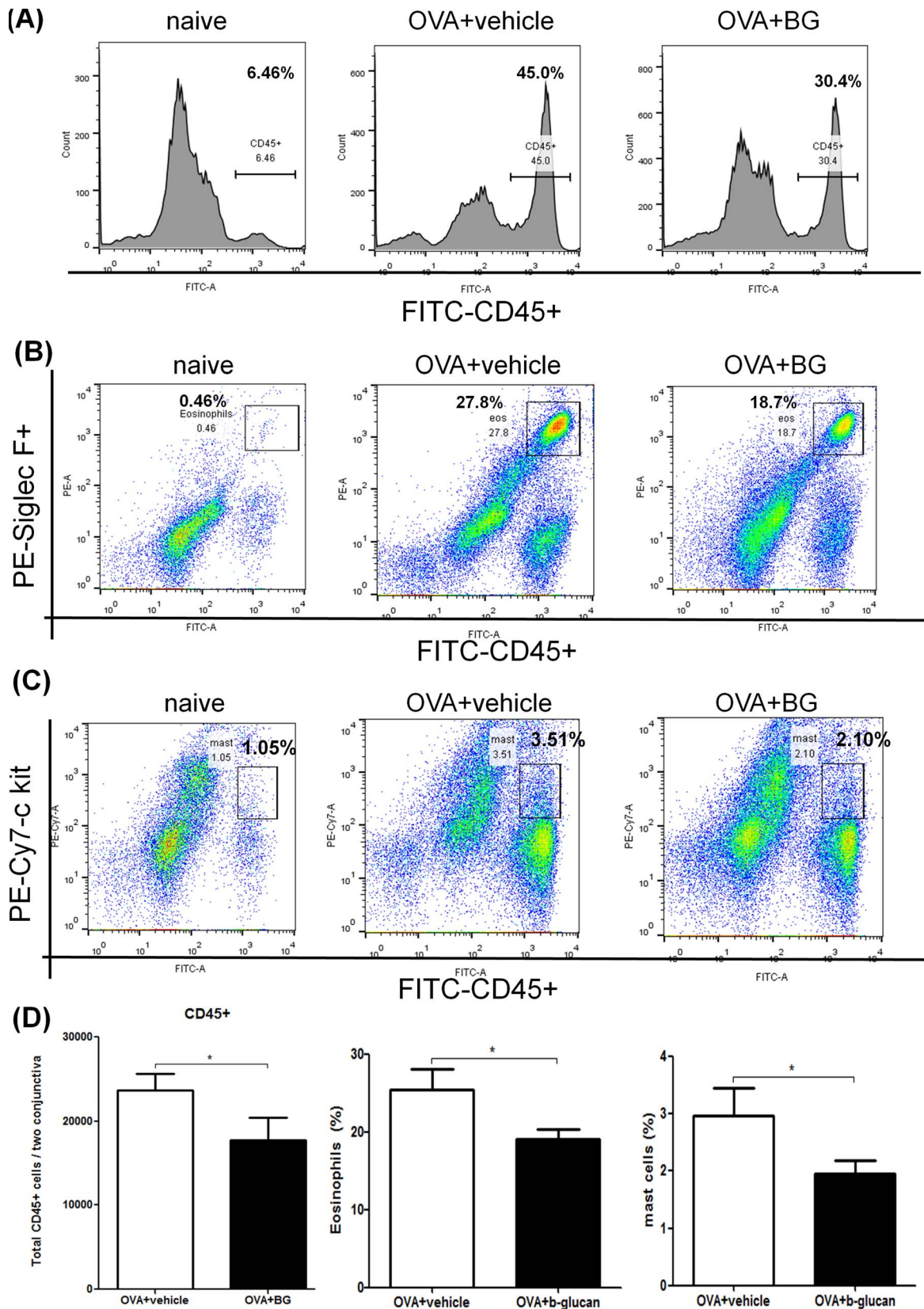


FIGURE 2. β -1,3-glucan suppresses infiltration of inflammatory cells into the conjunctivae. Topical BG or vehicle eye drops, applied 5 minutes after topical OVA challenge, were instilled in the right eye of OVA-sensitized mice once a day for 13 days. Conjunctivae were harvested from these mice or naive controls and prepared for flow cytometry analysis of CD45+ cells (A), eosinophils ([B]; CD45+ Siglec-F+), and mast cells ([C]; CD45+ c-kit+). Figures represent multiple independent experiments. (D) Data are representative of three independent experiments ($n = 2$ mice each group/each experiment) and data are presented as mean and standard error of the mean. * $P < 0.05$. ** $P < 0.01$.

