

## Suppression of IgE-mediated mast cell activation and mouse anaphylaxis via inhibition of Syk activation by 8-formyl-7-hydroxy-4-methylcoumarin, 4μ8C



Seung Taek Nam<sup>a</sup>, Young Hwan Park<sup>a</sup>, Hyun Woo Kim<sup>a</sup>, Hyuk Soon Kim<sup>a</sup>, Dajeong Lee<sup>a</sup>, Min Bum Lee<sup>a</sup>, Young Mi Kim<sup>b,\*\*</sup>, Wahn Soo Choi<sup>a,\*</sup>

<sup>a</sup> Department of Immunology, College of Medicine, Konkuk University, Chungju 27478, Republic of Korea

<sup>b</sup> College of Pharmacy, Duksung Women's University, Seoul 01369, Republic of Korea

### ARTICLE INFO

#### Article history:

Received 7 March 2017

Revised 8 July 2017

Accepted 17 July 2017

Available online 20 July 2017

#### Keywords:

8-Formyl-7-hydroxy-4-methylcoumarin

4μ8C

Mast cells

Allergy

Anaphylaxis

Lyn

Fyn

### ABSTRACT

Mast cells trigger IgE-mediated allergic reactions by releasing various allergic mediators. 8-Formyl-7-hydroxy-4-methylcoumarin, also called 4μ8C, was originally known as an inositol-requiring enzyme 1 (IRE1) suppressant, but no study has examined its relationship with mast cells and allergic diseases. Therefore, the purpose of this study was to determine whether 4μ8C is effective in suppressing allergic reactions in mast cells and in IgE-mediated allergic animal model. 4μ8C suppressed the degranulation of IgE-mediated mast cells ( $IC_{50} = 3.2 \mu M$ ) and the production of cytokines such as tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-4 (IL-4) in a dose-dependent manner. 4μ8C also suppressed passive cutaneous anaphylaxis (PCA) in mice ( $ED_{50} = 25.1 \text{ mg/kg}$ ). In an experiment on mast cell signaling pathways stimulated by antigen, the phosphorylation and activation of Syk was decreased by 4μ8C, and phosphorylation of downstream signaling molecules, such as linker for activated T cells (LAT), Akt, and the three MAP kinases, ERK, p38, and JNK, were suppressed. Mechanistic studies showed that 4μ8C inhibited the activity of Lyn and Fyn in vitro. Based on the results of those experiments, the suppressor mechanism of allergic reaction by 4μ8C involved reduced activity of Lyn and Fyn, which is pivotal in an IgE-mediated signaling pathway. In summary, for the first time, this study shows that 4μ8C inhibits Lyn and Fyn, thus suppressing allergic reaction by reducing the degranulation and the production of inflammatory cytokines. This suggests that 4μ8C can be used as a new medicinal candidate to control allergic diseases such as seasonal allergies and atopic dermatitis.

© 2017 Elsevier Inc. All rights reserved.

### 1. Introduction

Allergic diseases, such as rhinitis, asthma, food allergy, and atopic dermatitis, are increasing worldwide (Begin and Nadeau, 2014). The mast cell is known to be a critical effector cell that causes allergic diseases (Bischoff, 2007; Gregory and Brown, 2006). If antigen (Ag) binds to IgE associated with FcεRI on the cell membranes of mast cells, allergy-inducing substances (e.g., histamine, heparin, leukotriene, and

prostaglandins) that exist in the granules of the mast cell are secreted, and the formation and secretion of inflammatory cytokines, such as interleukin-(IL)-4 and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) is expedited (Galli, 1993). Immunosuppressive steroidal or antihistamine medications that inhibit the activation of histamine secreted from mast cells are generally used to treat allergic diseases (Holgate and Polosa, 2008; Simons and Simons, 2011). Antihistamine medication is a suppressor of symptoms, but repetitive administration creates tolerance toward this medication, and long-term administration can cause other side effects (Holgate and Polosa, 2008). Therefore, many researchers are conducting research to find new allergy medicines.

The signaling pathway for activation of mast cells is triggered when Ag combines with an IgE connected to the FcεRI, an IgE high affinity receptor, on mast cells. The Src family kinases, such as Lyn and Fyn, are involved in the initial signaling events. When mast cells are stimulated by Ag, the immunoreceptor tyrosine-based activation motifs (ITAMs) of FcεRI are phosphorylated by Lyn and cytosolic spleen tyrosine kinase (Syk) binds to the phosphorylated ITAMs of FcεRI (Mocsai et al., 2010). The bound Syk is activated, which then stimulates directly or

**Abbreviations:** BMMC, Bone marrow-derived mast cell; RBL, Rat basophilic leukemia; PCA, Passive cutaneous anaphylaxis; ITAM, Immunoreceptor tyrosine-based activation motif; DNP-BSA, Dinitrophenol-conjugated bovine serum albumin; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; PIPES, 1,4-Piperazinediethanesulfonic acid; Syk, Spleen tyrosine kinase; Gab2, Grb2-associated binder 2; LAT, Linker for activation of T cells; MAP kinase, Mitogen-activated protein kinases; ERK, Extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

\* Correspondence to: W.S. Choi, School of Medicine, Konkuk University, Chungju 27478, Republic of Korea.

\*\* Corresponding author.

E-mail addresses: [kym123@duksung.ac.kr](mailto:kym123@duksung.ac.kr) (Y.M. Kim), [wahnchoi@kku.ac.kr](mailto:wahnchoi@kku.ac.kr) (W.S. Choi).

indirectly the downstream signaling molecules such as linker for activated T cells (LAT), phospholipase C- $\gamma$  (PLC- $\gamma$ ), and mitogen-activated protein (MAP) kinases (Gilfillan and Rivera, 2009). The degranulation and secretion of inflammatory cytokines by the Ag-activated signaling molecules lead to allergic responses. Therefore, recently, initial signaling molecules, including Src family kinase members or Syk, have been suggested as target proteins for allergic diseases (Bradding, 2008).

