

# Benzoxazole derivatives suppress lipopolysaccharide-induced mast cell activation

KYUNG-AH CHO<sup>1</sup>, MINHWA PARK<sup>1</sup>, YU-HEE KIM<sup>1</sup>, HEA-YOUNG PARK CHOO<sup>2</sup> and KYUNG HO LEE<sup>3</sup>

<sup>1</sup>Department of Microbiology, School of Medicine, Ewha Womans University, Seoul 07985; <sup>2</sup>College of Pharmacy and Graduate School of Pharmaceutical Sciences, Ewha Womans University, Seoul 03760; <sup>3</sup>Department of Dermatology, Bucheon St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Bucheon-si 14647, Republic of Korea

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**Abstract.** Mast cells are central regulators of allergic inflammation that function by releasing various proallergic inflammatory mediators, including histamine, eicosanoids and proinflammatory cytokines. Occasionally, bacterial infections may initiate or worsen allergic inflammation. A number of studies have indicated that activation of lipoxygenase in mast cells positive regulates allergic inflammatory responses by generating leukotrienes and proinflammatory cytokines. In the present study, the effects of benzoxazole derivatives on the lipopolysaccharide (LPS)-induced expression of proinflammatory cytokines, production of histamine and surface expression of co-stimulatory molecules on bone marrow-derived mast cells (BMMCs) were studied. The benzoxazole derivatives significantly reduced the expression of interleukin (IL)-1 $\beta$ , IL-6, IL-13, tumor necrosis factor- $\alpha$ , perilipin (PLIN) 2, and PLIN3 in BMMCs treated with LPS. Furthermore, histamine production was suppressed in BMMCs treated with LPS, or treated with phorbol-12-myristate-13-acetate/ionomycin. Benzoxazole derivatives marginally affected the surface expression of cluster of differentiation (CD)80 and CD86 on BMMCs in the presence of LPS, although LPS alone did not increase the expression of those proteins. Therefore, benzoxazole derivatives inhibited the secretion of proinflammatory cytokines in mast cells and may be potential candidate anti-allergic agents to suppress mast cell activation.

## Introduction

Mast cells (MCs) are secretory cells that are strategically located at host-environment interfaces, including the skin,

lungs and mucosal surfaces. MCs release granules following activation and are part of the innate immune system, being involved in the first line of defense against pathogens, including bacteria and parasites (1). Activated MCs produce a broad spectrum of inflammatory cytokines, chemokines, lipid compounds and vasoactive amines, all of which are involved in immune responses (2).

Lipopolysaccharide (LPS) released from Gram negative bacteria is of great significance to mast cells, for the innate immune response and, additionally, as it is able to aggravate an existing inflammatory state in allergic airway inflammation via T helper cell 2 (Th2) cytokine production (3). Toll-like receptor (TLR) 4 is a receptor for LPS that serves an important role in inflammation and is a risk factor for MC-mediated asthma. MCs directly respond to LPS by producing inflammatory cytokines including interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-13 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (4). These mediators exert acute inflammatory effects through endothelial cells and leukocytes. Activation of TLR4 by LPS on MCs leads to the generation of Th2 cytokines (e.g. IL-4, IL-5 and IL-13), which enhances the allergic inflammation that may occur in asthma (5,6). In addition to allergic airway inflammation, MCs trigger inflammatory responses in the skin when exposed to LPS. For example, LPS-stimulated MCs serve an important role in edema formation by releasing factors that increase vascular permeability, including histamine and leukotrienes (LTs) (7).

Lipoxygenases (LOXs) are a family of enzymes that catalyze the incorporation of oxygen into unsaturated fatty acids (8). LOXs are expressed in immune and epithelial cells and they are involved in a variety of conditions, including inflammatory skin disease and allergic asthma, wherein they generate LTs and proinflammatory mediators (9,10). MCs were suggested to be one of the principal cell types responsible for the action of 5-/12-LOX during the progression of asthmatic inflammation due to their production of LTs and IL-13 (11,12). Considering that LTs and IL-13 are potent proinflammatory mediators, LOX may serve a role in allergic inflammation mediated by MCs.

Previously, benzoxazoles have been reported to act as 5-lipoxygenase or IL-6 inhibitors (13-16). To investigate the effects of benzoxazole derivatives on the LPS-induced inflammatory response in MCs, the expression of proinflammatory

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*Correspondence to:* Dr Kyung Ho Lee, Department of Dermatology, Bucheon St. Mary's Hospital, College of Medicine, The Catholic University of Korea, 327 Sosa-ro, Wonmi-gu, Bucheon-si 14647, Gyeonggi-do, Republic of Korea  
E-mail: beauty4u@catholic.ac.kr

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cytokines, the levels of histamine secreted from MCs under LPS stimulation, and the cell-surface localization of co-stimulatory molecule in the presence or absence of benzoxazole derivatives was assessed.

## Materials and methods

**Synthesis of benzoxazole derivatives.** A total of four derivatives with the benzoxazole moiety were synthesized starting from the commercially available 2-amino-4-nitro (or 4-methyl) phenol (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and substituted-phenyl isothiocyanate (Sigma-Aldrich; Merck KGaA; Fig. 1). The thiourea 2 was cyclized to benzoxazole anti-itch agent provided by Park [(ICP)-1, 2, 5, and 13, Laboratory of Dr Park, Ewha Womans University, Seoul, Korea] by oxidation with 5 eq  $\text{KO}_2$  (Alfa Aesar, Haverhill, MA, USA). This procedure to prepare the benzoxazoles had an 80-90% yield (13-16). For the preparation of ICP-1 and 5, the nitro group was reduced to amino group with catalytic hydrogenation using 5% Pd/C (Sigma Aldrich; Merck KGaA) as catalyst.