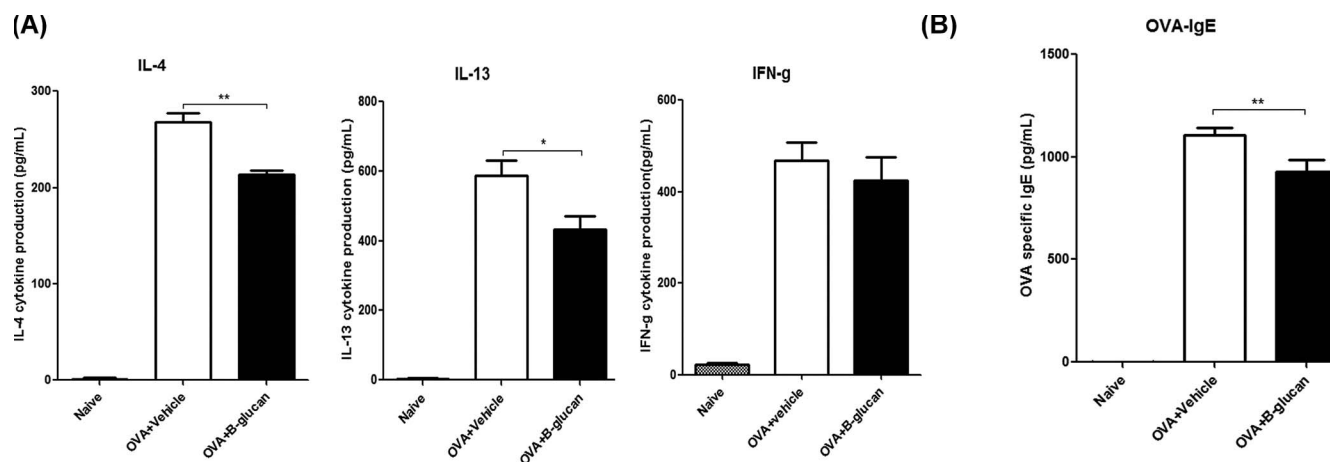


FIGURE 3. Topical BG application ameliorates allergic responses in AC. (A) Draining lymph nodes were harvested and T cells were purified with magnetic bead sorting ($n = 3$ mice/group). Purified T cells were stimulated with OVA-pulsed bone marrow dendritic cells for 72 hours and then restimulated with phorbol myristate acetate/ionomycin for up to 4 hours. Cytokines in culture supernatants were quantified by ELISA. (B) Topical BG led to impaired OVA-specific IgE levels. Sera were collected from mice ($n = 2$ mice/group) and OVA-specific IgE levels were measured via ELISA. Data presented from three to four independent experiments as the mean and standard error of the mean. * $P < 0.05$. ** $P < 0.01$.

GL113; BioLegend) was added into each well in this assay daily for 3 days.

ELISA Measurement of T-Cell Cytokines

Cells were plated as indicated above. Following 72-hour OVA stimulation, cultures were restimulated with phorbol myristate acetate/ionomycin (Sigma-Aldrich Corp.) for up to 6 hours. The interferon gamma (IFN- γ), IL-4, and IL-13 cytokines in the collected supernatant were measured by ELISA per the manufacturer's instructions (Ready-SET-Go! ELISA kit; eBioscience, San Diego, CA, USA).

Flow Cytometric Analyses of Treg Cells

Following topical challenge, ipsilateral LNs (cervical and submandibular) were collected and pooled from freshly euthanized mice. Cells were washed and stained for fluorescein-conjugated anti-CD4, PE-conjugated anti-CD25, or PE/Cy5-conjugated anti-FoxP3 antibodies (eBioscience). Isotype control was stained with the appropriate matched antibodies (eBioscience). For nuclear staining of FoxP3, a cell fixation/permeabilization kit (eBioscience) was used as per the manufacturer's instruction.