8-Formyl-7-hydroxy-4-methylcoumarin, generally called 4 $\mu$ 8C, has been known to suppress the ribonuclease activity of IRE1, which is activated by endoplasmic reticulum stress, by directly combining with its active site (Cross et al., 2012; Ma et al., 2016). It was reported that 4 $\mu$ 8C inhibited the production of IL-4, IL-5, and IL-13, which are critical for allergic responses, by suppressing the activity of IRE1 in CD4<sup>+</sup> T cells (Kemp et al., 2013). 4 $\mu$ 8C was also reported to inhibit inflammatory responses induced by the activation of toll-like receptor on macrophages (Lubamba et al., 2015; Qiu et al., 2013). However, its effect on mast cells or allergic responses has not yet been examined. Therefore, to determine the effect of 4 $\mu$ 8C on the activation of mast cells and allergic responses in this study, *in vitro* and *in vivo* experiments were performed. As a result, we show that activation of mast cells and allergic reactions by Ag was inhibited by suppressing the activation of Syk and Syk-mediated signaling pathways.

## 2. Materials and methods

### 2.1. Reagents

8-Formyl-7-hydroxy-4-methylcoumarin (4 $\mu$ 8C) and PP2 were purchased from Calbiochem (La Jolla, CA). Monoclonal dinitrophenol (DNP)-specific IgE, DNP-human serum albumin (HSA), Evans blue, cetirizine, and toluidine blue were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies, such as phospho-Syk, phospho-LAT, phospho-Akt, phospho-ERK1/2, phospho-p38, phospho-JNK, and Actin, were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies, such as Syk and LAT, were from Santa Cruz Biotechnology (Santa Cruz, CA). The culture media used for cell culture were from GIBCO/Life Technologies, Inc. (Rockville, MD).

### 2.2. Animal

The mice used in the experiment were 5- to 6-week old male BALB/c mice obtained from Orient Bio, Inc. (Gyeonggi-do, Korea). The mice were used to prepare bone marrow-derived mast cells (BMMC) and to perform passive cutaneous anaphylaxis (PCA) experiments. All experiments with mice were conducted according to institutional guidelines after receiving approval from Konkuk University Institutional Animal Care Committee (IACUC).

### 2.3. Preparation of mast cells and cell culture

Rat basophilic leukemia (RBL)-2H3 cells were purchased from the American Type Culture Collection (ATCC), and were cultured in minimal essential medium (MEM) that contained 4 mM L-glutamine, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 15% fetal bovine serum (FBS). For BMBCs, bone marrow cells were collected from mouse femurs, cultured in Roswell Park Memorial Institute (RPMI) 1640 medium that contained 4 mM L-glutamine, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 25 mM HEPES, 10% FBS, and 10 ng/ml IL-3. The cells were used as BMBCs for experiments after 4 to 6 weeks of culturing.

### 2.4. Measurement of $\beta$ -hexosaminidase release in RBL-2H3 cells and BMBCs

After RBL-2H3 cells were dispensed in a 24-well plate ( $1.8 \times 10^5$  cells/well), they were cultured in MEM containing 20 ng/ml DNP-

specific IgE for 12 h. After washing the cells twice with 1,4-piperazinediethanesulfonic acid (PIPES) buffer, also called S-buffer (25 mM PIPES, pH 7.2, 119 mM NaCl, 5 mM KCl, 0.4 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 5.6 mM glucose, and 0.1% fatty acid-free fraction V bovine serum albumin (BSA)), cells were treated for 30 min with 4 $\mu$ 8C or PP2 at varying concentrations before incubating with 25 ng/ml of Ag (DNP-BSA) for 15 min. After stimulating with Ag, the supernatant and cell lysate were allowed to react with 1 mM *p*-nitrophenyl-*N*-acetyl- $\beta$ -D-glucosaminide at 37 °C for 1 h. Absorbance was then measured at 405 nm with a microplate reader. The experiments with BMBCs utilized a Tyrode buffer solution (20 mM HEPES, pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5.6 mM glucose, and 0.1% BSA). Degranulation of mast cells was expressed as the ratio of  $\beta$ -hexosaminidase released in the culture medium to the total amount of  $\beta$ -hexosaminidase present in the culture medium and cell lysates.

### 2.5. Measurement of cell viability

RBL-2H3 cells ( $2 \times 10^4$  cells/well) were cultured in a 96-well plate for 12 h and subsequently treated with 4 $\mu$ 8C for 4 h. Cell viability was measured with a Cell Counting Kit-8 (CKK-8) (Dojindo Laboratories, Kumamoto, Japan). The experiment was performed according to the manufacturer's protocol.

### 2.6. Reverse transcriptase-polymerase chain reaction

Total RNA was extracted from RBL-2H3 cells using an Easy-spin™ Total RNA Extraction Kit (iNtRON Biotechnology, Inc.). For reverse transcription, a SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA) was used. The PCR was performed for 30 cycles at 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 60 s. The primers were: rat TNF- $\alpha$  forward 5'-CACCACGCT CTTCTGTCTACTGAAC-3'; rat TNF- $\alpha$  reverse: 5'-CCGGACTCCGTGATGTCTAAGTACT-3'; rat IL-4 forward 5'-ACC TTGCTGCACCTGTTC-3'; rat IL-4 reverse 5'-T TGTGAGCGTGACTCAT TC-3'; rat GAPDH forward 5'-GTGGAGTCTACTGGCGTCTTC-3'; and rat GAPDH reverse 5'-CCAAGGCTGTGGCAAGGTCA-3'.

### 2.7. Measurement of secreted cytokines by ELISA

IgE-sensitized RBL-2H3 cells ( $5 \times 10^5$  cells/well) were stimulated with 25 ng/ml Ag for 3 h, with or without 4 $\mu$ 8C or PP2. The amount of TNF- $\alpha$  and IL-4 in the cultured media were determined using ELISA kits, according to the manufacturer's protocol (BD Biosciences, San Jose, Calif.).

### 2.8. Western blot analysis

RBL-2H3 cells ( $1 \times 10^6$  cells/well) were sensitized with 20 ng/ml DNP-IgE for 12 h and washed twice with fresh culture medium. After pretreating with 4 $\mu$ 8C at varying concentrations for 30 min, the cells were stimulated with 25 ng/ml Ag for 7 min. The reaction was then stopped on ice. After washing with cold phosphate buffered saline twice, the cells were lysed in 100  $\mu$ l lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 60 mM octyl  $\beta$ -glucopyranoside, 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM *p*-nitrophenyl phosphate, 0.7 mg/ml pepstatin, and protease-inhibitor cocktail). The cell lysates were centrifuged at 15,000  $\times g$  for 5 min. The supernatants were mixed with a 3 $\times$  Laemmli buffer and denatured by heating for 5 min at 95 °C. Protein was separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. After incubating with primary antibody in TBS-T buffer that contained 5% BSA and then washing with TBS-T, the membrane was incubated with a secondary antibody tagged with horseradish peroxidase (HRP). After washing with TBS-T, the blot was detected with an ECL detection kit.

