Original Research

The antiallergic mechanisms of *Citrus sunki* and bamboo salt (K-ALL) in an allergic rhinitis model

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Abstract

The antiallergic effects of traditional medicines have long been studied. Traditional Korean medicine, *Citrus sunki* and bamboo salt, has been used for the treatment of allergic diseases in Korea. K-ALL, composed of *Citrus sunki* and bamboo salt, is a newly prepared prescription for allergic patients. To develop the new antiallergic agent, we examined the effects of K-ALL through *in vivo* and *in vitro* models. K-ALL and naringin (an active compound of K-ALL) significantly inhibited histamine release from rat peritoneal mast cells. This inhibitory effect of K-ALL on histamine release was higher than effects from other known histamine inhibitors such as bamboo salt, *Citrus sunki* or disodium cromoglycate. K-ALL significantly inhibited systemic anaphylactic shock induced by the compound 48/80 and passive cutaneous anaphylaxis induced by the IgE. K-ALL also inhibited production and mRNA expression of inflammatory cytokines induced by phorbol 12-myristate 13-acetate and the calcium ionophore A23187 on HMC-1 cells (a human mast cell line). In the ovalbumin-induced allergic rhinitis animal model, rub scores, histamine, IgE, inflammatory cytokines and inflammatory cell counts were all reduced by the oral or nasal administration of K-ALL (pre and posttreatment). These results indicate the great potential of K-ALL as an active immune modulator for the treatment of mast cell-mediated allergic diseases.

Keywords: K-ALL, naringin, systemic anaphylactic shock, passive cutaneous anaphylaxis, allergic rhinitis

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Introduction

Allergic rhinitis (AR) is a common and very serious public health problem with a high and increasing prevalence.[1–3] AR is primarily characterized by nasal symptoms such as sneezing, itching, rhinorrhea, nasal congestion and post-nasal drip.[4] AR was formerly classified as seasonal allergic rhinitis (SAR), which occurs in the presence of seasonal allergens (such as grass and tree pollens), and perennial allergic rhinitis (PAR), which occurs throughout the year and is related to the presence of non-seasonal allergens (such as house dust mites, animal dander and mould).[3,4] Both SAR and PAR are IgE-mast cell-mediated hypersensitivity reactions and are divided into two phases with the mast cell playing a central role.[5,6] Mast cells are activated by IgE, and then potent inflammatory mediators such as histamine, proteases, interleukin (IL)-1β, IL-6, thymic stromal lymphopoietin (TSLP), tumor necrosis factor (TNF)-α and metabolites of arachidonic acid are released.[7] Chemotactic factors released by mast cells induce the production of IgE from B-cells, enhance the production of Th2-lymphocytes, attract eosinophils and activate vascular endothelial corneal and conjunctival cells to release chemokines and adhesion molecules.[8,9] The chemokines (macrophage inflammatory protein-2; MIP-2) and adhesion molecules (intercellular adhesion molecule-1; ICAM-1) mediate the infiltration of eosinophils, basophils, neutrophils and Th2-lymphocytes to sites of inflammation and couple with newly formed mediators to sustain mast-cell activation in the late phase response.[10–14]

Mast-cell stabilizers, antihistamines, antihistamine–vasoconstrictor combinations and dual action agents (with mast-cell stabilizing and antihistaminic properties) are widely used to manage AR.[15] However, current conventional medications for allergic disorders always have adverse effects for this chronic disease.[16] It is therefore urgent to find new and safe treatments for AR as it has become a major health problem. Traditional medicine therapies are generally considered safe and effective by the general population and are increasingly attractive for the treatment of various diseases.[16] We have previously found that various traditional medicines used in the treatment of

AR have antiallergic, anti-inflammatory and immune modulation activities.[17–19] Among others tested with bamboo salt against allergic responses, *Citrus sunki* had substantial activity. Therefore, K-ALL is composed of bamboo salt and *Citrus sunki*. We investigated the antiallergic effects of K-ALL and one of its components, naringin (NG), in mast cells and ovalbumin (OVA)-induced AR mice.

