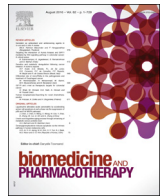




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Bupleurum chinense extract ameliorates an OVA-induced murine allergic asthma through the reduction of the Th2 and Th17 cytokines production by inactivation of NFκB pathway



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ABSTRACT

Bupleurum chinense belongs to the *Bupleurum* spp. family that has been used in traditional herbal medicine for over thousand years. It has been reported to have anti-inflammatory, anti-oxidant, hepatoprotective, antipyretic, analgesic, anti-fibrotic and immunomodulatory effect. However, the effect of *B. Chinense* on allergic asthma remains unclear. This study investigated the immunomodulatory effects of *B. Chinense* extracts (BCE) on airway inflammation in asthmatic mice model. In the ovalbumin (OVA)-induced allergic asthma model, we evaluated the number of total cells, differential inflammatory cells and the production of proinflammatory cytokines in bronchoalveolar lavage fluid (BALF) and lung homogenate as well as histological structure. The levels of NFκB p65, IκBα, p-NFκB p65, p-IκBα and the total immunoglobulin (Ig) E, anti-OVA IgE, anti-OVA IgG were also examined. The oral administration of 200 mg/kg BCE inhibited the accumulation of inflammatory cells especially eosinophils in BALF. Also, BCE regulated the imbalance of Th1, Th2 and Th17-related production, with attenuated the expression of GATA3, IL-1β, IL-4, IL-5, IL-6, TNF-α and RORγt, IL-17A in BALF and lung homogenate, meanwhile, up-regulated the secretion of INF-γ in lung homogenate. The levels of IgE, anti-OVA IgE, anti-OVA IgG1 and anti-OVA IgG2a were also suppressed by BCE treatment in serum. Furthermore, BCE inhibited the proinflammatory cytokines via inactivation of NFκB p65 phosphorylation and IκBα degradation in cytoplasm. The histological analysis showed that the infiltration of inflammatory cells, mucus hypersecretion and collagen fiber deposits were ameliorated in BCE treated mice. In addition, BCE induced the functional differentiation of naive CD4+ T cells forward to Th1 and Tr1 through producing INF-γ and IL-10. These results suggest that BCE may have therapeutic potential for treating allergic asthma through inhibiting Th2/Th17 cytokines production by inactivation of NFκB pathway.

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1. Introduction

Asthma is a chronic disease and is one of the major global health problems [1]. Asthma occurs in people of all ages and affects more

than 334 million people worldwide today and prevalence is rising [2]. Pathologically, patients are found to have bronchial mucosal thickening by edema, bronchial wall remodeling, mucus overproduction and eosinophil infiltration [3]. Inhaled corticosteroids are currently the most effective drugs available for the treatment of asthma [4,5]. However, long-term use of inhaled steroids is often accompanied by undesirable adverse effects, particularly when high doses are used [6]. In addition, corticosteroids also does not address all issues and in all patients [7].

Naive conventional CD4 T cells can differentiate into different effector subsets, such as Th1, Th2 and Th17 cells, depending on the cytokines in the microenvironment [8]. Th1 induced by intracellular pathogens secrete predominantly interferon (IFN)-γ. Th2 cells are involved in host defense against extracellular

Abbreviations: BCE, *Bupleurum chinense*; Dex, Dexamethasone; OVA, ovalbumin; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; BALF, bronchoalveolar lavage fluid; Ig, immunoglobulin; IL, interleukin; TNF-α, tumor necrosis factor alpha; INF-γ, interferon gamma; NFκB, Nuclear factor kappa B; IκBα, Nuclear factor kappa B alpha inhibitor; RORγt, Retinoic-related orphan receptor gamma t; GATA3, GATA binding protein 3.

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pathogens and produce interleukin (IL) IL-4, IL-5, IL-13 as a result of the action of GATA3 and STAT6 transcription factors [9]. On the other hand, Th17 cells are characterized by their production of IL-17. Retinoid-related orphan receptor γ t (ROR γ t) is required for Th17 cell differentiation [10]. Previous research reported that these allergic inflammatory responses are mediated by T-helper type 2 (Th2) cells, mast cells, B cells and eosinophils [11,12]. Other studies shown that the pathophysiology of asthma is related to a Th1/Th2 cell imbalance in the airways [13,14]. Thus, targeted therapies have been toward modulating Th2 responses. In other words, the balance of Th1/Th2 cells may reveal improvement of inflammatory responses. In recent years, the discovery of Th17 introduced complexity into the existing Th1/Th2 balance paradigm and expanded our understanding of the pathogenesis of allergic [15]. Th17 cells and their effector cytokine IL-17 have been showed to play proinflammatory roles in the development of autoimmune disorders and chronic inflammation [16]. The increased Th17 cells and IL-17 expression have also been found in asthmatic patients [17]. On the other hand, NF- κ B regulates the expression of a large number of genes involved in the immune and inflammatory responses [18]. NF κ B plays a crucial role for the production of Th2 cytokines and recruitment of inflammatory cells in the airways of murine asthma models [19,20]. In addition, NF κ B is a transcription factor that has pivotal roles in inflammation, immunity, cell proliferation [21]. While in an inactivated state, NF κ B is located in the cytosol complexed with the inhibitory protein I κ B α . The main activated form of NF κ B consisted of p50 or p52 subunit and the trans-activating subunit p65 [22]. NF- κ B is activated by various stimuli, such as cytokines IL-1 β and TNF- α [23]. NF κ B is also observed increase of cytokines in the airway epithelium, such as IL-1 β , IL-4, IL-5, IL-6 and TNF- α [24]. Furthermore, I κ B α is one member of a family of cellular proteins that function to inhibit the NF- κ B transcription factor [25]. Therefore, we examined the NF κ B p65 phosphorylation and I κ B α degradation to investigate the effect of BCE treatment on the inflammatory reactions as well as identified whether or not it relates to the expression of Th2 (IL-1 β , IL-4, IL-5, IL-6 and TNF- α) proinflammatory cytokines.

Bupleurum chinense belongs to the *Bupleurum* spp. family, locally known as “Bei Chaihu” in China that is one of the most important traditional herbal medicines for over thousand years. It has been reported to have anti-inflammatory, anti-oxidant, hepato-protective, antipyretic, analgesic, anti-fibrotic and immunomodulatory effect [26,27]. Some research reported that saikosaponin as the main pharmacodynamic active ingredient of *B. Chinense* shows their anti-inflammatory effects through regulating nicotinate, nicotinamide metabolism and arachidonic acid metabolism [28]. Saikosaponin A also have the anti-inflammatory and antifibrotic actions on CCl₄-induced liver damage in rat and hepatic proinflammatory cytokines TNF- α , IL-1 β and IL-6 were significantly inhibited [22]. Nevertheless, antiasthmatic effects of *B. Chinense* have been currently unclear. Based on these information, this study aims to investigate the effects of *B. Chinense* water extract (BCE) on ovalbumin (OVA)-induced allergic asthma mouse model.

2. Materials and methods

2.1. Animals

Six-week-old male BALB/c mice were obtained from the Damool Science (Dae-jeon, Korea). All procedures of experiments were approved accordance with the guidelines of the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School (CBN 2013-0011) and were approved by the National Institutes of Health.