**Generation of bone marrow-derived MCs (BMMCs) and in vitro stimulation.** For bone marrow cell isolation, 5 female C57BL/6 mice were purchased at 10 weeks of age (weight, 20-22 g) from OrientBio, Inc., (Emsung, Korea). All animals were maintained at 21-23°C with 51-54% humidity under pathogen-free conditions on a 12-h light/dark cycle with free access to food and water. All procedures were approved by the Ewha Womans University College of Medicine Animal Care and Use Committee (Seoul, Korea; ESM 15-0309). Bone marrow cells were isolated from the femurs of female C57BL/6 mice and grown in Iscove's Modified Dulbecco's Medium (IMEM; Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS; Welgene), 50  $\mu\text{M}$   $\beta$ -mercaptoethanol, 2 mM L-glutamine, 100  $\mu\text{g}/\text{ml}$  streptomycin and 100 U/ml penicillin, and 10 ng/ml IL-13 (PeproTech, Inc., Rocky Hill, NJ, USA). Non-adherent cells were transferred to fresh medium every 2-3 days to remove adherent macrophages and fibroblasts. After 5 weeks, the cells were identified as MCs using flow cytometric analyses of high affinity immunoglobulin  $\epsilon$  receptor subunit  $\gamma$  (Fc $\epsilon$ RI) and cluster of differentiation (CD)117 (c-kit) expression and toluidine blue staining. For toluidine blue staining cytospin slides were stained for 3 min at room temperature. The image was captured by light microscope with magnification, x1,000 (Olympus BX50, Olympus Corporation; Tokyo, Japan). MCs at 5-6 weeks following initiation of culture were used for the experiments. For treatment with LPS, BMMCs were seeded at a density of  $6 \times 10^5$  cells/well in 96-well plates and stimulated with LPS (Sigma-Aldrich; Merck KGaA) at a concentration of 1  $\mu\text{g}/\text{ml}$ . A total of four types of benzoxazole derivatives (ICP-1, 2, 5 and 13) were separately added to the BMMCs at a concentration of 10  $\mu\text{M}$  each to observe the effects on LPS-induced BMMCs.

**Flow cytometry.** The surface expression of Fc $\epsilon$ RI and CD117 on BMMCs following 5 weeks of culture was detected by staining with Alexa 488-conjugated anti-Fc $\epsilon$ RI antibody (Clone MAR-1; cat. no. 134329; BioLegend, Inc., San Diego,

CA, USA) and PerCP-conjugated anti-CD117 antibody (Clone 2B8; cat. no. 105821; BioLegend, Inc.) at a concentration of 5 mg/ml on ice for 30 min followed by washing using PBS containing 0.5% FBS and 0.1% sodium azide. The surface expression of CD80 and CD86 were additionally analyzed using fluorescein isothiocyanate-conjugated anti-CD80 antibody (Clone 16-10A1; cat. no. 553768; BD Biosciences, San Jose, CA, USA) and phycoerythrin-conjugated anti-CD86 antibody (Clone GL1; cat. no. 553692; BD Biosciences) at a concentration of 5 mg/ml on ice for 30 min. The level of nonspecific staining was performed using the corresponding isotype antibody as described above; Armenian Hamster IgG (cat. no. 400923; Biolegend, Inc.) for anti-Fc $\epsilon$ RI antibody, Rat IgG2b,  $\kappa$  for anti-CD117 antibody (cat. no. 400629; Biolegend, Inc.). Each sample was quantified on a NovoCyt<sup>TM</sup> Flow Cytometer (ACEA Biosciences, San Diego, CA, USA) and the data were analyzed using FlowJo software (version 8.7, FlowJo LLC, Ashland, OR, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from BMMCs with TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Complementary DNA was synthesized by incubation at 30°C for 10 min, at 42°C for 20 min and at 99°C for 5 min using the First Strand cDNA Synthesis kit (Toyobo Life Science, Osaka, Japan). Amplification was performed in duplicate by 40 cycles of 15 sec denaturation step at 95°C and a 1 min amplification and signal acquisition step at 60°C using StepOnePlus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) using SYBR<sup>®</sup> green (Kapa Biosystems, Inc., Wilmington, MA, USA).

All gene expression values were normalized to the expression of the *GAPDH* gene using the following primers: 5'-GACCTTGTGTCTCCGCTTAT-3' forward, and 5'-CAA CCGCAATTTGTGGCTC-3' reverse for mouse perilipin 2 (*Plin2*; 157 bp); 5'-ATGTCTAGCAATGGTACAGATGC-3' forward, and 5'-CGTGGAAGTACTGATAAGAGGCAGG-3' reverse for mouse *Plin3* (114 bp); 5'-GCAACTGTTCTGAA CTCAACT-3' forward, and 5'-ATCTTTTGGGGTCCGTCA ACT-3' reverse for mouse *Iilb* (89 bp); 5'-CTGCAAGAG ACTTCCATCCAG-3' forward, and 5'-AGTGGTATAGAC AGGTCTGTTGG-3' reverse for mouse *Iil6* (131 bp); 5'-CCT GGCTCTTGCTTGCCCTT-3' forward, and 5'-GGTCTTGTG TGATGTTGCTCA-3' reverse for mouse *Iil3* (116 bp); 5'-CCT GTAGCCCACGTCGTAG-3' forward, and 5'-GGGAGTAGA CAAGGTACAACCC-3' reverse for mouse *Tnfa* (148 bp); and 5'-GGTAAAGTGGATATTGTTGCCATCAATG-3' forward, and 5'-GGAGGGATCTCGCTCCTGGAAGATGGTG-3' reverse for mouse *GAPDH* (173 bp). The relative fold expression and changes were calculated  $2^{-\Delta\Delta C_q}$  method (17).

