

Ellagic Acid Attenuates Immunoglobulin E-Mediated Allergic Response in Mast Cells

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Received January 13, 2009; accepted February 27, 2009; published online March 10, 2009

Ellagic acid is known to have anti-oxidant, anti-carcinogenic and anti-mutagenic effects. However, roles of ellagic acid in the immediate-type allergic reactions have not yet been investigated. In the present study, we have demonstrated that ellagic acid attenuates immunoglobulin (Ig)E-mediated allergic response in mast cells and *in vivo*. Oral administration of ellagic acid inhibited anti-dinitrophenyl (DNP) IgE-mediated passive cutaneous anaphylaxis. Ellagic acid dose-dependently reduced histamine release and the expression and secretion of pro-inflammatory cytokines, such as tumor necrosis factor- α and interleukin-6 in rat peritoneal mast cells (RPMC) activated by anti-DNP IgE. Moreover, ellagic acid suppressed an increase in intracellular calcium ion concentration ($[Ca^{2+}]_i$) in RPMC. Furthermore, ellagic acid decreased the activation of nuclear factor-kappa B (NF- κ B). Decreased NF- κ B activity as well as reduced $[Ca^{2+}]_i$ might be involved in the inhibitory effect of ellagic acid on the secretory response. Our findings provide possibility that ellagic acid may serve as an effective therapeutic agent for allergic diseases.

Key words mast cell; allergic response; ellagic acid; histamine; calcium; nuclear factor-kappa B

Several studies have demonstrated the potential of flavonoids for the prevention and/or intervention of various allergic diseases. Ellagic acid (2,3,7,8-tetrahydroxy[1]benzopyrano[5,4,3-cde][1]benzopyran-5,10-dione) is a polyphenol antioxidant found in certain fruits and nuts, such as raspberries, strawberries, walnuts, longan seed, mango kernel and pomegranate (Fig. 1).¹⁾ Ellagic acid has a variety of biological activities, including potent anti-oxidant,²⁾ anti-cancer³⁾ and anti-mutagen properties⁴⁾ in a number of *in vitro* and small animal models.

Asthma, allergic rhinitis, atopic dermatitis and atopic eczema are among the common causes of chronic illness. Mast cells play a key role in the immediate-type allergic diseases through the release of a number of mediators and cytokines. The secretory response of mast cells can be induced by aggregation of their cell surface-specific receptors (Fc ϵ RI) for immunoglobulin (Ig)E by the corresponding antigen. Activated mast cells can produce histamine, as well as a wide variety of other inflammatory mediators such as leukotrienes, prostaglandins, proteases and several pro-inflammatory and chemotactic cytokines such as tumor necrosis factor (TNF)- α , and interleukins (IL)-6, IL-4, IL-13, IL-8.⁵⁾ Among the inflammatory substances released from mast cells, histamine remains the best-characterized and most potent vasoactive mediator implicated in the acute phase of immediate hypersensitivity.⁶⁾

In the earlier work, ellagic acid has been suggested to

ameliorate allergic asthma by suppressing eosinophilic inflammation in a murine model.⁷⁾ However, a direct relationship between ellagic acid and the mast cell-mediated anaphylactic reaction has not yet been understood. The objective of the present study is to investigate whether ellagic acid can inhibit IgE-mediated allergic response in mast cells and *in vivo*, and its mechanism of action. Our results will give an insight into the prevention or treatment of mast cell-dependent allergic diseases.

MATERIALS AND METHODS

Materials Ellagic acid, azelastine, disodium cromoglycate (DSCG), anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), anti-actin antibody and *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). Percoll solution was purchased from Pharmacia (Uppsala, Sweden). Ellagic acid was dissolved in dimethyl sulfoxide (DMSO) and freshly diluted in phosphate-buffered saline (PBS) for all *in vitro* experiments.

Experimental Animals Male Sprague-Dawley rats (8 weeks old, 230–280 g) were purchased from Damool Science (Daejeon, Korea). Animals were housed 3–5 per cages in laminar air-flow cabinet maintained at $22 \pm 1^\circ\text{C}$ and relative humidity of $55 \pm 10\%$ throughout the study. All experiments were performed in compliance with the guidelines approved by Institutional Animal Care and Use Committee of Yanbian University School of Medical Sciences.

