



Morus bombycis extract suppresses mast cell activation and IgE-mediated allergic reaction in mice

Ju Dong Kim^{a,1}, Do Kyun Kim^{a,1}, Hyuk Soon Kim^a, A-Ram Kim^a, Bokyung Kim^a, Erk Her^a, Kyung Hee Park^b, Hyung Sik Kim^c, Young Mi Kim^d, Wahn Soo Choi^{a,*}

^a Institute of Functional Genomics and College of Medicine, Konkuk University, Chungju 380–701, Republic of Korea

^b Department of Microbiology, College of Medicine, Konkuk University, Chungju 380–701, Republic of Korea

^c College of Pharmacy, Pusan National University, Busan 609–735, Republic of Korea

^d College of Pharmacy, Duksung Women's University, Seoul 132–714, Republic of Korea

ARTICLE INFO

Article history:

Received 8 November 2012

Received in revised form

26 December 2012

Accepted 29 December 2012

Available online 9 January 2013

Keywords:

Morus bombycis extract (MBE)

Mast cells

Allergy

Syk kinase

Passive cutaneous anaphylaxis (PCA)

ABSTRACT

Ethnopharmacological relevance: *Morus bombycis* Koidzumi (MB) is widely distributed throughout Korea, where it is used as a traditional folk remedy for the treatment of allergic diseases including asthma. However, the pharmacological effect and the mechanistic study of MB have not been investigated. We aimed to investigate the anti-allergic activity of MB in vitro and in vivo and the mechanism of its action on mast cells.

Materials and methods: The anti-allergic activity of MB extract (MBE) was assessed using passive cutaneous anaphylaxis (PCA) in mice and mouse bone marrow-derived mast cells (BMMCs) in vitro. The effects of MBE on mast cell activation were evaluated by using the β -hexosaminidase release assay, reverse transcriptase-polymerase chain reaction, enzyme-linked immunosorbent assay, and western blotting analysis.

Results: MBE reversibly inhibited degranulation and generation of cytokines (TNF- α and IL-4) in antigen-stimulated mast cells. With regard to its mechanism of action, MBE inhibited the activation of Lyn and Syk, which have essential roles in degranulation and the production of various inflammatory cytokines. MBE also inhibited the activating phosphorylation of mitogen-activated protein (MAP) kinases, Erk1/2, p38, JNK, and Akt. In agreement with its in vitro effect, MBE significantly inhibited mast cell-mediated PCA reactions in IgE-sensitized mice.

Conclusions: The present results strongly suggest that MBE exerts an anti-allergic effect, both in vitro and in vivo by inhibiting the Lyn and Syk pathways in mast cells. Therefore, MBE may be useful for the treatment of allergic diseases, including atopic dermatitis and allergic asthma.

© 2013 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Allergic diseases are common diseases caused by hypersensitive immune disorders. It is observed in 10–20% of the world's population, and the number of allergy patients has steadily increased in recent years (Flohr et al., 2009; Erb, 2009). Most patients with allergies are genetically predisposed to produce IgE in the body. Under allergic conditions, mast cells are the major effector cells sensitized by IgE through the IgE high-affinity receptor (Fc ϵ RI) on their membrane (Metzger, 2004).

The aggregation of Fc ϵ RI by antigens activates a variety of signaling molecules, which result in the release of mediators such

as histamine, products of arachidonic acid metabolism, and an array of inflammatory cytokines (Gilfillan and Beaven, 2011). The antigen stimulation of mast cells activates Src family kinases (SFKs) such as Lyn, Fyn, and Fgr (Gilfillan and Beaven, 2011; Lee et al., 2011). Subsequently, the association of Syk with the tyrosine-phosphorylated immunoreceptor tyrosine-based activation motif (ITAM) of Fc ϵ RI γ results in a conformational change that increases its enzymatic activity (Siraganian et al., 2002; Mócsai et al., 2010). This leads to the downstream propagation of signals, such as tyrosine phosphorylation of Linker for activation of T cells (LAT), phospholipase C γ , and Ca²⁺ influx (Gilfillan and Rivera, 2009). These pathways are essential for the secretion of allergic mediators (Beaven, 2009; Gilfillan and Beaven, 2011).

Morus bombycis Koidzumi (MB), which belongs to the Moraceae family, is a species of forest tree. It is largely distributed throughout eastern Asia, including Korea. The bark of this tree is appreciably grayish brown, and the branches appear as thorns with stinging hairs. MB extract (MBE) has been used as an

* Correspondence to: Department of Immunology, College of Medicine, Konkuk University, Chungju 380–701, Republic of Korea. Tel.: +82 43 840 3788; fax: +82 43 851 9329.

E-mail address: wahnchoi@kku.ac.kr (W. Soo Choi).

¹ The authors are equally contributed to this work.

Oriental folk medicine for allergic diseases including asthma, tuberculosis, arthritis, stroke, and asthenia (Mun, 2011). Although anti-arthritis (Kim et al., 2011), anti-obesity (Kim et al., 2010), and anti-oxidant effects (Jin et al., 2005) of MBE have been reported, its effect on allergic responses remained to be investigated.

In this study, we demonstrate for the first time that a traditional folk medicine, MBE, suppresses antigen-stimulated mast cell activation and mast cell-mediated allergic responses. The current study suggests that MBE can be a new herbal medicine for treating various allergic diseases, including asthma and atopic dermatitis.

2. Materials and methods

2.1. Reagents

Dinitrophenol (DNP)-specific IgE, DNP-conjugated bovine serum albumin (BSA, antigen), Evans blue, and cetirizine were obtained from Sigma-Aldrich (St. Louis, MO). (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl) pyrazolo[3,4-d]pyrimidine (PP2) and thapsigargin were obtained from Calbiochem (La Jolla, CA). Antibodies against Syk and actin were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies against the phosphorylated forms of Akt, Erk1/2, p38, JNK, Syk, linker for activation of T cells (LAT), and phosphotyrosine (pY) were obtained from Cell Signaling Technology Inc. (Danvers, MA). Enzyme-linked immunosorbent assay (ELISA) kits for assessing TNF- α and IL-4 in media were obtained from Invitrogen to Biosource Cytokine & Signaling (Camarillo, CA). Cell-culture media and other components were obtained from GIBCO/Life Technologies Inc. (Rockville, MD).