## 2.9. Immunoprecipitation and protein tyrosine kinase assay

After treating RBL-2H3 cells ( $5 \times 10^6$  cells/well) with 4 $\mu$ 8C at the indicated concentrations for 30 min (for the Syk activation in cells) or without 4 $\mu$ 8C (for the Lyn and Fyn activity in vitro), they were stimulated with 25 ng/ml Ag for 7 min. Reactions were then stopped by placing the cells on ice. After washing twice with ice-cold PBS, the cells were lysed in 0.5 ml lysis buffer. Cell lysates were centrifuged at  $15,000 \times g$  at 4 °C for 15 min. Supernatants were incubated with 5  $\mu$ g each primary antibody for 3 h and then with 50  $\mu$ l protein A/G-agarose at 4 °C overnight. After washing three times with lysis buffer including 0.1% Nonidet P-40, the activity of tyrosine kinase was analyzed using an ELISA-based Universal Tyrosine Kinase Assay Kit (GenWay Biotech Inc., San Diego, CA), according to the manufacturer's protocol. For the measurement of tyrosine kinase activity with immunoprecipitated Lyn and Fyn, the immunoprecipitates were pretreated with 4 $\mu$ 8C for 30 min and then subjected to the assay.

## 2.10. Passive cutaneous anaphylaxis

Twelve hours after injecting 0.5  $\mu$ g of DNP-IgE into the ear of each mouse, 4 $\mu$ 8C (10, 50, or 100 mg/kg) and cetirizine (50 mg/kg) dissolved in 5% gum Arabic was orally administrated. After 1 h, 250  $\mu$ l of Ag solution (1 mg/ml in 5 mg/ml Evans blue solution) was administered i.v. into the tail. The mouse was euthanized after one hour. The ear was then excised and placed in 700  $\mu$ l formamide. The Evans blue dye of the ear was extracted at 63 °C for 12 h. The absorbance of the extracted dye was measured at 620 nm and calculated from a standard curve established with known amounts of Evans blue dye.

## 2.11. Histological analysis

Each mouse ear was excised and fixed in 4% paraformaldehyde. The tissue of the fixed ear was dehydrated with ethanol and subsequently embedded in paraffin. The paraffin tissue was sliced into 6  $\mu$ m sections and stained with toluidine blue. Percent of degranulated mast cells was calculated as the ratio of degranulated mast cells to total mast cells in tissue.

## 2.12. Statistical analysis

The experimental values are expressed as mean  $\pm$  SEM from three or more independent experiments. Statistical analyses were conducted with ANOVA and Dunnett's tests. All statistics were calculated using Sigma Stat software (Systat Software, Inc., Point Richmond, CA), with  $p < 0.05$  considered significant.

## 3. Results

### 3.1. Effect of 4 $\mu$ 8C on degranulation in Ag-stimulated RBL-2H3 cells and BMMCs

To determine the allergy suppressor effect of 4 $\mu$ 8C (Fig. 1) in mast cells, the release of  $\beta$ -hexosaminidase, an indicator of degranulation, was examined. Degranulation by Ag in RBL-2H3 cells and BMMCs was decreased by 4 $\mu$ 8C in a concentration-dependent manner (Fig. 2A).

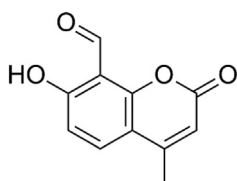


Fig. 1. Compound structure of 4 $\mu$ 8C, 8-formyl-7-hydroxy-4-methylcoumarin.

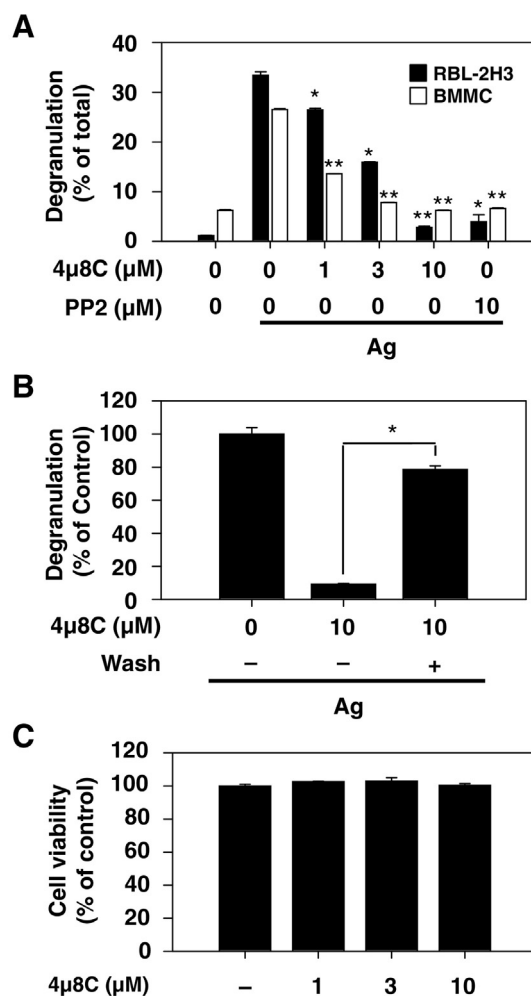


Fig. 2. 4 $\mu$ 8C reversibly inhibits degranulation in Ag-stimulated mast cells. (A) RBL-2H3 cells ( $1.8 \times 10^5$  cells/well) and BMMCs ( $2.5 \times 10^5$  cells/tube) were primed with 20 ng/ml DNP-specific IgE overnight. The cells were stimulated with 25 ng/ml DNP-BSA for 10 min after pre-incubating with 4 $\mu$ 8C (1, 3, or 10  $\mu$ M) or PP2 (10  $\mu$ M) for 30 min. (B) RBL-2H3 cells were washed five times after pre-incubating with 4 $\mu$ 8C (10  $\mu$ M) for 30 min. The cells were stimulated with 25 ng/ml DNP-BSA for 10 min. (C) After incubating RBL-2H3 cells ( $2.0 \times 10^4$  cells/well) with 4 $\mu$ 8C for 4 h, CCK-8 solution (at a 1:10 ratio of CCK-8 to media) was added to each well of the plate. The plate was incubated in a CO<sub>2</sub> incubator at 37 °C for 1 h.  $\beta$ -hexosaminidase release and cell viability were measured at 450 nm as described in the Materials and Methods. The values are mean  $\pm$  SEM from three independent experiments. (A) Significant differences with Ag-only groups are indicated, \* $p < 0.05$  and \*\* $p < 0.01$ . (B–C) Control means the value without 4 $\mu$ 8C.