Anti-DNP IgE-Mediated Passive Cutaneous Anaphylaxis (PCA) Rats were sensitized in the right dorsal skin by the intradermal injection of 500 ng anti-DNP IgE in 50 μ l PBS and were given a sham PBS injection in the left dorsal skin. Twenty-four hours later, the rats received into the penile vein an injection of 200 μ l of PBS containing 100 μ g DNP-HSA with 1% Evans blue. Ellagic acid [10, 50, 100 mg/kg body weight (BW)] was administered orally at 1 h before the

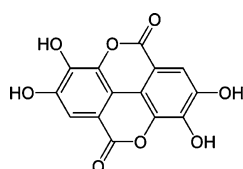


Fig. 1. Chemical Structure of Ellagic Acid

Ellagic acid is a polyphenolic compound found in grapes, nuts and fruits, especially pomegranates and red berries.

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antigen challenge. As a control, an anti-histamine and mast cell-stabilizing agent, azelastine, was orally given at 1 h prior to the challenge with antigen. Thirty minutes after the challenge, the rats were killed by terminal anaesthesia, tissue sections around the intradermal injection site excised and weighed, followed by extraction of extravasated Evans blue dye by incubation of biopsies in 1 ml formamide at 55 °C for 24 h and measurement of absorbance at 620 nm using a spectrophotometer (Spectra MAX PLUS, Molecular Devices, CA, U.S.A.). Tissue Evans blue concentrations were quantified by interpolation on a standard curve of dye concentrations in the range of 0.01 to 30 µg/ml.

Preparation of Rat Peritoneal Mast Cells (RPMC) Suspension Rats were anesthetized with ether and injected with 10 ml of calcium-free HEPES-Tyrod buffer into the peritoneal cavity, and the abdomen was gently massaged for about 90 s. The peritoneal cavity was opened, and the fluid was aspirated using a Pasteur pipette, and RPMC were purified by using a Percoll density gradient as described in detail elsewhere.⁸⁾ RPMC preparations were at least 95% pure as assessed by toluidine blue staining and at least 98% of these cells were viable as judged by trypan blue exclusion. Purified mast cells (1×10^6 cells/ml) were resuspended in HEPES-Tyrod buffer.

Assay of Histamine Release Mast cell suspensions (2×10^5 cells in 200 µl) were sensitized with 10 µg/ml anti-DNP IgE for 6 h and preincubated with ellagic acid (50, 100, 200 µM), the vehicle (0.1% DMSO, used as a control) or DSCG at 37 °C for 5 min prior to the challenge with DNP-HSA (100 ng/ml). Following centrifugation at $150 \times g$ for 10 min, the amount of histamine in the supernatant was determined by the radioenzymatic method.⁹⁾ The inhibition percentage of histamine release was calculated using the following formula:

$$\text{inhibition (\%)} = \{1 - (T - B)/(C - N)\} \times 100$$

Control (C): anti-DNP IgE (+), ellagic acid (−); Normal (N): anti-DNP IgE (−), ellagic acid (−); Test (T): anti-DNP IgE (+), ellagic acid (+); Blank (B): anti-DNP IgE (−), ellagic acid (+).

Assay of TNF-α and IL-6 Secretion RPMC were sensitized with 10 µg/ml anti-DNP IgE for 6 h and then stimulated with 100 ng/ml DNP-HSA for 30 min with or without ellagic acid. TNF-α and IL-6 concentrations in the supernatant were determined by using commercial ELISA kits, according to the manufacturer's instructions (Invitrogen-Biosource Cytokine & Signaling, Camarillo, CA, U.S.A.). The formula for calculating the inhibition rate of the production of TNF-α and IL-6 by ellagic acid is the same as that for estimating the inhibition rate of histamine release.

Western Blot Analysis RPMC pellets were lysed in ice-cold 1% Nonidet P-40-containing lysis buffer (20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 25 µM *p*-nitrophenyl *p'*-guanidinobenzoate, 1 µM pepstatin and 0.1% sodium azide) immediately after stimulation and centrifuged. Protein concentrations of cell lysates were determined using the Bradford reagent (Bio-Rad, Hercules, CA, U.S.A.). Samples (30 µg of protein per lane) were loaded on a 12% SDS-PAGE gel. After electrophoresis at 120 V for 90 min, separated proteins were transferred to

polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.) by the wet transfer method (250 mA, 90 min). The amount of TNF-α and IL-6 was determined using anti-TNF-α (Serotec Ltd., Oxford, United Kingdom) and -IL-6 antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.). Immunodetection was done using enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

