



Anti-Inflammatory Effect of 3-Bromo-4,5-Dihydroxybenzaldehyde, a Component of *Polysiphonia morrowii*, *In Vivo* and *In Vitro*

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3-Bromo-4,5-dihydroxybenzaldehyde (BDB) is a natural bromophenol compound that is most commonly isolated from red algae. The present study was designed to investigate the anti-inflammatory properties of BDB on atopic dermatitis (AD) in mice induced by 2,4-dinitrochlorobenzene (DNCB) and on lipopolysaccharide (LPS)-stimulated murine macrophages. BDB treatment (100 mg/kg) resulted in suppression of the development of AD symptoms compared with the control treatment (induction-only), as demonstrated by reduced immunoglobulin E levels in serum, smaller lymph nodes with reduced thickness and length, a decrease in ear edema, and reduced levels of inflammatory cell infiltration in the ears. In RAW 264.7 murine macrophages, BDB (12.5, 25, 50, and 100 μ M) suppressed the production of interleukin-6, a pro-inflammatory cytokine, in a dose-dependent manner. BDB also had an inhibitory effect on the phosphorylation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and signal transducer and activator of transcription 1 (STAT1; Tyr 701), two major signaling molecules involved in cellular inflammation. Taken together, the results show that BDB treatment alleviates inflammatory responses in an atopic dermatitis mouse model and RAW 264.7 macrophages. These results suggest that BDB may be a useful therapeutic strategy for treating conditions involving allergic inflammation such as atopic dermatitis.

Key words: 3-Bromo-4,5-dihydroxybenzaldehyde, Inflammation, Atopic dermatitis, 2,4-Dinitrochlorobenzene, Immunoglobulin E, Interleukin-6

INTRODUCTION

Allergic skin inflammation, such as atopic dermatitis, is characterized by skin barrier dysfunction, edema, and infiltration of various types of inflammatory cells. Atopic dermatitis (AD) is a chronic skin disease associated with skin hyper-reactivity, including edema and itching, which affects

approximately 10~20% of children and 1~3% of adults worldwide (1). This systemic disorder is characterized by thickening of the papillary dermis, parakeratosis, skin barrier dysfunction, epidermal hyperplasia, severe skin dehydration, and T-cell hyper-proliferation. The skin lesions in AD patients are characterized by the proliferation and infiltration of various inflammatory cells, especially eosinophils, mast cells, basophils, and T-cells (2,3).

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Abbreviations: BDB, 3-Bromo-4,5-dihydroxybenzaldehyde; AD, Atopic dermatitis; DNCB, 2,4-Dinitrochlorobenzene; IgE, Immunoglobulin E; NF- κ B, Nuclear factor kappa-light-chain-enhancer of activated B cells; STAT, Signal transducer and activator of transcription 1.

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Mast cells play an important role in inflammatory and anaphylactic reactions. Cross-linking with Fc3RI, the high-affinity surface receptor for immunoglobulin E (IgE), activates mast cells, causing them to secrete a variety of inflammatory mediators, such as cytokines, serotonin, and histamine (4,5).

T-cells are a type of lymphocytes that play a key role in cell-mediated immunity. These cells can differentiate into one of several subtypes, such as T-helper 1 (Th1), Th2, Th9, Th17, and regulatory T-cells, all of which express different types of cytokines to facilitate different immune responses. Chronic AD is characterized by Th1-mediated immune

responses. Th1 cells, which are regulated by the transcription factor T-box expressed in T-cells (T-bet), express large amounts of interferon-gamma (IFN- γ), which can suppress the development of Th2 cells (6,7). Acute AD is characterized by Th2-mediated immune responses. Th2 cells, which are regulated by GATA-binding protein-3 (GATA-3), secrete a variety of cytokines, such as interleukin-4 (IL-4), IL-5, IL-6, IL-9, and IL-13, which regulate the development of Th1 cells (8,9).