2.2. Plant material

The *Morus bombycis* Koidzumi (MB) was collected from Jeju Island in Korea, and the sample was authenticated by the Plant Extract Bank at the Korea Research Institute of Bioscience and Biotechnology (KRIBB, Daejeon, Korea). The methanol extract of MB was prepared using the dried stem of the plant, according to the institute's standard protocol (Kim et al., 2011). The extraction yield was ~15% relative to the dry starting material. A voucher specimen (0004-040) was deposited at the Plant Extract Bank and Konkuk University. The extract was dissolved in dimethyl sulfoxide (DMSO) for the in vitro assay and suspended in 5% gum arabic for the in vivo animal study.

2.3. Culture of bone-marrow-derived mast cells (BMMCs) and rat basophilic leukemia (RBL)-2H3 mast cells.

BMMCs were prepared with bone marrow cells from Balb/c mice, according to a previous report with minor modification (Jensen et al., 2006). The BMMCs were cultured for 4 weeks in a medium (RPMI 1640, containing 2 mM L-glutamine, 0.1 mM nonessential amino acids, antibiotics, and 10% fetal bovine serum (FBS) containing 10 ng/mL IL-3. RBL-2H3 cells were grown in minimum essential medium (MEM) with Earle's salts, supplemented with glutamine, antibiotics, and 15% FBS.

2.4. Measurement of degranulation in mast cells

The degree of degranulation of mast cells was determined by β -hexosaminidase (granule marker protein) release assay. Briefly, the mast cells on 24-well cluster plates (2×10^5 cells/0.5 mL/well) were sensitized overnight with 50 ng/mL of DNP-specific IgE. The cells were washed twice, and then buffered solution was added (0.2 mL/well), as required. Experiments were performed in

1,4-piperazinediethanesulfonic acid (PIPES)-buffered medium [25 mM PIPES (pH 7.2), 159 mM NaCl, 5 mM KCl, 0.4 mM MgCl₂, 1 mM CaCl₂, 5.6 mM glucose, and 0.1% fatty acid-free fraction V from bovine serum] for RBL-2H3 cells or in Tyrode buffer [20 mM HEPES (pH 7.4), 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.05% bovine serum albumin (BSA)] for BMMCs. The cells were incubated for 30 min with or without MBE before treatment with 25 ng/mL of antigen for 10 min or for the indicated times. The release of *p*-nitrophenol from *p*-nitrophenyl-*N*-acetyl-*b*-D-glucosaminide was measured for β -hexosaminidase activity. Degranulation of mast cells was determined by calculating the ratio of β -hexosaminidase activity in culture media to the total activity in the media and cell lysate (Ozawa et al., 1993).

2.5. Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from RBL-2H3 cells by using Trizol Reagent (Invitrogen, Carlsbad, CA) and reverse transcribed using the Superscript first-strand synthesis system (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. PCR was performed at 94 °C for 45 s, at 55 °C for 45 s, and at 72 °C for 60 s for 30 cycles. The specific primers used were as follows: rat TNF- α forward 5'-CACCACGCTCTTCTGTCTACTGAAC-3'; rat TNF- α reverse: 5'-CCGGACTCCGTGATGCTAAGTACT-3'; rat IL-4 forward 5'-ACCTTGCTGTCAACCTGTTC-3'; rat IL-4 reverse 5'-TGTGAGCGTGGACTCATTC-3'; rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward 5'-GTGGAGTCTACTGGC-GTCTTC-3'; rat GAPDH reverse: 5'-CCAAGGCTGTGGCAAGGTCA-3'.

2.6. ELISA of TNF- α or IL-4

IgE-primed RBL-2H3 cells were stimulated with 25 ng/mL DNP-BSA for 6 h, with or without MBE. The level of TNF- α and IL-4 secreted from the cells was measured in the media by using an ELISA kit (Invitrogen, Carlsbad, CA).

2.7. Generation of passive cutaneous anaphylaxis (PCA) in mice

A total of 0.5 μ g of DNP-specific IgE was injected subcutaneously into the right ears of BALB/c mice (male, 4 weeks old) 24 h before antigen challenge. After IgE-sensitization, either MBE (0, 100, 300, and 1000 mg/kg) or cetirizine (20 mg/kg) was orally administered to the mice. After 60 min, the mice were intravenously injected with 250 μ g of antigen in 250 μ L of $1 \times$ phosphate-buffered saline (PBS) containing Evans blue (5 mg/mL). The ear tissues were removed after mice were euthanized 1 h later. Dye extravasated into the ear tissue was extracted overnight in 700 μ L of formamide at 63 °C. The absorbance of the formamide solution containing Evans blue was measured at 620 nm, and the amount of dye was calculated by using a standard curve (Kim et al., 2009). The animal study was performed using a protocol pre-approved by the Institutional Animal Care and Use Committee (IACUC) at Konkuk University.

2.8. Immunoprecipitation and immunoblotting analysis

For preparation of the cell lysate, RBL-2H3 cells were harvested, washed twice with $1 \times$ PBS, and lysed with lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 60 mM octyl β -glucoside, 10 mM NaF, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2.5 mM nitrophenylphosphate, 0.7 mg/mL pepstatin, and a protease-inhibitor cocktail tablet). After lysis, cell lysates were kept on ice for 30 min and then centrifuged at $13,000 \times g$ for 10 min at 4 °C. The supernatant protein solution was "precleared" by the addition of 50 μ L of protein G-agarose, and the supernatant solution of equal protein content was used for immunoprecipitation.

For immunoprecipitation, whole cell lysates were incubated with anti-Syk antibody by overnight incubation (at 4 °C with gentle rocking) and then with protein G-agarose. The agarose was washed 5 times with a washing buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, 10 mM NaF, 1 mM Na₃VO₄, 1 mM PMSF, 2.5 mM nitrophenylphosphate, 0.7 mg/mL pepstatin, and protease inhibitor cocktail tablet), and the precipitates were boiled at 95 °C for 5 min in 2 × Laemmli buffer (Laemmli, 1970). Immunoblot analysis of the cell lysates were performed using specific antibodies. Immunoreactive proteins were detected with horseradish peroxidase (HRP)-coupled secondary antibodies and enhanced chemiluminescence according to the manufacturer's protocol (Amersham Biosciences, Piscataway, NJ, USA).