Materials and methods

Reagents

We purchased phorbol 12-myristate 13-acetate (PMA), A23187 (calcimycin; $C_{29}H_{37}N_3O_6$), compound 48/80, anti-dinitrophenyl (DNP)-IgE, DNP-human serum albumin (HSA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), disodium cromoglycate (DSCG), 2′-azinobis (3-ethylbenzithiazoline-6-sulphonic acid) tablets substrate (ABTS), dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), bovine serum albumin (BSA), *o*-phthaldialdehyde (OPA), bicinchoninic acid (BCA), avidin–peroxidase, OVA and Evans blue from Sigma Chemical Co. (St. Louis, MO, USA); Isocove's modified Dulbecco's medium (IMDM), penicillin, streptomycin and fetal bovine serum (FBS) from Gibco BRL (Grand Island, NY, USA); antimouse IgE/TSLP/IL-1β/IL-4/IL-6/ICAM-1/MIP-2/TNF-α/IFN-γ antibody (Ab), biotinylated anti-mouse IgE/TSLP/IL-1β/IL-4/IL-6/ICAM-1/MIP-2/TNF-α/Inferon (IFN)-γ Ab, recombinant mouse (rm) IgE/TSLP/IL-1β/IL-4/IL-6/ICAM-1/MIP-2/TNF-α/IFN-γ, antihuman IL-1β/IL-6/TSLP/TNF-α Ab, biotinylated anti-human IL-1β/IL-6/TSLP/TNF-α Ab and recombinant human (rh) IL-1β/IL-6/TSLP/TNF-α from Pharmingen (Sandiego, CA, USA) and R&D Systems (Minneapolis, MN, USA); and Ab for cyclooxygenase (COX)-2 and actin from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of K-ALL

K-ALL was provided by Tae-sung Food Inc. (Jeonbuk, Republic of Korea). The dried herb, *Citrus sunki* Hort. ex Tanaka (100 g), was extracted by decocting with boiling distilled water for approximately 2 h 30 min. The decoction was then filtered, lyophilized and kept at 4°C. The yield of dried extract from starting materials was about 11% (w/w). Powdered *Citrus sunki* was then prepared by dissolving with a bamboo salt solution (0.9%) and filtered through a 0.22-μm syringe filter. Dilutions of K-ALL were made in bamboo salt solution (0.9%). We performed the preliminary test to determine the K-ALL concentration in the various doses and used final concentration of 0.01, 0.1 and 1 mg/mL (or g/kg) of K-ALL for *in vitro* or *in vivo* study. K-ALL was analysed by high-performance liquid chromatography. NG is a major constituent of K-ALL with an approximate concentration of 7.00 ± 1.72 mg/g (data not shown).

Culture of HMC-1 cells

The human mast-cell line (HMC-1) was generously provided by Eichi Morri (Osaka University, Japan). HMC-1 cells were grown in IMDM supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin and 10% heat-inactivated FBS at 37°C in 5% CO_2 with 95% humidity.

Animals

The original stock of male ICR (4 weeks old) and male SD rats (7 weeks old) were purchased from the Dae-Han Experimental Animal Center (Eumsung, Chungbuk, Republic of Korea). The animal care and experimental procedures were performed with the approval from the Animal Care Committee of Kyung Hee University.

Preparation of rat peritoneal mast cells

We maintained male SD rats under pathogen-free conditions. In brief, rats were anesthetized with ether and injected with 30 mL of Tyrode buffer B (137 mM NaCl, 5.6 mM glucose, 12 mM $NaHCO_3$, 2.7 mM KCl and 0.3 mM Na_2HPO_4) containing 0.1% gelatin into the peritoneal cavity, and the abdomen was gently massaged for about 2 min. The peritoneal cavity was carefully opened, and the fluid-containing peritoneal cells were aspirated with a Pasteur pipette. Thereafter, peritoneal cells were sedimented at $150 \times g$ for 10 min at room temperature and resuspended in Tyrode buffer B. To separate mast cells from the major components of rat peritoneal cells (e.g. macrophages and small lymphocytes), suspended peritoneal cells were layered on 2 mL of 22.5% metrizamide (density, 1.12 g/mL) and centrifuged at $400\times$ g for 10 min at room temperature. The cells remaining at the buffer metrizamide interface were aspirated and discarded, while the cells in the pellet were washed and resuspended in 1 mL of Tyrode buffer A (10 mM HEPES, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 0.1% bovine serum albumin) containing calcium. Mast-cell preparations, about 95% pure, were assessed with toluidine blue staining. More than 97% of the cells were viable, as judged by trypan blue uptake.

Histamine assay

The rat peritoneal mast cells (RPMCs) (2×10^5) were treated with K-ALL (0.01, 0.1 or 1 mg/mL), bamboo salt (0.9%), *Citrus sunki* (1 mg/mL), DSCG (10 μg/mL) and NG (0.1 or 1 mM) for 40 min prior to stimulation with compound 48/80 (6 μg/mL) and incubated for 25 min. Histamine content in the serum and supernatant was measured by the OPA spectrofluorometric procedure.[20] The fluorescent intensity was measured at 440 nm (excitation at 360 nm) with a spectrofluorometre.

Compound 48/80-induced systemic anaphylaxis

Mice were given an intraperitoneal (i.p.) injection of compound 48/80 (8 mg/kg), a mast cells degranulator. The period for observing mortality was based on the control mice, which died within 20 min of receiving compound 48/80. Mortality was monitored for 30 min after induction of anaphylactic shock.