IL-6 is well known as a multifunctional cytokine and is a soluble mediator of inflammatory responses, immune responses, and a variety of biological functions. Previous studies have shown that IL-6 is involved in various diseases, including rheumatoid arthritis, and that IL-6 amplifies Toll-like receptor (TLR)-induced cytokine and chemokine production during inflammatory responses, such as those associated with rheumatic inflammatory disease. IL-6 is also produced by many cell types and is regulated by various receptors, including cytokine receptors, antigen receptors, and TLRs (10-16). Upon stimulation with inflammatory agents, LPS binding initiates cell activation via kinase cascades, such as those involving the TLR member TLR4 and downstream signaling mechanisms. LPS-mediated TLR4 signaling activates the nuclear factor- κ B (NF- κ B) complex and signal transducer and activator of transcription 1 (STAT1) in macrophages, monocytes, astrocytes, and other cells. These signaling pathways are responsible for TLR-mediated induction of inflammatory cytokines and changes in gene expression, including expression of IL-6 (17-25).

Recent studies have investigated the active components of several natural marine resources to identify novel pharmaceutical applications and nutritional value (26-29). Several previous studies examined the anti-allergenic effects of 3-bromo-4,5-dihydroxybenzaldehyde (BDB) isolated from marine red algae, including *Polysiphonia morrowii* and *P. urceolata*. BDB protects human keratinocytes against UVB-induced oxidative stress by scavenging ROS and has potential as an antiviral therapeutic agent in fish (30,31). The beneficial effects of BDB have been described in many studies; however, the mechanism by which it modulates the immune system and inflammatory reactions is poorly understood. Therefore, the aim of the present study was to examine whether BDB suppresses a variety of inflamma-

tory responses. The ability of BDB to modulate inflammatory reactions was investigated using a mouse model of AD and murine macrophages.

MATERIALS AND METHODS

Reagents. BDB (purity > 95%) was purchased from Matrix Scientific (Colombia, SC, USA). 3-Bromo-4,5-dihydroxybenzaldehyde (BDB) was purchased from Matrix Scientific (Colombia SC) and the purity of BDB is > 95 percent (Fig. 1). 2,4-Dinitrochlorobenzene (DNCB) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). LPS (*E. coli* 0111:B4) was purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Invitrogen-GIBCO (Grand Island, NY, USA). Mouse IL-6 Duoset enzyme-linked immunosorbent assay (ELISA) kits were obtained from R&D Systems (St. Louis, MO, USA). Phospho-STAT1 antibodies (Tyr701 and Ser727) and NF- κ B p65 and phospho-p65 antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA); NF- κ B p50 (E-10) antibody was purchased from Santa Biotechnology, Inc. (Santa Cruz, CA, USA); anti-STAT1 was purchased from Becton Dickinson (San Diego, CA, USA); and anti- β -actin was obtained from Sigma-Aldrich Chemical Co. All other reagents were of reagent grade.

Experimental animals. BALB/c mice (female, 7-week-old) were purchased from Orient Bio (Orient Bio Inc., Seongnam, Korea) and maintained under pathogen-free conditions in the animal facility of Jeju National University. All animal experiments were approved by the Jeju National University Animal Care and Use Committee.

DNCB application to induce AD. Mice were divided into four groups (n = 5 per group): saline (normal), AD (induction-only), AD + Hydrocort cream, and AD + BDB. Mice were sensitized by spreading 1% DNCB or vehicle (an acetone:olive oil mixture [3:1 vol/vol]) on their abdomen as the first sensitization (day 0). On day 7, mice were challenged again by applying 0.3% DNCB to the ears on every other day for 30 days. Starting on day 19, the mice were treated with hydrocort cream (Green Cross, Korea) containing 2 mg/g hydrocortisone valerate and BDB (2.5 mg in acetone; 30 μ L/ear), which was applied on their ears every other day (32,33). The mice were killed on day 38, and the mandibular lymph nodes (LNs) were photographed to record morphological changes.