Determination of Intracellular Calcium Ion Concentration ($[Ca^{2+}]_i$) RPMC were incubated at 37 °C for 30 min with 5 µM fura 2-AM (Molecular Probes, Eugene, OR, U.S.A.) in HEPES-Tyrod solution after 6 h sensitization with anti-DNP IgE. After washing the dye from the cell surface, aliquots of the cell suspension were placed in a cuvette and incubated with ellagic acid at 37 °C for 5 min, and then 100 ng/ml DNP-HSA was added. Fluorescence was recorded using a fluorometer (Molecular Devices, Sunnyvale, CA, U.S.A.) at an excitation of 340 nm and an emission of 510 nm. Ellagic acid did not exhibit autofluorescence nor interfere with fura 2 fluorescence.

Cytosolic and Nuclear Protein Extractions for Analysis of Nuclear Factor-Kappa B (NF-κB) RPMC were sensitized with 10 µg/ml anti-DNP IgE for 6 h and preincubated with ellagic acid or the vehicle at 37 °C for 5 min prior to the challenge with DNP-HSA (100 ng/ml). The cells were harvested and washed twice with ice-cold PBS. According to the method described by Lee *et al.*, cytosolic and nuclear proteins were obtained from the washed cell pellets.¹⁰⁾ For Western blotting analysis, samples were processed by the procedure mentioned above. The NF-κB activation was assayed using anti-NF-κB p65 antibody (Upstate Biotech, Lake Placid, NY, U.S.A.).

Statistical Analysis The results obtained were expressed as mean ± S.E.M. for the number of experiments. Statistical evaluation of the results was performed using one-way ANOVA, followed by Duncan's multiple range tests. Results with $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

PCA represent models of acute allergic reactions in which mast cells appear to be essential.¹¹⁾ In these models, IgE of defined allergen specificity is injected into the skin of rats, and 24 h thereafter, the specific antigen is administered intravenously. The ensuing FcεRI aggregation of mast cells results in the secretion of multiple preformed mediators. These products in turn produce the local effects including increased cutaneous blood flow, enhanced vascular permeability leading to tissue swelling and itching due to the stimulation of cutaneous sensory nerves by histamine.¹²⁾ The present study indicates that physiological responses associated with PCA were less severe in ellagic acid-treated rats (Table 1). Likewise, azelastine exhibited significant inhibition of PCA at the dose of 10 mg/kg BW. In agreement with this *in vivo* experiment, ellagic acid inhibited the release of preformed mediators (histamine) from RPMC triggered by anti-DNP IgE (Fig. 2). Ellagic acid alone did not affect spontaneous histamine release (data not shown). Thus, we suggest that ellagic acid might inhibit IgE-mediated anaphylaxis by down-regulating mast cell activation.

Activation of mast cells also stimulates cytokine release.

Table 1. Inhibitory Effect of Ellagic Acid on Anti-DNP IgE-Mediated Passive Cutaneous Anaphylaxis in Rats

Treatment (mg/kg BW)	Anti-DNP IgE	Amount of Evans blue ($\mu\text{g/g}$)	Inhibition ^{a)} (%)
None (PBS)	—	51.30 $\pm$ 4.16	—
Ellagic acid 10	—	50.58 $\pm$ 3.72	—
50	—	48.41 $\pm$ 5.69	—
100	—	52.16 $\pm$ 4.33	—
Azelastine 10	—	50.95 $\pm$ 2.54	—
None (PBS)	+	269.57 $\pm$ 18.13	—
Ellagic acid 10	+	225.34 $\pm$ 14.95	19.93
50	+	188.15 $\pm$ 10.60*	35.98
100	+	169.63 $\pm$ 8.21*	46.18
Azelastine 10	+	153.47 $\pm$ 12.29*	53.06

Ellagic acid was administered orally at 1 h prior to the challenge with antigen (DNP-HSA). Each amount of Evans blue represents the mean $\pm$ S.E.M. of five independent experiments. *a)* Inhibition (%) = $\{1 - (T - B)/(C - N)\} \times 100$. Control (C): anti-DNP IgE (+), ellagic acid (-); Normal (N): anti-DNP IgE (-), ellagic acid (-); Test (T): anti-DNP IgE (+), ellagic acid (+); Blank (B): anti-DNP IgE (-), ellagic acid (+). * $p < 0.05$, significantly different from the control value. Azelastine (10 mg/kg) was used as a typical anti-allergic control drug.

