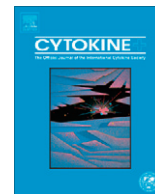




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Bee venom ameliorates ovalbumin induced allergic asthma via modulating CD4⁺CD25⁺ regulatory T cells in mice

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ABSTRACT

Asthma is a potentially life-threatening inflammatory disease of the lung characterized by the presence of large numbers of CD4⁺ T cells. These cells produce the Th2 and Th17 cytokines that are thought to orchestrate the inflammation associated with asthma. Bee venom (BV) has traditionally been used to relieve pain and to treat chronic inflammatory diseases. Recent reports have suggested that BV might be an effective treatment for allergic diseases. However, there are still unanswered questions related to the efficacy of BV therapy in treating asthma and its therapeutic mechanism. In this study, we evaluated whether BV could inhibit asthma and whether BV inhibition of asthma could be correlated with regulatory T cells (Treg) activity. We found that BV treatment increased Treg populations and suppressed the production of Th1, Th2 and Th17-related cytokines in an *in vitro* culture system, including IL2, IL4, and IL17. Interestingly, production of IL10, an anti-inflammatory cytokine secreted by Tregs, was significantly augmented by BV treatment. We next evaluated the effects of BV treatment on allergic asthma in an ovalbumin (OVA)-induced mouse model of allergic asthma. Cellular profiling of the bronchoalveolar lavage (BAL) and histopathologic analysis demonstrated that peribronchial and perivascular inflammatory cell infiltrates were significantly lowered following BV treatment. BV also ameliorated airway hyper-responsiveness, a hallmark symptom of asthma. In addition, IL4 and IL13 levels in the BAL fluid were decreased in the BV treated group. Surprisingly, the beneficial effects of BV treatment on asthma were eradicated following Treg depletion by anti-CD25 antibody injection, suggesting that the major therapeutic targets of BV were Tregs. These results indicate that BV efficiently diminishes bronchial inflammation in an OVA-induced allergic asthma murine model, and that this effect might correlate with Tregs, which play an important role in maintaining immune homeostasis and suppressing the function of other T cells to limit the immune response. These results also suggest that BV has potential therapeutic value for controlling allergic asthma responses.

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1. Introduction

Asthma is an inflammatory disease of the lungs that is characterized by increased infiltration of leukocytes, especially eosinophils, into the airways and reduced respiratory function. This inflammation leads to bronchoconstriction, increased airway hyper-responsiveness (AHR), and mucus production [1]. The prevalence of asthma is rapidly increasing worldwide, especially in young children, and it has become a significant cause of morbidity and mortality in developed countries [2]. Inhaled glucocorticosteroids, inhaled β_2 -agonists and oral systemic glucocorticosteroids

are crucial for treating the disease, but they cannot be used for long periods of time because of frequently occurring side effects. Therefore, there is a need for safer and improved strategies for providing long-term relief from asthmatic symptoms.

The underlying process that drives and maintains the asthmatic inflammatory response appears to be an imbalance of the equilibrium between T helper cell type 1 (Th1) and T helper cell type 2 (Th2) immune response, with a bias towards an increased Th2 response [3]. The cytokines produced in Th2 immune response, including IL-4, IL5 and IL-13 are associated with allergic reactions and eosinophilia. These cytokines are of major importance because IL-4 and IL-13 induce the production of IgE by B cells and IL-5 regulates the growth, differentiation, and activation of eosinophils, which are directly associated with the inflammatory process and AHR [4–6]. Although this hypothesis is still thought to be valid, therapies directed at inhibiting Th2 processes in mouse models and in clinical trials have generally met with only partial success.

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These observations suggest that an impaired general ability to regulate inflammation could be an important contributing factor in asthma and perhaps other atopic disorders. The search for immunoregulatory mechanisms has recently centered on a specific subset of regulatory T cells (CD4⁺CD25⁺Foxp3⁺ Treg cells) that limit inflammatory responses and promoting immune tolerance in a variety of experimental systems and clinical disorders [7].

The Treg plays an important role in regulating the immune response. Tregs can reduce inflammation and allergic reactions through their inhibitory functions. Using a murine model of asthma Dongaci et al. [8] provided evidence that the diminished levels of Tregs resulted in increased airway hyperreactivity, thus linking Tregs to airway physiology [7]. Given the capacity of Tregs to suppress disease-promoting immune responses, enhancing Treg function in patients represents an attractive therapeutic strategy for treating allergic diseases, including asthma.

Bee venom (BV) therapy is one of the most frequently used traditional complementary and alternative therapeutic methods and has long been believed to be effective in the treatment of many diseases, including rheumatic arthritis, bursitis, tendinitis, shingles (herpes zoster), multiple sclerosis, wounds, gout, burns, infections [9] and allergic asthma. Several animal experiments have suggested that BV is effective in reducing pain and improving the symptoms of arthritis [10–12]. Although BV has been widely used for treating allergic disorders, including asthma [13], only a limited number of controlled studies have assessed the efficacy of BV as an allergy treatment, and the mechanism of action has thus far remained elusive.

In this study, we investigated whether BV could diminish allergic asthma inflammation and whether the beneficial effects of BV could be correlated with an effect on CD4⁺CD25⁺ regulatory T cells.

2. Methods

2.1. Ethics statement

All experiments were approved by the Kyung Hee University Animal Care and Use Committee (KHUASP (SE) – 11-025).

2.2. Animals

Foxp3^{EGFP} Balb/c (C. Gg-Foxp3^{tm2Tch}/J) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Balb/c mice were purchased from Orient Bio (Orient Bio, Seongnam, Korea). All mice were kept under pathogen-free conditions with air conditioning and a 12-h light/dark cycle. All mice had free access to food and water during the experiments.

2.3. Reagents

BV (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in saline at the respective concentration mentioned for each experiment.

2.4. Staining for CD4⁺CD25⁺Foxp3⁺ Treg in vitro

For immunofluorescence, splenocytes were stimulated with anti-mouse CD3 and anti-mouse CD28 antibodies. Cells treated with PBS or BV (0.1, 1 µg/ml) were cultured in complete RPMI1640 media containing 10 µg/ml of anti-mouse CD3 antibody and 2 µg/ml of anti-mouse CD28 antibody for 72 h. Single-cell suspensions of splenocytes incubated with fluorescently tagged Abs directed against a panel of cell surface markers were stained with CD4 and CD25 (eBioscience, San Diego, CA, USA). The staining was performed in accordance with the manufacturer's instructions. All FACS data were acquired on a FACS Calibur flow cytometer (BD

Biosciences, San Jose, CA, USA) and analyzed using CellQuest Pro (BD Biosciences, San Jose, CA, USA).

2.5. Measurement of cytokines in culture supernatants

Splenocytes stimulated by anti-mouse CD3 antibody and anti-mouse CD28 antibody were treated with various concentrations of Bee Venom for 3 days. The culture supernatants were collected, centrifuged at 4 °C and 300 rcf for 10 min, and stored at –70 °C for subsequent use. The level of cytokines was normalized against the level of cytokines of the stimulated control group. Cytokines were measured using the mouse cytometric bead array (CBA) (BD Biosciences, San Jose, CA, USA). Briefly, 50 µl of samples or known concentrations of standard samples (0–5000 pg/ml) was added to a mixture of 50 µl each of capture antibody bead reagent and phycoerythrin (PE)-conjugated detection antibody. The mixture was subsequently incubated for 2 h at room temperature in the dark and was then washed to remove unbound detection antibody. Data were acquired by flow cytometry (FACS Calibur; BD Biosciences Corp., San Jose, CA, USA) and analyzed on a computer (CBA software 1.1; BD Biosciences Corp.).