Macroscopic edema and histology. In the experimental AD mouse model, DNCB stimulation elicited ear edema, and ear thickness was measured using a digital thickness gauge (Mitutoyo, Kawasaki, Japan). Ear tissues were fixed in 10% formalin, and then embedded in paraffin. Paraffin sections

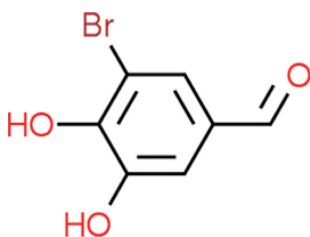


Fig. 1. Chemical structure of 3-bromo-4,5-dihydroxybenzaldehyde (BDB) from *Polysiphonia urceolata*.

(3 μm each) were stained with hematoxylin and eosin (H&E).

Cell culture and cell viability. Murine macrophage RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were cultured in DMEM supplemented with 10% (vol/vol) FBS and 100 U/mL penicillin-streptomycin (GIBCO, Grand Island, NY, USA). The cells (1.5×10^5 cells/mL) were pre-incubated with DMEM/10% FBS for 18 hr. Cell viability was then determined from the culture supernatants of cells treated with LPS (1 $\mu\text{g}/\text{mL}$) in the absence or presence of BDB (100 μM in dimethyl sulfoxide; 12.5, 25, 50, 100 μM /group) for 24 hr. The cells were maintained in 5% CO_2 humidified atmosphere at 37°C in an incubator. Cell viability was determined using EZ-CyTox (WST-1) assays (its-BIO, Korea) following the manufacturer's protocol. The light absorbance of each well was quantified using a VersaMax ELISA microplate reader (Molecular Devices, CA, USA) at 450 nm.

ELISA. The production of IL-6 proteins was measured using ELISA kits following the manufacturer's protocol. The optical density was measured using a VersaMax ELISA microplate reader at 450 nm.

Western blot analysis. The cells were washed twice with ice-cold phosphate-buffered saline (PBS). The cells were lysed in the lysis buffer (iNtRON Biotechnology, Gyeonggi, Korea) for 30 min on ice. Protein in the supernatant was quantified using the Bradford assay (Bio-rad, Hercules, CA, USA). Aliquots of the lysates were separated on a NuPAGE 4–12% bis-Tris gel (Invitrogen, Carlsbad, CA, USA). The proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane using an iBlot gel transfer device (Invitrogen). Membranes were blocked with 5% non-fat skim milk diluted in Tween 20-tris buffered saline (TTBS), and the membranes were incubated with primary antibodies diluted in 1% bovine serum albumin (BSA)-TTBS buffer at 4°C overnight. After washing, the membrane was incubated with secondary HRP-linked anti-rabbit or anti-mouse IgG respectively for 90 min at room temperature. After washing again, immunoreactive proteins were detected with WEST-ZOL (plus) western blot detection system (iNtRON Biotechnology) following the manufacturer's instructions.

Statistical analysis. Quantity One version 4.2.1 and Image-Pro plus version 4.5 software (Bio-rad) were used to transform images into numerical values. Student's t-test and two-way analysis of variance (ANOVA) were used to determine the statistical significance of differences between experimental and control groups. Data are shown as mean \pm standard deviation. The p -values less than 0.05 indicated statistical significance.