Passive cutaneous anaphylaxis

An IgE-dependent passive cutaneous anaphylaxis (PCA) was generated by sensitizing the skin with an intradermal injection of anti-DNP-IgE, followed 48 h later with an injection of DNP-HSA (diluted in PBS) into the mice tail vein. The mice were intradermally injected with 100 ng of anti-DNP-IgE into each of three dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. After 48 h, each mouse received an injection of 200 μL of a 1 : 1 mixture of 1 mg/mL DNP-HSA in PBS and 4% Evans blue via the tail vein. One hour before this injection, K-ALL (0.01, 0.1 or 1 g/kg) and NG (0.1 or 1 mg/kg) were orally administered. The mice were euthanized 40 min after the intravenous challenge. The dorsal skin of each mouse was removed to measure the pigment area. The amount of dye was then colorimetrically determined after extraction with 0.5 mL of 1 M KOH and 4.5 mL of a mixture of acetone and phosphoric acid (5 : 13 ratio, respectively). The absorbent intensity of the extraction was measured at 620 nm in a spectrophotometre, and the amount of dye was calculated with the Evans blue measuring line.

Enzyme-linked immunosorbent assay

HMC-1 cells (3×10^5) were treated with K-ALL (0.01, 0.1 or1 mg/mL), bamboo salt (0.9%), *Citrus sunki* (1 mg/mL), dexamethasone (100 nM) or NG (0.1 or 1 mM) for 1 h prior to stimulation with PMA and A23187 for 8 h. Cytokines in the serum, mucosa, spleen tissue and supernatant were measured by enzyme-linked immunosorbent assay (ELISA). The ELISA was performed using 96-well plates coated with 1 μg/well of capture Ab. The coated plates were washed twice with 1 × PBS containing 0.05% Tween-20 (PBST). All reagents and coated wells used in this assay were incubated for 2 h at room temperature. The standard curve was generated from known concentrations of the cytokine examined, as provided by the manufacturer. After exposure to the media, the assay plates were sequentially exposed to the proper biotin-conjugated secondary antibodies, then AP and an ABTS substrate solution containing 30% H_2O_2. The plates were read at 405 nm. Appropriate specificity controls were included, and all samples were run in duplicate. Cytokine levels in the spleen and nasal mucosa were divided according to the total protein. Protein amounts were determined using the BCA assay.

MTT assay

HMC-1 cell aliquots (3×10^5 cells/mL) were cultured in microplate wells for 8 h after treatment with K-ALL (0.01, 0.1 or 1 mg/mL), bamboo salt (0.9%), *Citrus sunki* (1 mg/mL), dexamethasone (100 nM) or NG (0.1 or 1 mM) and incubated with 20 μL of an MTT solution (5 mg/mL) for an additional 4 h at 37°C under 5% CO_2 and 95% air. Afterwards, 250 μL of DMSO was added to extract the MTT formazan, and the absorbance of each well was read by an automatic microplate reader at 540 nm.

OVA-induced AR animal model

The method described by Jeong *et al.*[13] was used to confirm the antiallergic effect of K-ALL in AR animal model. We maintained 6-week-old female BALB/c mice under pathogen-free conditions. We sensitized mice on days 1, 5 and 14 by an i.p. injection of 100 μg OVA emulsified in 20 mg of aluminum hydroxide (Sigma), and we challenged mice with 1.5 mg of OVA. Under pretreatment conditions, mice received K-ALL (0.01, 0.1 or 1 g/kg) or NG (0.1 or 1 mg/kg) for 10 days by oral or nasal administration before the intranasal OVA challenge. Under post-treatment conditions, mice received K-ALL (1 g/kg) for 10 days by oral or nasal administration after the intranasal OVA challenge. Nasal symptoms were evaluated by counting the number of nasal rubs that occurred in 10 min after OVA i.n. provocation at 10 days after the challenge. Five mice were in each group.