Statistical Analyses

Data are expressed as the mean $\pm$ standard error of the mean. The significance of the difference between groups was analyzed with the analysis of variance or two-tailed Student's t -test as indicated throughout. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Impaired Development of Clinical Signs of AC and Conjunctival Infiltration of Eosinophils and Mast Cells With BG Treatment

We first aimed to elucidate whether topical application of BG could modulate immune responses in AC. Previously, we had demonstrated robust AC responses to topical OVA eye drops in mice that were sensitized with intraperitoneal injection of

OVA.⁹ In this study, we applied topical BG or vehicle eye drops with topical OVA challenge once daily to OVA-sensitized mice for 13 days. To evaluate AC responses, clinical signs of AC (lid edema, conjunctival chemosis, hyperemia, and discharge/tearing) were scored in a masked fashion about 20 minutes post challenge.⁸ Results from this experiment showed that topical BG led to an improvement of clinical scoring of allergic responses relative to the levels seen in the vehicle-treated group (Fig. 1). To determine the effect of BG on conjunctival inflammatory responses in the AC model, conjunctiva were harvested to count CD45+ cells (Fig. 2A); eosinophils (CD45+ Siglec-F+; Fig. 2B); and mast cells (CD45+ Siglec-F+; Fig. 2C) using flow cytometry. Mice treated with BG displayed significantly decreased infiltrations of CD45+ cells ($P = 0.038$ versus vehicle); eosinophils ($P = 0.022$ versus vehicle); and mast cells ($P = 0.029$ versus vehicle) into allergic conjunctivae, relative to the vehicle group (Fig. 2D).

BG Suppresses Th2 Responses and OVA-Specific Serum IgE Production in AC

To address whether BG leads to allergy modulation in AC, draining LNs were harvested 13 days after daily OVA and BG/vehicle instillation and purified T cells were stimulated with OVA and BMDC in vitro. Secretions of cytokines into the supernatants were quantified via ELISA. The levels of Th2 cytokines, such as IL-4 ($P = 0.001$ versus vehicle) and IL-13 ($P = 0.018$ versus vehicle), were significantly reduced, compared with those in the vehicle-treated mice, but not the Th1 cytokine IFN- γ ($P = 0.217$ versus vehicle; Fig. 3A). In addition, we found a statistically significant reduction in OVA-specific IgE in BG-treated mice, relative to the vehicle group ($P = 0.002$ versus vehicle; Fig. 3B).

BG Increases IL-10 Producing CD4+ T Cells

We set out to determine the mechanism by which BG treatment may modulate the allergic response. Draining LNs were harvested 13 days after daily OVA with BG/vehicle application. It has previously been demonstrated that BG could induce IL-10-producing CD4+ T cells and modulate the asthmatic response in mice models. Thus, we next examined the numbers of IL-10 in CD4+ T cells at draining LNs via intracellular staining and flow cytometry. Mice treated with BG