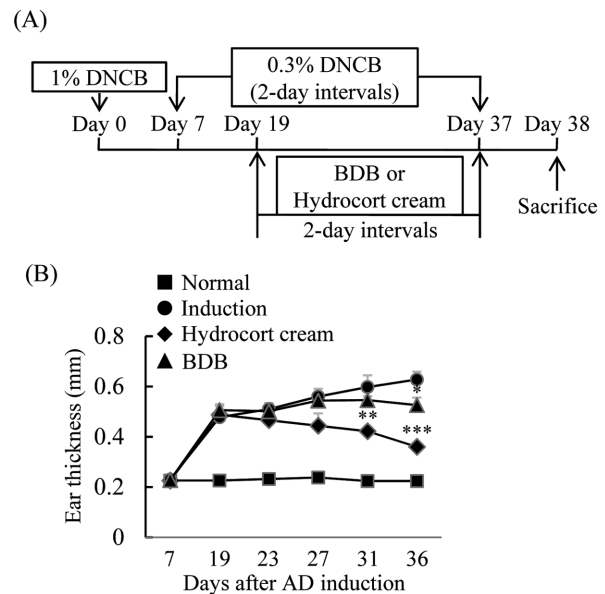


Fig. 2. 3-Bromo-4,5-dihydroxybenzaldehyde (BDB) suppresses the expression of serum IgE. (A) Mice were sensitized by applying 1% 2,4-dinitrochlorobenzene (DNCB) or vehicle on their abdomen (day 0). On day 7, mice were challenged again by applying 0.3% DNCB to the ears every other day for up to 30 days. Starting on day 19, the mice were treated with hydrocort cream and BDB (100 mg/kg) on their ears every other day. The mice were killed on day 38. (B) After killing, IgE levels in mouse serum were measured by ELISA. AD: atopic dermatitis. Data are representative of 5 mice per group. Values represent the mean \pm SD. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared to mice stimulated with DNCB alone (induction group).

RESULTS

BDB decreases serum IgE levels. To experimentally induce AD, mice were subjected to an initial sensitization with 1% DNCB applied to the abdomen. They were then re-sensitized by applying 0.3% DNCB to the ears every other day for up to 30 days. Starting on day 19, the mice were treated with hydrocortisone cream and BDB (100 mg/kg) on the ears every other day. All mice were killed on day 38 (Fig. 2A). IgE is a crucial therapeutic target for AD because it is the major activator of mast cells, which release histamine, tryptase, and cytokines (34). Therefore, we measured serum IgE levels in AD mice using ELISA. The BDB-treated group had significantly decreased levels of IgE ($p < 0.05$) compared to those of the induction-only group (mice exposed to DNCB instead of BDB; Fig. 2B).

BDB suppresses the development of experimental AD. The skin lesions associated with AD are characterized by infiltration of various inflammatory cells (35). Therefore, we measured whether BDB treatment alleviates the degree of inflammatory cell infiltration in the ears of mice with