Reverse transcription-polymerase chain reaction

Total RNA was isolated from the nasal mucosa according to the manufacturer's specification using the easy-BLUETM RNA extraction kit (iNtRON Biotech, Korea). The concentration of total RNA in the final eluates was determined by spectrophotometry. Total RNA (2.5 μg) was heated at 65°C for 10 min and then chilled on ice. Each sample was reverse-transcribed to cDNA for 90 min at 37°C using a cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA). PCR was performed with the following primers for human TSLP (5′ TAT GAG TGG GAC CAA AAG TAC CG 3′; 5′ GGG ATT GAA GGT TAG GCT CTG G 3′), human IL-1β (5′ GGG GTA CCT TAG GAA GAC ACA AAT TG 3′; 5′ CCG GAT CCA TGG CAC CTG TAC GAT CA 3′), human IL-6 (5′ GAT GGA TGC TTC CAA TCT GGA T 3′; 5′ AGT TCT CCA TAG AGA ACA ACA TA 3′), human TNF-α (5′ CAC CAG CTG GTT ATC TCT CAG CTC 3′; 5′ CGG GAC GTG GAG CTG GCC GAG GAG 3′) and human GAPDH (5′ CCT GCT TCA CCA CCT TCT TG 3′; 5′ CAA AAG GGT CAT CAT CTC TG 3′). GAPDH was used to verify that equal amounts of RNA were used for reverse transcription and PCR amplification from different experimental conditions. An annealing temperature of 50°C was used to detect human IL-1β, IL-6 and TNF-α, while an annealing temperature of 60°C was used to detect human TSLP and GAPDH. Products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Western blot analysis

The nasal mucosa tissue was prepared by a detergent lysis procedure. Samples were heated at 95°C for 5 min and briefly cooled on ice. Following centrifugation at 15,000 × g for 5 min,g aliquots were resolved by 10% SDS–PAGE. Resolved proteins were electrotransferred overnight to nitrocellulose membranes in 25 mM Tris, pH 8.5, 200 mM glycerin and 20% methanol at 25 V. Blots were blocked for at least 2 h with PBST containing 5% nonfat dry milk and then incubated with primary antibodies for 1 h at room temperature. Blots were developed and probed with peroxidase-conjugated secondary antibodies, and proteins were visualized by enhanced chemiluminescence procedures

(Amersham Bioseciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

Histological examination

Tissue samples were immediately fixed with 10% formaldehyde and embedded in paraffin. Nasal mucosa samples were sectioned to a 4-μm thickness. Each section was stained with hematoxylin and eosin (H&E, for eosinophils) before dewaxing and dehydration. The numbers of eosinophils on both sides of the septal mucosa were counted. Sections were coded and randomly analysed by two blinded observers.

Confocal laser scanning microscopy

Tissue samples were fixed with 4% formaldehyde and embedded in paraffin. After dewaxing and dehydration, sections were blocked with bovine serum albumin followed by 60 min of incubation with an antimouse c-Kit (Santa Cruz Biotechnology) at a concentration of 1 μg/mL. The secondary antibody, tetramethylrhodamine-5-(and-6-)-isothiocyanate (TRITC)-conjugated antimouse IgG (Invitrogen), was added to the incubation for 30 min. The mounting medium, containing 4′,6-diamidino-2-phenylinodole (DAPI, Vector Laboratories, Burlingame, CA, USA), was used to counterstain the DNA. All specimens were examined with a confocal laser-scanning microscope. The numbers of mast cells on both sides of the septal mucosa were counted. Sections were coded and randomly analysed by two blinded observers.

Statistical analysis

The experiments shown are a summary of the data from at least three experiments, and statistical analyses were performed using SPSS statistical software (SPSS 11.5, Armonk, NY, USA). Treatment effects were analysed by an independent *t*-test and one-way ANOVA with a Tukey's *post hoc* test. Significance was determined for $P < 0.05$.

Results

Effect of K-ALL on the histamine release from RPMCs

To clarify the effect of K-ALL on the degranulation of RPMCs, we measured histamine release from RPMCs. The RPMCs were pretreated with K-ALL (0.01, 0.1 or 1 mg/mL), bamboo salt (0.9%), *Citrus sunk* (1 mg/mL), DSCG (10 μg/mL) or NG (0.1 or 1 mM) for 40 min prior to stimulation with compound 48/80 for 25 min. DSCG (mast cell stabilizer) was used as a reference drug. The K-ALL, bamboo salt, *Citrus sunki*, DSCG and NG significantly inhibited compound 48/80-induced histamine release from RPMCs (Figure 1, $P < 0.05$). The inhibitory effect of K-ALL (1 mg/mL) was higher than bamboo salt, *Citrus sunki*, DSCG or NG on histamine release. K-ALL, bamboo salt,