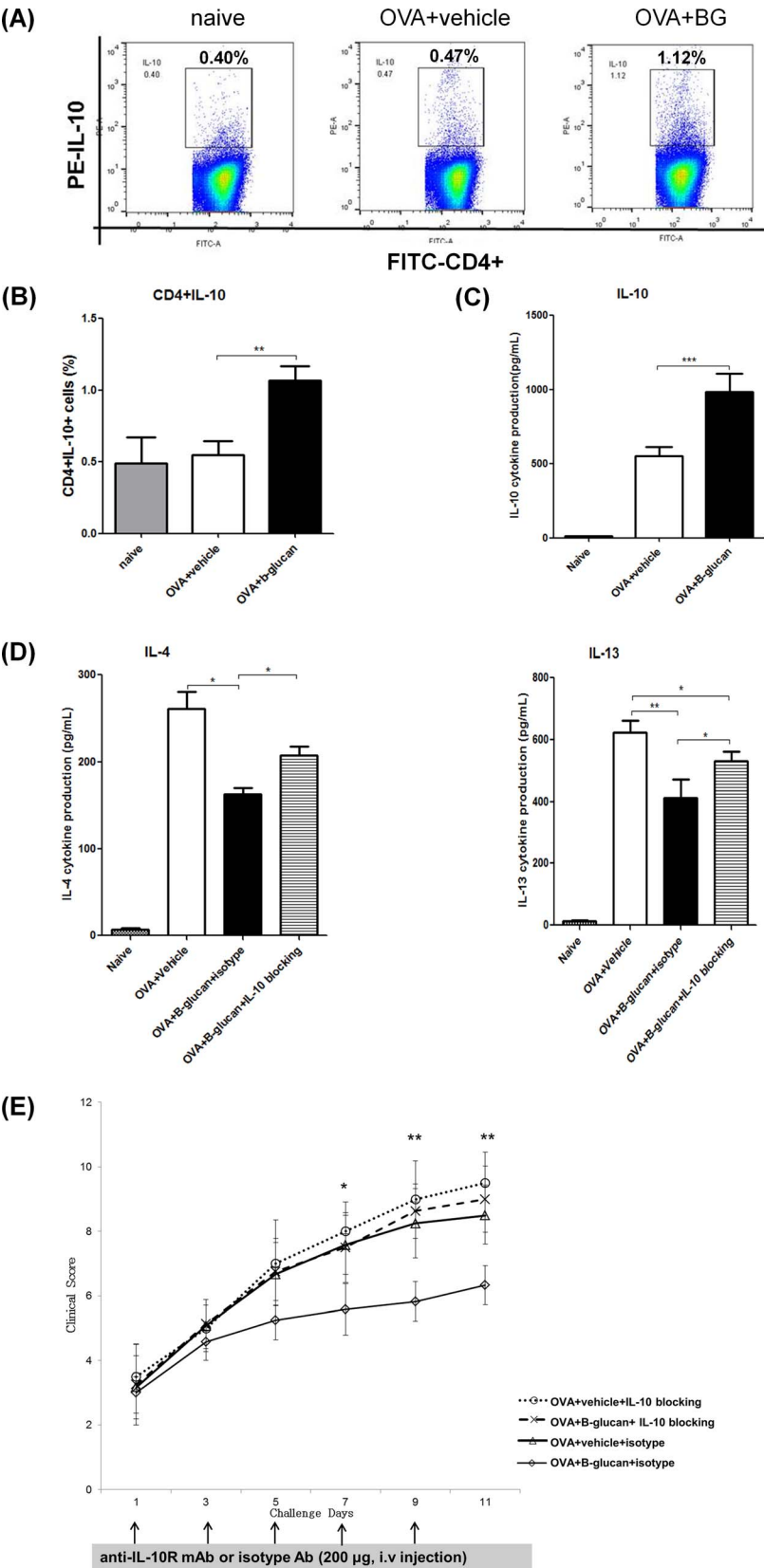


FIGURE 4. Allergy modulation in AC via BG application is associated with increased levels of IL-10+ CD4+ T cells in draining LNs. **(A)** Topical BG or vehicle eye drops, applied 5 minutes after topical OVA challenge, were applied to the right eye of OVA-sensitized mice once a day for 13 days. Draining LNs in mice were harvested and analyzed with flow cytometry. The data presented here as the percentages for the population of CD10+ CD4+ T cells in total FITC-CD4+ T cells of draining LNs. Application of BG was associated with increased frequencies of IL-10+ CD4+ T cells in draining LNs. **(B)** Graphs represent three independent experiments and data are presented as mean and standard error of the mean ($n = 6/\text{group}$).