2.6. In vivo and ex vivo Treg suppression assay

Foxp3^{EGFP} Balb/c (C. Gg-Foxp3^{tm2Tch}/J) mice or Balb/c mice were treated with intraperitoneal (i.p.) injections of 0.1 mg/ml of BV (1 mg/kg body wt) every day for 5 days. Control mice were treated with i.p. injections of PBS alone (*n* = 5/group). On the 5th day, the mice were sacrificed, splenocytes were collected and CD4⁺CD25⁺ Foxp3⁺ Treg percentages were analyzed using a FACS Calibur and CellQuest software (BD Bioscience). For *ex vivo* Treg suppression assay, using magnetic bead separation (CD4⁺CD25⁺ Treg cell kit; Miltenyi Biotec, Bergisch Gladbach, Germany), CD4⁺CD25⁺ Treg and CD4⁺CD25[–] T cells were isolated from spleens obtained from Balb/c mice that had received PBS or BV. CD4⁺CD25[–] T cells from PBS received mice were labeled with e670 fluorescence dye (eBioscience, San Diego, CA, USA), plated at a density of 10 × 10⁵ cells/well (responder only) or 8 × 10⁵ cells/well in 48 well plates and activated with 5 µg/ml coated anti-CD3 and 2 µg/ml soluble anti-CD28 antibody. CD4⁺CD25⁺ Treg from mice that had received PBS or BV were added at a density of 2 × 10⁵ cells/well. The cultures were incubated for 96 h, and the fluorescence intensity of responder cells was measured. Following the acquisition of sample data on a FACScalibur flow cytometer (BD Biosciences), the sample results were generated in graphical and tabular formats using the FlowJo software (Tree Star Inc., Ashland, OR, USA).

2.7. Murine allergic asthma experimental protocol and design

Six-week-old female Foxp3^{EGFP} Balb/c (C. Gg-Foxp3^{tm2Tch}/J) mice were used for this experiment. On the 0th day, the mice were divided into a negative control (PBS-treated) group (*n* = 8) and an OVA-induced asthmatic group. The OVA-induced asthmatic group was further separated into four groups (the OVA group; the OVA + BV group; the OVA + Treg depletion group; the OVA + Treg depletion + BV group) (*n* = 8/group). Allergic asthma was induced in the OVA-induced asthmatic groups according to a modification of the methods of Doganci et al. [8]. Briefly, mice were sensitized by intraperitoneal (i.p.) injections on the 0th and the 14th day with 100 µg of ovalbumin (OVA) (Sigma–Aldrich, St. Louis, MO, USA) combined with 20 mg of aluminum hydroxide in 100 µl of PBS. The mice were challenged by administering 1% OVA in 50 µl PBS directly into the nostrils using a micropipetor six times between the 20th and the 31st day. Negative control mice were sensitized and challenged with PBS alone. The Treg depletion group mice were treated with i.p. injections of 0.25 mg anti-CD25 antibody

on the 1st, 8th and 15th day. Anti-mouse CD25 rat IgG1 (anti-CD25; clone PC61) was generated in-house from hybridomas obtained from American Type Culture Collection (Manassas, VA, USA). The efficacy of Treg depletion was confirmed by flow cytometry analysis using PE-anti-mouse CD25 and fluorescein isothiocyanate-anti-mouse CD4.

The BV challenge groups were treated with i.p. injections of 0.1 mg/ml of BV (1 mg/kg body wt) six times from the 3rd to the 17th day. On the 34th day, the mice were sacrificed and various tissues were collected for analysis (Fig. 1).

2.8. Fluorescence-activated cell sorting (FACS) and flow cytometric analyses

The lungs were removed and washed with PBS to remove blood, after which the tissue was minced and erythrocytes were lysed in 1% RBC lysis buffer (BD, Pharmingen) for 5 min. After RBC lysis, the lung tissues were forced through a 25- μ m cell strainer. The cells were washed three times and resuspended in FCM buffer (PBS with 2% FBS and 0.1% NaN₃). Single-cell suspensions and pneumocytes obtained from Foxp3-EGFP Balb/C mice were labeled with anti-CD4-APC and anti-CD25-PE mAbs using standard staining methods, and the percentage of cells stained with each reagent was analyzed by FACS Calibur using CellQuest software (BD Bioscience).

2.9. Bronchoalveolar lavage fluid (BALF)

BALF was collected by infusion and extraction of 1 ml of ice-cold PBS for three times, and the lavages were pooled (mean volume, $2.0 \pm$ ml). Recovered BALF (70–80%) was centrifuged at 1300 rpm for 10 min. The cell pellets were resuspended in 1 ml of PBS and adhered to glass slides using cytocentrifugation. The total viable cell counts were determined in a hemocytometer using trypan blue exclusion. Differential counts of eosinophils, neutrophils, lymphocytes, and macrophages were determined on cytospin smears of BALF samples ($5 \times 10^5/200$ μ l cells) from individual mice stained with Diff-Quick (Life Technologies, Auckland, New Zealand) after counting 500 cells. The BALF was then centrifuged, and the supernatants were kept at -70 °C. The results are expressed as the total cell number $\times 10^4$.

2.10. Assessment of Th2 cytokines in BALF using Enzyme-Linked Immunosorbent Assay (ELISA)

The concentration of the Th2-associated cytokines IL-4 and IL-13 in the BALF was measured with a quantitative sandwich enzyme-linked immunoassay kit (BD, San Diego, USA for IL-4 and R&D, Minneapolis, USA for IL-13). A 96-well microtiter plate

(Costar, NY, USA) was incubated overnight at 4 °C with anti-mouse IL-4 or IL-13 monoclonal antibodies in coating buffer, washed with PBS containing 0.05% Tween 20 (Sigma, MO, USA) and blocked with 5% FBS in PBS and 1% BSA in PBS for 1 h at 4 °C and room temperature, each. Subsequently, wells were loaded with 100 μ l of BAL fluids and incubated for 2 h at room temperature. After washing, a secondary peroxidase labeled biotinylated anti-mouse IL-4 or IL-13 monoclonal antibody in assay diluents was added for 1 h. Finally, the plates were treated with TMB substrate solution (KPL, San Diego, CA, USA) for 30 min, and the reaction was stopped by the addition of 50 μ l TMB stop solution per well. The optical density was measured at 450 nm in a microplate reader (SOFT max PRO, version 3.1 software, CA, USA). Detection limits for the IL-4 and IL-13 ELISAs were 500 and 100 ng/ml, respectively.

2.11. Determination of serum IgE titers using ELISA

Blood was collected from the retro-orbital plexus of mice while under ether anesthesia on the 32nd day. Serum samples were obtained by centrifugation and stored at -20 °C until assayed. For serum analysis, 96-well immuno microplates (Costar, NY, USA) were coated with an anti-mouse IgE monoclonal antibody. The serum was diluted 1:250 with 5% FBS in PBS (assay diluent), and IgE was measured using a standardized sandwich ELISA (BD Pharmingen) according to the manufacturer's protocol. Optical density was measured at 450 nm in a microplate reader (SOFT max PRO, version 3.1 software, CA, USA). The detection limit for the IgE ELISA was 100 ng/ml.