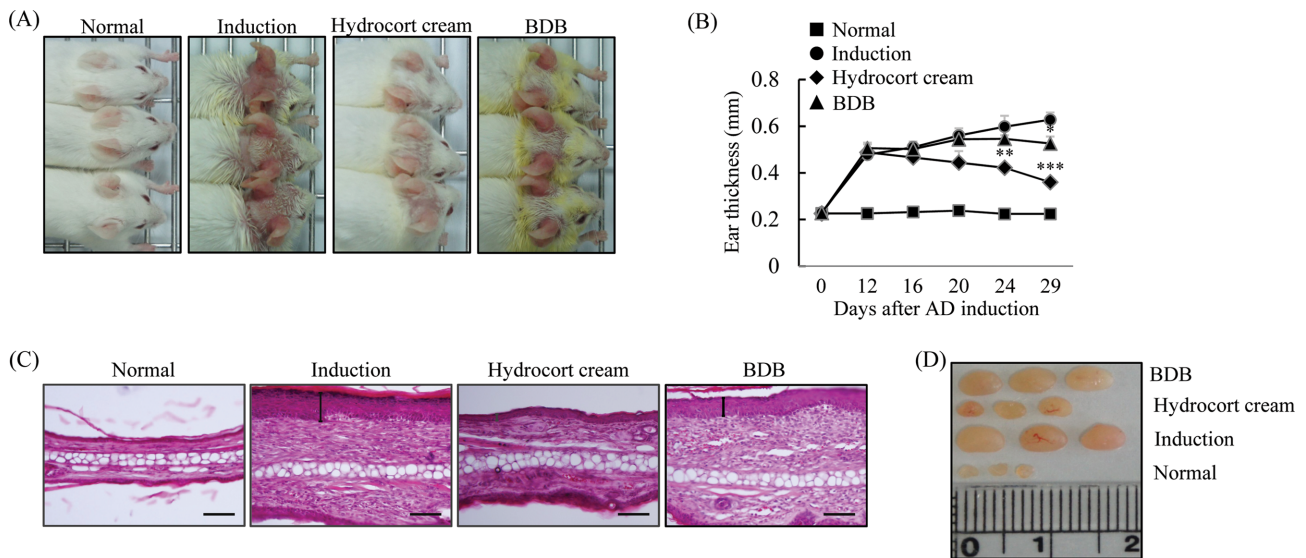


Fig. 3. 3-Bromo-4,5-dihydroxybenzaldehyde (BDB) suppresses experimental atopic dermatitis (AD). (A) Macroscopic views of the ears and (B) ear thickness measured on days 7, 19, 23, 27, 31, and 36. (C) Paraffin-embedded sections of ear tissue were stained with hematoxylin and eosin. (D) The lymph nodes (LNs) were photographed to record morphologic changes. $N = 5$ mice per group. Scale bar = 0.1 mm. Values represent the mean \pm SD. * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ compared to mice stimulated with 2,4-dinitrochlorobenzene (DNCB) alone (induction-only group).

experimental AD. We also tested skin swelling as a measure of AD progression. We found that cutaneous edema in BDB-treated mice reduced on day 36 compared with that of the induction-only mice ($p < 0.05$; Fig. 3A, 3B). We next examined the effect of BDB on the infiltration of inflammatory cells by H&E staining of ear tissue sections. Epidermal thickness and the degree of inflammatory cell infiltration were significantly lower in the BDB-treated group than in the induction-only group (Fig. 3C). LNs have a crucial role in cell-mediated immunity by regulating the activity of T and B-cells (36). Therefore, we examined morphologic changes in the LNs of AD mice. The LNs in mice in the induction-only group were swollen, whereas those in the BDB-treated mice were smaller (Fig. 3D).

BDB inhibits LPS-induced IL-6 production in RAW 264.7 cells. To assess the anti-inflammatory effect of BDB, we examined the inhibitory activity of BDB on LPS-induced IL-6 production, an important pro-inflammatory cytokine (10,11), in RAW 264.7 cells. To confirm the effect of BDB, we simultaneously determined cell viability at various concentrations of BDB. We found that BDB was not cytotoxic at the tested concentrations (Fig. 4). Next, we assayed the inhibitory effect of BDB on LPS-induced IL-6 production. As shown in Fig. 4, BDB inhibited the production of IL-6 in a dose-dependent manner at concentrations of 25, 50, and 100 μ M.

BDB inhibits NF- κ B phosphorylation in LPS-stimulated RAW 264.7 cells. LPS presentation on macrophages may mediate the activation of the NF- κ B pathway

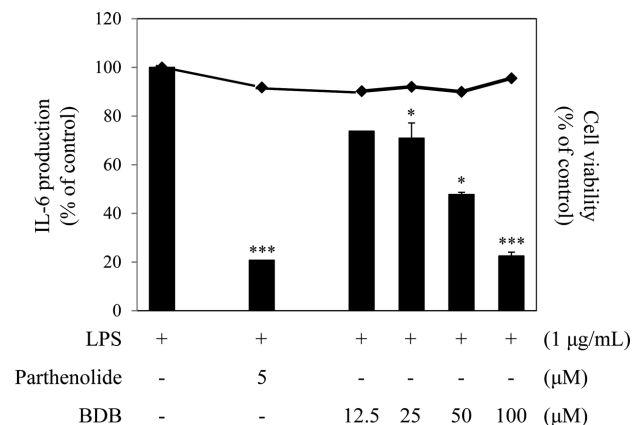


Fig. 4. Effects of 3-bromo-4,5-dihydroxybenzaldehyde (BDB) on cell viability and interleukin-6 (IL-6) production in RAW 264.7 murine macrophages. Cells (1.5×10^5 cells/mL) were pre-incubated with DMEM/10% FBS for 18 hr, after which IL-6 production was analyzed from the supernatants of cells stimulated with lipopolysaccharide (LPS; 1 μ g/mL) in the absence or presence of BDB for 24 hr (12.5 to 100 μ M). IL-6 production was determined by ELISA. The measurement of IL-6 was obtained in triplicate. Error bars indicate \pm SD. * $p < 0.05$, *** $p < 0.001$ compared with LPS-positive control.