**** $P < 0.01$.** (C) T cells were purified and stimulated with OVA-pulsed bone marrow dendritic cells for 72 hours and then restimulated with phorbol myristate acetate/ionomycin for up to 4 hours. Interleukin 10 secretion in culture supernatants was quantitated by ELISA. (D) We cocultured CD4⁺ cells from draining LNs and OVA-pulsed BMDCs with anti-IL-10R blocking antibody for 72 hours and then IL-4 and IL-13 in the supernatant were measured via ELISA. Graphs represent three independent experiments (mean and SEM). ***** $P < 0.001$.** (E) Mice were injected intravenously via tail vein with 200 μ g of anti-IL-10R mAb or isotype control antibody every other day, 5 minutes after daily topical OVA with BG or vehicle challenge. Graphs are representative of two independent experiments and data are presented as mean and standard deviation ($n = 4$ –6/group). *** $P < 0.05$.** **** $P < 0.01$.**

showed a significantly increased population of IL-10⁺ CD4⁺ T cells in draining LNs, compared with the vehicle group ($P = 0.003$ versus vehicle; Figs. 4A, 4B). T cells were purified from draining LNs with magnetic bead sorting at day 13 and these purified T cells were stimulated with OVA and BMDCs in vitro for 72 hours and the IL-10 concentration in the supernatant was analyzed by ELISA. Treatment with BG led to increased IL-10 production from purified T cells, relative to the vehicle group ($P = 0.0008$ versus vehicle; Fig. 4C). To evaluate inhibitory function of IL-10 CD4⁺ T cells in vitro, CD4⁺ cells from draining LNs and OVA-pulsed BMDCs were cocultured with anti-IL-10R blocking antibody. Suppressive level of IL-4 and IL-13 in BG was partially reversed by adding anti-IL-10R blocking antibody (Fig. 4D; $P = 0.023$ versus anti-IL-10R blocking, $P = 0.033$ versus anti-IL-10R blocking, respectively). In addition, we analyzed the immunomodulatory effect of IL-10 by BG in vivo, mice were injected intravenously with anti-IL-10R mAb or isotype control antibody after topical OVA with BG or vehicle challenge. Clinical scoring was significantly aggravated by injecting anti-IL-10R blocking antibody on topical BG-treated mice, but there was no significant differences among anti-IL-10R blocking antibody treated BG group, isotype/or anti-IL-10R blocking antibody-treated vehicle group (Fig. 4E; $P = 0.018$ versus isotype on BG at day 7, $P = 0.0016$ versus isotype on BG at day 9, and $P = 0.0011$ versus isotype on BG at day 11, respectively). Also systemic treatment of anti-IL-10R blocking antibody on BG mice led to increase conjunctival infiltration of CD45⁺ cells and eosinophils (CD45⁺ Siglec-F⁺), relative to levels seen in the isotype-treated BG mice (Supplementary Fig. S1).

Next, we examined whether BG can increase the number of Treg cells. Draining LNs and spleen were collected and intranuclear staining was performed. The group treated with BG showed an increased population of CD4⁺ CD25⁺ FoxP3⁺

Treg cells in draining LNs, compared with the vehicle group ($P = 0.046$ versus vehicle; Figs. 5A, 5B), but not in the spleen (Supplementary Fig. S2). These data suggested a possible immune-modulating role for IL-10-producing CD4⁺ T cells and CD4⁺ CD25⁺ FoxP3⁺ Treg cells in the BG-induced suppression of AC.

BG Modulates Allergic Responses in Mice With Advanced AC

In this study, we have shown that BG could attenuate the development of AC responses to topical OVA in OVA-sensitized mice; we next tried to show whether BG could modulate AC responses in advanced AC states. We started application of topical BG or vehicle eye drops once daily, 5 days after topical OVA challenge in OVA-sensitized mice. Topical BG resulted in improved clinical scoring of allergic responses (Fig. 6A) and decreased conjunctival infiltration of CD45⁺ cells (Fig. 6B) and eosinophils (CD45⁺ Siglec-F⁺; Fig. 6C), relative to levels seen in the vehicle group. In addition, BG-treated mice displayed a significantly reduced production of OVA-specific IgE in sera, relative to the vehicle group ($P = 0.022$ versus vehicle; Fig. 6D).