**Immunoblotting.** Cell culture supernatant from non-treated BMMCs, BMMCs stimulated with LPS (1  $\mu\text{g}/\text{ml}$ ), and BMMCs stimulated with LPS in the presence of the benzoxazole derivatives (ICP-1, 2, 5, and/or 13) were collected and prepared in a sample buffer [0.5 M Tris/HCl (pH 6.8), 25% glycerol, 10% SDS, 500 mM DTT, 1% bromophenol blue; Sigma-Aldrich; Merck KGaA].

Equal amounts (40  $\mu\text{l}$ ) cell lysate were resolved by 10% SDS-PAGE and transferred to Immobilon-P polyvinylidene

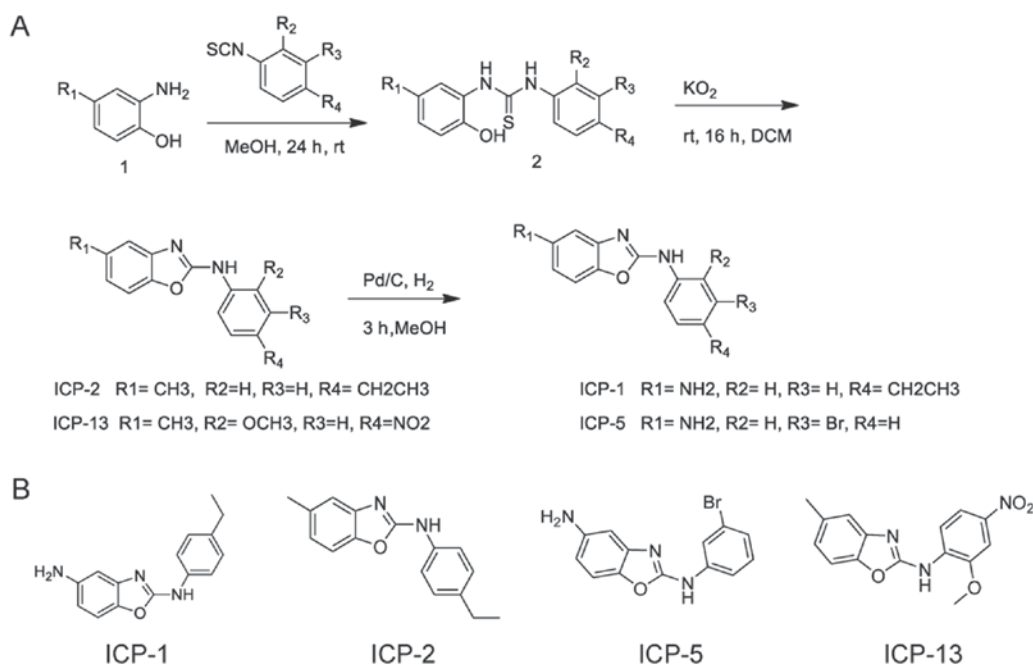


Figure 1. Synthesis of benzoxazole derivatives. (A) Synthetic procedure for benzoxazole derivatives. (B) Structures of ICP-1, ICP-2, ICP-5, and ICP-13. ICP, anti-Itch agent by Park; DCM, dichloromethane; rt, room temperature; Pd/C, palladium on carbon catalyst.

fluoride membranes (EMD Millipore, Billerica, MA, USA). Membranes were blocked with 5% skim milk in TBS containing 0.1% Tween-20 (TBS-T) solution for 1 h at room temperature and were subsequently incubated with primary antibodies overnight at 4°C. The following primary antibodies were used: IL-1 $\beta$  (1:200, diluted in 3% bovine serum albumin (BSA; Bovogen Biologicals, Pty. Ltd., East Keilor, Victoria, Australia) containing TBST; cat. no. sc-7884; Santa Cruz Biotechnology, Inc., Dallas, TX, USA); and  $\beta$ -actin [1:3,000, diluted in 3% BSA containing TBST; cat. no. A1978; mouse; Sigma-Aldrich; Merck KGaA]. The membranes were washed 3 times for 10 min in TBST and incubated with anti-rabbit (cat. no. BR170-6515; Bio-Rad Laboratories, Inc.) or anti-mouse (cat. no. BR170-6516; Bio-Rad Laboratories, Inc.) horseradish peroxidase-conjugated secondary antibodies (1:3,000, diluted in TBST) for 1 h at room temperature. Following incubation, membranes were washed 3 times for 10 min in TBST and developed using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce; Thermo Fisher Scientific, Inc.). Images were obtained using ImageQuant LAS 4000 (GE Healthcare Life Sciences, Little Chalfont, UK). The band pixel densities of the precursor form of IL-1 $\beta$  and the active form of IL-1 $\beta$  were divided by the pixel densities of the corresponding  $\beta$ -actin bands for quantitation using UN-SCAN-IT-gel 6.1 software (Silk Scientific Inc., Orem, UT, USA).