2.12. Histological examination

Trachea and lung tissues were removed from the mice and fixed in 4% paraformaldehyde, embedded in paraffin after dehydration, cut into 6- μ m sections, and stained with Hematoxylin and Eosin (H&E) or Periodic Acid Schiff reagent (PAS). For immunohistochemistry detection of myosin light chain 2 (MLC2) 6- μ m sections of the lower trachea and lung were treated with 0.3% H₂O-methanol for 20 min to block endogenous peroxidases. The sections were subsequently incubated at 4 °C overnight with anti-MLC rabbit polyclonal antibody (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After the slides were incubated with avidin-biotin peroxidase complex (ABC kit, Vector Laboratories, Burlingame, CA, USA), color was developed using 3,3'-diaminobenzidine tetrachloride (DAB; Zymed Laboratories, South San Francisco, CA, USA). After immunohistochemical staining, the slides were counterstained with Harris's hematoxylin for 1 min and mounted with Canada balsam (Show Chemical Co., Ltd., Tokyo, Japan).

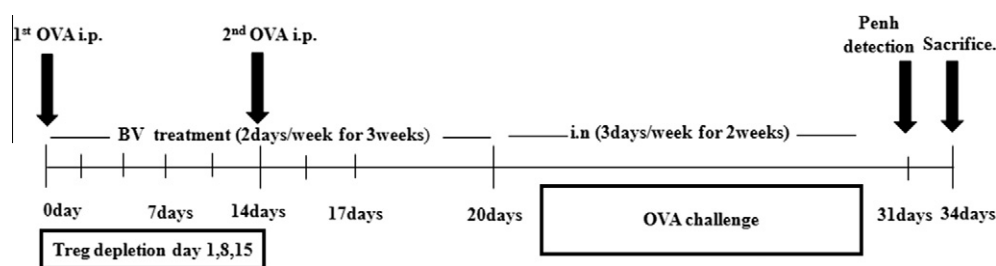


Fig. 1. Schematic diagram of the experimental protocol. On the 0th day, mice were divided into negative control groups ($n = 8$) and OVA-induced asthmatic groups. OVA-induced asthmatic groups were separated into four groups (OVA group, OVA + BV group, OVA + Treg depletion group and OVA + Treg depletion + BV group) ($n = 8/\text{group}$). OVA-induced asthmatic groups were sensitized i.p. injection with 100 μ g of OVA precipitated with 20 mg of aluminum hydroxide in 100 μ l of PBS on 0th and the 14th days. The mice were challenged by administering 1% OVA in 50 μ l PBS directly into the nostrils using a micropipet 3 days per week during the 2 weeks from the 20th to the 31st days. Negative control mice were sensitized and challenged with PBS alone. Treg depletion groups mice were treated i.p. injection with 0.25 mg of anti-CD25 antibody on the 1st, the 8th and the 15th days. BV challenge groups were treated i.p. injection with 1 mg/kg of BV 2 days per week during the 3 weeks from the 3rd to the 17th days. On 31th days, mice were analyzed using (non-invasive) lung function measurements (All Medicus, Seoul, Korea) to assess AHR. On the 34th days, the mice were sacrificed and various tissues were collected for analyses.

2.13. Measuring AHR to methacholine

The day after the final challenge of 1% OVA in 50 μ l PBS, the animals were analyzed using non-invasive lung function measurements (All Medicus, Seoul, Korea) to assess AHR. Mice were placed in a barometric plethysmographic chamber (All Medicus, Seoul, Korea), and baseline readings were taken for 3 min. The enhanced pause (Penh) was calculated according to the manufacturer's protocol [i.e., (expiratory time/relaxation time – 1) $\times$ (peak expiratory flow/peak inspiratory flow)]. Penh is a dimensionless parameter that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and is a function of the timing of expiration. Results were expressed as the percentage increase in Penh following challenge with each increasing concentration of methacholine (0, 50 and 100 mg/ml).

2.14. Statistical analysis

Statistical analysis of the data was conducted using the Prism 5 software (Graph Pad Software Inc., CA, USA). All values are presented as the means $\pm$ S.E.M (standard error of the mean). Differences between the means of the control and treatment samples were determined by one-way ANOVA or Student's *t* test. In all cases, *p* < 0.05 was considered to be statistically significant.

3. Results

3.1. Effect of BV on CD4⁺CD25⁺Foxp3⁺ Treg and inflammatory cytokine production by splenocytes *in vitro*

The total number of CD4⁺CD25⁺Foxp3⁺ Treg increased in BV treated splenocytes compared to that in saline treated splenocytes.

The percentage of CD4⁺CD25⁺Foxp3⁺ Treg also increased significantly in BV treated splenocytes compared to that in saline treated splenocytes (Fig. 2). IL-2 and IL-17A levels were significantly decreased in BV treated splenocytes supernatants compared to those in saline treated splenocytes supernatants. A decrease in IL-2 levels implies that BV could suppress inflammation by B cell immune response. Levels of IL-10, a Treg-associated cytokine, increased significantly after BV treatment of splenocytes supernatants as compared to saline treated splenocytes supernatants. However, BV treatment did not significantly affect the levels of IL-4, IL-6, INF- γ , or TNF- α (Fig. 3).

3.2. BV increases the proportion of Treg in CD4⁺ T cells and BV-treated Treg are suppressive *ex vivo*

To determine the effects of BV on the proportion of Treg *in vivo*, mice received BV (1 mg/kg) or PBS for 5 days and the frequency and phenotype of the Treg populations in splenocytes were analyzed (Fig. 4A–D). The percentage of CD4⁺CD25⁺Foxp3⁺ Treg increased significantly following BV injection (Fig. 4A, B). Additionally, BV treatment induced the increase of absolute number of CD4⁺CD25⁺Foxp3⁺ T cells (1.26 ± 0.16 vs. 3.86 ± 1.69 , *p* < 0.05; Fig. 4C). The suppressive ability of BV-treated Treg was tested in *ex vivo* proliferation assays using e670 fluorescent dye. In these assays, donor-derived CD4⁺CD25⁺ cells were freshly isolated from BV or PBS-treated mice and then co-cultured with responder-derived CD4⁺CD25⁺ cells from PBS-treated mice. Treg from BV-treated mice showed maintenance in the functional capacity to suppress responder T cell (CD4⁺CD25⁺) proliferation (Fig. 4D).

These results confirmed that BV could affect the population of CD4⁺CD25⁺Foxp3⁺ Treg, which suggested that BV might have the capability to attenuate the allergic diseases by increasing Treg population.