DISCUSSION

Allergic conjunctivitis is caused by the allergen-specific response of type 2 helper T cells and degranulation of vasoactive amines from mast cells and eosinophils, and it presents with itching sensation, mucus secretion, conjunctival swelling/redness, and lid swelling. To date, most therapies for AC have focused on either suppressing mast cell degranulation, or inhibiting the function of inflammatory vasoactive amines using steroids, antihistamines, or mast cell stabilizers.^{4,10}

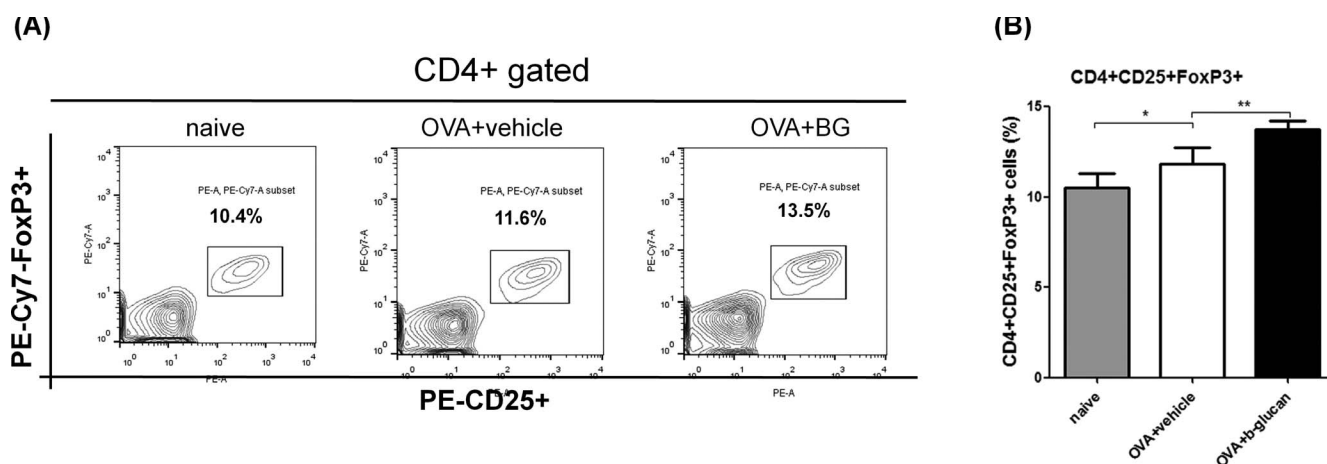


FIGURE 5. Application of BG led to an increase of the Treg cell population in draining LNs. (A) Topical BG or vehicle with OVA was applied to the right eye of OVA sensitized mice once a day for 13 days. Draining LNs in mice were collected and analyzed with flow cytometry. The data presented here as the percent population of PE-Cy7⁺ FoxP3⁺ PE-CD25⁺ cells in total FITC-CD4⁺ T cells of draining LNs. β-1,3-glucan BG led to an increase in the frequencies of CD4⁺ CD25⁺ FoxP3⁺ cells in draining LNs. Figures represent multiple independent experiments. (B) Graphs represent at least 4 to 5 independent experiments and data are presented as mean and SEM ($n = 8$ –10/group). *** $P < 0.05$.**