2.9. *In vitro* kinase assay

Lyn, Fyn, Fgr, and Syk immunoprecipitates were assayed for kinase activity using an ELISA-based Universal Tyrosine Kinase Assay Kit (Gen Way, San Diego, USA) according to the manufacturer's instructions.

2.10. Statistical analysis

The results were expressed as the mean ± SEM from 3 or more independent experiments. Statistical analysis was performed by one-way analysis of variance (ANOVA) and the Dunnett's test. All statistical calculations (**p* < 0.05 and ***p* < 0.01) were performed using the SigmaStat software (Systat Software, Inc., Point Richmond, CA).

3. Results

3.1. Effect of MBE on degranulation in antigen-stimulated mast cells

Mast cell-derived mediators play a central role in the initiation of the inflammatory reactions associated with atopic asthma and other allergic disorders (Gilfillan and Beaven, 2011; Beaven, 2009). In this study, 2 types of mast cells, RBL-2H3 cells and BMMCs, were used to assess the effect of MBE on degranulation. MBE inhibited antigen-induced degranulation in RBL-2H3 cells and BMMCs in a dose-dependent manner (Fig. 1A). There was no effect on cell viability within the tested range of MBE concentrations (data not shown). In addition, the inhibitory effect of MBE on mast cell degranulation was recovered by washing the cells with the reaction buffer after incubation with MBE for 30 min (Fig. 1B), indicating that its effect is reversible.

3.2. Effect of MBE on the antigen-stimulated expression and secretion of TNF-α and IL-4 in mast cells

Several cytokines, including TNF-α and IL-4, are critical for the allergic response (Theoharides and Kalogeromitros, 2006). In this study, we investigated whether MBE suppressed the expression and secretion of TNF-α and IL-4 in the cells. The antigen-stimulated increases in IL-4 and TNF-α mRNA levels were largely suppressed by MBE (Fig. 2A). MBE also inhibited antigen-stimulated secretion of TNF-α and IL-4 in a dose-dependent manner (Fig. 2B). The inhibitory effect of 100 μg/mL of MBE was similar to or even more potent than that of 10 μM of PP2, a typical Src family kinase (SFK) inhibitor.

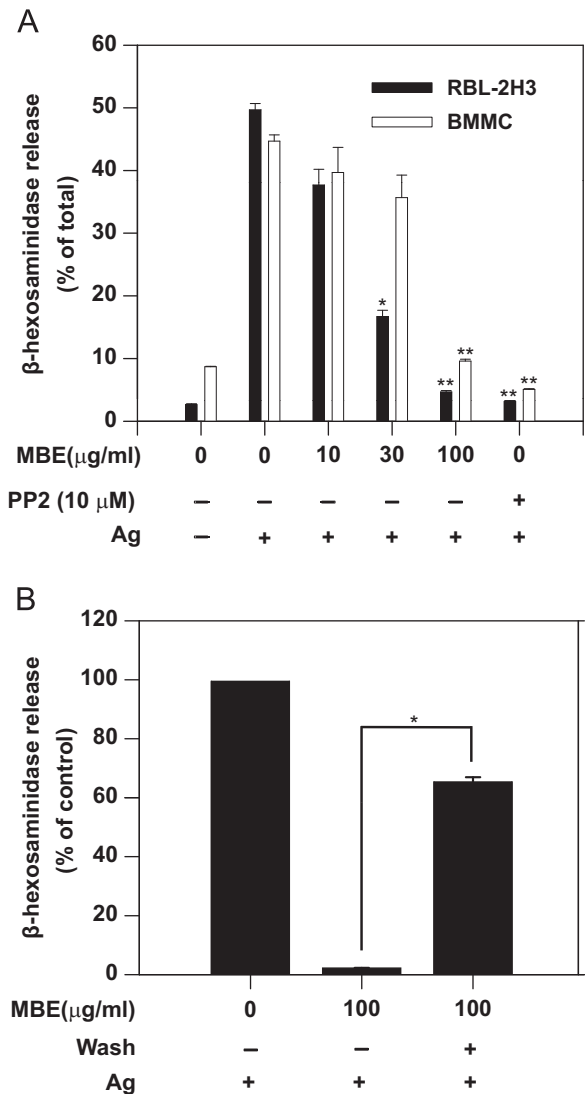


Fig. 1. Effect and reversibility of MBE on antigen-stimulated degranulation in mast cells. (A) RBL-2H3 cells (2×10^5 cells/well) and BMMCs (2×10^5 cells/well) were incubated overnight in 24-well cluster plates with 20 ng/mL DNP-specific IgE in complete growth medium. The medium was replaced with PIPES (for RBL-2H3 cells) or Tyrode buffer (for BMMCs) that contained the indicated concentration of MBE. The cells were stimulated with 25 ng/mL antigen (Ag) for 15 min and then assayed for the release of β-hexosaminidase. PP2 (10 μM) is a general Src-family kinase inhibitor. (B) Reversibility of the MBE effect in RBL-2H3 cells. The cells (2×10^5 cells/well) were pre-incubated for 30 min, with or without 100 μg/mL MBE. MBE-treated cells were washed 5 times with PIPES buffer and then stimulated for 15 min with Ag. The data represent mean ± S.E.M. of 3 independent experiments. The asterisks indicate significant differences from Ag-stimulated controls, which were not treated with MBE (A) or as indicated (B): **p* < 0.05, ***p* < 0.01.

3.3. Effect of MBE on mast cell-mediated passive cutaneous anaphylaxis reaction in mice

Next, we used a mast cell-mediated allergic mouse model, the passive cutaneous anaphylaxis (PCA) model, to test whether MBE inhibited the allergic response (Wershil et al., 1987). IgE was injected into an ear, and this was followed by an injection of antigen containing 4% Evans blue 24 h later. The PCA response was successfully induced, as shown in Fig. 3A. The antigen-induced PCA response was suppressed by the oral treatment of MBE in a dose-dependent manner (Fig. 3B).