**ELISA.** For histamine measurements, 80 nM phorbol-12-myristate-13-acetate (PMA) (Ebioscience; Thermo Fisher Scientific, Inc.) and ionomycin (1  $\mu$ M; Ebioscience; Thermo Fisher Scientific, Inc.) were added in the presence of the benzoxazole derivatives, and the cultures were maintained for 24 h at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. Cell culture supernatants were collected from non-treated BMMCs and BMMCs stimulated with LPS (1  $\mu$ g/ml) in the presence of the benzoxazole derivatives (ICP-1, 2, 5, and/or 13). The levels

of secreted histamine were determined using a histamine ELISA kit in accordance with the manufacturer's recommended protocol (Abnova; cat. no. KA2589; Taipei, Taiwan).

**Statistical analysis.** Data are presented as the mean  $\pm$  standard error of the mean (n=3). Statistical significance was determined by one-way analysis of variance (ANOVA) in conjunction with Dunnett's post hoc test, or two-way ANOVA in conjunction with Sidak's post-hoc test compared with the normal group using GraphPad Prism, version 7 software (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

## Results

**Generation of BMMCs from mice.** BMMCs were generated from C57BL/6 mice. As exhibited in Fig. 2A, low expression of the mast cell markers Fc $\epsilon$ RI and c-kit was observed prior to BMMC differentiation on the cell surface of fresh mouse bone marrow cells. However, following 5 weeks of culture in the presence of IL-3, >90% of the cells expressed Fc $\epsilon$ RI and c-kit (Fig. 2B). In addition, toluidine blue staining demonstrated differentiated MCs that contained granules in the cytoplasm (Fig. 2C).

**Benzoxazole derivatives attenuate the LPS-mediated gene expression of *Il1b*, *Il6*, *Il13*, *Tnfa*, *Plin2* and *Plin3* in BMMCs.** A number of studies have indicated that MCs produce proinflammatory cytokines, including IL-1 $\beta$ , IL-6, IL-13 and TNF- $\alpha$  when exposed to LPS (4,18-20). LOX activation positively regulates inflammatory responses, including LPS-induced inflammation (21,22) and LPS is a direct inducer of 5-LOX in immune cells, including monocytes (23). As it was reported that benzoxazoles are 5-LOX and IL-6 inhibitors (13), benzoxazole derivatives were synthesized and LPS-stimulated



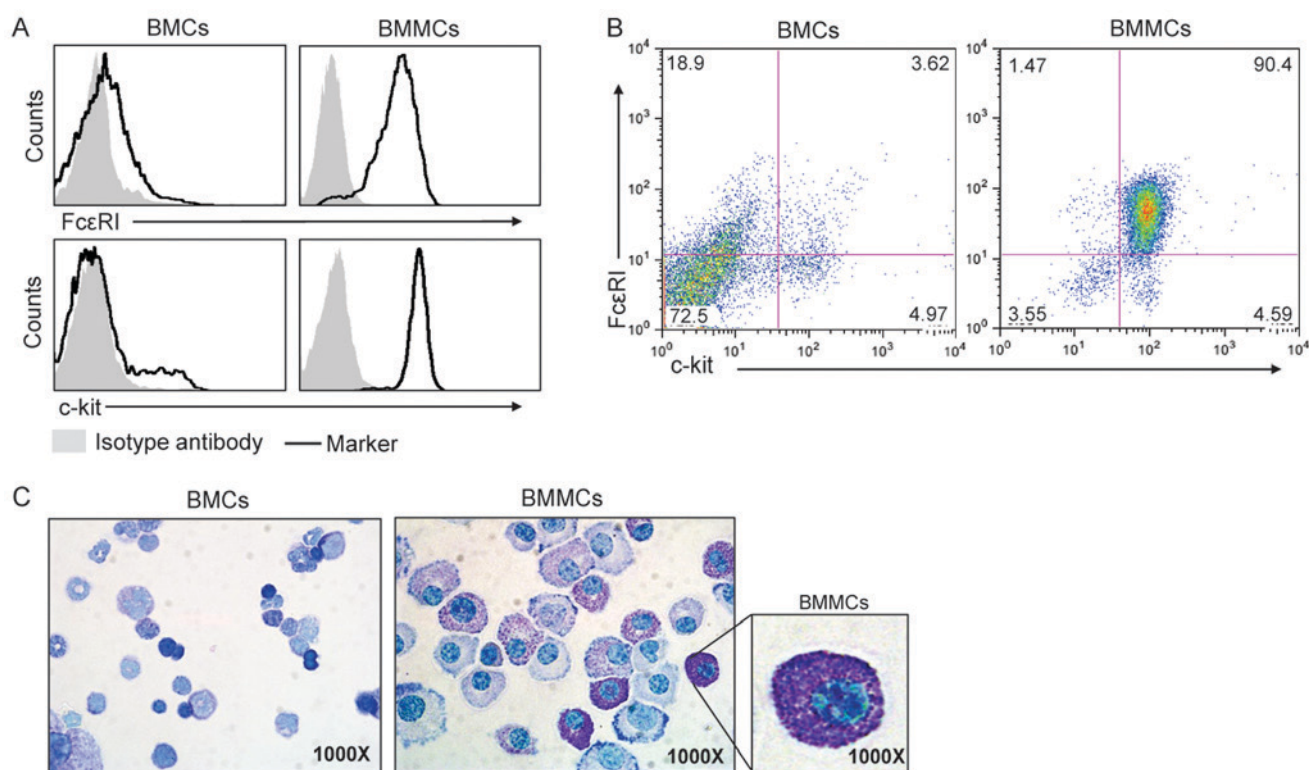


Figure 2. Generation of BMMCs. (A) The surface expression of FcεRI and c-kit on BMCs and BMMCs are exhibited as representative histograms using flow cytometry. (B) The surface expression of FcεRI and c-kit on BM cells and BMMCs are exhibited as representative dot plots from the flow cytometry. (C) BM cells (left) and BMMC (right) were distinguished by toluidine blue stain. BMMCs, bone marrow-derived mast cells; c-kit, cluster of differentiation 17; BMCs, bone marrow cells; FcεRI, high affinity immunoglobulin ε receptor subunit γ.