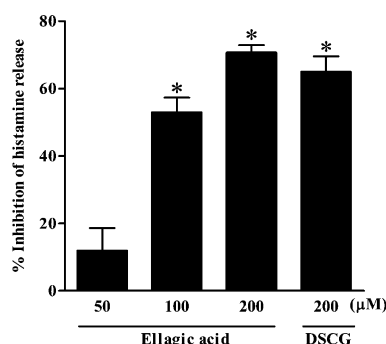
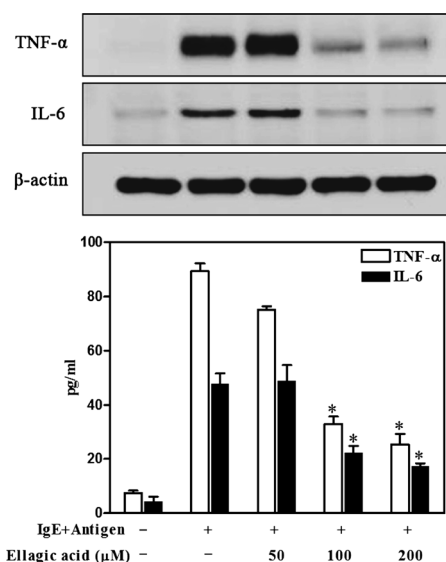


Fig. 2. Inhibitory Effect of Ellagic Acid on Anti-DNP IgE-Mediated Histamine Release from Rat Peritoneal Mast Cells (RPMC)

RPMC were preincubated with ellagic acid at 37 °C for 5 min prior to the incubation with DNP-HSA. Each data value represents the mean $\pm$ S.E.M. of five independent experiments. * $p < 0.05$, significantly different from the control value.

Thus, we first determined whether ellagic acid could modify the protein expression of cytokines. Ellagic acid dose-dependently inhibited the antigen-stimulated expression of protein for TNF- α and IL-6 (Fig. 3). To further prove the above results, we next tested whether ellagic acid suppressed the secretion of both cytokines in the cells. In consistent with the Western blotting results, in the presence of ellagic acid, a dose-dependent suppression of the secretion of both cytokines was shown (Fig. 3). Ellagic acid significantly inhibited the release of TNF- α and IL-6 at 100 μM (69.4 and 57.9% inhibition, respectively), and at 200 μM of ellagic acid, the inhibition was 77.7 and 68.5% inhibition, respectively. Pro-inflammatory cytokines, particularly TNF- α and IL-6, are known to play a central biological role in triggering and sustaining allergic inflammation in mast cells.^{13,14)} From this, reduction of pro-inflammatory cytokines from mast cells is considered to be one of the key indicators of attenuated allergic symptom. Therefore, our findings propose that ellagic acid could suppress acute allergic inflammation through the regulation of cytokines production in mast cells.

To gain insight into the mechanism of mast cell inhibition by ellagic acid, we investigated its effects on calcium signaling and NF- κB activation in mast cells. As noted, increased

Fig. 3. Inhibitory Effect of Ellagic Acid on the Anti-DNP IgE-Mediated Protein Expression and Production of Cytokines (TNF- α and IL-6) in Rat Peritoneal Mast Cells (RPMC)

RPMC were sensitized with anti-DNP IgE and preincubated at 37 °C for 5 min in the absence or presence of ellagic acid before the challenge with DNP-HSA. The protein expression of these cytokines was measured by Western blotting. A representative result was presented among at least five separate experiments with similar results. The level of cytokine in the supernatant was measured by using ELISA. Each data value represents the mean $\pm$ S.E.M. of five independent experiments. * $p < 0.05$, significantly different from the control value.