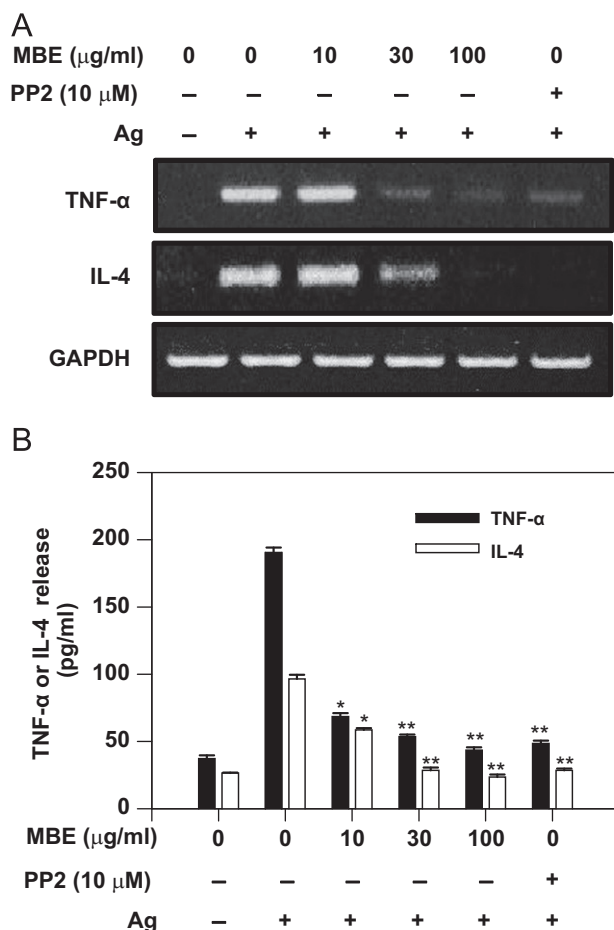


Fig. 2. Effect of MBE on the expression and secretion of TNF- α and IL-4 in antigen-stimulated mast cells. (A) The RBL-2H3 cells (1×10^6 cells/well) were incubated overnight in 6-well plates with 20 ng/mL DNP-specific IgE in a complete growth medium. The cells were stimulated with 25 ng/mL DNP-BSA, with or without MBE for 15 min, and total RNA was isolated and reverse-transcribed. The polymerase chain reaction was performed using primers specific for TNF- α , IL-4, or GAPDH, as described in the Materials and Methods. (B) IgE-primed RBL-2H3 cells were stimulated with 25 ng/mL antigen (Ag) for 6 h, with or without MBE as indicated. Next, the amounts of TNF- α and IL-4 in the culture media were determined using commercial ELISA kits. Values are shown as Mean $\pm$ S.E.M. from 3 independent experiments. Asterisks indicate significant differences from antigen-stimulated controls, which were not treated with MBE, * $p < 0.05$ and ** $p < 0.01$. PP2 is a general Src-family kinase inhibitor.

3.4. MBE inhibits antigen-induced activation of Syk and LAT in mast cells

Spleen tyrosine kinase (Syk) is a key regulatory protein in the IgE-mediated signaling pathway in mast cells. Syk becomes phosphorylated and following binding to the ITAMs of the Fc ϵ RI γ , it becomes fully active (Siraganian et al., 2002). Therefore, we investigated whether MBE inhibited Syk phosphorylation by antigen stimulation in mast cells. The results show that MBE significantly and dose-dependently inhibited the tyrosine phosphorylation of Syk in RBL-2H3 cells (Fig. 4A), as well as in BMMCs (Fig. 4B). Furthermore, the activation of Syk was inhibited by the treatment of MBE in antigen-stimulated BMMCs (Fig. 4C). In addition, the Syk downstream molecule, LAT, was also inhibited by MBE (Fig. 4B).

3.5. Effect of MBE on the activation of MAP kinases and Akt in antigen-stimulated RBL-2H3 cells

MAP kinases play critical roles in the expression and secretion of inflammatory cytokines in antigen-stimulated mast cells. It is

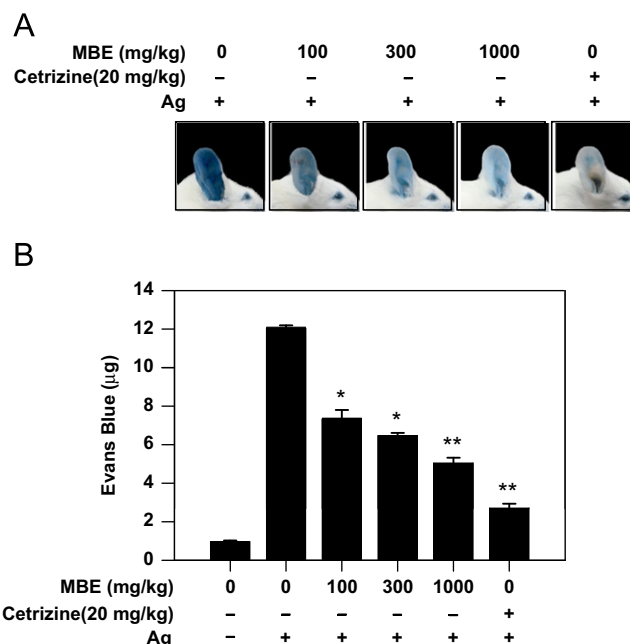


Fig. 3. Effect of MBE on passive cutaneous anaphylaxis in mice. DNP-specific IgE (0.5 mg) was intradermally injected into the mouse ear. An injection of 250 μ g of antigen (Ag, 1 mg/mL in PBS containing 5 mg/mL Evans blue) was administered into the tail vein 24 h later. MBE (100, 300, and 1000 mg/kg) was administered 1 h before Ag treatment. The mouse was euthanized 1 h after Ag challenge, and the ear was removed to measure the amount of dye extravasated by Ag stimulation. The values are expressed as mean $\pm$ S.E.M. from 3 independent experiments ($n = 6$ for each group). Significant differences are indicated as follows: * $p < 0.05$ or ** $p < 0.01$, compared with Ag-induced release of Evans blue without inhibitors. Cetirizine (20 mg/kg) is a typical antihistamine drug.

generally accepted that MAP kinases are downstream signaling molecules of the Syk/LAT pathway (Law et al., 1996; Saitoh et al., 2003). Thus, we investigated whether MBE inhibited the signaling molecules, including Akt, and Erk1/2, p38, and JNK MAP kinases. The results show that Akt and the 3 MAP kinases were inhibited by MBE in a dose-dependent manner (Fig. 5).