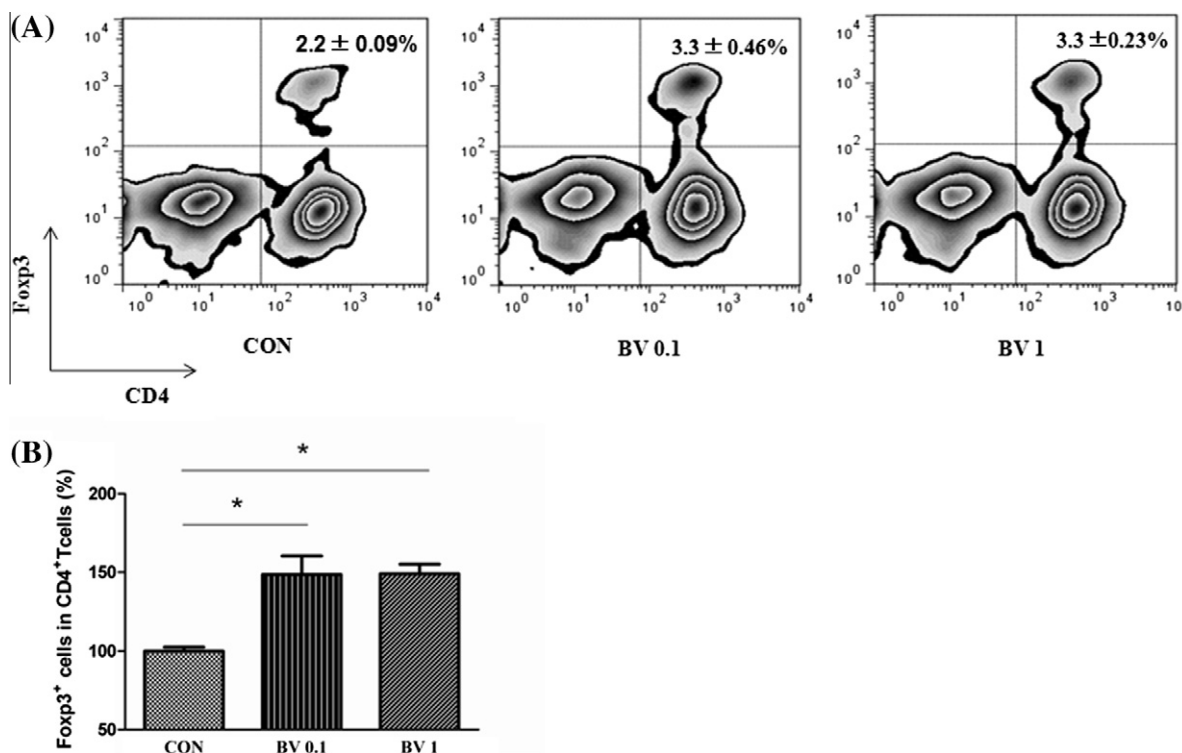


Fig. 2. The CD4⁺CD25⁺Foxp3⁺ Treg population in splenocytes *in vitro*. Splenocytes stimulated by anti-mouse CD3 antibody and anti-mouse CD28 antibody were treated with various concentrations of Bee Venom for 3 days. The values were normalized against the value of the stimulated control group. A) CD4⁺CD25⁺Foxp3⁺ Treg from splenocytes treated with control or BV (0.1, 1 μ l/ml). The numbers in the dot plots indicate the percent of cells within each quadrant. B) The percentage of CD4⁺CD25⁺Foxp3⁺ Treg increased significantly in the BV treated group compared to the control group. Data are shown as mean $\pm$ S.E.M. Statistical analysis was conducted by one-way ANOVA followed by the Newman–Keuls Multiple Comparison test. (**P* < 0.05, ***P* < 0.01, ****P* < 0.001, *n* = 3).

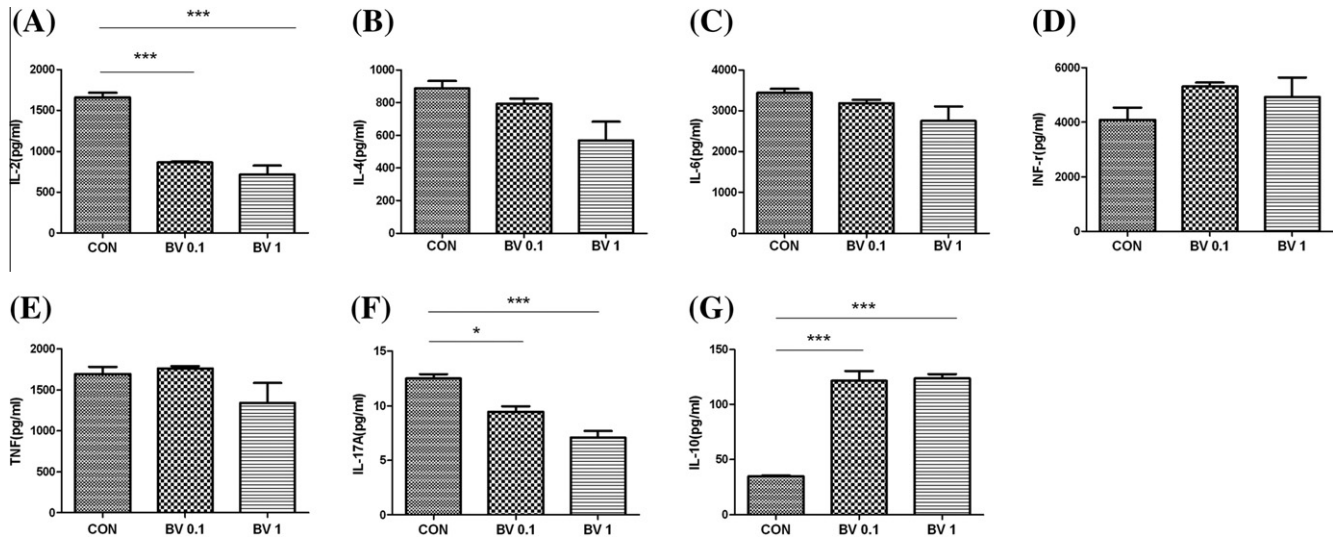


Fig. 3. Effects of BV on cytokine production. Splenocytes stimulated with anti-mouse CD3 antibody and anti-mouse CD28 antibody were treated with various concentrations of Bee Venom for 3 days. The culture supernatants were collected, centrifuged at 4 °C and 300 rcf for 10 min, and stored at –70 °C for subsequent use. The values were normalized against the value of the stimulated control group. Cytokines were measured by flow cytometry using the cytometric bead array (CBA): (A) IL-2; (B) IL-4; (C) IL-6; (D) INF-γ; (E) TNF-α; (F) IL-17A; (G) IL-10. Data are shown as mean ± S.E.M. Statistical analysis was conducted by one-way ANOVA followed by the Newman–Keuls Multiple Comparison test. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 3$).