(21,22,37), leading to the generation of pro-inflammatory cytokines, including IL-6 and TNF- α . We determined the phosphorylation status of NF- κ B with and without BDB treatment by western blot analysis. BDB inhibited LPS-induced phosphorylation of NF- κ B at concentrations of 50

and 100 μM (Fig. 5). These results suggest that the inhibitory effect of BDB on the production of IL-6 occurs through inhibition of NF- κB phosphorylation.

BDB inhibits STAT1 phosphorylation in LPS-stimulated RAW 264.7 cells. The STATs are a family of nuclear proteins that mediate the action of several cytokines, including ILs and IFNs. Suppressor of cytokine signaling 1 (SOCS1) is a negative-feedback regulator of Janus kinase (JAK)-STAT signaling (25,38). Therefore, we examined the effect of BDB on the activation of STAT1 and SOCS in LPS-treated RAW 264.7 cells. LPS induced STAT1 phosphorylation at both Tyr701 and Ser727 residues, and had no effect on SOCS activation. BDB (100 μM) strongly inhibited the phosphorylation of STAT1 at Tyr701 (Fig. 6).

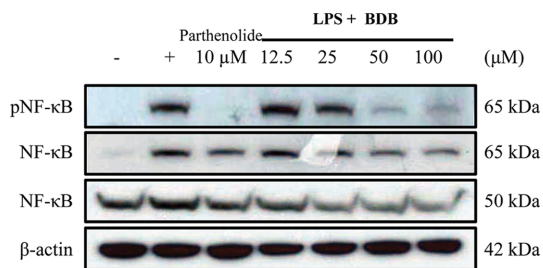


Fig. 5. Effects of 3-bromo-4,5-dihydroxybenzaldehyde (BDB) on NF- κB phosphorylation in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. RAW 264.7 cells (5.0×10^5 cells/mL) were pre-incubated for 18 hr. Cells were pretreated with BDB (12.5 to 100 μM) for 120 min, and then stimulated with LPS (1 $\mu\text{g}/\text{mL}$) for 5 min. Expression of NF- κB phosphorylation (p65 and p50) and β -actin was determined by western blotting of whole cell lysates with the indicated antibodies. The data are representative of two independent experiments.

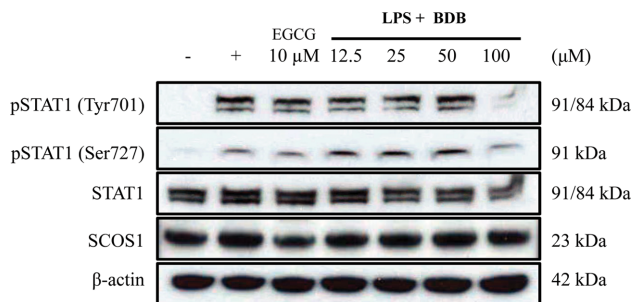


Fig. 6. Effects of 3-bromo-4,5-dihydroxybenzaldehyde (BDB) on the phosphorylation of STAT1 at Tyr701 and Ser727 residues in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. RAW 264.7 cells (5.0×10^5 cells/mL) were pre-incubated for 18 hr. Cells were pretreated with BDB (12.5 to 100 μM) for 120 min and stimulated with LPS (1 $\mu\text{g}/\text{mL}$) for 120 min. Expression of phosphorylated STAT1 at Tyr701 and Ser727, STAT1, SOCS1, and β -actin was determined by western blotting of whole cell lysates with the indicated antibodies.

DISCUSSION

Marine plants have been the focus of recent studies in the search for active components for use in the pharmaceutical and functional food industries (26-29). BDB is a compound mainly isolated from marine red algae, including *P. morrowii* and *P. urceolata*.