3.6. Effect of MBE on the activity of Src-family kinases

In general, the phosphorylation of Syk is directly or indirectly dependent on the upstream Src-family kinases, including Lyn, Fyn, and Fgr (Gilfillan and Beaven, 2011 and Lee et al., 2011). In this way, Syk kinase is initially phosphorylated by Lyn kinase and/or other Src-family kinases, and it is further phosphorylated by its auto-phosphorylation (Siraganian et al., 2002). Therefore, we tested whether MBE inhibited the activations of Lyn, Fyn, and Fgr in antigen-stimulated mast cells. Of interest, we found that MBE inhibited only Lyn, but not Fgr or Fyn (Fig. 6).

4. Discussion

Mast cells are associated with many allergic diseases such as allergic asthma and allergic rhinitis (Galli and Tsai, 2012). Stimulation of mast cells by antigen initiates the process of secretion, resulting in the release of mediators such as histamine and an array of inflammatory cytokines that cause allergic symptoms (Gilfillan and Beaven, 2011; Beaven, 2009). In the present study, we demonstrated for the first time that MBE suppressed mast cell-mediated PCA in mice through inhibiting the activation of the Lyn/Syk signaling pathway in mast cells.

Syk is a critical signaling molecule that activates many downstream signaling molecules such as LAT and NTAL and molecules

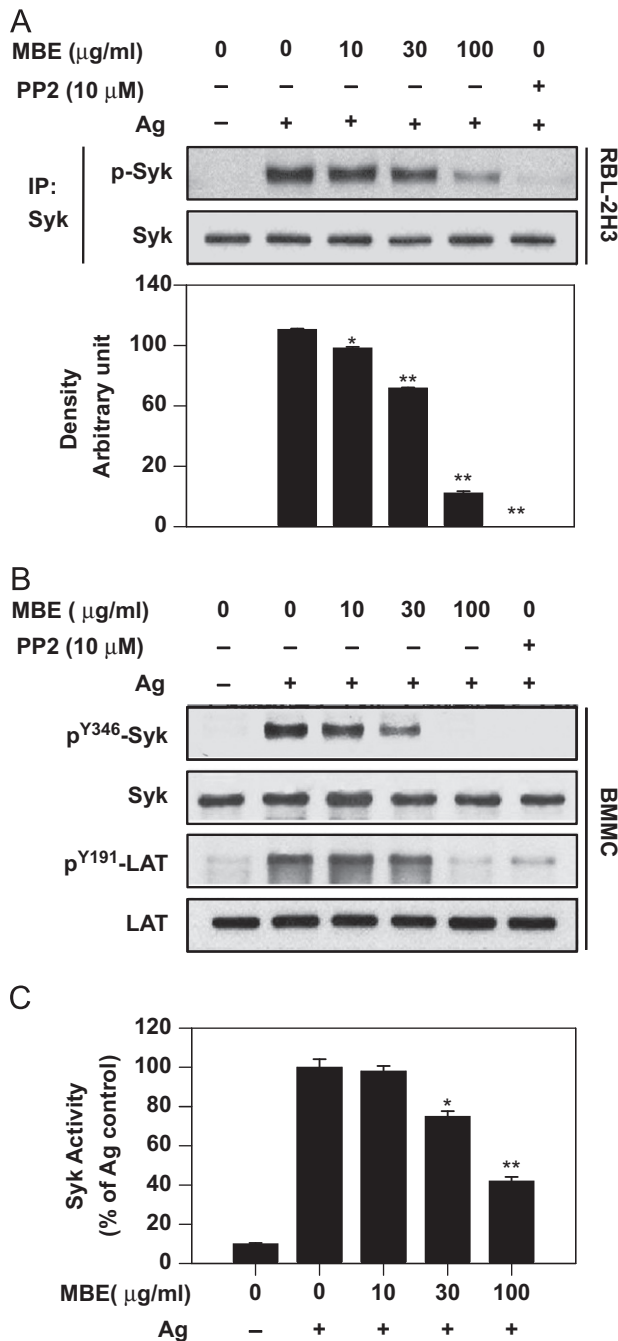


Fig. 4. Effect of MBE on the phosphorylation of Syk and LAT in mast cells. (A) The RBL-2H3 cells (5×10^6 cells/well) were incubated overnight in 100-mm cell culture dishes with 20 ng/mL DNP-specific IgE in a complete growth medium. The cells were stimulated with 25 ng/mL antigen, with or without MBE, for 7 min. Syk kinase was immunoprecipitated with specific antibodies and subjected to immunoblot analysis to detect phosphorylated or total proteins. (B) The BMMCs (5×10^6 cells/well) were incubated with 20 ng/mL DNP-specific IgE in complete growth media overnight. The cells were stimulated with 25 ng/mL antigen, with or without MBE for 7 min. The cell lysates were subjected to immunoblot analysis to detect the phosphorylation of Syk and LAT. PP2 ($10\text{ }\mu\text{M}$) is a general Src-family kinase inhibitor. Representative images are shown from 3 independent experiments. (C) The immunoprecipitated Syk from the cell lysates, as shown in panel B, was subjected to the tyrosine kinase assay as described in the Materials and Methods. The data are presented as mean $\pm$ S.E.M. from 3 independent experiments. Asterisks indicate significant differences from antigen-stimulated controls, which were not treated with MBE, $*p < 0.05$ and $**p < 0.01$. PP2 is a general Src-family kinase inhibitor.

that regulate activation of the GTPase Ras, phospholipase (PL) C- γ , and phosphatidylinositol-3 kinase (PI3K) pathways of mast cell activation (Kalesnikoff and Galli, 2008; Gilfillan and Rivera, 2009).