2.2. Asthmatic model establishment and treatment

Mice were divided into five groups ($n=6$ each group), including control group (saline sensitization/challenge/treatment), saline/BCE group (saline sensitization/challenge, 200 mg/kg BCE oral treatment), OVA/saline group (asthma model, OVA sensitization/challenge, saline treatment), OVA/BCE group (OVA sensitization/challenge, 200 mg/kg BCE oral treatment) and OVA/dexamethasone (Dex) group (OVA sensitization/challenge, 2.5 mg/kg Dex oral treatment). Dex is a type of corticosteroid medication, has anti-inflammatory and immunosuppressant effects [29]. Dex was used as a positive control in this study for comparing with BCE treatment.

The procedures of an OVA-induced asthma mouse model and treatment were established as described in Fig. 1. Briefly, OVA-induced asthma mice were sensitized on day 1 with an intraperitoneal injection of 200 μ L saline along with 50 μ g OVA (GradeV, Sigma, St. Louis, MO, USA) and 1 mg aluminum hydroxide (Thermo Scientific, Rockford, MD, USA). On day 14, mice were sensitized 200 μ L saline containing 50 μ g OVA without aluminum hydroxide. After sensitization, on days 27, 28 and 29, mice received an airway challenge with OVA (5%, w/v, in saline) for 20 min using an ultrasonic nebulizer (NE-U17, Omron Co., Tokyo, Japan). Mice in the control and saline/BCE group were sensitized and challenged with 200 μ L saline on days 1 and 14. On days 15–26, mice in the OVA/BCE group and Dex group were orally administered once daily with BCE and Dex at dose of 200 and 2.5 mg/kg, respectively; control group and OVA/saline group were given saline. Mice were sacrificed 24 h after the last OVA challenge.

2.3. Preparation of BCE

BCE was provided by research grants from the Korea Food Research Institute and managed with specimen number (KFRI-SL-2147). The dried root BCE powder was kept at 4 °C until used. For asthmatic mice model treatment, BCE (200 mg/kg) was dissolved in 0.1% dimethylsulfoxide (DMSO) (Sigma, France) in saline and was prepared fresh daily before each treatment. For *in vitro* spleen cell culture, BCE dose-dependently (1, 10, 100 and 200 μ g/mL) was dissolved in saline.

2.4. Bronchoalveolar lavage fluid collection and cell count

Twenty-four hours after the final OVA challenge, mice were sacrificed, and bronchoalveolar lavage fluid (BALF) was collected by flushing 1 mL of ice-cold PBS into the trachea and withdrawn through the cannula. BALF was centrifuged at 1000 RPM/min for 10 min at 4 °C. The supernatant was stored at –70 °C for ELISA analysis. The cell pellets were resuspended in PBS and the total cell numbers were counted in a hemocytometer.

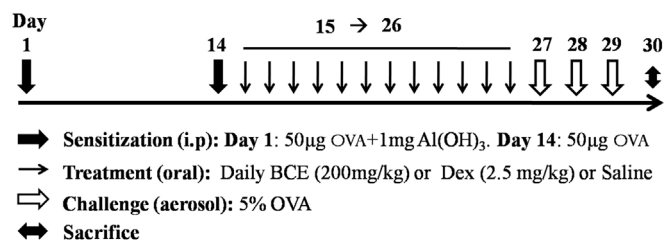


Fig. 1. Protocol for asthma allergic mouse model and treatment with BCE. Mice were sensitized on days 1 and 14; challenged on days 27, 28 and 29 by ovalbumin (OVA). Mice in control group were sensitized and challenged by saline. Mice in OVA/saline group were sensitized and challenged by OVA. Mice in the OVA/BCE or OVA/Dex treatment groups were sensitized and challenged by OVA and administered orally once a day at 200 mg/kg BCE or 2.5 mg/kg dexamethasone for 12 days.

To determine differential cell counts, 150 μ L of BALF was centrifuged onto slides using a cytopsin device (Centrifuge 5403, Eppendorf, Hamburg, Germany) (1000 rpm, 10 min, 4 °C). Diff-Quik Staining reagent (1-5-1 Wakinohama-Kaigandori, Chuo-Ku, Kobe, Japan) was used for the cell staining according to the protocols from the manufacturer.

2.5. Histological examination

The middle lobe of the lung was removed and fixed in 10% (v/v) neutral buffered formalin. The specimen was dehydrated with ethyl alcohol and xylene. Tissues were embedded in paraffin, sectioned at 5 μ m thickness. The lung tissues were stained with hematoxylin and eosin (H&E) (Sigma, St. Louis, MO), congo red (Sigma, St. Louis, MO), periodic acid-Schiff (PAS) (Sigma, St. Louis, MO) and Masson trichrome (Sigma, St. Louis, MO) to assess inflammatory cell infiltration, eosinophil, goblet cell and mucus production, collagen deposition, respectively.

2.6. Measurement of cytokines, NF κ B p65, I κ B α , p-NF κ B p65, p-I κ B α in BALF and lung tissue; immunoglobulins in serum.

Lung tissue was homogenized (Omni Prep Rotor Stator Generator, Omni International, USA) and centrifuged to obtain supernatant. The BALF was centrifuged to collect the supernatant. Blood was collected from vena cava and serum was obtained by centrifugation and stored at –70 °C. The levels of cytokines such as IFN- γ , IL-1 β , IL-6, IL-10, IL-14, IL-5, IL-17A and TNF- α , the levels of transcription factors GATA3 (Th2-associated responses) and ROR γ t (Th17-associated responses), the levels of total IgE, anti-OVA IgE, anti-OVA IgG1, anti-OVA IgG2a were quantified by using ELISA kits (R&D Systems Inc. USA) according to the manufacturers' instruction. NF κ B p65, I κ B α , phosphorylation of NF κ B p65 (p-NF κ B p65) and phosphorylation of I κ B α (p-I κ B α) in BALF and lung homogenate were examined by using ELISA kits (eBioscience Inc. USA)

according to the protocols from the manufacturer. The optical density of 96-well culture plates was measured at 450 nm by using ELISA reader.

2.7. Differentiation of naive CD4⁺ T cells

Primary splenocytes and lymphocytes were prepared by aseptically removing the spleens and mesenteric lymph nodes isolated from normal BALB/c mice, respectively. The tissues were homogenized, and naive CD4⁺ T cells from these cells were isolated by magnetic-activated cell sorting (MACS) system (Miltenyi Biotec, Bergisch-Gladbach, Germany). The number of naive CD4⁺ T cells was adjusted to a cell density of 1×10^6 cells/mL in RPMI 1640 medium by using the trypan blue exclusion method. Naive CD4⁺ T lymphocytes were cultured with T cell receptor (TCR) stimulation (10 μ g/mL anti-CD3 mAb, 10 μ g/mL anti-CD28 mAb and IL-2) in the absence or presence of BCE (1, 10, 100 and 200 μ g/mL) at 37 °C for seven days in a humidified incubator with 5% CO₂ to induce primed CD4⁺ T lymphocytes. And then primed CD4⁺ T lymphocytes were re-stimulated with only TCR stimulation (10 μ g/mL anti-CD3 mAb and 10 μ g/mL anti-CD28 mAb, and IL-2) for three days. Next, the supernatants of activated CD4⁺ T lymphocytes were harvested, and the levels of IL-4, IL-10, IL-17, IFN- γ and TGF- β were measured using ELISA kits according to the manufacturer's instruments.