The suppressor effect on degranulation at the highest concentration of 4 $\mu$ 8C (i.e., 10  $\mu$ M) was similar to that of PP2, an inhibitor of the Src-family kinases ( $IC_{50} \approx 3.2 \mu$ M). When RBL-2H3 cells were treated with 10  $\mu$ M 4 $\mu$ 8C for 30 min and then washed with an S-buffer, degranulation was restored, indicating that the suppressor effect of 4 $\mu$ 8C was reversible (Fig. 2B). Using a CCK-8 kit, it was determined that the concentration range of 4 $\mu$ 8C used in this experiment was not toxic to the cells (Fig. 2C).

### 3.2. Effect of 4 $\mu$ 8C on expression and secretion of inflammatory cytokines

The inflammatory cytokines, TNF- $\alpha$  and IL-4, released from mast cells are known to be important effectors in the progress of allergic disease (Tete et al., 2012). Therefore, RT-PCR was conducted to examine whether the TNF- $\alpha$  and IL-4 generated from Ag-stimulated mast cells could be inhibited. As a result, it was demonstrated that 4 $\mu$ 8C inhibited the expression of TNF- $\alpha$  and IL-4 in a concentration-dependent manner

(Fig. 3A). Consistently, in ELISA assay, the secretions of TNF- $\alpha$  and IL-4 were also suppressed by 4 $\mu$ 8C in Ag-stimulated mast cells in a dose-dependent manner (Fig. 3B).

### 3.3. Effect of 4 $\mu$ 8C on Ag-stimulated mast cell signaling pathways

To examine the inhibitory effect of 4 $\mu$ 8C on Ag-stimulated mast cell signal transduction pathways, western blotting analyses were used. The activations of Syk and LAT, which are involved in the initial signaling pathway in RBL-2H3 cells, were decreased by 4 $\mu$ 8C in a concentration-dependent manner. It was also shown that the phosphorylation of Akt, ERK1/2, p38, and JNK was inhibited by 4 $\mu$ 8C (Fig. 4A). We also confirmed the inhibitory effect of 4 $\mu$ 8C on the activation of Syk and LAT in BMMCs (Fig. 4B).

### 3.4. Effect of 4 $\mu$ 8C on activity of Syk in Ag-stimulated mast cells

In mast cells that are stimulated by Ag, the activity of Syk is essential for degranulation and cytokine production. To verify that the decrease in phosphorylation of Syk protein led to a decrease in activity of Syk in mast cells, the activity of the protein tyrosine kinase was measured

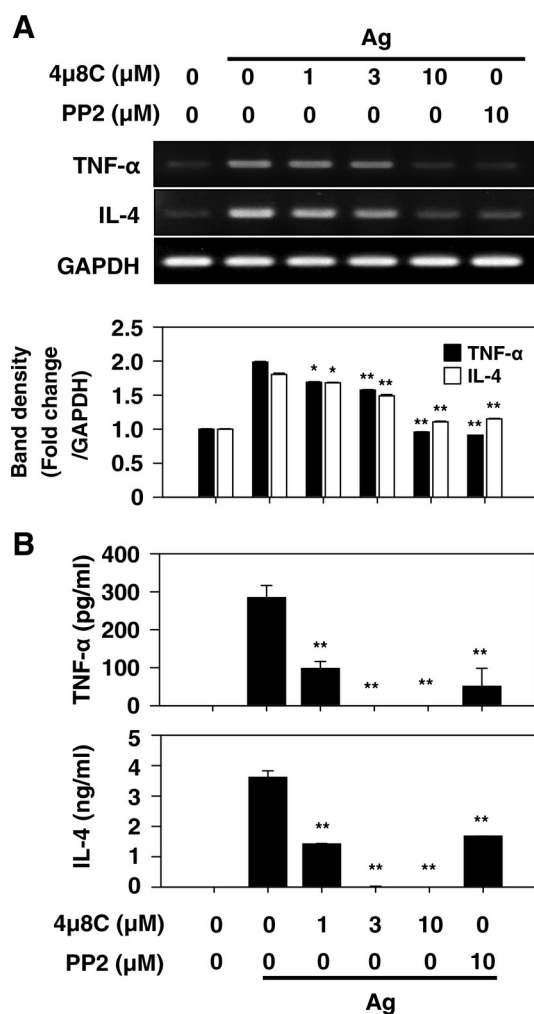


**Fig. 3.** 4 $\mu$ 8C inhibits production of TNF- $\alpha$  and IL-4 by Ag in mast cells. IgE-primed RBL-2H3 cells ( $1 \times 10^6$  cells/well) were stimulated with 25 ng/ml Ag for 15 min (for RT-PCR) or 3 h (for ELISA) after pre-incubating with or without 4 $\mu$ 8C (1, 3, or 10  $\mu$ M) or PP2 (10  $\mu$ M) for 30 min. (A–B) RT-PCR (A) and ELISA (B) were performed to measure the expression and secreted amount of TNF- $\alpha$  and IL-4, respectively, as described in the “Materials and methods” section. The values are presented as the mean  $\pm$  SEM from three independent experiments. Significant differences with Ag-only groups are indicated, \* $p < 0.05$  and \*\* $p < 0.01$ .