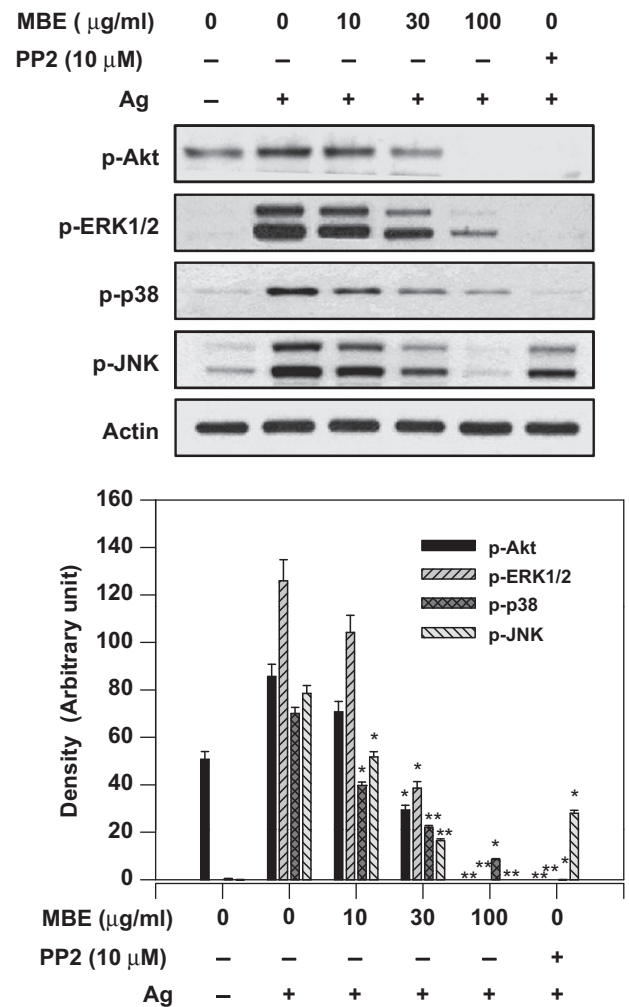


Fig. 5. Effect of MBE on the phosphorylation of MAP kinases and Akt in antigen-stimulated RBL-2H3 cells. The RBL-2H3 cells (1×10^6 cells/well) were incubated overnight in 6-well plates, with 20 ng/mL DNP-specific IgE, in complete growth media. The cells were stimulated with 25 ng/mL DNP-BSA, with or without MBE, for 7 min. The proteins derived from the RBL-2H3 cell lysates were subjected to immunoblot analysis to detect phosphorylated forms of MAP kinases and Akt or Actin. PP2 ($10\text{ }\mu\text{M}$) is a general Src-family kinase inhibitor. Representative images are shown from 3 independent experiments. The values for band density are presented as mean $\pm$ S.E.M. from 3 independent experiments. Asterisks indicate significant differences from antigen-stimulated controls, which were not treated with MBE, $*p < 0.05$ and $**p < 0.01$.

Therefore, it is well accepted that Syk has a pivotal role in the induction of IgE/mast cell-mediated allergic responses. In this study, MBE inhibited the phosphorylation and activation of Syk in antigen-stimulated mast cells (Fig. 4), suggesting that the inhibitory effect of MBE on mast cells was derived from the suppression of Syk activation. However, whether MBE inhibits directly Syk remains to be determined.

After the aggregation of Fc ϵ RI by antigen, the Src-family kinase Lyn is thought to be the first enzyme that phosphorylates the ITAMs of the receptor, leading activation of other substrate molecules such as Syk, LAT, and other downstream signaling molecules (Rivera and Gilfillan, 2006; Siraganian et al., 2002; Masuda and Schmitz, 2008), seemingly has a positive role of Lyn on mast cell functions. However, Lyn^{−/−} BMMCs have normal or enhanced antigen-stimulated degranulation, suggesting that other tyrosine kinases could substitute for Lyn. Such a kinase could be Fyn, which is also activated by Fc ϵ RI stimulation (Parravinci et al., 2002; Odom et al., 2004). Recently, another Src-family kinase, Fgr, was found to be activated by antigen

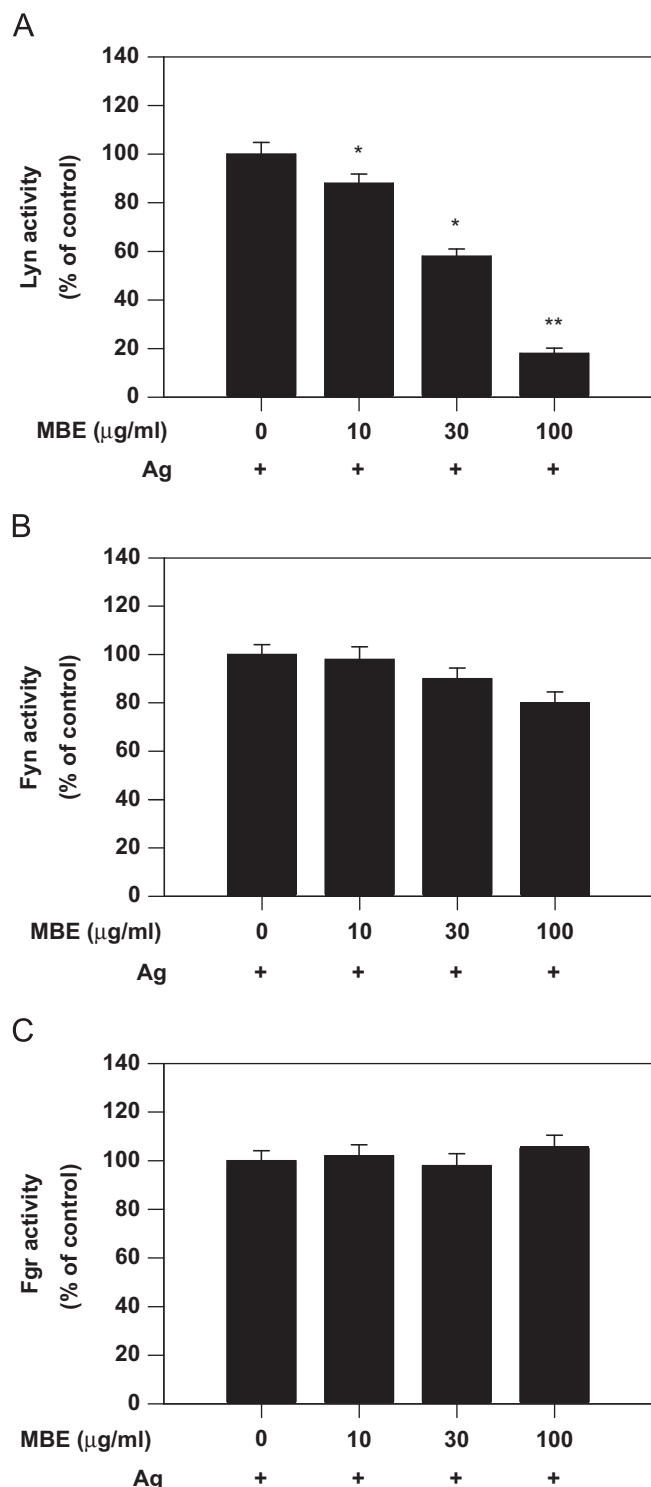


Fig. 6. Effect of MBE on tyrosine kinase activity of Lyn (A), Fyn (B), or Fgr (C) in mast cells. IgE-primed BMMCs (5×10^6 cells) were stimulated with 25 ng/mL antigen (Ag) for 7 min, with or without pre-treating with MBE for 30 min, as indicated. Lyn, Fyn, or Fgr were immunoprecipitated with specific antibodies, as described in the Materials and Methods section. The immunoprecipitates were assayed for protein tyrosine kinase activity by using an ELISA-based Universal Tyrosine Kinase Assay Kit (GenWay, San Diego, CA) according to the manufacturer's instructions. The values shown represent the Mean $\pm$ S.E.M. from 3 independent experiments. Asterisks indicate significant differences from Ag-stimulated controls, which were not treated with MBE, * $p < 0.05$ and ** $p < 0.01$.