2.8. Cytotoxicity assay

To test the cytotoxicity of human mast cell-1 (HMC-1), the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay was performed. HMC-1 (2×10^5 cells/well) were incubated with various concentrations (1, 10, 100 and 200 μ g/mL) of BCE at 37 °C for 3 h. After addition of MTT (1000 μ g/mL), cells were incubated at 37 °C for 1 h. The crystallized MTT was dissolved, and the absorbance was measured at 570 nm with a spectrophotometer.

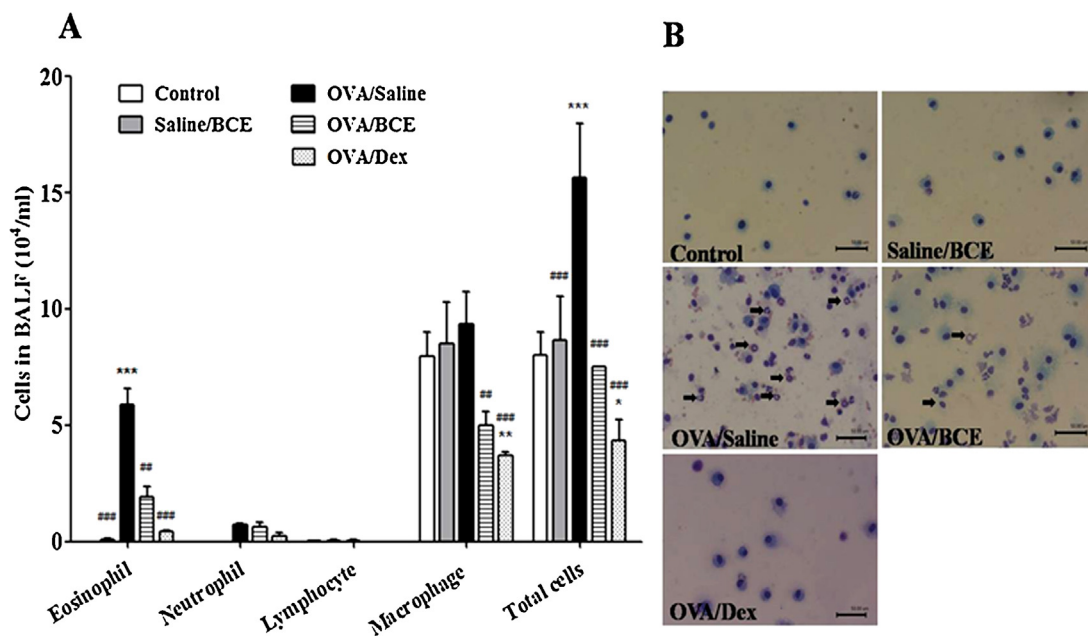


Fig. 2. Effect of BCE on the differential cells and total cell number (A) and infiltration of inflammatory cells (B) in BALF. Control group: saline challenge/treatment. Saline/BCE group: saline challenge, BCE 200 mg/kg. OVA/Saline group: OVA challenge. OVA/BCE group: OVA challenge, BCE 200 mg/kg. OVA/Dex group: OVA challenge, dexamethasone 2.5 mg/kg. (A) The values represent the mean $\pm$ S.E.M ($n=6$ /group). Significant differences at *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ compared with the control group. ### $P < 0.001$, ## $P < 0.01$ compared with the OVA/saline group. (B) BALF was collected 24 h after the last OVA challenge, the cell was isolated by cytopsin and stained with Diff-Quick. Black arrows indicated as eosinophils. Scale bar: 50 μ m.

2.9. Statistical analysis

The results were performed by using Graph Pad Prism software (v5.0, La Jolla, CA, USA). Data were expressed as means $\pm$ SEM. The differences among the groups were analyzed with one-way ANOVAs followed by Tukey's test. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. Effect of BCE on the number of eosinophils and differential inflammatory cells in BALF

To determine whether BCE could suppress the airway inflammation, the number of total cell and various inflammatory cells in BALF were measured. Mice in OVA/saline group were significantly elevated the infiltration and the accumulation of total cells, macrophages and especially eosinophils compared to control group ($P < 0.001$). In contrast, BCE treatment (200 mg/kg) significantly reduced the increase of these cells when compared to the mice in OVA/saline group ($P < 0.01$), similar results with Dex treatment (2.5 mg/kg) ($P < 0.001$). While, the neutrophils and lymphocytes number did not show statistical difference among groups (Fig. 2).

3.2. Effect of BCE on histopathological changes in lung tissue

In the OVA/saline group, the bronchus showed significantly histopathological changes compared with the control group (Fig. 3A,C). H&E staining indicated that the bronchial epithelium was edema and the smooth muscle layer was thickened, in

addition, showed the infiltration of leukocytes in peribronchial and alveolar septal regions in asthmatic mice (Fig. 3C1). In contrast, for the mice in the OVA/BCE (200 mg/kg) and OVA/Dex (2.5 mg/kg) treatment group, airway epithelium and smooth muscles were thinner than those in the OVA/saline group, and the treatment with BCE and Dex considerable decreased the leukocytes infiltration in peribronchial and perivascular compared with the asthmatic mice (Fig. 3D1,E1). Mucus hypersecretion in the OVA/saline group was observed as a violet color in the bronchial airways compared with the control group (Fig. 3C3). However, Dex and BCE-treated mice significantly inhibited the mucus overproduction in the bronchial airways compared with that observed in the OVA/saline group (Fig. 3D3). Masson trichrome staining revealed that administration of BCE suppressed collagen deposition, that was observed as a blue color in peribronchial and perivascular when compared with the asthmatic mice (Fig. 3D4). The histopathological features in saline/BCE group did not find a difference compared to control group (Fig. 3B).

3.3. Effect of BCE on Th1, Th2 and Th17 responses in BALF and lung tissue

We examined the cytokines level of INF- γ ; transcription factors GATA3, IL-4, IL-5; and transcription factors ROR γ t, IL-17A as well as IL-10, IL-1 β , IL-6, TNF- α productions in BALF and lung homogenate to investigate the effect of BCE on asthmatic mice. In the OVA/saline group; the levels of GATA3, IL-1 β , IL-4, IL-5, TNF- α , IL-17A and ROR γ t were significantly higher than those in both BALF and lung homogenate of Control group; except IL-6 only significantly increase in BALF; while IFN- γ in lung tissue significantly reduced compared to the control group (Fig. 4). In contrast; BCE-treated