BMMCs were treated with the inhibitors for 24 h. Benzoxazole derivatives had an inhibitory effect on the expression of the *Il1b*, *Il6*, *Il13* and *Tnfa* genes in BMMCs. In particular, a higher fold increase in *Il1b* and *Tnfa* expression, compared with *Il6* and *Il13*, following LPS stimulation was observed. A total of three types of cytokines (*Il1b*, *Il13* and *Tnfa*) were significantly downregulated by all four types of benzoxazole derivatives (ICP-1, 2, 5 and 13;  $P < 0.05$ ; Fig. 3A).

In addition, lipid droplets (LD) from MCs are involved in cell signaling and the generation of biologically active lipid mediators (e.g. prostaglandin D<sub>2</sub>, leukotriene B<sub>4</sub> and C<sub>4</sub>) evoked by inflammatory and infectious conditions (24,25). The most well-known LD proteins are members of the PLIN family. Out of the five PLINs (PLIN1, PLIN2, PLIN3, PLIN4 and PLIN5), PLIN2 and PLIN3 are expressed in developing and mature human MCs (26), and the constitutive expression of the *Plin2* and *Plin3* genes in BMMCs was confirmed in the present study (data not shown). A modest, although significant, inhibition and induction of *Plin2* and *Plin3*, respectively by ICP-1 and 5, following treatment with LPS was observed ( $P < 0.05$ ; Fig. 3A).

As the activation of MCs via the NACHT, LRR and PYD domains-containing protein 3 (NLRP3) inflammasome may be linked to IL-1β production (27), the protein expression levels of IL-1β in the cell culture supernatant were tested. Following 24 h of treatment with LPS in the presence or absence of benzoxazole derivatives, the secreted active form of IL-1β was most abundant in the BMMC supernatant; however, benzoxazole derivatives suppressed the secretion of the protein. Instead, a precursor form of IL-1β (pro-IL-1β)

accumulated, particularly under treatment with the benzoxazole derivatives ICP-2 and ICP-5 (Fig. 3B).

*Benzoxazole derivatives suppress histamine production in BMMCs.* LPS-induced inflammation was demonstrated to increase histamine secretion, possibly by directly stimulating histamine-forming enzyme (histidine decarboxylase) activity (28). Therefore, it was hypothesized that MC activation by LPS may be followed by histamine secretion. Furthermore, the suppressive effect of the benzoxazole derivatives was additionally investigated. The secretion of histamine from BMMCs was triggered by PMA and ionomycin as a positive control. As exhibited in Fig. 4A, LPS stimulation alone for 24 h promoted histamine production in BMMCs. Although the increases in histamine production with ICP-1, 2 and 5 were not statistically significant, benzoxazole derivative ICP-13 significantly inhibited the increase ( $P < 0.05$ ). The BMMCs treated with PMA and ionomycin generated significantly increased histamine levels, which was suppressed by all the inhibitors ( $P < 0.05$ ; Fig. 4B).

*Benzoxazole derivatives do not affect the surface expression of co-stimulatory molecules on BMMCs.* In addition to their contribution to host defense via innate mechanisms, MCs additionally promote adaptive immune responses through interactions with CD4<sup>+</sup> and CD8<sup>+</sup> T cells (29). It was demonstrated that MCs may be primed to express functional class II major histocompatibility complex molecules and co-stimulatory molecules, and may serve as antigen presenting cells for CD4<sup>+</sup> T lymphocytes, including helper T cells (30). Therefore mouse BMMCs were examined by flow cytometry for the