stimulation in mast cells, which is also critical for the activation of Syk (Lee et al., 2011). Of note, Lyn, unlike Fyn, also plays an important role as a negative regulator in mast cells, presumably

through the regulation of immunoreceptor tyrosine based inhibition motifs (ITIMs) (Gilfillan and Rivera, 2009). Therefore, it is generally accepted that aggregation of FcεRI results in the engagement of both Lyn and Fyn for the optimal activation of mast cells, although there was a recent controversial report that Syk is the major regulator, whereas Fyn has a minor role, for mast cell activation (Barbu et al., 2010). In this study, MBE differentially suppressed the Lyn and Syk pathway, not Fyn or Fgr (Fig. 4C and Fig. 6), suggesting that the inhibition of Syk was caused by the inhibition of Lyn.

MAP kinase cascades are important signaling pathways in various immune cells, including mast cells (Arbabi and Maier, 2002). Erk1/2 is an essential signal in the production of IL-5, TNF-α, IL-3, and IL-13 in mast cells (Lorentz et al., 2003). p38 MAP kinase stimulates IL-4 production in BMMCs (Frossi et al., 2007). The activation of JNK is also responsible, at least partially, for the expression and production of several cytokines such as TNF-α, IL-2, and IL-6 in mast cells (Kawakami et al., 1998; Hata et al., 1998). In our study, MBE inhibited TNF-α and IL-4 expression (Fig. 2A), as well as the activation of 3 typical MAP kinases (Fig. 5), suggesting that these effects might be connected.

It is well established that mast cells are the effector cells for the acute phase of IgE-associated allergic disorders (Wershil et al., 1987; Mukai et al., 2005). The PCA animal model is generally used to evaluate anti-allergic effects in mice (Franke et al., 1995; Ishizuka et al., 1996). MBE effectively suppressed the PCA reaction in mice in a dose-dependent manner (Fig. 3). A significant inhibition occurred with 100 mg/kg of MBE administered orally. Further suppression was demonstrated with 300 and 1000 mg/kg MBE, indicating a potential use of MBE in treating mast cell-mediated allergic symptoms, similar to the typical antihistamine drug, namely, cetirizine.

In summary, this study is the first to show that MBE suppresses antigen-stimulated degranulation and cytokine production in mast cells and furthermore, inhibits mast cell-mediated allergic responses in mice. The results strongly suggest the use for MBE in anti-allergic herbal therapy. To achieve this goal, further studies regarding bioavailability, toxicity, and active components of MBE are required.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (No. 2011-0028873).

References

- Arbabi, S., Maier, R.V., 2002. Mitogen-activated protein kinases. *Critical Care Medicine* 30, S74–S79.
- Barbu, E.A., Zhang, J., Siraganian, R.P., 2010. The limited contribution of Fyn and Gab2 to the high affinity IgE receptor signaling in mast cells. *Journal of Biological Chemistry* 285, 15761–15768.
- Beaven, M.A., 2009. Our perception of the mast cell from Paul Ehrlich to now. *European Journal of Immunology* 39, 11–25.
- Erb, K.J., 2009. Can helminths or helminth-derived products be used in humans to prevent or treat allergic diseases? *Trends in Immunology* 30, 75–82.
- Flohr, C., Quinzel, R.J., Britton, J., 2009. Do helminth parasites protect against atopy and allergy disease? *Clinical & Experimental Allergy* 39, 20–32.
- Franke, T.F., Yang, S.L., Chan, T.O., Datta, K., Kazlauskas, A., Morrison, D.K., Kaplan, D.R., Tsichlis, P.N., 1995. The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* 81, 727–736.
- Frossi, B., Rivera, J., Hirsch, H.E., Pucillo, C., 2007. Selective activation of Fyn/PI3K and p38 MAPK regulates IL-4 production in BMMC under nontoxic stress condition. *Journal of Immunology* 178, 2549–2555.
- Galli, S.J., Tsai, M., 2012. IgE and mast cells in allergic disease. *Nature Medicine* 18, 693–704.
- Gilfillan, A.M., Beaven, M.A., 2011. Regulation of mast cell responses in health and disease. *Critical Reviews™ in Immunology* 31, 475–530.