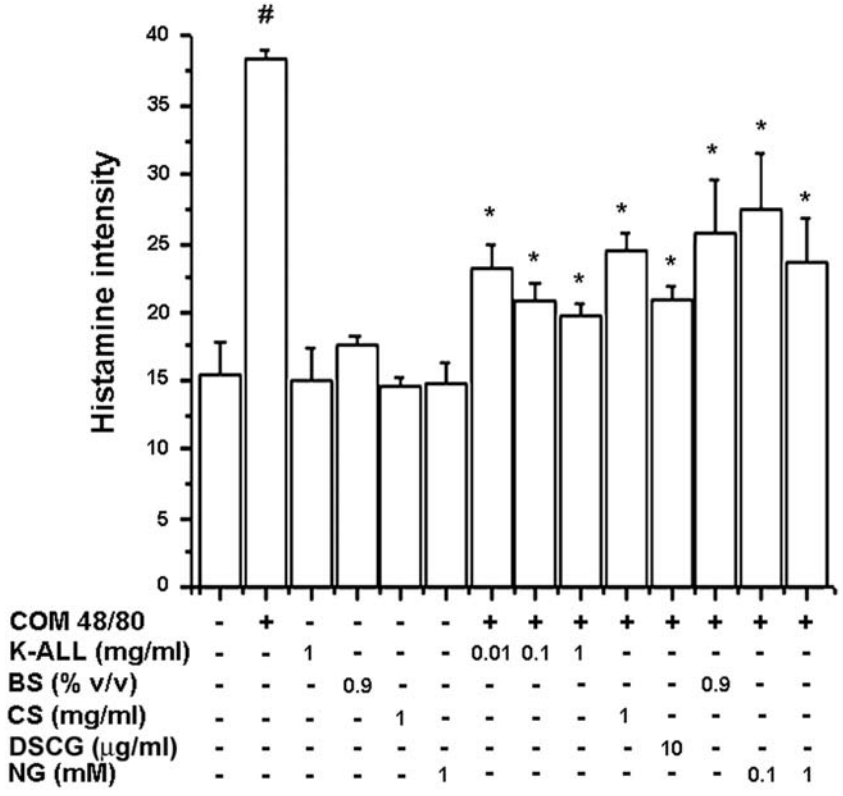

Figure 1 Effect of K-ALL on the compound 48/80-induced histamine release from RPMCs. RPMCs (2×10^5 cells/mL) were treated with K-ALL (0.01, 0.1 or 1 mg/mL), bamboo salt (0.9%), *Citrus sunki* (1 mg/mL), DSCG (10 μg/mL) or naringin (0.1 or 1 mM) for 40 min prior to stimulation with compound 48/80 (6 μg/mL) for 25 min. Histamine release was measured from culture supernatants. Values are the mean ± SEM of duplicate determinations from three separate experiments. $^{\#}P < 0.05$, significantly different from non-stimulated cells; $^{*}P < 0.05$, significantly different from compound 48/80 alone. BS: bamboo salt; COM 48/80: compound 48/80; CS: *Citrus sunki*; DSCG: disodium cromoglycate; NG: naringin; RPMCs: rat peritoneal mast cells

Citrus sunki, DSCG or NG alone had no effect on histamine release from non-stimulated cells.

Effect of K-ALL on the systemic anaphylaxis

To examine the contribution of K-ALL in anaphylactic reactions, we used an *in vivo* model of systemic anaphylactic reactions. Compound 48/80 (8 mg/kg) was used as a systemic fatal anaphylaxis inducer. After injection with compound 48/80, the mice ($n = 10$ per group) were monitored for 30 min, and mortality rate was determined. The oral administration of saline as a control induced a fatal reaction in 100% of each group (Figure 2a). However, when K-ALL or NG was orally administered for 1 h, the mortality from compound 48/80 was significantly reduced (Figure 2a, $P < 0.05$). Treatment with K-ALL (0.1 or 1 g/kg) or NG (0.1 or 1 mg/kg) alone (without Compound 48/80) did not appear to induce physiological differences. Histamine levels in serum after systemic anaphylaxis were significantly reduced by K-ALL or NG (Figure 2b, $P < 0.05$).

Effect of K-ALL on the PCA reaction

PCA is one of the most important murine models of anaphylaxis in allergic reactions. To confirm the antiallergic effect of K-ALL *in vivo*, we performed the PCA reaction on the dorsal skin of mice. When K-ALL (0.01, 0.1 or 1 g/kg) was orally administered, the PCA reaction was significantly inhibited. We also tested the effect of NG (0.1 or 1 mg/kg) on the PCA reaction. The PCA reaction was significantly inhibited by NG (Figure 3a, $P < 0.05$). K-ALL (1 g/kg) or NG (1 mg/kg) alone had no effect on the PCA reaction (data not shown). We also analysed levels of inflammatory cytokines in serum after PCA. It may help to clarify whether K-ALL or NG has an anti-inflammatory effect. The levels of IL-1β, IL-6, TSLP and TNF-α in the serum of IgE-induced PCA mice were more highly increased compared to normal mice (Figure 3b). However, mice administered with K-ALL or NG significantly inhibited IL-6 levels compared with IgE-induced PCA mice (Figure 3b, $P < 0.05$).