[Ca²⁺]_i functions as an essential trigger for the secretion of inflammatory mediators, activation of transcription factors and elaboration of cytokines by IgE-stimulated mast cells. This Ca²⁺ response is supposed to occur in (at least) two steps: first one is calcium release from the internal store and second one is calcium influx from the external environment.¹⁵⁾ Ellagic acid suppressed the increase in [Ca²⁺]_i in RPMC activated by IgE-mediated antigen stimulation (Fig. 4A). This effect is consistent with the inhibition of degranulation and cytokine production by ellagic acid. Next, an additional experiment was performed to determine which type of Ca²⁺ response is impaired by ellagic acid in the IgE-sensitized RPMC. In a Ca²⁺-free solution, DNP-HSA induced a transient increase in [Ca²⁺]_i that may be attributable to Ca²⁺ release from the internal store. This increase was dose-dependently inhibited by ellagic acid (Fig. 4B). After cessation of the DNP-HSA-induced Ca²⁺ release, the re-addition of Ca²⁺ to the store-depleted cells led to a fast [Ca²⁺]_i increase and a plateau indicating the presence of an influx pathway for Ca²⁺. This increase was also attenuated by ellagic acid in a dose-dependent way (Fig. 4B). From these, ellagic acid was found to suppress both the Ca²⁺ release from the internal store and the following Ca²⁺ influx.

Previous reports have demonstrated that NF- κB plays a critical role in immune and inflammatory responses, including asthma and anaphylaxis.¹⁶⁾ Activation of NF- κB has also been observed after Fc ϵ RI-mediated allergic response in mast cells.¹⁷⁾ Determination of NF- κB protein level in nuclear extracts has revealed that this protein level is substantially increased in IgE-stimulated RPMC, suggesting that NF- κB is activated (Fig. 5). However, the NF- κB p65 levels in nucleus were decreased by the treatment of ellagic acid. In contrast, levels of NF- κB p65 protein in cytosolic extracts

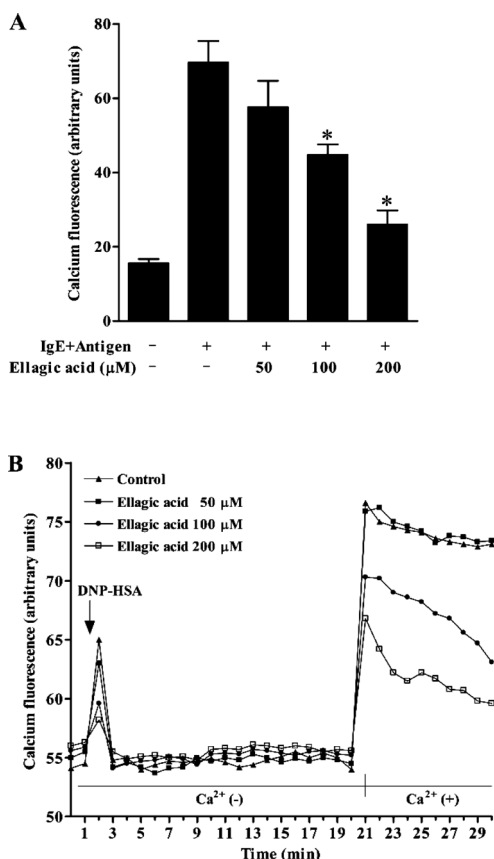


Fig. 4. Effect of Ellagic Acid on Ca^{2+} Signaling (A, Increase in $[\text{Ca}^{2+}]$; B, Ca^{2+} Release and Influx) in Anti-DNP IgE-Stimulated Rat Peritoneal Mast Cells (RPMC)

RPMC were sensitized with anti-DNP IgE and preincubated at 37°C for 5 min in the absence or presence of ellagic acid before the challenge with DNP-HSA. The level of calcium fluorescence was recorded using fluorometer. Each data value represents the mean $\pm$ S.E.M. of five independent experiments. * $p < 0.05$, significantly different from the control value.

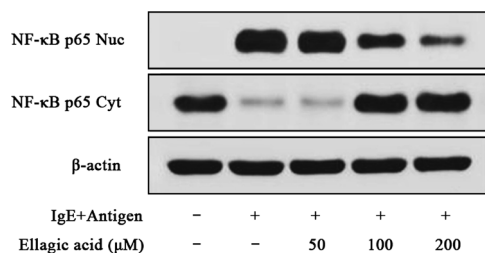


Fig. 5. Effect of Ellagic Acid on NF- κ B Expression in Nuclear and Cytosolic Protein Extracts from Anti-DNP IgE-Stimulated Rat Peritoneal Mast Cells (RPMC)

NF- κ B p65 expression in nuclear (Nuc) and cytosolic (Cyt) protein extracts from RPMC was shown. A representative result was presented among at least five separate experiments with similar results.

from IgE-stimulated RPMC were decreased compared with the levels in the normal group. The NF- κ B p65 levels in cytosol preparations were increased by the treatment of ellagic acid. These results indicate that ellagic acid inhibits NF- κ B

activity by preventing translocation of this transcription factor into the nucleus. Several evidences have also shown that expression of TNF- α and IL-6 is dependent on the activation of NF- κ B.¹⁸⁾ Therefore, these data suggest that the inhibition of NF- κ B activity by ellagic acid modulates allergic inflammatory processes through suppression of the production of inflammatory cytokines.