- Gilfillan, A.M., Rivera, J., 2009. The tyrosine kinase network regulating mast cell activation. *Immunological Reviews* 228, 149–169.
- Hata, D., Kawakami, Y., Inagaki, N., Lantz, C.S., Kitamura, T., Khan, W.N., Maeda-Yamamoto, M., Miura, T., Han, W., Hartman, S.E., Yao, L., Nagai, H., Goldfeld, A.E., Alt, F.W., Galli, S.J., Witte, O.N., Kawakami, T., 1998. Involvement of Bruton's tyrosine kinase in FcεRI-dependent mast cell degranulation and cytokine production. *Journal of Experimental Medicine* 187, 1235–1247.
- Ishizuka, T., Oshiba, A., Sakata, N., Terada, N., Johnson, G.L., Gelfand, E.W., 1996. Aggregation of the FcεpsilonRI on mast cells stimulates c-Jun amino-terminal kinase activity. A response inhibited by wortmannin. *Journal of Biological Chemistry* 271, 12762–12766.
- Jensen, B.M., Swindle, E.J., Iwaki, S., Gilfillan, A.M., 2006. Generation, isolation, and maintenance of rodent mast cells and mast cell lines. *Current Protocols in Immunology* 3, 3–23.
- Jin, Y.S., Sa, J.H., Shim, T.H., Rhee, H.I., Wang, M.H., 2005. Hepatoprotective and antioxidant effects of *Morus bombycis* Koidzumi on CCl4-induced liver damage. *Biochemical Biophysical Research Communication* 329, 991–995.
- Kalesnikoff, J., Galli, S.J., 2008. New developments in mast cell biology. *Nature Immunology* 9, 1215–1223.
- Kawakami, Y., Hartman, S.E., Holland, P.M., Cooper, J.A., Kawakami, T., 1998. Multiple signaling pathways for the activation of JNK in mast cells: involvement of Bruton's tyrosine kinase, protein kinase C, and JNK kinases, SEK1 and MKK7. *Journal of Immunology* 161, 1795–1802.
- Kim, H.S., Kim, A.R., Park, H.J., Park, D.K., Kim, D.K., Ko, N.Y., Kim, B., Choi, D.K., Won, H.S., Shin, W.S., Kim, Y.M., Choi, W.S., 2011. *Morus bombycis* Koidzumi extract suppresses collagen-induced arthritis by inhibiting the activation of nuclear factor-κB and activator protein-1 in mice. *Journal of Ethnopharmacology* 136, 392–398.
- Kim, J.W., Lee, J.H., Hwang, B.Y., Mun, S.H., Ko, N.Y., Kim, D.K., Kim, B., Kim, H.S., Kim, Y.M., Choi, W.S., 2009. Morin inhibits Fyn kinase in mast cells and IgE-mediated type I hyperpersensitivity response in vivo. *Biochemical Pharmacology* 77, 1506–1512.
- Kim, Y.S., Lee, Y.M., Kim, H., Kim, J., Jang, D.S., Kim, J.H., Kim, J.S., 2010. Anti-obesity effect of *Morus bombycis* root extract: anti-lipase activity and lipolytic effect. *Journal of Ethnopharmacology* 130, 621–624.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Law, C.L., Chandran, K.A., Sidorenko, S.P., Clark, E.A., 1996. Phospholipase C-γ1 interacts with conserved phosphotyrosyl residues in the linker region of Syk and is a substrate for Syk. *Molecular and Cellular Biology* 16, 1305–1315.
- Lee, J.H., Kim, J.W., Kim, D.K., Kim, H.S., Park, H.J., Park, D.K., Kim, A.-Ram., Kim, B.K., Beaven, M.A., Park, K.L., Kim, Y.M., Choi, W.S., 2011. The Src family kinase Fgr is critical for activation of mast cells and IgE-mediated anaphylaxis in mice. *Journal of Immunology* 187, 1807–1815.
- Lorentz, A., Klopp, I., Gebhardt, T., Manns, M.P., Bischoff, S.C., 2003. Role of activator protein 1, nuclear factor-κappaB, and nuclear factor of activated T cells in IgE receptor-mediated cytokine expression in mature human mast cells. *Journal of Allergy and Clinical Immunology* 111, 1062–1068.
- Masuda, E.S., Schmitz, J., 2008. Syk inhibitors as treatment for allergic rhinitis. *Pulmonary Pharmacology & Therapeutics* 21, 461–467.
- Metzger, H., 2004. The high affinity receptor for IgE, FcεpsilonRI. *Novartis Foundation Symposium* 257, 51–59.
- Mócsai, A., Ruland, J., Tybulewicz, V.L., 2010. The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nature Reviews Immunology* 10, 387–402.
- Mukai, K., Matsuoka, K., Taya, C., Suzuki, H., Yokozeki, H., Nishioka, K., Hirokawa, K., Etori, M., Yamashita, M., Kubota, T., 2005. Basophils play a critical role in the development of IgE-mediated chronic allergic inflammation independently of T cells and mast cells. *Immunity* 23, 191–202.
- Mun, S.Y., 2011. *Morus bombycis* Koidzumi. In: Han, D.H. (Ed.), *Korean Herbal Medicines*. Yemoondang Publishing Inc., Seoul, pp. 298–299.
- Odom, S., Gomez, G., Kovarova, M., Furumoto, Y., Ryan, J.J., Wright, H.V., Gonzalez-Espinosa, C., Hibbs, M.L., Harder, K.W., Rivera, J., 2004. Negative regulation of immunoglobulin E-dependent allergic responses by Lyn kinase. *Journal Experimental Medicine* 199, 1491–1502.
- Ozawa, K., Szallasi, Z., Kazanietz, M.G., Blumberg, P.M., Mischak, H., Mushinski, J.F., Beaven, M.A., 1993. Ca(2+)-dependent and Ca(2+)-independent isozymes of protein kinase C mediate exocytosis in Antigen-stimulated rat basophilic RBL-2H3 cells. Reconstitution of secretory responses with Ca²⁺ and purified isozymes in washed permeabilized cells. *Journal of Biological Chemistry* 268, 1749–1756.
- Parravinci, V., Gadina, M., Kovarova, M., Odom, S., Gonzalez-Espinosa, C., Furumoto, Y., Saitoh, S., Samelson, L.E., O'Shea, J.J., Rivera, J., 2002. Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. *Nature Immunology* 3, 741–748.
- Rivera, J., Gilfillan, A.M., 2006. Molecular regulation of mast cell activation. *Journal Allergy and Clinical Immunology* 117, 1214–1225.
- Saitoh, S., Odom, S., Gomez, G., Sommers, C.L., Young, H.A., Rivera, J., Samelson, L.E., 2003. The four distal tyrosines are required for LAT-dependent signaling in FcεpsilonRI-mediated mast cell activation. *The Journal of Experimental Medicine* 198, 831–843.
- Siraganian, R.P., Zhang, J., Suzuki, K., Sada, K., 2002. Protein tyrosine kinase Syk in mast cell signaling. *Molecular Immunology* 38, 1229–1233.
- Theoharides, T.C., Kalogeromitros, D., 2006. The critical role of mast cells in allergy and inflammation. *Annals of the New York Academy of Sciences* 1088, 78–99.
- Wershil, B.K., Mekori, Y.A., Murakami, T., Galli, S.J., 1987. 125I-fibrin deposition in IgE-dependent immediate hypersensitivity reactions in mouse skin. Demonstration of the role of mast cells using genetically mast cell-deficient mice locally reconstituted with cultured mast cells. *Journal of Immunology* 139, 2605–2614.