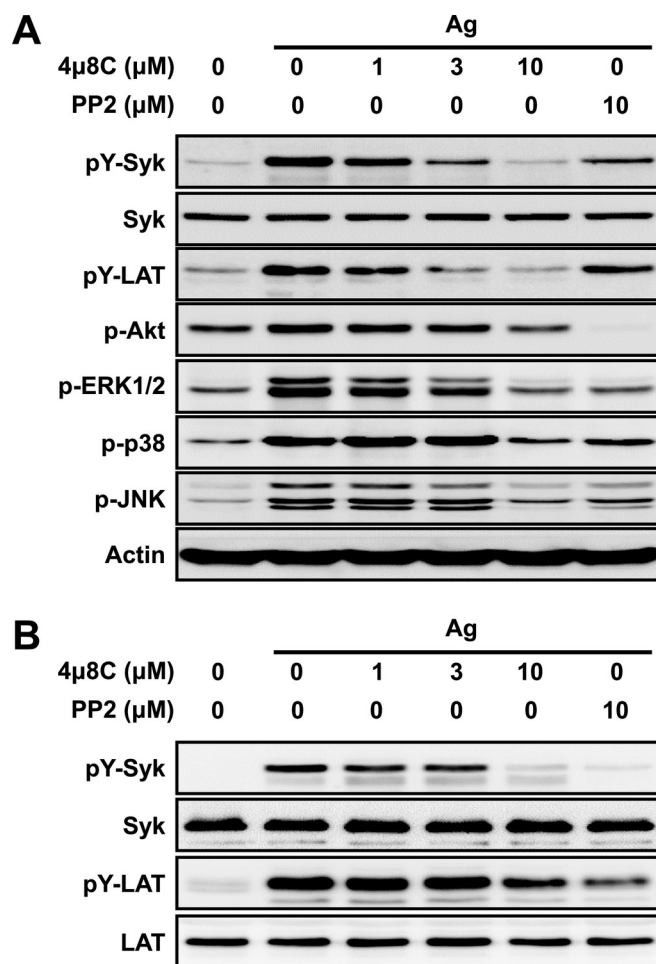


**Fig. 4.** 4 $\mu$ 8C inhibits phosphorylation of Syk and downstream signaling proteins by Ag in mast cells. (A) RBL-2H3 cells ( $1.0 \times 10^6$  cells/well) and (B) BMMCs ( $5.0 \times 10^6$  cells/tube) were sensitized with 20 ng/ml DNP-specific IgE overnight. After pre-incubating with 4 $\mu$ 8C (1, 3, or 10  $\mu$ M) or PP2 (10  $\mu$ M) for 30 min, the cells were stimulated with 25 ng/ml Ag for 10 min. The cell lysates were subjected to western blot analysis as described in the “Materials and methods”. Representative images from three independent experiments are shown.

with an in vitro kinase assay. The Syk protein was purified by immunoprecipitation and its activity was measured. As a result, it was demonstrated that the activity of Syk that was increased by Ag stimulation was suppressed by 4 $\mu$ 8C in a concentration-dependent manner (Fig. 5).

### 3.5. Effect of 4 $\mu$ 8C on activity of Lyn and Fyn in vitro

During the activation of mast cells by antigen, Lyn and Fyn are well known as the initially activated protein kinases in the signaling process. These activated Lyn and Fyn stimulate the phosphorylation and activation of Syk (Draber et al., 2016; Yu et al., 2006). Our results showed that 4 $\mu$ 8C inhibited the phosphorylation and activation of Syk by antigen in mast cells (Figs. 4 and 5). Based on these experimental results, we examined whether 4 $\mu$ 8C inhibited the activity of Lyn and Fyn in vitro to find the direct target protein of 4 $\mu$ 8C. As the results, 4 $\mu$ 8C inhibited the activity of both Lyn and Fyn in a dose-dependent manner (Fig. 6). These observations suggested that 4 $\mu$ 8C suppressed mast cells by inhibiting the activity of Lyn and Fyn by antigen.

### 3.6. Effect of 4 $\mu$ 8C on passive cutaneous anaphylaxis (PCA)

To corroborate the allergy suppressor effect of 4 $\mu$ 8C in an animal allergy model, PCA was conducted. PCA is a representative animal model of allergy that stimulates local allergic response by injection of IgE into

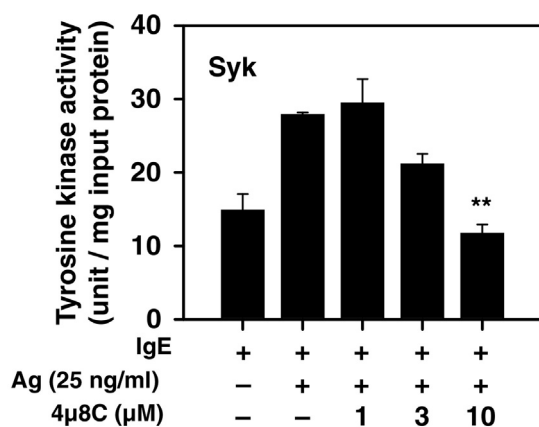


**Fig. 5.** 4μ8C inhibits the activation of Syk by Ag in mast cells. (A) RBL-2H3 cells ( $5.0 \times 10^6$  cells/well) were sensitized with 20 ng/ml DNP-specific IgE overnight. After pre-incubating with 4μ8C (1, 3, or 10 μM) for 30 min, the cells were stimulated with 25 ng/ml DNP-BSA for 10 min. Syk was immunoprecipitated with specific antibody, as described in the [Materials and methods](#) section. The values shown are mean  $\pm$  SEM from three independent experiments. Significant differences with Ag-only groups are indicated, \*\* $p < 0.01$ .

the ears of mice and intravenous injection of Ag into the tails. After IgE injection, cetirizine (a typical antihistamine drug, 50 mg/kg, as a positive control) and 4μ8C (10, 50, or 100 mg/kg) were orally administered. The Ag was then injected to induce PCA. The Evans blue dye in the ears of PCA-induced mice administered 4μ8C was visibly reduced in a concentration-dependent manner compared to the dye in the ears of PCA-induced mice administered vehicle ([Fig. 7A](#)). In addition, the amount of Evans blue dye extracted from the ears of PCA-induced mice was decreased by 4μ8C in a concentration-dependent manner

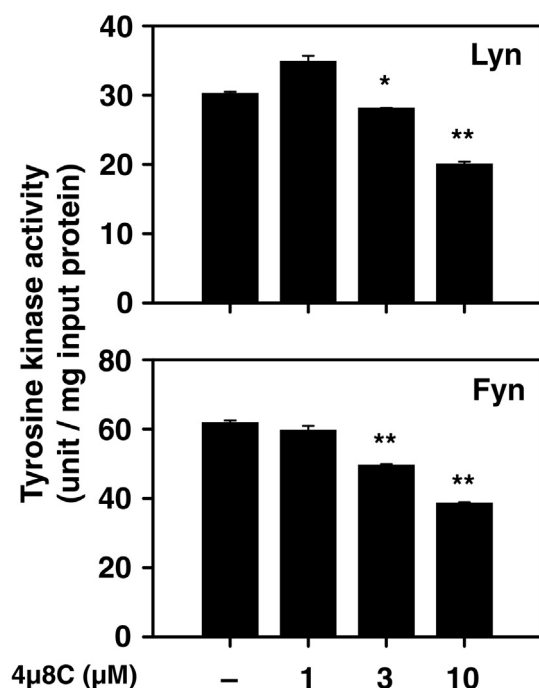


**Fig. 6.** 4μ8C inhibits Lyn and Fyn in vitro. (A) RBL-2H3 cells ( $5.0 \times 10^6$  cells/well) were sensitized with 20 ng/ml DNP-specific IgE overnight. The cells were stimulated with 25 ng/ml Ag for 10 min. Lyn and Fyn were immunoprecipitated with each specific antibody. The immunoprecipitated Lyn and Fyn were pretreated with 4μ8C (0, 1, 3, 10 μM) for 30 min and then subjected to the protein kinase assay, as described in the [Materials and methods](#) section. The values shown are mean  $\pm$  SEM from three independent experiments. Significant differences with Ag-only groups are indicated, \* $p < 0.05$  and \*\* $p < 0.01$ .