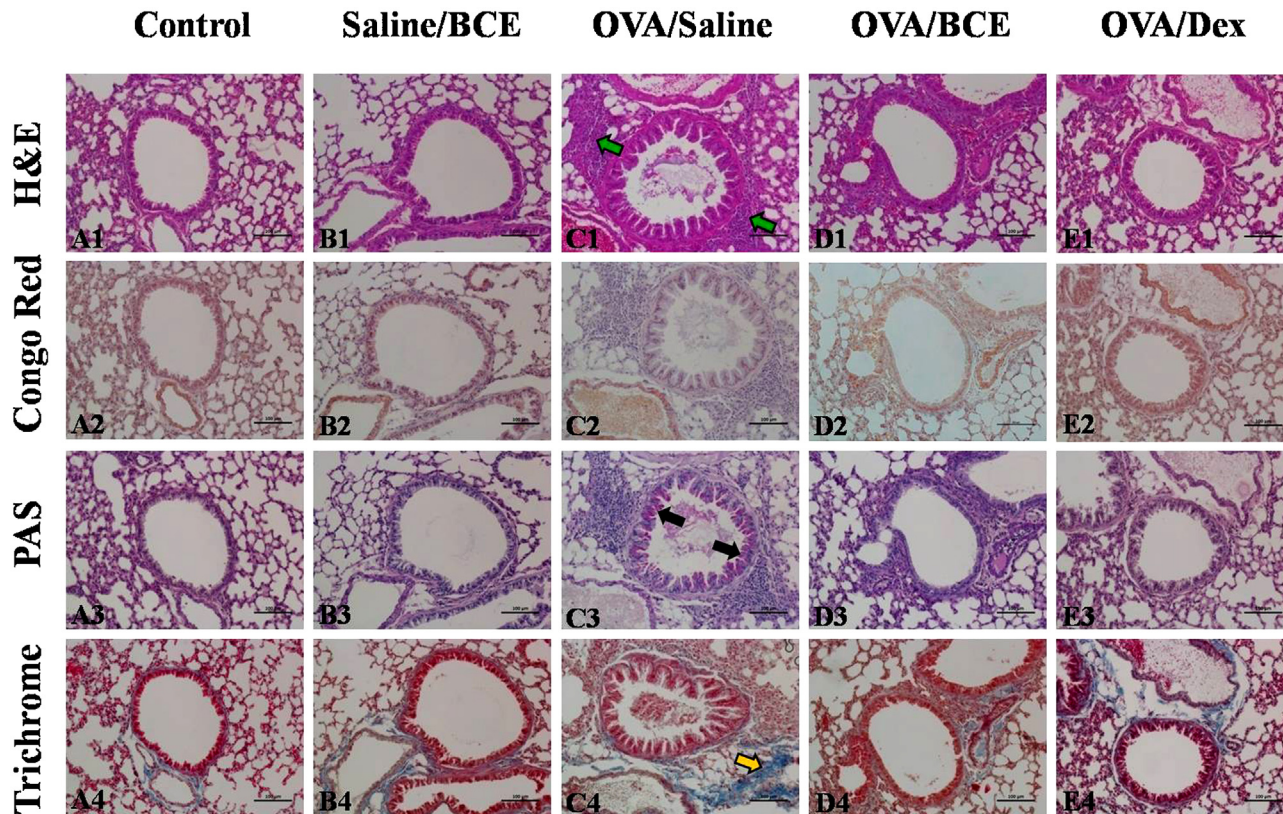


Fig. 3. Histologic features of the lung tissue. A1, A2, A3, A4: Control group. B1, B2, B3, B4: Saline/BCE group (200 mg/kg BCE, saline challenge). C1, C2, C3, C4: OVA/Saline group. D1, D2, D3, D4: OVA/BCE group (200 mg/kg BCE, OVA challenge). E1, E2, E3, E4: OVA/Dex group (2.5 mg/kg dexamethasone, OVA challenge). The sections were stained with H&E, congo red, PAS and Masson trichrome. Green arrows indicated as leukocytes infiltration. Black arrows indicated as mucus hypersecretion, that was observed as a violet color in the bronchial airways. Yellow arrows indicated as collagen fiber deposits, that was observed as a blue color in peribronchial and perivascular. Scale bar: 100 μ m.

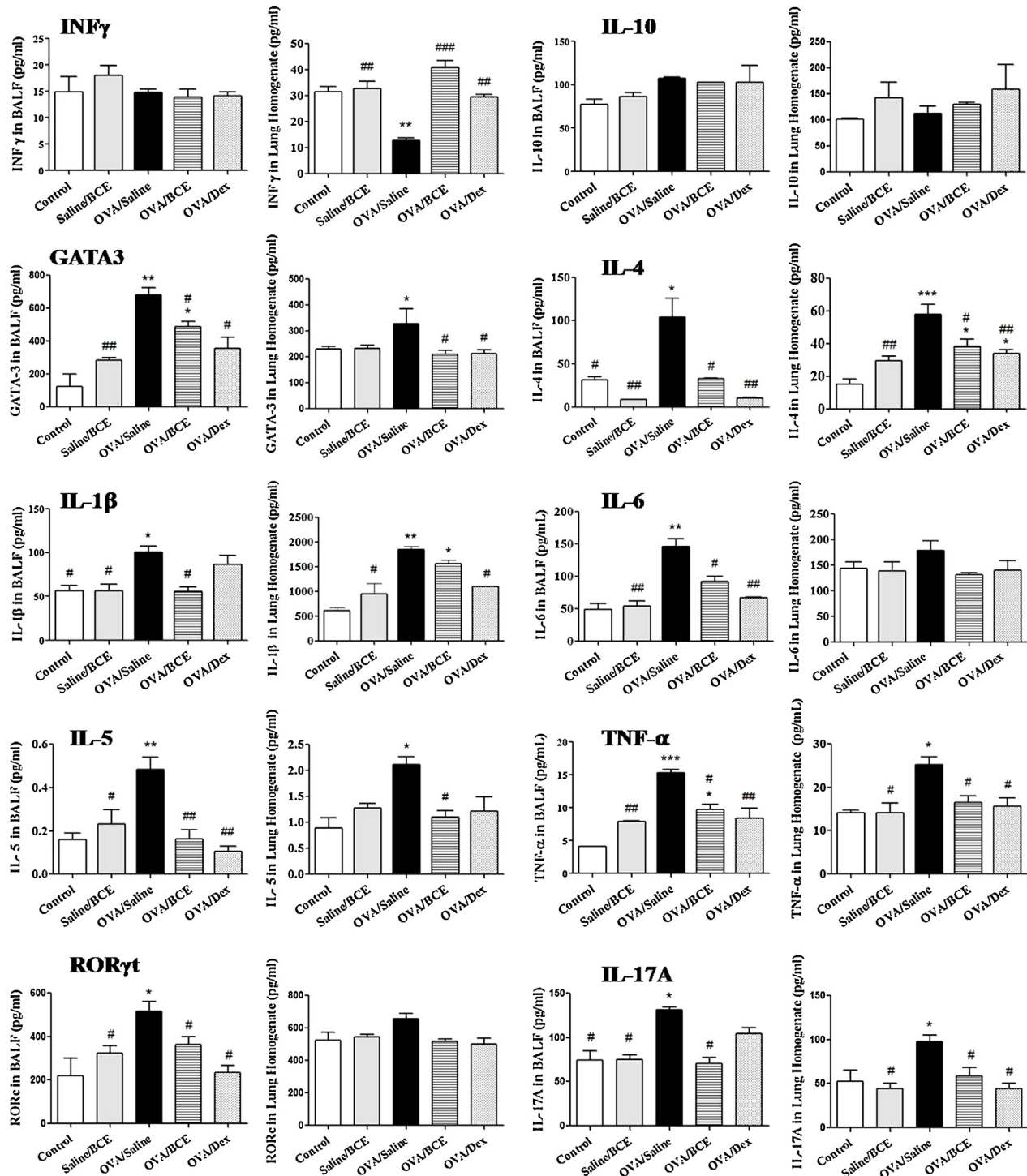


Fig. 4. Effect of BCE on cytokine levels in BALF and lung homogenate. The concentrations of IL-10, INF- γ , GATA3, IL-1 β , IL-4, IL-5, IL-6, TNF- α , ROR γ t and IL-17A in the BALF and lung homogenate were determined by ELISA kits. Control group: saline challenge. Saline/BCE group: saline challenge, BCE 200 mg/kg. OVA/Saline group: OVA challenge. OVA/BCE group: OVA challenge, BCE 200 mg/kg. OVA/Dex group: OVA challenge, dexamethasone 2.5 mg/kg. The values represent the mean $\pm$ S.E.M ($n=6$ /group). Significant differences at *** $P < 0.001$, ** $P < 0.01$ and * $P < 0.05$ compared with the control group. ## $P < 0.01$ and # $P < 0.05$ compared with the OVA/saline group.