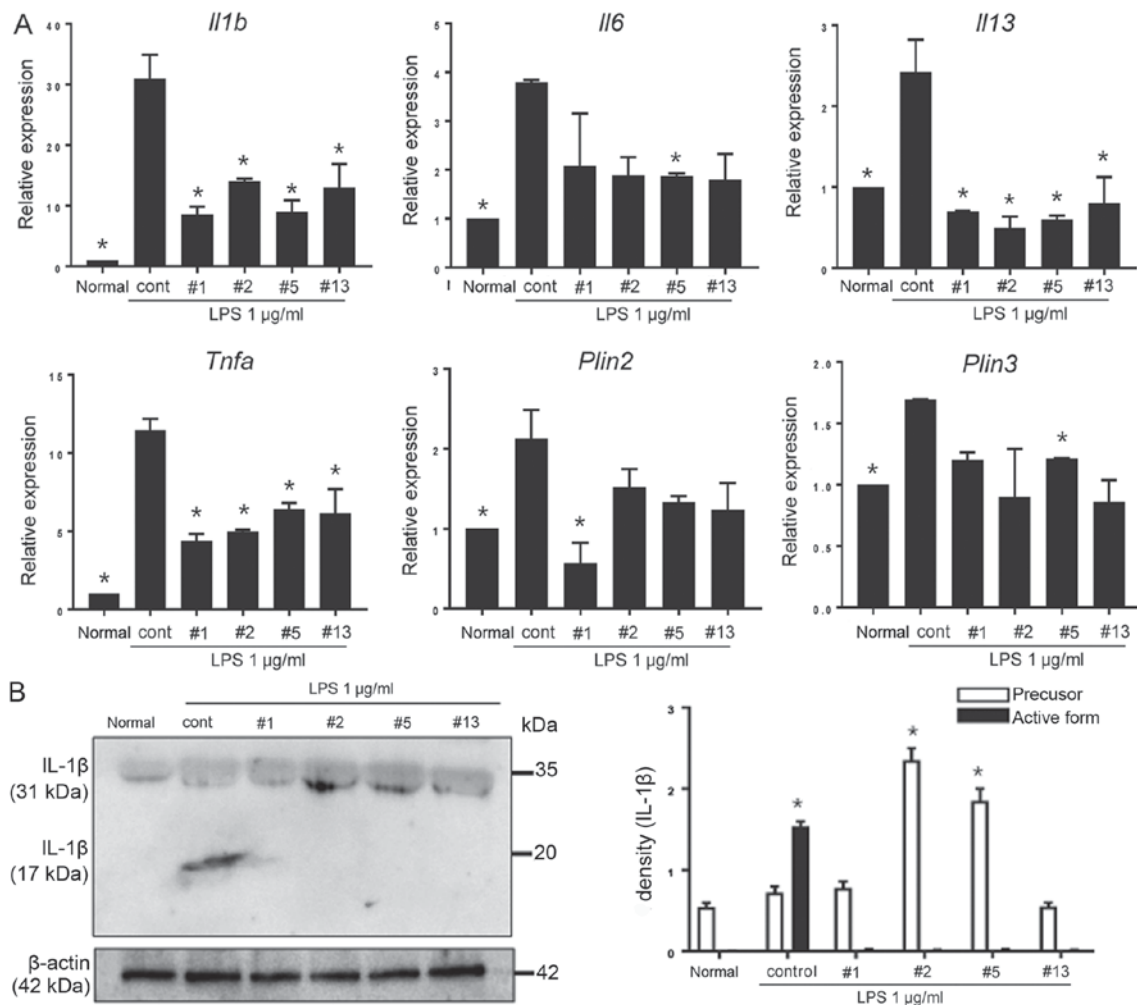


Figure 3. Gene expression profiles in BMMCs. (A) Non-treated normal BMMCs, BMMCs cultured with LPS, and BMMCs cultured with LPS in the presence of benzoxazole derivatives (10 µM each) were collected, and the mRNA expression of *Il1b*, *Il6*, *Il13*, *Tnfa*, *Plin2* and *Plin3* was analyzed by reverse transcription-quantitative polymerase chain reaction. The benzoxazole derivatives ICP-1, ICP-2, ICP-5 and ICP-13 are indicated as #1, #2, #5 and #13, respectively. Data are presented as the mean ± SEM. \*P<0.05 vs. control. (B) The level of secreted IL-1β from non-treated normal BMMCs, and BMMCs cultured with LPS stimulation and each benzoxazole derivative (10 µM each) were compared using the cell culture supernatant. The precursor form of IL-1β was indicated by the 31 kDa band and the active form of IL-1β was indicated by the 17 kDa band (upper). The pixel densities of each band were divided by the corresponding β-actin bands for normalization (lower). Data are presented as the mean ± SEM. \*P<0.05 vs. normal. SEM, standard error of the mean; BMMCs, bone marrow derived mast cells; LPS, lipopolysaccharide; *Il*/IL, interleukin; *Tnfa*, tumor necrosis factor-α; cont, control; *Plin*, perilipin.