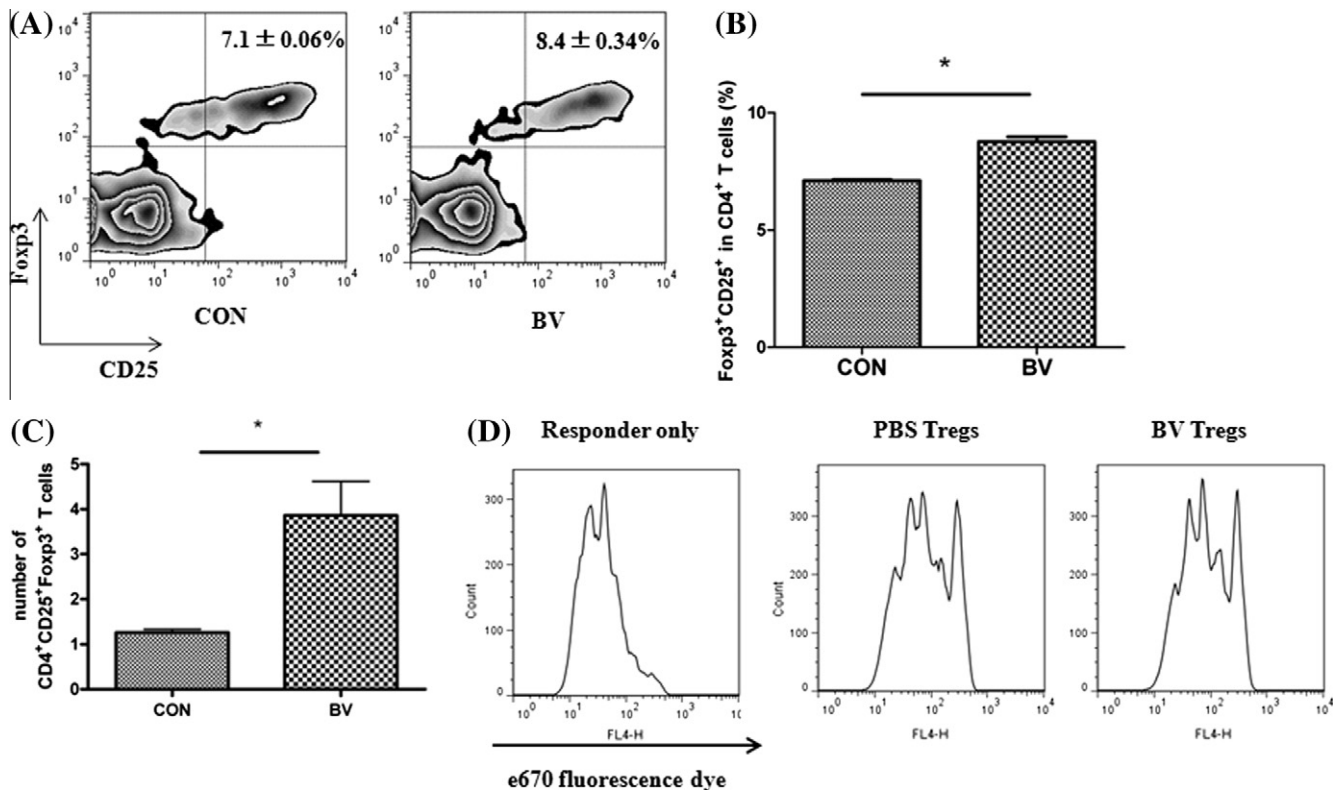


Fig. 4. The splenic population of CD4⁺CD25⁺Foxp3⁺ Treg. Foxp3^{EGFP} Balb/c (C. G-Foxp3^{tm2Tch}/J) mice or Balb/c mice were injected i.p. with 1 mg/kg of BV every day for 5 days. Control mice were injected i.p. with PBS alone. Splenocytes were isolated from each mouse after sacrifice and analyzed by flow cytometry (FACSscan Calibur, BD Biosciences). (A) CD4⁺CD25⁺Foxp3⁺ Treg from mouse treated with control or BV (1 mg/kg), representative results from five mice. The numbers in the dot plots indicate the percentage of cells within each quadrant. (B) The percentage of CD4⁺CD25⁺Foxp3⁺ Treg. (C) For measurement of CD4⁺CD25⁺Foxp3⁺ Treg number, splenocytes were stained with anti-CD4-FITC mAb and anti-CD25-PE mAb using standard staining methods and nuclear antigen such as Foxp3 was stained with anti-Foxp3-PE-Cy5 using Bestprotocol methods (eBioscience). (D) For ex vivo suppression assay, CD4⁺CD25⁺ T cells (responder) stained with e670 fluorescence dye were co-cultured with freshly isolated CD4⁺CD25⁺ T cells from mice that had received BV or PBS in the presence of anti-CD3/28 for 96 h. Proliferation of responder T cells was measured by e670 dye dilution by flow cytometry. Data are shown as mean ± S.E.M. Statistical analysis was conducted by two-tailed Student's *t*-test. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 5$).

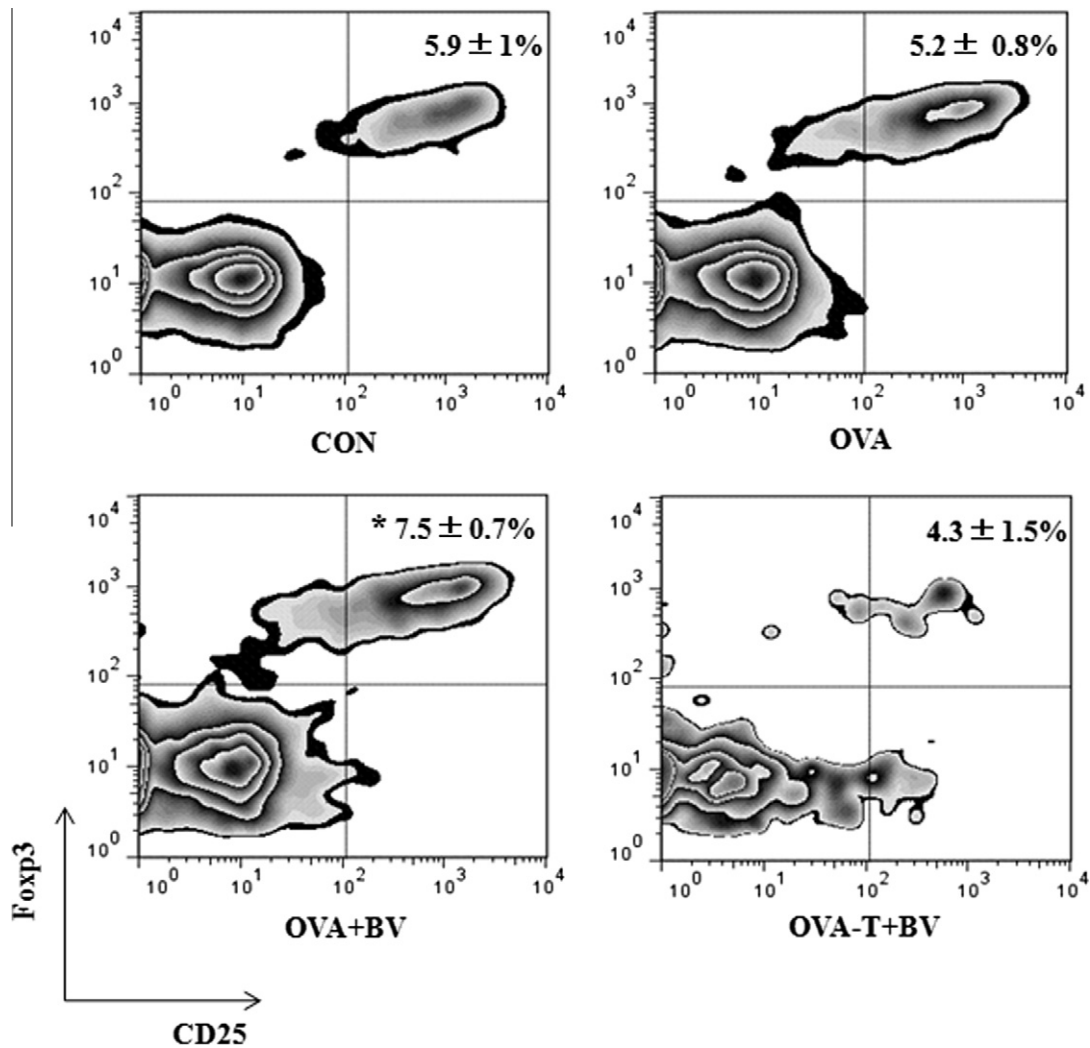


Fig. 5. Assessment of CD4⁺CD25⁺Foxp3⁺ Treg population in the OVA-induced asthmatic groups PBS treatment alone (Control); OVA induction alone (OVA); BV injection with OVA induction (OVA + BV), and BV injection with OVA induction and anti-CD25 Ab injection (OVA_T + BV). Isolated pneumocytes were stained with anti-CD4 allophycocyanin and anti-CD25 PE and analyzed by flow cytometry. Data are shown as mean $\pm$ S.E.M. Statistical analysis was conducted by one-way ANOVA followed by the Newman–Keuls Multiple Comparison test ($n = 8$).