mice markedly decreased the levels of GATA3, IL-4, IL-5 and TNF- α in both BALF and lung homogenate compared with OVA-induced mice. Except IL-17A, IL-1 β and IL-6 levels only significantly decreased in lung homogenate; and ROR γ t only significantly decreased in BALF (Fig. 4). BCE-treated mice was also no significant

difference the concentrations of IL-10 production in BALF and lung homogenate, and IFN- γ in BALF when compared with those in the control group (Fig. 4). Similarly; OVA/Dex group also significantly decreased the levels of GATA3, IL-1 β , IL-4, IL-5, IL-6 and TNF- α ; except IL-1 β , IL-17A in BALF and ROR γ t, IL-5, IL-6 in lung tissue

(Fig. 4). There was no significant difference between saline/BCE group and control group of Th1, Th2 and Th17 responses in both BALF and lung homogenate (Fig. 4).

3.4. Effect of BCE on total IgE and antigen-specific-antibody in serum

Th2 cytokines play an important role on the allergic responses via the regulation on IgE and other antigen-specific-antibody levels, we examined the levels of total IgE, anti-OVA IgE, anti-OVA IgG1 and anti-OVA IgG2a in serum. The level of total IgE, anti-OVA IgE and anti-OVA IgG2a were significantly up-regulated in OVA-induced mice compared with those in the control group. Although anti-OVA IgG1 showed tendency to overexpression in the OVA/saline group, there was no statistically significant difference. By contract, BCE treated-mice led to a marked decrease in levels of total IgE, anti-OVA IgE, anti-OVA IgG1 and anti-OVA IgG2a in serum compared with those in the OVA/saline group. Saline/BCE group did not find difference data with Control group. Dex treated-mice significantly attenuated only anti-OVA IgG2a compared to OVA/saline group in serum (Fig. 5).

3.5. Effect of BCE on OVA-induced activation NFκB p65 and IκBα degradation

We assessed the NFκB p65 (Ser536) activation and IκBα (Ser32/36) degradation whether or not BCE could inhibit OVA-induced inflammation. Fig. 5 shows OVA/saline group was increased the expression of NFκB p65 and significantly decreased the expression

of IκBα compared with control group in both BALF and lung homogenate. However, BCE treated mice shown NFκB p65 was inhibited and the expression of IκBα was significantly higher than OVA-induced mice in BALF and lung homogenate. Furthermore, we saw an elevation in the expression of p-NFκB p65 and p-IκBα in OVA/saline group when compared with the control group in BALF and lung homogenate. Nevertheless, BCE significantly suppressed the degradation of IκBα and also inhibited NFκB p65 activation compared to OVA-induced mice (Fig. 6).

3.6. Effect of BCE on the functional differentiation of CD4⁺T cells

To reveal the mechanism by which BCE ameliorates OVA-induced allergic asthmatic symptoms, we were investigated whether BCE regulates the functional differentiation of naive CD4⁺ T cells. Each INF-γ, IL-4, IL-17A, TGF-β or IL-10 used as an index such as Th1, Th2, Th17, Treg, or Tr1, respectively. The result showed that the treatment of BCE dose-dependently significantly increased the INF-γ production which is associated with the Th1 cell differentiation and the IL-10 production which is related to the Tr1 cell differentiation. However, IL-17A, IL-4 and TGF-β levels did not show statistical difference by BCE treatment (Fig. 7).

3.7. Effect of BCE on the cytotoxicity of HMC-1

MTT assay was used to determine the cytotoxicity of HMC-1 exposed to BCE. As shown in Fig. 8, the viable cells were almost

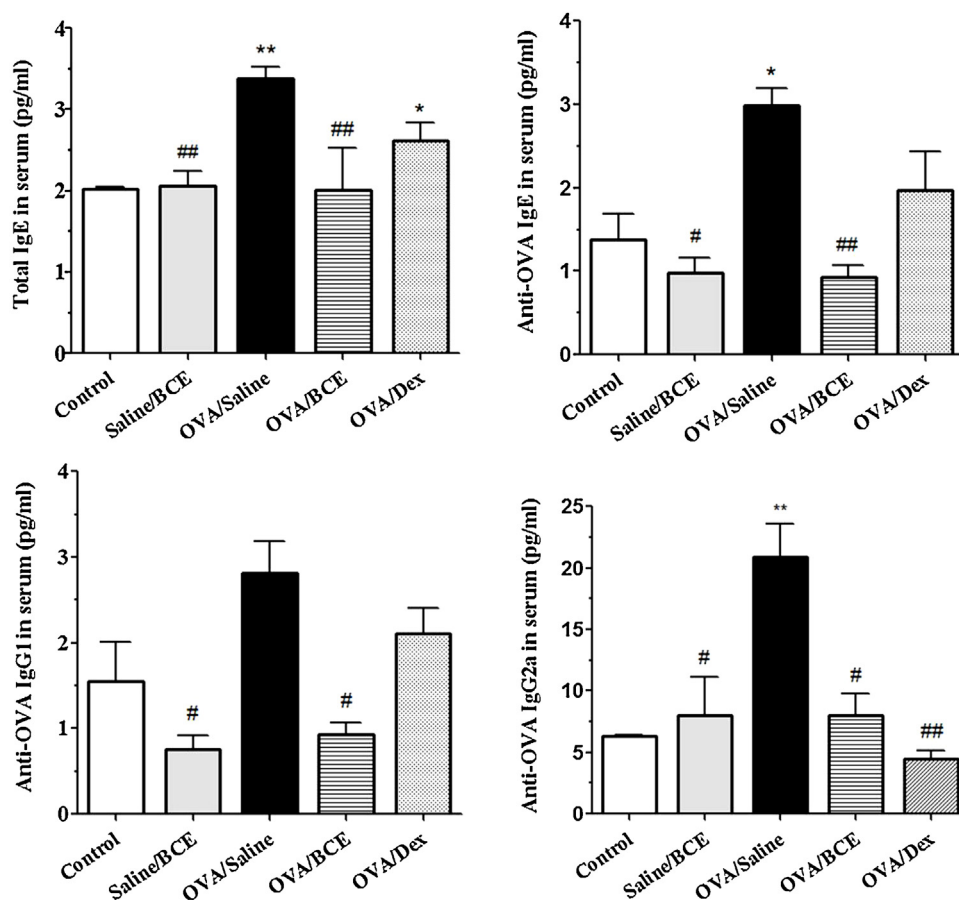


Fig. 5. Effect of BCE on the levels of total IgE, anti-OVA IgE, anti-OVA IgG1 and anti-OVA IgG2a in the serum. Control group: saline challenge. Saline/BCE group: saline challenge, BCE 200 mg/kg. OVA/Saline group: OVA challenge. OVA/BCE group: OVA challenge, BCE 200 mg/kg. OVA/Dex group: OVA challenge, dexamethasone 2.5 mg/kg. The values represent the mean $\pm$ S.E.M ($n = 6$ /group). Significant differences at $^{*}P < 0.01$ and $^{*}P < 0.05$ compared with the control group. $^{##}P < 0.01$ and $^{#}P < 0.05$ compared with the OVA/saline group.