([Fig. 7B](#)). 4μ8C was more effective than cetirizine at the same concentration of 50 mg/kg ([Fig. 7B](#)). To measure the degranulation of mast cells in the ear tissue after inducing the allergic reaction, the tissue was fixed and stained with toluidine blue to examine mast cells histology. The ratio of mast cells that showed degranulation by Ag decreased significantly in a dose-dependent manner of 4μ8C ([Fig. 7C](#)). Therefore, these results have shown that the allergy suppressing effect of 4μ8C is active in the allergy animal model.

#### 4. Discussion

In recent years, the incidence of allergic disease has increased around the world ([Galli, 1993](#)). The activation of mast cells is involved in the induction of allergic diseases ([Theoharides and Kalogeromitros, 2006](#)) by releasing intracellular granules and various cytokines, such as TNF-α, transforming growth factor-β (TGF-β), IL-1β, IL-4, and IL-6 ([Beaven, 2009](#)). Currently, the medicines mainly used for allergy treatment are immunosuppressants or antihistamines, which focus on the relief of symptoms rather than the treatment of causes ([Holgate and Polosa, 2008; Simons and Simons, 2011](#)). Therefore, as a new treatment approach for mast cell-mediated allergic diseases, studies on the Src family kinases and Syk, an initial signaling molecule for mast cell activation, are in progress. This study revealed that 4μ8C decreased activation of Syk, thus inhibiting the allergic reaction mediated by mast cells in vitro and in vivo.

Coumarin is a natural compound isolated from various plants. Coumarin and its derivatives are known for various pharmacological activities, including anti-coagulation effects, anti-cancer activities, and anti-inflammatory effects ([Sandhu et al., 2014](#)). Among coumarin derivatives, selinidin ([Kishiro et al., 2008](#)), 3-aryl coumarin derivatives ([De Souza Santos et al., 2013](#)), and cnidicin ([Ryu et al., 2001](#)) were reported as inhibitors of Ag-stimulated degranulation in RBL-2H3 mast cells. However, the mechanisms of their actions, their anti-allergic effects in animal models, and their effects on the activation of tissue mast cells in vivo are unknown.

Another coumarin derivative, 4μ8C, has been recognized as an IRE1 inhibitor that suppresses the activity of this ribonuclease by binding directly to it. According to existing reports, 4μ8C is known to inhibit the production of Type 2 cytokines, such as IL-4 and IL-13 ([Kemp et al., 2013](#)), and suppressing the inflammatory reaction of alveolar macrophages by inhibiting IRE1 in CD4<sup>+</sup> T cells ([Lubamba et al., 2015](#)). In addition, it was reported that 4μ8C inhibits arthritis ([Qiu et al., 2013](#)) and weakens tight junctions by decreasing ZO-1 and occludin, tight junction-related proteins in retinal epithelial cells ([Ma et al., 2016](#)). However, since the effect of 4μ8C on mast cells and allergic disease in animals is not yet known, the present study was undertaken. In our study, the inhibitory effect of 4μ8C on mast cells was reversible ([Fig. 2B](#)) and was caused by the suppression of Syk activation and Syk-dependent signaling in mast cells ([Figs. 4 and 5](#)). Of note, 4μ8C suppressed the allergic response by Ag in PCA mice via inhibition of the activation of local tissue mast cells ([Fig. 7](#)). Our results demonstrate for the first time that 4μ8C shows anti-allergic activity in vivo by regulating tissue mast cells. This warrants further development of 4μ8C as an anti-allergic agent for mast cell-mediated allergic diseases.

Some cytokines such as TNF-α and IL-4 that are secreted by Ag stimulus in mast cells are known to play critical roles in prompting allergic symptoms in later stages of allergic diseases ([Tete et al., 2012](#)). TNF-α induces inflammation and could recruit immune cells near inflamed areas ([Hide et al., 1997](#)). IL-4 boosts the proliferation of plasma cells and the generation of IgE ([Geha et al., 2003](#)), and can contribute to allergic symptoms through the inflammation and contraction of smooth muscles ([Li-Weber and Krammer, 2003](#)). Therefore, the production of pro-inflammatory cytokines in mast cells is used as an important indicator for the severity of mast cell-mediated allergic symptoms. In this study, it was verified that the increase of expression of TNF-α and IL-4 by Ag could be inhibited by 4μ8C in a concentration-dependent way

(Fig. 3). This is a strong evidence that 4 $\mu$ 8C could inhibit the chronic progression of allergic diseases.

In the production of cytokines from mast cells, MAP kinases, through Ag stimulation, play critical roles. The activation of typical MAP kinases (i.e., ERK1/2, p38, and JNK) stimulates transcription factors, leading to the expression of various cytokines. MAP kinase signal transduction promotes transcription by activating, for example, c-Jun and ATF-2, transcription factors of TNF- $\alpha$  and IL-4. As a result, this produces TNF- $\alpha$  and IL-4 (Blank et al., 2014; Ishizuka et al., 1997). Therefore, through this study, it was shown that the inhibition of cytokine production by 4 $\mu$ 8C could be caused by the inhibition of MAP kinases, such as ERK1/2, p38, and JNK (Fig. 4A).