Effect of K-ALL on the inflammatory cytokine production in HMC-1 cells

To assess the inhibitory effect of K-ALL on cytokine production in mast cells, we examined the effect of K-ALL or NG on PMA plus A23187-induced IL-1 β, IL-6, TSLP and TNF-α production in HMC-1 cells. The HMC-1 cells were pretreated with K-ALL, bamboo salt, *Citrus sunki*, dexamethasone or NG for 1 h prior to stimulation. Increased IL-1 β, IL-6, TSLP and TNF-α production by PMA plus A23187 was significantly inhibited by K-ALL treatment in a dose-dependent manner (Figure 4a, $P < 0.05$). NG, bamboo salt, *Citrus sunki* or dexamethasone also significantly inhibited inflammatory cytokine production. K-ALL alone (1 mg/mL) had no effect on cytokine production in HMC-1 cells. Increased IL-1β, IL-6, TSLP and TNF-α mRNA levels by PMA plus A23187 were also reduced by K-ALL or NG (Figure 4b). We also performed an MTT assay for 24 h to evaluate the cytotoxicity of drugs (K-ALL, NG, bamboo salt, *Citrus sunki* or dexamethasone) on mast cells. These drugs did not affect the cell viability of HMC-1 cells (Figure 4c).

Effects of K-ALL in the AR animal model

To assess the regulatory effects of K-ALL in the AR animal model, we orally or nasally administered mice with K-ALL or NG. The clinical symptoms were significantly elevated in

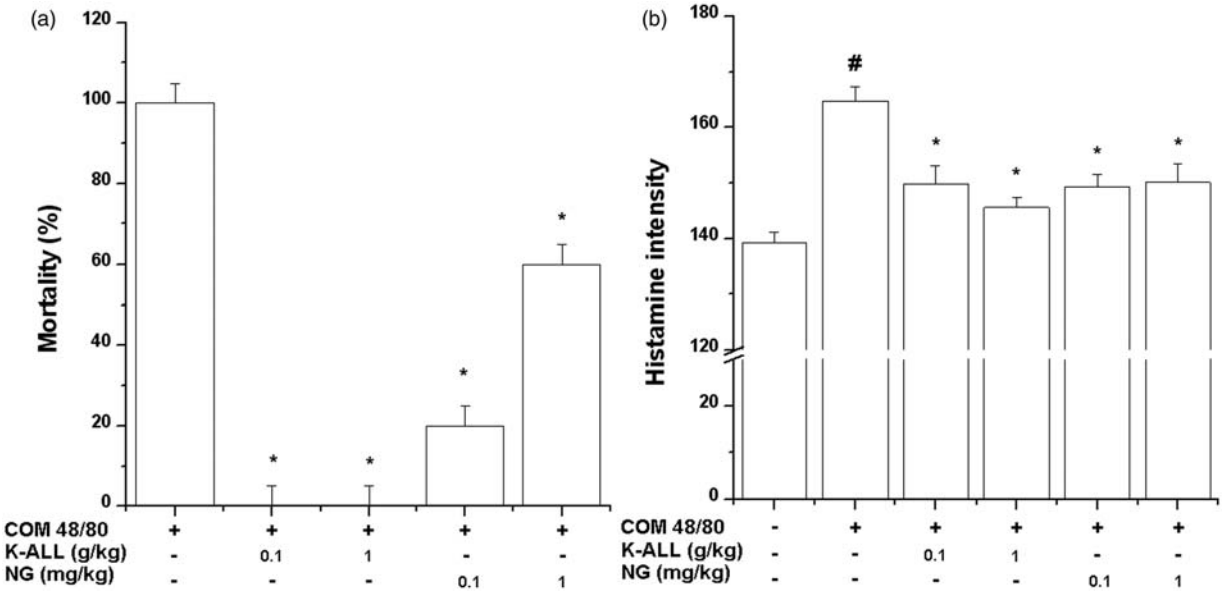

Figure 2 Effect of K-ALL on the compound 48/80-induced systemic anaphylaxis. (a) Mice orally administered with K-ALL (0.1 or 1 mg/kg) or naringin (0.1 or 1 mg/kg) 1 h before compound 48/80 (8 mg/kg) injection. Mortality (%) is presented as the number of dead mice × 100/total number of experimental mice. (b) Serum histamine was analysed by the histamine assay. Each datum represents the mean ± SEM. $n = 10$. *$P < 0.05$, significantly different from mice treated with compound 48/80 alone. COM 48/80: compound 48/80; NG: naringin