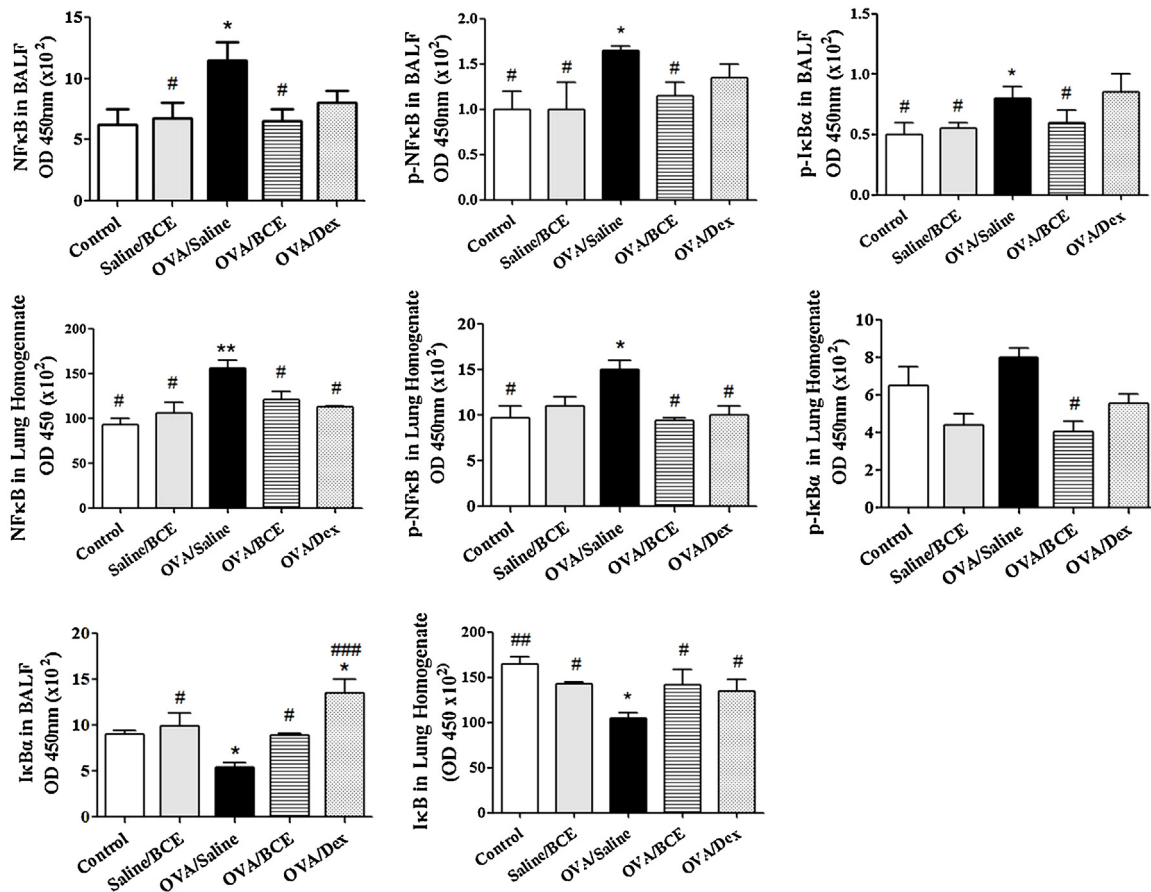


Fig. 6. Effect of BCE on the levels of NFκB p65, p-NFκB p65, IκBα and p-IκBα expression in BALF and lung homogenate. Control group: saline challenge. Saline/BCE group: saline challenge, BCE 200 mg/kg. OVA/Saline group: OVA challenge. OVA/BCE group: OVA challenge, BCE 200 mg/kg. OVA/Dex group: OVA challenge, dexamethasone 2.5 mg/kg. The values represent the mean $\pm$ S.E.M ($n = 6$ /group). Significant differences at $^*P < 0.05$ compared with the control group. $^{***}P < 0.001$, $^{\#}P < 0.05$ compared with the OVA/Saline group.

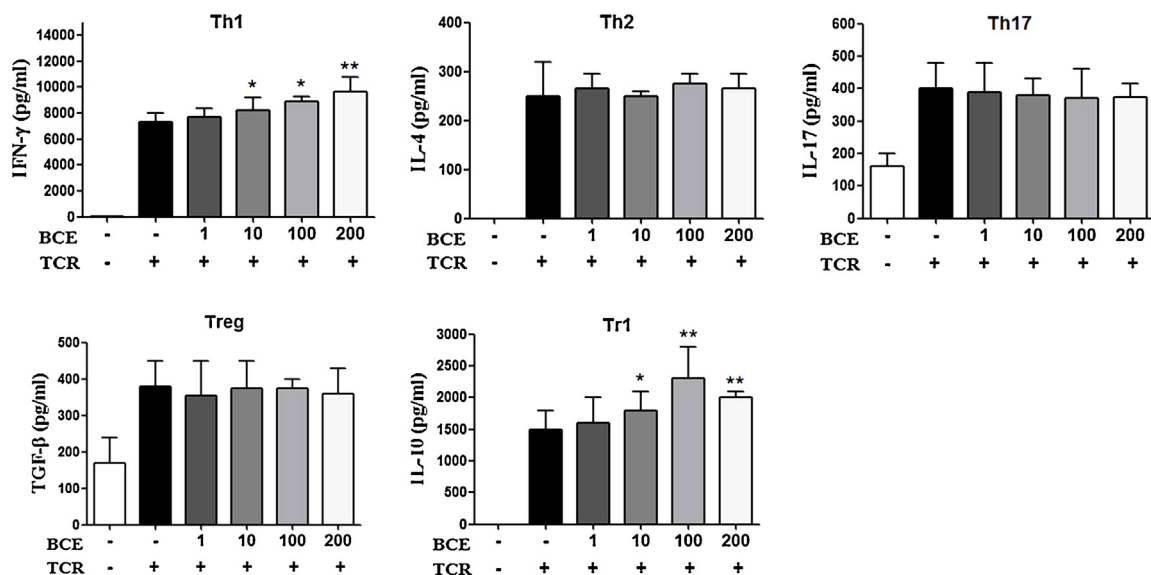


Fig. 7. Effect of BCE on the functional differentiation of CD4⁺ T cells. Naïve CD4⁺ T cells were isolated from the spleens and mesenteric lymph nodes of normal BALB/c mice. Naïve CD4⁺ T lymphocytes were cultured with TCR stimulation (10 μg/mL anti-CD3 mAb, 10 μg/mL anti-CD28 mAb and IL-2) in the absence or presence of BCE (1, 10, 100 and 200 μg/mL) for seven days. And then primed CD4⁺ T lymphocytes were re-stimulated with only TCR stimulation (concentration as above) for three days. Supernatants were collected for measuring the cytokines. The treatment of BCE could induce the functional differentiation of naïve CD4⁺ T cells forward to Th1 and Tr1 through producing INF-γ and IL-10. The values represent the mean $\pm$ S.E.M ($n = 6$ /group). Significant differences at $^*P < 0.01$ and $^{\#}P < 0.05$ compared with the control group. $^{**}P < 0.01$ and $^{\#}P < 0.05$ compared with the OVA/Saline group.