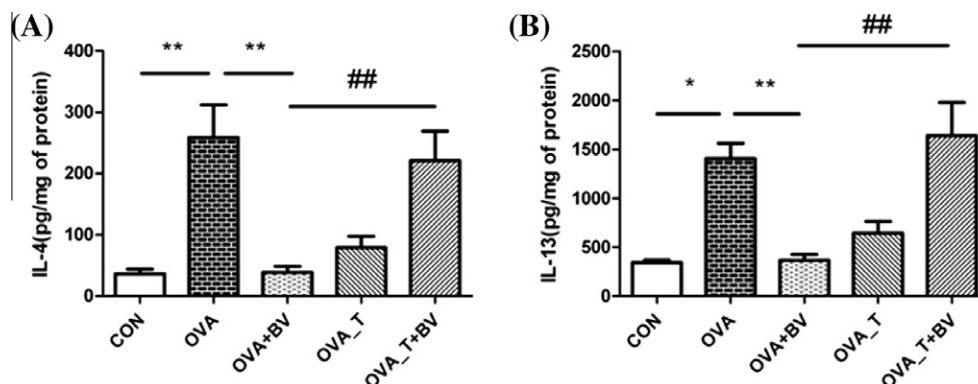


Fig. 6. IL-4 and IL-13 production in the BAL Fluid. Th2 inflammatory cytokines increased in the OVA and OVA_T + BV groups compared to the OVA + BV and control groups. Data are shown as mean $\pm$ S.E.M. Statistical analysis was conducted by one-way ANOVA followed by Newman–Keuls Multiple Comparison test ($***p < 0.001$, $**p < 0.05$ vs. control, $n = 8$).

3.3. Effect of BV on CD4⁺CD25⁺Foxp3⁺ Treg in OVA-induced asthmatic mice

The population of CD4⁺CD25⁺Foxp3⁺ Treg in isolated pneumocytes was not changed dramatically upon OVA sensitization

compared to the CON g group. However, the population of CD4⁺CD25⁺Foxp3⁺ Treg increased significantly in the OVA + BV group compared to the OVA group, suggesting the possible association of BV and Treg. The population was diminished in the OVA_T + BV groups compared to the CON, OVA and OVA + BV

groups due to the effects of anti-CD25 antibody injection (Fig. 5).

3.4. Effects of BV on Th2 cytokines (IL-4 and IL-13) levels in BALF

Th2 cytokines were remarkably reduced in the OVA + BV group at 48 h after the sampling compared to the OVA and OVA_T + BV groups. The control and OVA groups showed profoundly different results, which implied that asthma induction had been successful (Fig. 6).

3.5. Effect of BV on serum IgE titers

The IgE concentration in serum was significantly increased in the OVA group compared to the CON group, which implied that asthma induction had been successful. Serum IgE concentrations were significantly decreased in the OVA + BV group compared to the OVA, OVA_T and OVA_T + BV groups. Although BV could inhibit serum IgE concentrations from increasing in OVA-induced allergic asthma mice, this effect was not observed following Treg depletion (Fig. 7).

3.6. Effect of BV on Total cells and inflammatory cells in the BALF

The number of total cells, eosinophils and lymphocytes increased significantly in the OVA group compared to the CON group, which implied that the induction of asthmas had been successful.

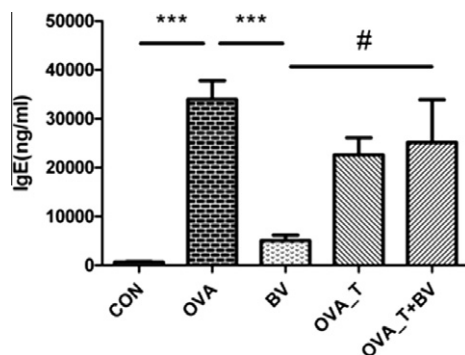


Fig. 7. Serum levels of IgE. Blood was collected by cardiac puncture, and the levels of IgE in the samples were evaluated by ELISA. Data are shown as mean $\pm$ S.E.M. Statistical analysis was conducted by one-way ANOVA followed by the Newman-Keuls Multiple Comparison test (* P < 0.05, ** P < 0.01 vs. control, n = 8).

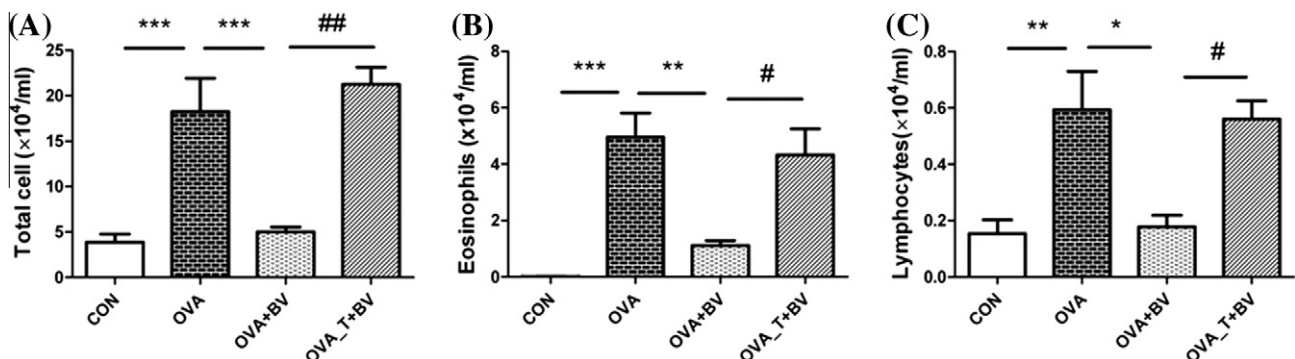


Fig. 8. Effect of BV treatment on leukocytes recruitment to the airways of OVA-induced asthmatic mice. BALF was obtained from the lungs of mice following the induction of allergic asthma. Total leukocytes, eosinophils and lymphocytes from the BALF were counted. (A) Cell numbers were assessed by direct counting under a light microscope in at least five squares of a hemocytometer after exclusion of dead cells stained with trypan blue. (B) The eosinophils are expressed as a percentage of the total leukocytes. Data are shown as mean $\pm$ S.E.M. Statistical analysis was conducted by one-way ANOVA followed by the Newman-Keuls Multiple Comparison test (* P < 0.05, ** P < 0.01, *** P < 0.001 vs. control and OVA, n = 8).

The numbers of total cells, eosinophils and lymphocytes were diminished significantly in the OVA + BV group compared to the OVA and OVA_T + BV groups. Although BV could suppress the increased BAL cell number and inflammatory cell numbers in OVA-induced allergic asthma mice, these effects were not observed following Treg depletion (Fig. 8).

3.7. Effect of BV on OVA-induced changes in lung morphology

H&E, PAS and immunohistochemical staining were performed on the lung tissues to analyze the effects of BV on the histological feature of asthma. Histological sections of lung tissue from mice exposed to OVA exhibited airway inflammation and were found to have infiltrating eosinophils in the peribronchial regions of the lung (Fig. 9). Conversely, airway inflammation was decreased in sections of lung tissue obtained from mice that were treated with BV and OVA_T + BV. Periodic acid Schiff (PAS)-positive mucus-counting goblet cells around the bronchial airway were detected in the OVA and OVA_T + BV-treated group. BV treatment substantially decreased PAS-positive goblet cells around the bronchial airway (Fig. 10). OVA-challenged mice showed expression of myosin light chain 2 (MLC2) in the peribronchial muscle layer of the lung, and BV treatment abrogated the expression of this protein (Fig. 11). These findings demonstrate that BV has the potential to counteract allergic asthma-associated airway remodeling.

3.8. Effect of BV on the airways during OVA-induced asthma

To evaluate the inhibitory effects of BV on airway hyperresponsiveness, the Pehn value was determined. Ovalbumin-sensitized mice showed significant increases in the Penh values compared to control mice at dosages of 50 and 100 mg/ml methacholine. BV treatment reduced the increased Penh values of the OVA-induced mice at 50 and 100 mg/ml methacholine to the level of control (* P < 0.05, ** P < 0.01) (Fig. 12). These effects were significantly reduced following Treg depletion by anti-CD25 antibody treatment.