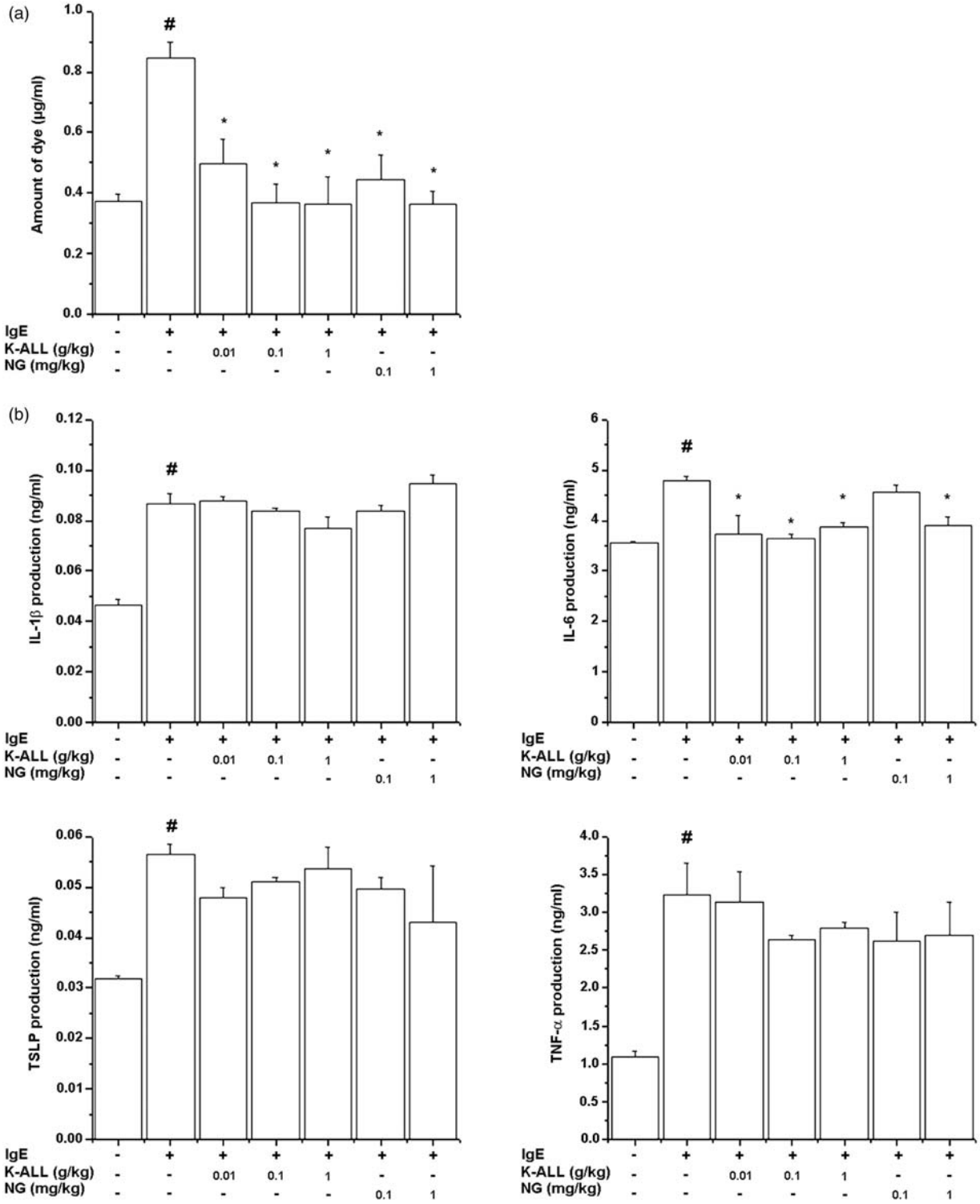

Figure 3 Effect of K-ALL on the passive cutaneous anaphylaxis. Mice were injected intradermally with 100 ng of anti-DNP-IgE. K-ALL (0.01, 0.1 or 1 g/kg) or naringin (0.1 or 1 mg/kg) were administered orally 1 h prior to the 48 h challenge with 1 mg/mL of antigen (DNP-HSA) containing 4% Evans blue (1:1) via the tail vein. (a) Amount of dye; (b) IL-1β, IL-6, TSLP and TNF-α protein levels were measured by the ELISA method in the PCA mice serum. $n = 5$. $^{\#}P < 0.05$, significantly different from normal mice; $^{*}P < 0.05$, significantly different from IgE-DNP + HSA mice. ELISA: enzyme-linked immunosorbent assay; IgE: IgE-DNP + HSA; NG: naringin; PCA: passive cutaneous anaphylaxis

the OVA-induced AR mice compared with normal mice. Increased rub scores, spleen weight, serum histamine and total IgE were significantly inhibited by the pretreatment with K-ALL (Figure 5a to d, $P < 0.05$). The post-treatment with K-ALL after OVA challenge also significantly reduced clinical symptoms ($P < 0.05$). Levels of inflammatory cytokines (IL-1β, IL-6 and TSLP) in serum were significantly inhibited by K-ALL or NG administration (Figure 5e to g, $P < 0.05$). To identify Th1/Th2 immune reactions in K-ALL or NG-administered mice, we measured IL-4 and IFN-γ