In conclusion, our results provide evidence that ellagic acid can inhibit the IgE-mediated anaphylactic reaction in *in vivo* and *in vitro* murine models. The inhibition of pro-inflammatory mediator release by ellagic acid appears to be involved in the suppression of $[\text{Ca}^{2+}]$ increases and NF- κ B activity. Ellagic acid may therefore be suitable for the treatment of allergic and inflammatory diseases. Further studies are needed to elucidate the possibility that ellagic acid may also be effective in the human mast cells and in the treatment of human allergic disorders.

Acknowledgments This study was supported by a Grant from Yanbian University Center of Medical Development.

REFERENCES

- 1) Yuce A., Atessahin A., Ceribasi A. O., *Basic Clin. Pharmacol. Toxicol.*, **103**, 186—191 (2008).
- 2) Hassoun E. A., Vodhanel J., Abushaban A., *J. Biochem. Mol. Toxicol.*, **18**, 196—203 (2004).
- 3) Whitley A. C., Stoner G. D., Darby M. V., Walle T., *Biochem. Pharmacol.*, **66**, 907—915 (2003).
- 4) Loarca-Pina G., Kuzmicky P. A., de Mejia E. G., Kado N. Y., *Mutat. Res.*, **398**, 183—187 (1998).
- 5) Galli S. J., Gordon J. R., Wershil B. K., *Curr. Opin. Immunol.*, **3**, 865—872 (1991).
- 6) Petersen L. J., Mosbech H., Skov P. S., *J. Allergy Clin. Immunol.*, **97**, 672—679 (1996).
- 7) Rogerio A. P., Fontanari C., Borducchi E., Keller A. C., Russo M., Soares E. G., Albuquerque D. A., Faccioli L. H., *Eur. J. Pharmacol.*, **580**, 262—270 (2008).
- 8) Hachisuka H., Nomura H., Sakamoto F., Mori O., Okubo K., Sasai Y., *Arch. Dermatol. Res.*, **280**, 158—162 (1988).
- 9) Harvima R. J., Harvima I. T., Fraki J. E., *Clin. Chim. Acta*, **171**, 247—256 (1988).
- 10) Lee K. S., Kim S. R., Park H. S., Jin G. Y., Lee Y. C., *J. Allergy Clin. Immunol.*, **114**, 1093—1099 (2004).
- 11) Kabu K., Yamasaki S., Kamimura D., Ito Y., Hasegawa A., Sato E., Kitamura H., Nishida K., Hirano T., *J. Immunol.*, **177**, 1296—1305 (2006).
- 12) Neel N. F., Creasy B. M., Rankin J. N., Pierce E. M., McCoy M. E., Daner R. H., Fowler J. A., Daniel J. C., Lantz C. S., *Immunol. Lett.*, **95**, 37—44 (2004).
- 13) Thomas P. S., *Immunol. Cell Biol.*, **79**, 132—140 (2001).
- 14) Mican J. A., Arora N., Burd P. R., Metcalfe D. D., *J. Allergy Clin. Immunol.*, **90**, 815—824 (1992).
- 15) Shumilina E., Lam R. S., Wolbing F., Matzner N., Zemtsova I. M., Sobiesiak M., Mahmud H., Sausbier U., Biedermann T., Ruth P., Sausbier M., Lang F., *J. Immunol.*, **180**, 8040—8047 (2008).
- 16) Siebenlist U., Franzoso G., Brown K., *Annu. Rev. Cell Biol.*, **10**, 405—455 (1994).
- 17) Klemm S., Ruland J., *Immunobiology*, **211**, 815—820 (2006).
- 18) Azzolina A., Bongiovanni A., Lampiasi N., *Biochim. Biophys. Acta*, **1643**, 75—83 (2003).