4. Discussion

Asthma is a chronic inflammatory disorder characterized by reversible airway obstruction, bronchial hyperresponsiveness and airway inflammation [14]. A skewing of the Th1/Th2 balance towards Th2 is thought to play an important role in the development of allergic asthma. The disease may result from functional or quantitative deficiencies in the regulatory T cells that control Th1 and Th2 immune responses [15]. In fact, Hartl et al. [15] demonstrated

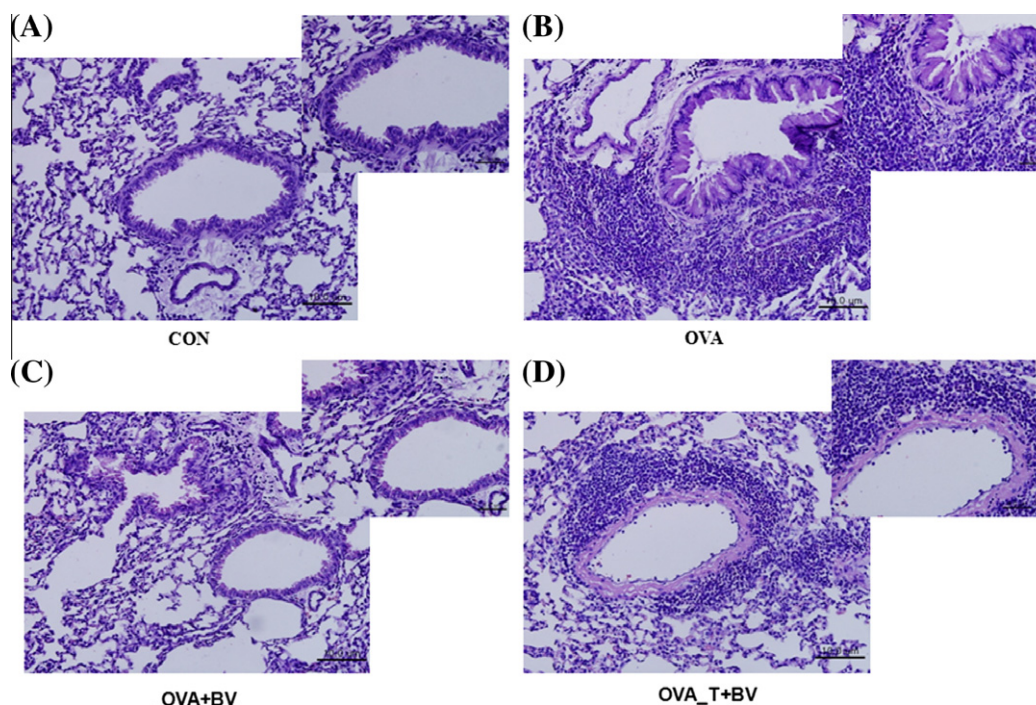


Fig. 9. Effect of BV treatment on the recruitment of inflammatory cells to lung tissues in OVA-induced allergic asthma mice. Foxp3^{EGFP} Balb/c (C. G-Foxp3^{tm2Tch}/J) mice were sensitized and challenged with OVA. Lung tissues were stained with hematoxylin and eosin (H&E). (Magnification 200 $\times$, 400 $\times$). (A) PBS-challenged mice treated with PBS (CON); (B) OVA-challenged mice treated with PBS (OVA); (C) OVA-challenged mice treated BV (1 mg/kg) (OVA + BV); (D) OVA plus anti-CD25 Ab-challenged mice treated with BV (1 mg/kg) (OVA_T + BV).

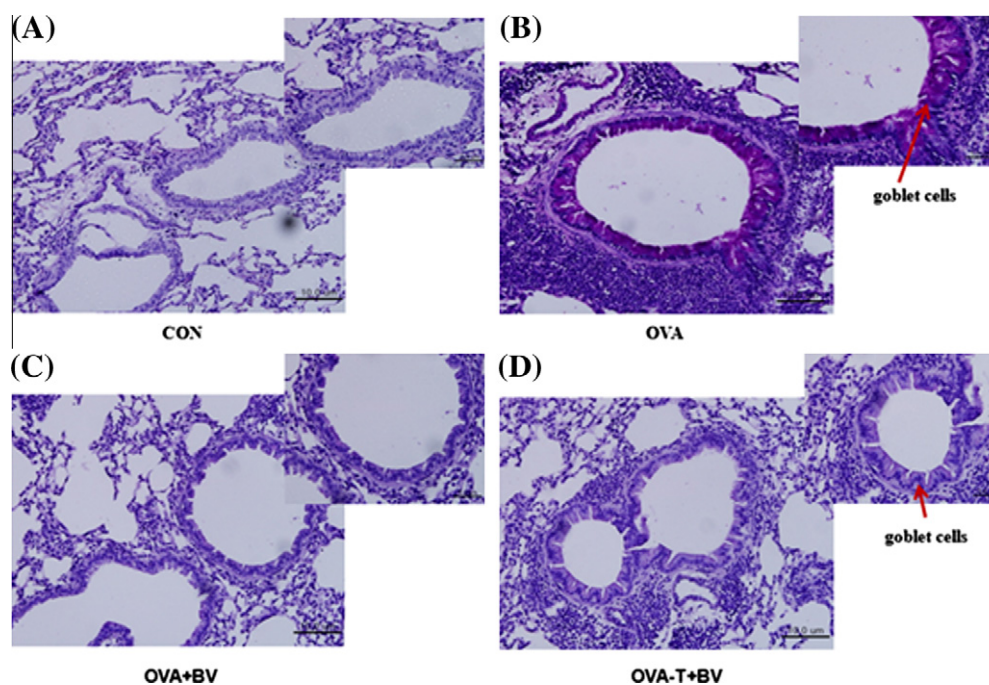


Fig. 10. Effect of BV treatment on the histopathologic changes in lung tissues during OVA-induced allergic asthma mice. Lung tissues stained with periodic acid-Schiff (PAS). (Magnification 200 $\times$, 400 $\times$). (A) PBS-challenged mice treated with PBS (CON); (B) OVA-challenged mice treated with PBS (OVA); (C) OVA-challenged mice treated BV (1 mg/kg) (OVA + BV); (D) OVA + anti-CD25 Ab-challenged mice treated with BV (1 mg/kg) (OVA_T + BV). The arrows indicate goblet cells.

that regulatory T cells were decreased in the BALF of asthmatic children compared with control subjects. Furthermore, many studies have demonstrated that CD4⁺CD25⁺ regulatory T cells play a pivotal role in the maintenance of tolerance in the immune system [16,17], thus helping to prevent autoimmune diseases [18] and allergy [19]. Due to the ability of Treg to suppress disease-promoting

immune responses, enhancing Treg function in patients is an attractive potential therapy for treating allergic asthma.