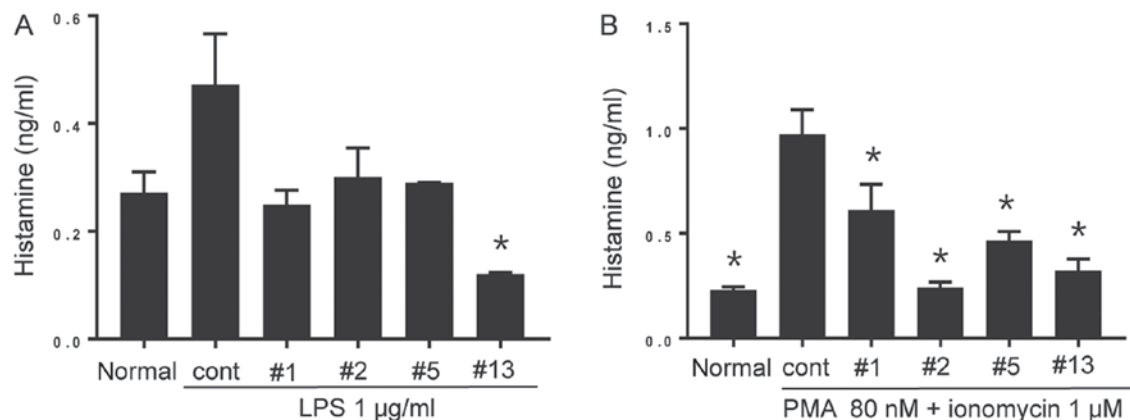


Figure 4. Histamine production of BMMCs. (A) The cell culture supernatant from non-treated normal BMMCs, BMMCs treated with LPS, and BMMCs treated with LPS in the presence of each of the benzoxazole derivatives (10 µM each) for 24 h was collected and the amount of secreted histamine was measured by ELISA. The benzoxazole derivatives ICP-1, ICP-2, ICP-5 and ICP-13 are indicated as #1, #2, #5 and #13, respectively. Data are presented as the mean ± SEM. \*P<0.05 vs. cont. (B) Cell culture supernatants from non-treated normal BMMCs, BMMCs treated with PMA and ionomycin, BMMCs treated with PMA and ionomycin in the presence of each benzoxazole derivative (10 µM each) for 24 h were collected, and the amount of secreted histamine was measured by ELISA. Data are presented as the mean ± SEM. \*P<0.05 vs. cont. SEM, standard error of the mean; BMMCs, bone marrow derived mast cells; LPS, lipopolysaccharide; PMA, phorbol-12-myristate-13-acetate; cont, control; ICP, anti-Itch agent by Park.

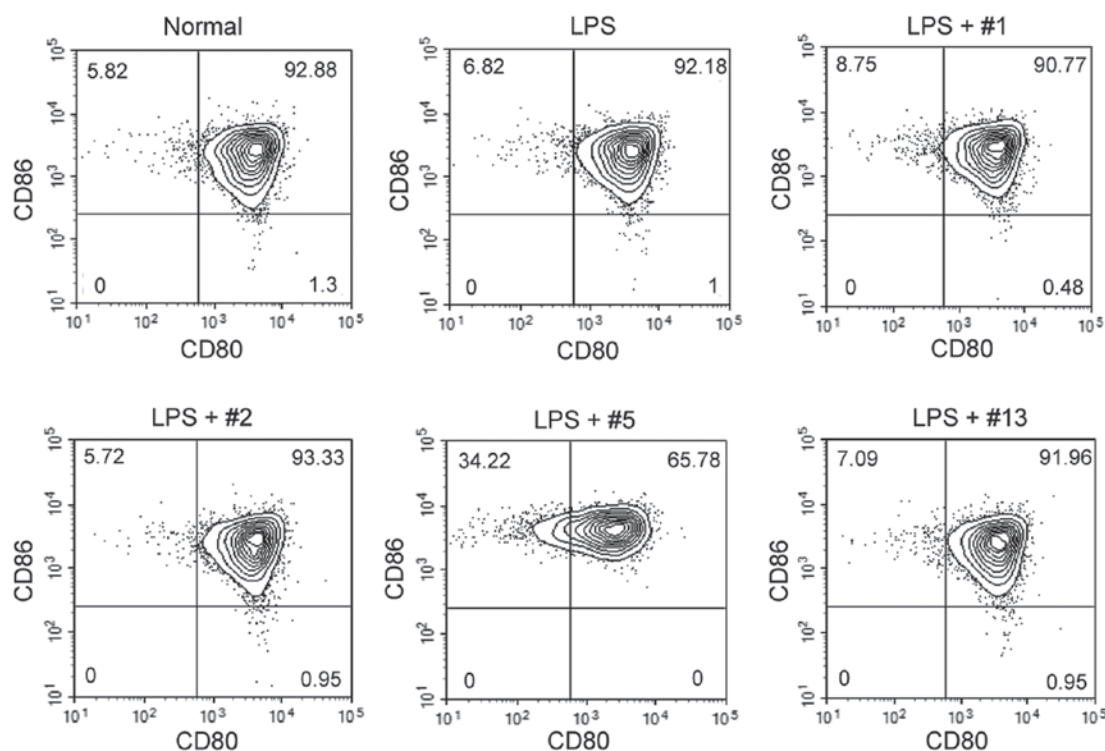


Figure 5. Surface expression of co-stimulatory molecules in BMMCs. Non-treated normal BMMCs, and BMMCs cultured in the presence of LPS (1  $\mu$ g/ml) and each benzoxazole derivative (10  $\mu$ M each) for 24 h were collected, and the surface expression of CD80 and CD86 was analyzed by flow cytometry. The benzoxazole derivatives ICP-1, ICP-2, ICP-5 and ICP-13 are indicated as #1, #2, #5 and #13, respectively. BMMCs, bone marrow derived mast cells; LPS, lipopolysaccharide; CD, cluster of differentiation, ICP, anti-Itch agent by Park.

surface expression of members of the B7 protein family, CD80 and CD86, which are potent co-stimulatory proteins for T cell activation. BMMCs highly expressed both CD80 and CD86 even in the absence of stimulation, and their expression was not altered following 24 h of treatment with LPS. Similarly, simultaneous treatment with benzoxazole derivatives and LPS did not affect the expression level of these surface proteins. However, ICP-5 downregulated the percentages of CD80-positive BMMCs (Fig. 5), by modifying CD80 expression rather than CD86 expression.

## Discussion

Previously, the authors reported that benzoxazoles act as 5-LOX or IL-6 inhibitors (13-16). As part of the authors' ongoing study on the use of benzoxazoles as anti-inflammatory agents, benzoxazole derivatives were synthesized and their effects on MCs were determined. In the present study, benzoxazole derivatives were demonstrated to attenuate LPS-induced BMMC activation. Specifically, it was demonstrated that the LPS-mediated gene expression of *Il1b*, *Il6*, *Il13*, *Tnfa*, *Plin2* and *Plin3* in BMMCs was downregulated by four benzoxazole derivatives. Furthermore, histamine secretion from BMMCs exposed to LPS or PMA/ionomycin was suppressed in the presence of benzoxazole derivatives. However, the inhibition of the surface expression of co-stimulatory molecules, including CD80 and CD86, was less evident.