In this study, we demonstrated that BDB, a natural bromophenol compound, inhibits various inflammatory responses in mice and in murine macrophages. To induce experimental AD, mice were sensitized by applying DNCB to the abdomen, and then re-sensitized by applying DNCB to the ears every other day. IgE is an important therapeutic target for AD, as it is the major activator of mast cells (34). Furthermore, IgE signaling through Fc ϵ RI can release histamine, tryptase, and cytokines from mast cells, which leads to potent induction of edema and itching (39,40). Therefore, we tested whether BDB can reduce serum IgE hyperproduction and cutaneous edema. BDB treatment reduced the levels of IgE and edema compared with induction only. H&E staining of ear tissue showed that BDB treatment alleviated the infiltration of inflammatory cells compared with induction alone. The LNs play an important role in regulating immune responses and contain a variety of immune cells. In addition, enlarged LNs are a key indicator of abnormalities in the immune system (41). We investigated the morphology of the LNs in an experimental AD model. The induction-only group had markedly enlarged LNs, whereas the LNs of BDB-treated mice were smaller. It is not yet clear why BDB has such strong anti-inflammatory effects. Thus, we are currently trying to identify the interrelationships between BDB and regulatory T cells.

To investigate the anti-inflammatory effect and the mechanisms of action of BDB *in vitro*, we stimulated RAW 264.7 cells with LPS, an inducer of the innate immune response. LPS binds to the myeloid differentiation factor-2 (MD-2)/TLR4 complex, activates a downstream signaling pathway, and releases various inflammatory mediators, including IL-6, inducible nitric oxide synthase (iNOS), and TNF- α . We examined the inhibitory effect of BDB on LPS-induced IL-6 production. BDB inhibited the production of IL-6 in a dose-dependent manner (Fig. 4). Several previous reports have shown that NF- κB and STAT1 act as important upstream signaling molecules for the production of IL-6 in LPS-stimulated monocytes or macrophages. In a previous study, we reported that N-tosyl-L-phenylalanine chloromethyl ketone, a specific inhibitor of NF- κB signaling, affects IL-6 production (23). NF- κB inhibition attenuates TLR4 expression in LPS-treated THP1 monocytes (22). Choi *et al.* (42) reported that azithromycin attenuated LPS-induced IL-6 production through inhibition of NF- κB , STAT1, and STAT3 in murine macrophages. Luu *et al.* (43) also reported that TLR4 induces phosphorylation of STAT1 at Ser727, and direct activation of STAT1 by TLR signaling is crucial

for the involvement of STAT1 in TLR-induced inflammation. In the present study, BDB strongly inhibited LPS-induced phosphorylation of NF- κ B and STAT1 (Tyr701). Similarly, Cho *et al.* reported that myricetin suppressed IL-6 and other pro-inflammatory mediators by activating NF- κ B and STAT1 in LPS-stimulated RAW 264.7 murine macrophages (44). Therefore, our results show that the inhibitory effect of BDB on the production of IL-6 occurs through inhibition of the phosphorylation of NF- κ B and STAT1.

In AD, macrophages are known to accumulate in acutely and chronically inflamed skin. Macrophages also exert pro-inflammatory functions, such as antigen-presenting phagocytosis and the production of inflammatory cytokines and chemokines that stimulate new capillary growth, collagen synthesis, and fibrosis (45,46). Monocytes are important macrophage precursor cells that are involved in AD-related skin inflammation. Monocytes invade the dermis and differentiate into macrophages, which can also act as antigen-presenting cells (47,48) Although the inter-relationships between BDB and regulatory T-cells are of interest to the field and are currently under investigation, the focus of the present study was to show that BDB alleviates inflammatory responses in macrophages and atopic dermatitis mice.

In conclusion, our results reveal that BDB, a component of *P. morrowii*, alleviated inflammatory responses in an atopic dermatitis mouse model and in LPS-stimulated RAW 264.7 murine macrophages. These results suggest that treatment with BDB may be a useful therapeutic strategy for allergic inflammation such as AD.

ACKNOWLEDGMENTS

This study was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIP) (2016R1A2B4014050).

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

Received July 19, 2017; Revised August 21, 2017; Accepted September 14, 2017

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