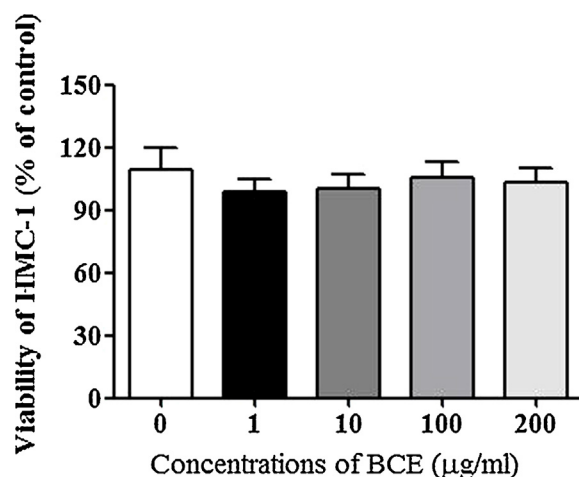


Fig. 8. Effect of BCE on the cytotoxicity of HMC-1. HMC-1 viability was performed by MTT conversion assay and percentage of the viability was calculated as a ratio of A570 of control cells. Each value represents the mean $\pm$ S.E.M ($n = 6$ /group).

100% after treatment with 200 μ g/mL BCE for 3 h. Thus, BCE had no cytotoxicity on HMC-1.

4. Discussion

In this study, the antiasthmatic activities of BCE using OVA-induced allergic asthma mice model were investigated. Allergic asthma is a chronic inflammatory disease of the airway that is characterized by airway inflammation, increase amounts of systemic IgE, intense eosinophilia, lymphocyte infiltration and mucus hypersecretion [30,31]. In addition, an imbalance between Th1 and Th2 cells leads to the clinical expression of this allergic disease [13].

On the other hand, Th17 cells have been associated with Th2-predominant allergic diseases. Increased Th17 responses have been found in asthma [32]. Thus, in order to evaluate the relative contribution of each functional T cell subset in the allergic inflammation, we assessed the proinflammatory cytokines production (IFN- γ , IL-10, IL-1 β , IL-4, IL-5, IL-6, IL-17A and TNF- α) involved with Th1/Th2/Th17 T cells, along with the expression of transcription factors associated with differentiated Th2 and Th17 T cells (GATA3 and ROR- γ t, respectively). Furthermore, we evaluated the number of total cells, differential inflammatory cells, histological structure, NF- κ B p65, I- κ B α , p-NF- κ B p65, p-I- κ B α as well as IgE, anti-OVA IgE, anti-OVA IgG1 and anti-OVA IgG2a levels in serum.

Our results shown that OVA-induced mice had an elevation of inflammatory cells number, increase IgE, anti-OVA IgE, anti-OVA IgG1, anti-OVA IgG2a production, imbalance of Th1/Th2/Th17 responses, mucus overproduction and eosinophil-rich leukocyte infiltration. By contrast, BCE oral administration resulted in a balance of Th1/Th2/Th17 responses. There were an up-regulation IFN- γ level in lung homogenate and a downregulation the levels of IL-1 β , IL-4, IL-5, IL-6, IL-17A and TNF- α in BALF; IL-4, IL-5, IL-17A and TNF- α in lung tissue. The levels of IgE, anti-OVA IgE, anti-OVA IgG1 and anti-OVA IgG2a were suppressed by BCE treatment in serum. The number of inflammatory cells in BALF as well as mucus hypersecretion also suppressed in BCE-treated mice when compare to OVA-induced mice. Also, BCE inhibited the proinflammatory cytokines via inactivation of NF- κ B p65 phosphorylation and I- κ B α degradation in cytoplasm. Accordingly, this suggests that BCE may have anti-inflammatory effects on OVA-induced airway inflammation in an asthmatic mouse model.

The infiltration of eosinophils into the airway is a characteristic of asthma, and increased number of these cells have been found in BALF. The proinflammatory mediators derived from eosinophils are major contributors to the inflammation observed in asthma, which includes airway epithelial cell loss and damage, airway dysfunction and mucus hypersecretion [33]. In addition, mucus hypersecretion, an important feature of asthma, is induced by eosinophils and mast cells products [34]. Recently, reported that IL-17A expression is associated with eosinophilic inflammation [35]. IL-4, and IL-5 regulate the growth and differentiation of eosinophils [36]. IL-17 producing Th2 cells resulted in profound goblet hyperplasia as well as elevated mucin production [37]. Additionally, NF- κ B signaling is important to eosinophil survival [38]. We found that asthmatic mice significantly increased eosinophil numbers in BALF and mucus overexpression, while oral treatment of BCE remarkably decreased the number of eosinophils in BALF and mucus hypersecretion in airway epithelial cells. Our results showed that Th2- and Th17-related cytokines and NF- κ B signaling were positively correlated to the frequency of eosinophilic inflammation and airway hypersecretion.

Th2 and Th17 cytokines have an important role in contributing to structural changes in bronchial epithelial and airway smooth muscle cells [36,37,39]. Our finding showed that the increase of Th2 and Th17 cytokines was positively correlated to the histopathological changes in asthma mice. Histologic features have been revealed that BCE treatment decreased the infiltration of inflammation cells and mucus overproduction of goblet cells in alveolar septal and peribronchial regions.

Allergic inflammation is characterized by a predominant Th2 response, while the Th1 response is thought to prevent this process [40]. Previous reports have shown that modulation of Th1/Th2 cytokines can inhibit airway inflammation in OVA-induced mice [41,42]. IFN- γ has been reported to play an important inhibitory role on recruitment of eosinophils to the lung, inhibit the synthesis of IgE and the differentiation of precursor cells to Th2 cells, increasing levels of IFN- γ have been used to explain the anti-allergic effects of therapeutic agents [43,44]. On the other hand, Th2 cells produce IL-4, IL-5 and provide help to B cells and as such, are believed to play a central role in the etiology of allergic disease [45]. IL-4 and IL-5 regulate the growth and differentiation of eosinophils, and serves as an essential signal for their movement into inflammatory tissue upon antigen exposure [36]. IL-6 has been found to be elevated in a number of inflammatory diseases. IL-6 has long been considered a general marker of inflammation together with TNF- α and IL-1 β , two other classical inflammatory cytokines [46]. IL-1 β can alter airway function by inducing cellular infiltrate, airway eosinophilia, mucus hyperplasia, airway wall thickening, fibrosis, collagen synthesis, IgE synthesis and is considered the host immune defensor [47–51]. The proinflammatory cytokine IL-1 β may directly stimulate fibrosis by inducing fibroblast proliferation and increased collagen deposition [52]. In support of these results, our findings showed that the OVA-induced mice were higher levels of IL-1 β , IL-4, IL-5, IL-6 and lower levels of IFN- γ compared with those in the Control group. In contrast, BCE oral treatment led to a downregulation of IL-1 β , IL-4, IL-5, IL-6 in BALF and lung homogenate, and upregulation of IFN- γ level in lung homogenate compared with the OVA-induced mice. Except that IL-10 production had no statistically significant difference between OVA-induced mice and BCE-treated mice. These findings demonstrated that BCE could attenuate the development of allergic inflammation responses via upregulated the Th1-related cytokines and downregulated Th2-related cytokines. Furthermore, TNF- α is a chemotactic cytokine for granulocytes including eosinophils and neutrophils [56]. Our data shown that TNF- α production was found to increase in the OVA-induced mice, while BCE treatment led to a decreased in both BALF and lung

tissue when compared to those in the OVA/saline group. Therefore, it is possible that the inhibition of bronchial wall thickening, mucus overproduction, collagen deposition and inflammatory cells infiltration, especially, eosinophil result from reduction of IL-1 β , IL-4, IL-5, IL-6 and TNF- α productions after oral treatment of BCE.