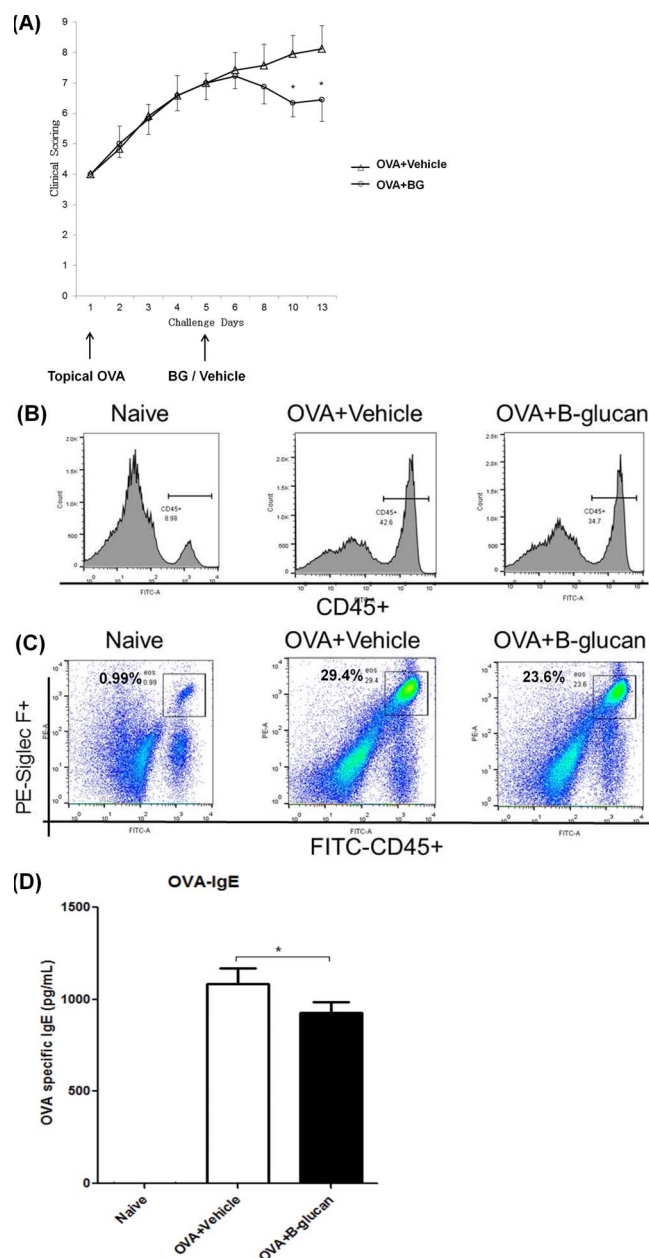


FIGURE 6. β -1,3-glucan suppressed allergic reaction of advanced AC. (A) Topical OVA eye drops were instilled into the right eye of OVA-sensitized mice via intraperitoneal injection of OVA/Al on day 1. Topical application of BG or vehicle eye drops with topical OVA once a day started from days 5 to 13. Clinical scoring was evaluated in a masked fashion. Graphs are representative of three independent experiments and data are presented as mean and standard deviation ($n = 6/\text{group}$). * $P < 0.05$. (B, C) Conjunctivae were harvested from treated mice or naïve controls at day 13 and prepared for flow cytometry to analyze the population of CD45+ cells (B) and eosinophils (C; CD45+ Siglec-F+). Figures represent at least two independent experiments ($n = 6/\text{group}$). (D) Sera were collected and were analyzed in duplicate or triplicate using an OVA-specific mouse IgE ELISA kit. Graphs represent at least two independent experiments and data are presented as the mean and SEM ($n = 6/\text{group}$). * $P < 0.05$.

β -glucans are polysaccharides composed of D-glucose monomers with β -glycosidic bonding. β -glucans are found in the form of cellulose in plants and the cell walls of yeast, fungi, bacteria, and mushrooms. Recently, BG derived from yeast and mushroom was found to have a distinctive ability to modulate

the immune reaction. The pattern recognition receptors for BG on the surface of innate immune cells are dectin-1 receptor, complement receptor 3, and toll-like-receptors.^{5,7,11,12} β -1,3-glucan can stimulate the immune system to enhance resistance to bacterial, viral, and fungal infection, as well as modulate adaptive immune responses. Inoue et al.¹³ demonstrated that BG extracted from mushroom decreases the activation of B cells and potentiates Th1-dominant cellular immunity by inducing the production of IFN- γ , IL-12, and IL-18 from spleen and lymph node cells. Yamada et al.⁶ also demonstrated that orally administered BG might contribute to the resolution of seasonal cedar pollen-induced allergy, and a decrease in serum IgE titers, observations that were well correlated with the binding of monocytes to BG.