The aggregation of Ag with Fc $\epsilon$ R1 of mast cells activates the Src-family kinases through Ag stimulus, which phosphorylates ITAM of Fc $\epsilon$ R1. Syk then binds and is phosphorylated (Paolini et al., 1991; Sada et al., 2001). If Syk is phosphorylated, it becomes fully activated, which then leads to the activation of various downstream signaling proteins, such

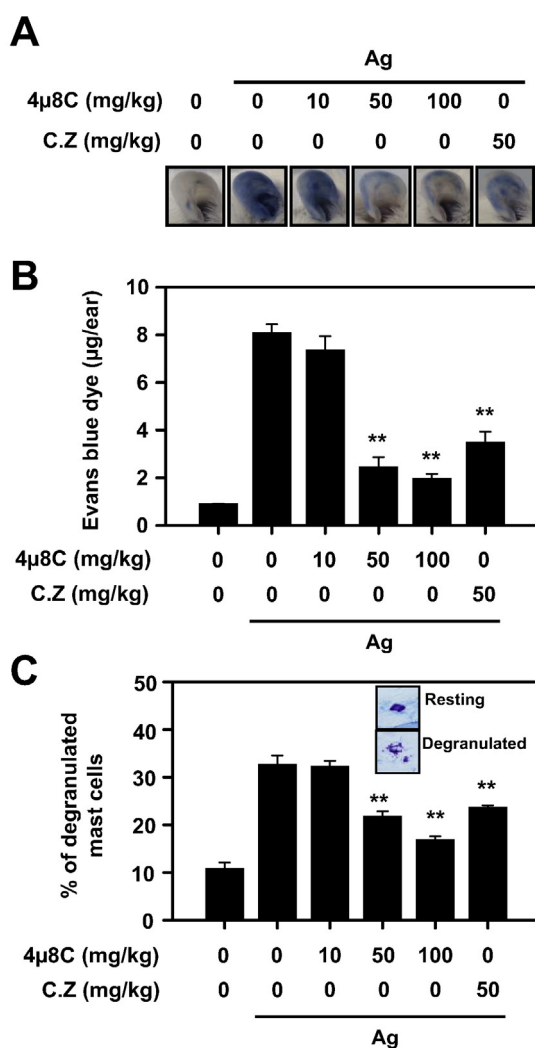


**Fig. 7.** 4 $\mu$ 8C inhibits PCA response and suppresses degranulation of mast cells by Ag in mice. The DNP-specific IgE (0.5  $\mu$ g) was intradermally injected into the ears of mice. After 12 h, 250  $\mu$ g Ag, containing 5 mg/ml Evans blue, was intravenously injected into the tails of the mice. 4 $\mu$ 8C (10, 50, or 100 mg/kg) or cetirizine (C.Z, 50 mg/kg) were orally administered to the mice 1 h before the injection of Ag. Each ear was excised 1 h after injection of Ag, and Evans blue dye was extracted in formamide overnight. The extracted dye was measured at 620 nm. (A and C) The representative images for ears (A) and resting or degranulated mast cells (C, inset) are shown. (B–C) The amount of Evans blue dye extracted from the ears (B) and the percent of degranulated tissue mast cells (C) are presented as mean  $\pm$  SEM from three independent experiments. Significant differences with Ag-only groups are indicated, \*\* $p < 0.01$ .

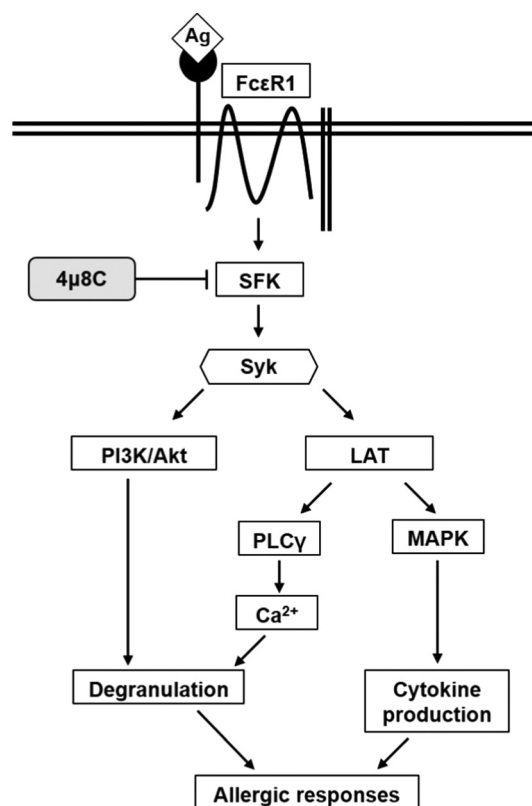


**Fig. 8.** Proposed scheme for the inhibitory mechanism of 4 $\mu$ 8C on mast cells and IgE-mediated allergic responses. SFK stands for Src-family kinase.

as LAT, PLC- $\gamma$ , and MAP kinases (Gilfillan and Tkaczyk, 2006). In this study, it was shown that 4 $\mu$ 8C inhibited the activation of Syk in a concentration-dependent manner (Figs. 4 and 5) and inhibited a variety of downstream signaling proteins (Fig. 4). Altogether, these results support that 4 $\mu$ 8C inhibits the degranulation and production of cytokines from mast cells by suppressing the activation of Syk.

In our results, 4 $\mu$ 8C inhibited the phosphorylation and activation of Syk by Ag in mast cells (Figs. 4 and 5). In the mast cell activation signaling pathway by Ag, Lyn and Fyn are well known as the upstream protein tyrosine kinases of Syk (Draber et al., 2016; Yu et al., 2006). Based on these reports and our results (Figs. 4 and 5), we tested whether 4 $\mu$ 8C directly inhibits the activity of Lyn and Fyn in vitro. As a result, 4 $\mu$ 8C significantly inhibited Lyn and Fyn in vitro in a dose-dependent manner (Fig. 6). Lyn and Fyn are protein tyrosine kinases belonging to Src-family kinases (Draber et al., 2016). Our results therefore indicated that 4 $\mu$ 8C is a Src-family kinase inhibitor as PP2, a typical Src-family kinase inhibitor. However, further studies on how 4 $\mu$ 8C inhibits Lyn and Fyn should be pursued in the future. For example, the binding region of 4 $\mu$ 8C and its inhibition kinetics for Lyn and Fyn are necessary to be determined.

PCA is a well-accepted animal model that acutely induces local allergic responses by tissue mast cells (El-Agamy, 2012). It was verified that the 4 $\mu$ 8C inhibited the allergic response in PCA mice in a dose-dependent manner (Fig. 7A and B). Of note, the degranulation of mast cells in the ear tissue was also decreased by 4 $\mu$ 8C (Fig. 7C). This study confirmed that the inhibition of mast cells by 4 $\mu$ 8C is effective both in vitro and in vivo.