Th2 cells produce IL-4, IL-5, as a result of the action of GATA3 and STAT6 transcription factors [53]. Th2 responses are completely dependent on the transcription factor GATA3 [54]. GATA3 transcripts are essential for the differentiation of Th2 cells and also relatively abundantly expressed in eosinophils [55]. In terms of the Th2 response, BCE significantly decreased the expression of transcription factor GATA3 in both BALF and lung homogenate (Fig. 4). These results were also consistent with decreasing of Th2-related cytokines in BALF and lung homogenate as well as eosinophil numbers.

In terms of the Th17 response, ROR γ t is a key transcription factor for the differentiation and effector function of the proinflammatory Th17 cells [57]. Overexpressing ROR γ t induces IL-17 production [58]. Our finding showed that ROR γ t expression and IL-17A levels in BALF and lung homogenate increased in the asthmatic mice. However, ROR γ t was downregulated in BALF and IL-17A was downregulated in lung homogenate with BCE treatment. Recently, Th17 cells have been associated with Th2-predominant allergic diseases [32]. IL-17 was demonstrated to upregulate the release of IL-1 β , IL-6, TNF- α by human macrophages [59]. These results correlated with our finding in decreasing of Th17-related cytokines (IL-17A) along with decreasing of IL-1 β , IL-6, TNF- α in BCE treatment.

IgE is a key target for the development of anti-asthma strategies, is one of the most important factors in the progression of allergic reactions [60]. Th2 cytokines play important role on the allergic responses via the regulation on IgE and other antigen-specific-antibody in serum. In this study, the increase of Th2-related cytokines including IL-4 and IL-5 could have effect on the levels of total IgE, anti-OVA IgE, anti-OVA IgG1 and anti-OVA IgG2a in the asthmatic mice model. Previous result shown that OVA-specific IgG in rat-sera, such as IgG1 and IgG2a increase on day 21 after OVA inhalation in asthmatic models induced by OVA [61]. Our finding shown that BCE treatment downregulated the over-expression of serum IgE, anti-OVA IgE, anti-OVA IgG1 and anti-OVA IgG2a in OVA-induced mice, indicating BCE oral treatment can inhibit the development of inflammation reactions. These findings are also consistent with the upregulation of Th1-related cytokine and the downregulation of Th2-related cytokines in BALF and lung homogenate.

NF- κ B has a central role in regulating the expression of inflammatory genes in airway cells. NF κ B is commonly found in the cytoplasm of various cells in its non-active state [62]. I κ B α inhibits NF κ B by masking the nuclear localization signals of NF κ B proteins and keeping them sequestered in an inactive state in the cytoplasm [25]. In this study, NF κ B p65 and I κ B α protein in cytoplasm was investigated, the expression of NF κ B p65 in the BCE treated mice was lower than OVA-induced mice. On the contrary, I κ B α levels in the BCE treatment was significantly higher than those compared with asthmatic mice. On the other hand, NF κ B and I κ B α phosphorylation play a critical role in contributing to inflammatory diseases. Thus, we evaluated the protective role of BCE administration via examination of NF κ B activation and I κ B α degradation. The results showed that OVA stimulation induced the phosphorylation of I κ B α and NF κ B p65 was elevated in cytoplasm. However, NF κ B activation and I κ B α degradation were significantly inhibited by BCE oral treatment. Previous study showed that several of these gene products, such as IL-1 β and TNF- α , also directly activate the NF κ B pathway to amplify and increase the inflammatory response [63]. Furthermore, when NF κ B is activated, resulting in the nuclear localization of NF κ B p65, that secretion of

inflammatory cytokines, such as IL-1 β , IL-4, IL-5, IL-6 and TNF- α , is increased [64–66]. This is consistent with our results; in the OVA-induced model, the expression of IL-1 β , IL-4, IL-5, IL-6 and TNF- α along with NF κ B p65, p-NF κ B p65, p-I κ B α was strongly increased and remarkably decreased after BCE treatment in BALF and lung homogenate. This means that the expression of NF κ B p65, p-NF κ B p65 and p-I κ B α was positively correlated to IL-1 β , IL-4, IL-5, IL-6 and TNF- α levels in the asthmatic mice model and BCE-treated mice, suggesting that BCE can inhibit the expression of proinflammatory cytokines via blocking the phosphorylation of I κ B α and NF κ B p65.

For safety testing of BCE at dose of 200 mg/kg, in the Saline/BCE group, lung tissue has not been found the histopathological changes compared to control group. Furthermore, there was no significant difference between saline/BCE group and control group in the Th1, Th2 and Th17 responses; NF κ B p65, I κ B α , p-NF κ B p65 and p-I κ B α in both BALF and lung homogenate as well as total IgE, anti-OVA IgE, anti-OVA IgG1 and anti-OVA IgG2a levels. This indicated that the dose administration of BCE at 200 mg/kg had no toxic effects on the treated mice and appropriate for allergic treatment. Also, BCE had no cytotoxicity of HMC-1 *in vitro* test.

To evaluate the mechanism by which BCE ameliorates OVA-induced allergic asthmatic symptoms, we investigated whether BCE regulates the functional differentiation of naive CD4⁺ T cells. As a result, the treatment of BCE dose-dependently increased the secretion of INF- γ which is associated with the Th1 cell differentiation and the secretion of IL-10 which is related to the Tr1 cell differentiation. However, IL-17A, IL-4 and TGF- β were not changed by BCE treatment. Therefore, these results demonstrated that the treatment of BCE could induce the functional differentiation of naive CD4⁺ T cells forward to Th1 and Tr1 through producing INF- γ and IL-10, resulting in attenuation of allergic asthma. Based on these results, we used the whole ethanol extract of *B. Chinense*, which is likely to contain the above-mentioned bioactive components including Saikosaponin-A [22]. Also other multiple compounds from BCE may have anti-inflammatory and anti-allergic activities. In the future, we are trying to purify other compounds from BCE to find additional biologically active compounds.

5. Conclusion

Our study demonstrated that oral administration of BCE significantly attenuated airway inflammation in OVA-induced mice model via suppressing inflammation cells such as eosinophils, goblet cells; reducing of mucus hypersecretion; ameliorating collagen fibers in lung tissue; regulating the balance of Th1, Th2, Th17-related cytokines; inhibiting expression of serum total IgE, anti-OVA IgE, anti-OVA IgG1 and anti-OVA IgG2a levels. In addition, BCE downregulated the Th2 proinflammatory cytokines through the inactivation of NF κ B p65 phosphorylation and I κ B α degradation. Also, BCE could induce the functional differentiation of naive CD4⁺ T cells forward to Th1 and Tr1, which is thought to prevent inflammatory mechanisms of Th2 cells. These results suggest that BCE may be provide a promising targets for allergic asthma treatment by inhibiting Th2/Th17 cytokines production via inactivation of NF κ B pathway.

Author disclosure statement

These authors declare no conflict of interest.

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