Bee venom (BV) is traditionally used for treating inflammatory diseases, including allergic asthma. Although Nam et al. demonstrated that the anti-allergic effects of BV may arise from direct regulation of Th1/Th2 lineage development [13], no groups have

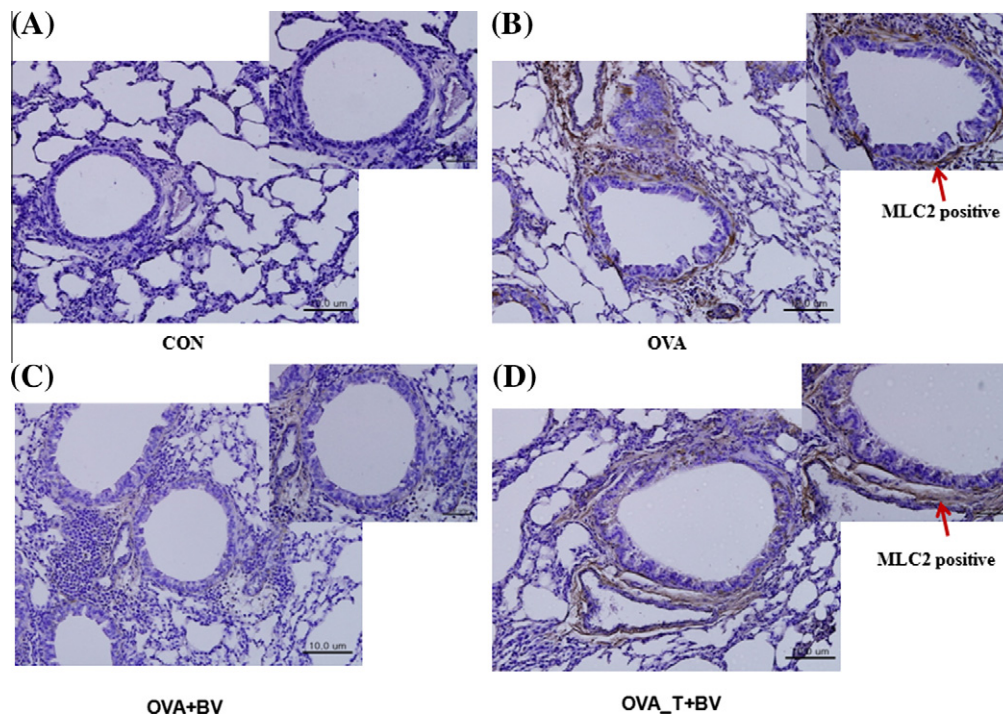


Fig. 11. Effects of BV treatment on airway remodeling in lung tissue, SMA immunohistochemistry in OVA-induced mice. Mouse lung sections stained immunohistochemically. Sections were incubated at 4 °C overnight with anti-MLC2 goat polyclonal antibody (1:50). The slides were then incubated with avidin–biotin peroxidase complex, and color was developed using 3,3'-diaminobenzidine tetrachloride. (Magnification 200 $\times$, 400 $\times$). (A) PBS-challenged mice treated with PBS (CON); (B) OVA-challenged mice treated with PBS (OVA); (C) OVA-challenged mice treated BV (1 mg/kg) (OVA + BV); (D) OVA + anti-CD25 Ab-challenged mice treated with BV (1 mg/kg) (OVA_T + BV). The arrows indicate MLC2 positive cells.

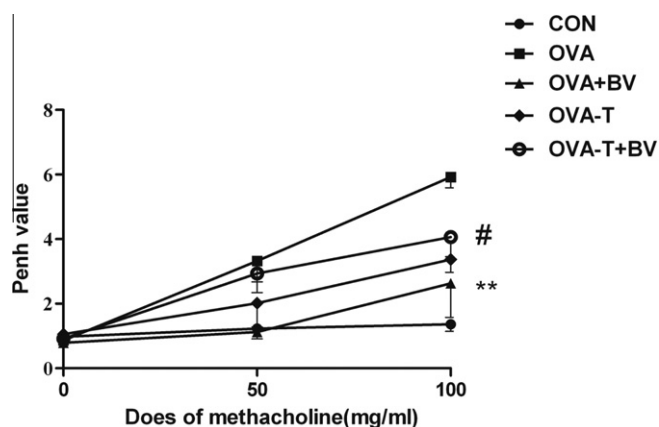


Fig. 12. Effect of BV treatment on airway hyperresponsiveness to methacholine in an OVA-induced murine asthma model. Airways hyper-responsiveness was measured as Penh (enhanced pause) in mice treated with BV or PBS after challenge with increasing concentrations of methacholine. CON: PBS treated mice treated with PBS, OVA: ovalbumin-challenged mice treated with PBS, OVA + BV: ovalbumin-challenged mice treated BV (1 mg/kg), OVA_T: ovalbumin challenged and treated with anti-CD25 Ab, OVA_T + BV: ovalbumin challenged and treated with anti-CD25 Ab plus BV (1 mg/kg). Data are mean $\pm$ S.E.M. of four mice. ** p < 0.01 for the OVA group vs. the OVA + BV group and # p < 0.05 for the CON group vs. the OVA_T + BV group.

examined the anti-asthmatic effects of BV and any possible involvement with Treg. Our group recently observed that bee venom treatment ameliorated the clinical symptoms of lupus nephritis in NZB/W F1 mice, which was associated with a significant increase in the CD4⁺CD25⁺ regulatory T cell population [14]. These results suggested that bee venom may induce immune tolerance via promoting Treg and thus might be effective in treating other types of immunological diseases, such as allergic asthma.

The *in vitro* results showed that BV significantly increased Treg and decreased inflammatory cytokines. We have shown that BV induced a significant increase in the percentage of Treg among the total number of CD4⁺ T cells *in vivo* and *in vitro*. Moreover, the increased proportion of Treg by BV treatment remained suppressive activity *ex vivo*. These data suggested that BV could inhibit asthmatic inflammation by increasing Treg during allergic asthma. BV also increased CD4⁺CD25⁺Foxp3⁺ numbers in an *in vivo* OVA-induced allergic asthma model. In addition, BV remarkably inhibited OVA-induced eosinophilia. Eosinophils contribute to several clinical features of allergic asthma, including tissue damage and AHR. Treg have been reported to directly suppress Th2 lineage development, and thus diminish Th2-associated cytokines. Among these cytokines, IL5 plays a key role in the maturation of eosinophils. Recent reports have also suggested that Treg directly block degranulation of mast cells and eosinophils.

Serum IgE concentration was also significantly decreased following bee venom treatment. Interestingly, Treg have been reported to suppress IgE production via direct regulation of B cells [20]. Accordingly, we believe that the systemic Th2 immune reaction observed in allergic asthma could be modified by BV treatment. In lung tissue histology, the OVA-treated group was characterized by dense peribronchial inflammation due to leukocyte infiltration and mucus hyperproduction by goblet cells within the bronchi, and these inflammatory changes were substantially decreased by BV treatment. However, the inhibitory effects of BV were abolished by Treg depletion, thus strongly suggesting that BV could diminish allergic asthma inflammation through increasing numbers of CD4⁺CD25⁺ regulatory T cells.

BV therapy has been reported to possess a broad range of immunosuppressive effects in both Th1 dominant diseases such as rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis and in Th2-skewed allergic asthma. Interestingly, venom immunotherapy studies have consistently demonstrated that

BV immunotherapy induces peripheral T cell tolerance though increased Treg, thus suppressing Th1/Th2 cells and increasing IL10 production [21–23]. Although these Treg were believed to be antigen-specific (Tr1), the broad range of immunosuppressive effects observed following BV treatment strongly suggested the involvement of other Treg populations such as naturally occurring Treg (nTreg) or inducible Treg (iTreg).

In summary, we demonstrated that BV inhibited the development of asthmatic inflammation in an OVA-induced murine allergic asthma model. Furthermore, we demonstrated that the allergic asthma modulating mechanism of BV was directly related to regulatory T cell-induced immune tolerance. Investigations to identify the major components of BV that are responsible for its inhibitory effects on allergic asthma are currently underway.

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