This study is the first demonstration that the topical application of BG alleviates AC in a murine model. Topical application of BG resulted in decreased infiltration of bone marrow-derived inflammatory cells such as mast cells and eosinophils into the conjunctivae, and reduction of OVA-specific IgE in serum. The study demonstrated that BG possesses the ability to suppress both the development and progression of AC. To establish the possible mechanism by which BG reduces the allergic response, we checked the activation of T cells in draining LNs. The production of Th2-dominant cytokines such as IL-4 and IL-13 was significantly higher after in vitro T-cell stimulation, but not the production of IFN- γ , a Th1-dominant cytokine. Previous studies had shown that one of the main immune-modulating actions of BG is the skewing of the Th1/Th2 balance toward Th1 activity; however, this could not explain the effects of BG in our model.^{7,10,13} Wu et al.¹⁴ also showed that levels of IL-4, IL-5, and tumor necrosis factor alpha significantly reduced after BG treatment, but the levels of IL-2 and IFN- γ were significantly elevated. However, we suggest that topical BG treatments did not drive the differentiation of T cells toward Th1 cells rather than the Th2 type in our AC model.¹⁴

The ability of intraperitoneally injected BG to modulate allergic airway inflammation through IL-10-secreting CD4+ T cells has been demonstrated recently.¹⁵ We also noted that the numbers of IL-10-positive CD4+ T cells in draining LNs and the secretion of IL-10 in the in vitro T cell assay significantly increased after topical BG application. Microbial infection or inflammatory mediators stimulate the secretion of inflammatory cytokines, such as IL-17, TGF- β , or IL-27, from antigen-presenting cells that promote the generation of IL-10-producing T cells.^{16,17} Some studies have suggested BG could induce Th17 cell differentiation and IL-17 plays an important role for induction of allergic inflammation, but also negatively regulates allergic response by suppressing eosinophil-chemokine eotaxin (chemokine [C-C Motif] ligand, CCL11) and thymus- and activation-regulated chemokine/CCL17.^{16–18} In addition, Mendel et al.¹⁹ demonstrated that development of IL-10-producing CD4+ T cells depends on IL-4 and signal transducer and activator of transcription-6 (STAT-6), and that STAT6 is important for both the differentiation of Th2 cells and the development of IL-10 producing CD4+ T cells.¹⁵ In addition, Kawashima et al.¹⁵ suggested that BG instructs antigen-presenting cells mediated by dectin-1 to induce development of IL-10-producing CD4+ T cells to modulate allergic inflammation.

Interleukin 10 is well known as a potent anti-inflammatory cytokine with the ability to suppress inflammatory and autoimmune responses.^{20,21} Interleukin 10 is generally secreted by monocytes, macrophages, dendritic cells, T cells, and B cells. It binds to two types of receptor complexes (i.e., two IL-10R1 chains and two IL-10R2), and especially the IL-10R1 chain, which is mainly expressed on T cells, B cells, monocytes, macrophages, dendritic cells, and natural killer

cells.^{22,23} The major immunosuppressive mechanisms of IL-10 involve inhibition of the production of proinflammatory mediators/chemokines, and suppression of the expression of major histocompatibility complex class II and costimulatory CD80/CD86 molecules on monocytes, dendritic cells, and macrophages. Furthermore, IL-10 inhibits T-cell proliferation and cytokine production.^{24,25} In this study, increased numbers of IL-10 at draining LNs suppressed the proliferation of Th2 cells and the production of the Th2 cytokines, IL-4 and IL-13, as described in previous reports.

Interleukin 10 is also an important factor in the development and suppressive function of inducible regulatory T cells.^{15,22,26,27} Kearley et al.²⁸ reported that CD4+CD25+ T cells can suppress the Th2 cell-driven response of airway inflammation to allergens in vivo by an IL-10-dependent mechanism. In our data, we observed that BG application induced an increase in the population of Foxp3+ CD4+ CD25+ Treg cells in the draining LNs. However, Kawashima et al.¹⁵ demonstrated that while BG induced IL-10-producing CD4+ T cells, the numbers of Foxp3+ conventional Tregs were not significantly different. Further studies, including the suppression assay of Foxp3+ Tregs or the numbers of Foxp3+ Tregs in the spleen in our model, might be needed to explain this discrepancy.^{27,29-31}

In conclusion, we have demonstrated that BG is capable of stimulating IL-10-producing CD4+ T cells, and suppressing the Th2 response in draining LNs and conjunctival eosinophil infiltration, demonstrating the therapeutic potential of topical BG for AC. Although further studies are required to understand the basis of the findings, our results should add new insight into the therapeutic potential of BG for allergic diseases including asthma or atopic dermatitis.

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