In summary, this study demonstrated for the first time that the coumarin derivative, 4 $\mu$ 8C, inhibits the activation of IgE-mediated mast cells both in vitro and in vivo. 4 $\mu$ 8C inhibited the activation of mast cells through the inhibition of Lyn and Fyn, and eventually decreased mast cell degranulation and allergic responses in mice (Fig. 8). Therefore, our results suggest that 4 $\mu$ 8C could be a new medicinal candidate for allergic diseases such as rhinitis and atopic dermatitis.

## Conflict of interest

The authors have declared that there is no conflict of interest.

## Acknowledgements

This research was supported by the National Research Foundation of Korea (NRF) grant (NRF-2016R1A2B3015840) and in part by Basic Research Laboratory Program (No. 2013R1A4A1069575) funded by the Korea government. Young Mi Kim was supported by the NRF grant (NRF-201700210001) in Korea.

## References

- Beaven, M.A., 2009. Our perception of the mast cell from Paul Ehrlich to now. *Eur. J. Immunol.* 39, 11–25.
- Begin, P., Nadeau, K.C., 2014. Epigenetic regulation of asthma and allergic disease. *Allergy Asthma Clin. Immunol.* 10, 27.
- Bischoff, S.C., 2007. Role of mast cells in allergic and non-allergic immune responses: comparison of human and murine data. *Nat. Rev. Immunol.* 7, 93–104.
- Blank, U., et al., 2014. Vesicular trafficking and signaling for cytokine and chemokine secretion in mast cells. *Front. Immunol.* 5, 453.
- Bradding, P., 2008. Novel approaches to the inhibition of mast cells in allergic disease. *Clin. Exp. Allergy* 38, 704–708.
- Cross, B.C., et al., 2012. The molecular basis for selective inhibition of unconventional mRNA splicing by an IRE1-binding small molecule. *Proc. Natl. Acad. Sci. U. S. A.* 109, E869–E878.
- De Souza Santos, M., et al., 2013. In situ screening of 3-arylcoumarin derivatives reveals new inhibitors of mast cell degranulation. *Arch. Pharm. Res.* 36, 731–738.
- Draber, P., et al., 2016. Signal transduction and chemotaxis in mast cells. *Eur. J. Pharmacol.* 778, 11–23.
- El-Agamy, D.S., 2012. Anti-allergic effects of nilotinib on mast cell-mediated anaphylaxis like reactions. *Eur. J. Pharmacol.* 680, 115–121.
- Galli, S.J., 1993. New concepts about the mast cell. *N. Engl. J. Med.* 328, 257–265.
- Geha, R.S., et al., 2003. The regulation of immunoglobulin E class-switch recombination. *Nat. Rev. Immunol.* 3, 721–732.
- Gilfillan, A.M., Rivera, J., 2009. The tyrosine kinase network regulating mast cell activation. *Immunol. Rev.* 228, 149–169.
- Gilfillan, A.M., Tkaczyk, C., 2006. Integrated signaling pathways for mast-cell activation. *Nat. Rev. Immunol.* 6, 218–230.
- Gregory, G.D., Brown, M.A., 2006. Mast cells in allergy and autoimmunity: implications for adaptive immunity. *Methods Mol. Biol.* 315, 35–50.
- Hide, I., et al., 1997. Suppression of TNF- $\alpha$  secretion by azelastine in a rat mast (RBL-2H3) cell line: evidence for differential regulation of TNF- $\alpha$  release, transcription, and degranulation. *J. Immunol.* 159, 2932–2940.
- Holgate, S.T., Polosa, R., 2008. Treatment strategies for allergy and asthma. *Nat. Rev. Immunol.* 8, 218–230.
- Ishizuka, T., et al., 1997. Mast cell tumor necrosis factor  $\alpha$  production is regulated by MEK kinase. *Proc. Natl. Acad. Sci. U. S. A.* 94, 6358–6363.
- Kemp, K.L., et al., 2013. The serine-threonine kinase inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ) promotes IL-4 production in T helper cells. *J. Biol. Chem.* 288, 33272–33282.
- Kishiro, S., et al., 2008. Selinidin suppresses IgE-mediated mast cell activation by inhibiting multiple steps of Fc  $\epsilon$ RI signaling. *Biol. Pharm. Bull.* 31, 442–448.
- Li-Weber, M., Krammer, P.H., 2003. Regulation of IL4 gene expression by T cells and therapeutic perspectives. *Nat. Rev. Immunol.* 3, 534–543.
- Lubamba, B.A., et al., 2015. X-box-binding protein 1 and innate immune responses of human cystic fibrosis alveolar macrophages. *Am. J. Respir. Crit. Care Med.* 192, 1449–1461.
- Ma, J.H., et al., 2016. The role of IRE-XBP1 pathway in regulation of retinal pigment epithelium tight junctions. *Invest. Ophthalmol. Vis. Sci.* 57, 5244–5252.
- Mocsai, A., et al., 2010. The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat. Rev. Immunol.* 10, 387–402.
- Paolini, R., et al., 1991. Phosphorylation and dephosphorylation of the high-affinity receptor for immunoglobulin E immediately after receptor engagement and disengagement. *Nature* 353, 855–858.
- Qiu, Q., et al., 2013. Toll-like receptor-mediated IRE1 $\alpha$  activation as a therapeutic target for inflammatory arthritis. *EMBO J.* 32, 2477–2490.
- Ryu, S.Y., et al., 2001. Cnidicin, a coumarin, from the root of *Angelica koreana*, inhibits the degranulation of mast cell and the NO generation in RAW 264.7 cells. *Planta Med.* 67, 172–174.
- Sada, K., et al., 2001. Structure and function of Syk protein-tyrosine kinase. *J. Biochem.* 130, 177–186.
- Sandhu, S., et al., 2014. Coumarin hybrids as novel therapeutic agents. *Bioorg. Med. Chem.* 22, 3806–3814.
- Simons, F.E., Simons, K.J., 2011. Histamine and H1-antihistamines: celebrating a century of progress. *J. Allergy Clin. Immunol.* 128, 1139–1150.
- Tete, S., et al., 2012. Role of mast cells in innate and adaptive immunity. *J. Biol. Regul. Homeost. Agents* 26, 193–201.
- Theoharides, T.C., Kalogeromitros, D., 2006. The critical role of mast cells in allergy and inflammation. *Ann. N. Y. Acad. Sci.* 1088, 78–99.
- Yu, M., et al., 2006. Scaffolding adapter Grb2-associated binder 2 requires Syk to transmit signals from Fc $\epsilon$ RI. *J. Immunol.* 176, 2421–2429.