MCs are particularly abundant in the skin, airway and gut mucosa, where they are strategically located for optimal

interaction with the environment. MCs are heterogeneous in terms of morphology, receptor expression, mediator content and reactivity towards various stimulants, according to their localization in the body and among animal species. Therefore, cellular pathways acting on MCs to produce key mediators involved in inflammation, regardless of the heterogeneity of cells, may be efficient targets to modulate MC responses (31). MCs have been demonstrated to express the majority of TLRs, and to respond to their agonists by secreting cytokines, chemokines and lipid mediators. There are similarities and differences between the cytokines produced upon stimulation with different TLRs. For example, TLR2 and TLR4 trigger the production of the Th2 cytokines IL-4, IL-5, IL-13, IL-6 and TNF- $\alpha$  from MCs via the c-Jun N-terminal kinase and p38 pathways (4). Additionally, the benzoxazole derivatives, particularly ICP-1, used in the present study were reported to suppress the production of interferon- $\gamma$ , IL-17, IL-4, IL-5 or IL-13 via suppression of the IL-6-signal transducer and activator of transcription 3 signaling pathway (16).

LPS stimulation of BMMCs induced the expression of *Il1b* mRNA in addition to the induction of the active form of IL-1 $\beta$ , which was suppressed by the benzoxazole derivatives. Notably, treatment with benzoxazole derivatives failed to induce expression of the cleaved form of active IL-1 $\beta$ . This result suggested that the benzoxazole derivatives used in the present study may inhibit the activation of the NLRP3 signaling pathway directly or indirectly, via a decrease in TNF- $\alpha$  (32) or PLIN (33) from BMMCs. Activation of the NLRP3 inflammasome requires two signals: Signal 1 from microbial components (e.g. LPS) or endogenous cytokines (e.g. TNF- $\alpha$ ), and signal 2 from

adenosine triphosphate or bacterial toxin stimulation of the NLRP3 inflammasome. Signal 1 leads to the upregulation of NLRP3 in addition to pro-IL-1 $\beta$  expression via nuclear factor (NF)- $\kappa$ B activation through TLR4. Signal 2 leads to the activation of the NLRP3 inflammasome followed by activation of caspase 1, which cleaves pro-IL-1 $\beta$  to the active form of IL-1 $\beta$ . Therefore, the benzoxazole derivatives that were tested possibly inhibited the NF- $\kappa$ B pathway and/or inflammasome activation, either by a direct effect on the NF- $\kappa$ B signaling pathway or an indirect effect by suppressing 5-LOX activity (13).

Inflammation is a fundamental protective response in higher eukaryotes against a variety of external stimuli, including environmental toxins, pathogens and allergens. These stimuli are encountered by immune cells including neutrophils, MCs or macrophages, which mediate the initial defense reaction. Therefore, the response of these cells upon activation is indispensable for maintaining host defenses and homeostasis. However, excessive or unresolved activation may induce pathophysiological processes resulting in the development of inflammatory disease. Inflammation is closely associated with oxidative processes and, therefore, oxidative enzymes, including 5-LOX, that are known to serve key roles in inflammation (34). 5-LOX generates a number of lipid and pro-inflammatory mediators that directly act on inflammation. Furthermore, the mediators generated by 5-LOX including LTs are involved in the activation of pro-inflammatory signal transduction pathways including the NF- $\kappa$ B signaling pathway that potentiates inflammatory status. Indeed, 5-LOX-generated pro-inflammatory products have been implicated in a number of human acute and chronic inflammatory diseases, including asthma, atherosclerosis, rheumatoid arthritis, inflammatory bowel diseases, urticaria and atopic dermatitis.

It was reported that 5-LOX enzymatic activity is inhibited by phenolic antioxidants including nordihydroguaiaretic acid and caffeic acid, suggesting a beneficial role of dietary polyphenol intake (35). Synthetic drugs that act on LOX are currently relatively limited. The 5-LOX inhibitor zileuton has been used successfully for the control of asthma. Currently, 5-LOX inhibitors are being developed by pharmaceutical companies (36). These inhibitors prevent the transportation of 5-LOX from the nucleus to the cytoplasm, leading to the suppression of 5-hydroperoxyeicosatetraenoic acid production.

In conclusion, the present study demonstrated that benzoxazole derivatives (ICP-1, 2, 5 and 13) suppressed the production of proinflammatory cytokines of BMMCs and LD proteins, including PLINs, following LPS stimulation. Therefore, agents, including benzoxazole derivatives that are able to inhibit acute inflammation resulting from the activation of MCs in allergic reactions or urticaria may be therapeutically important. Further studies are required to refine the mode of action of benzoxazole derivatives and their therapeutic effects *in vivo* to determine the most effective therapeutic strategy.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

KAC performed experiments and wrote the manuscripts. MP and YHK performed experiments and analyzed data. HP and KHL designed the experiments and wrote the manuscripts.

## Ethics approval and consent to participate

All procedures were approved by the Ewha Womans University College of Medicine Animal Care and Use Committee (Seoul, Korea; ESM 15-0309).

## Consent for publication

Not applicable.

## Competing interests

The authors declare they have no competing interests.

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