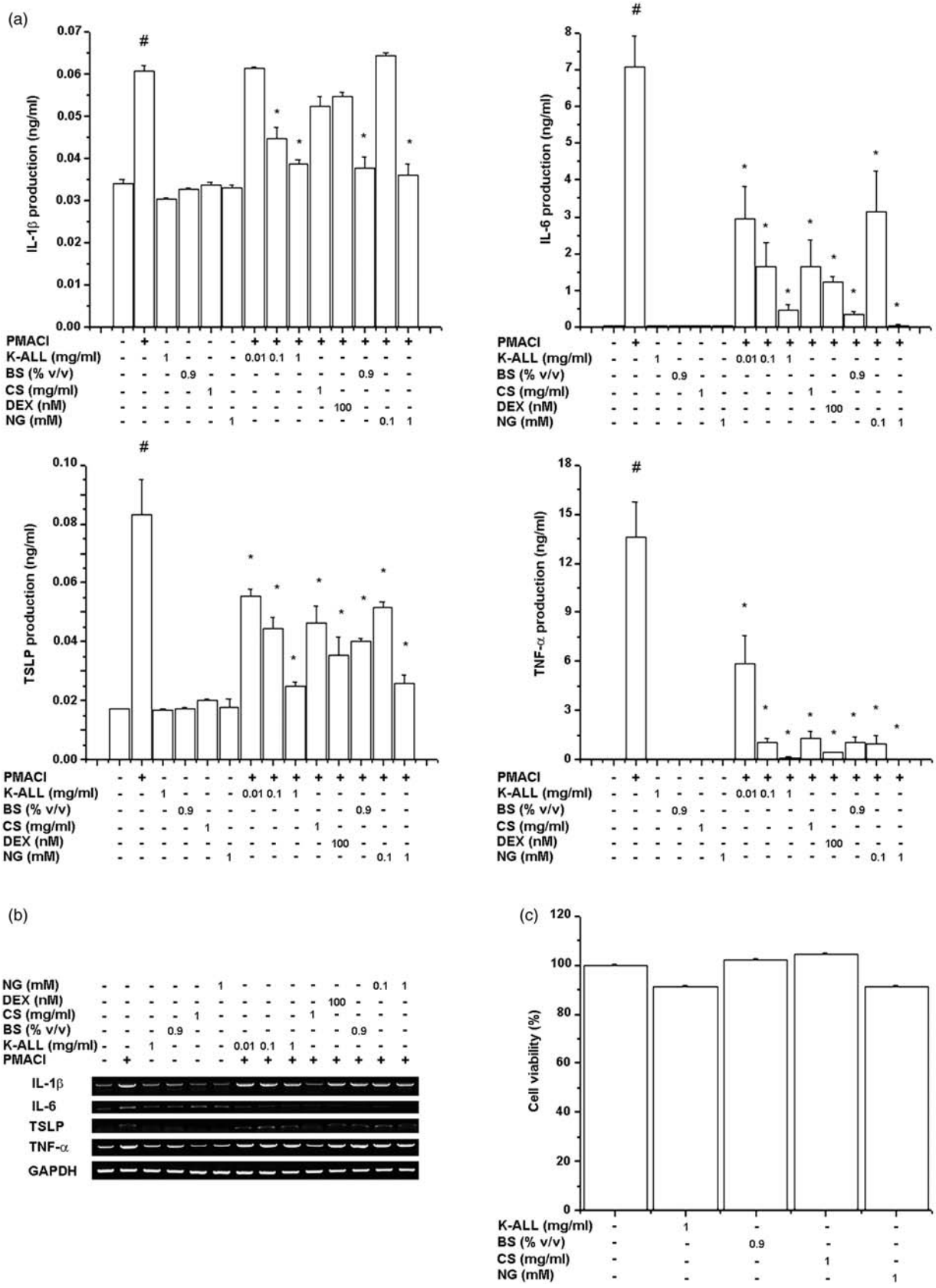

Figure 4 Effects of K-ALL on the production of pro-inflammatory cytokines in HMC-1 cells. HMC-1 cells were treated with K-ALL (0.01, 0.1 or 1 mg/mL), bamboo salt (0.9%), *Citrus sunki* (1 mg/mL), dexamethasone (100 nM) and naringin (0.1 or 1 mM) for 1 h and then stimulated with PMA + A23187 for 8 h. (a) IL-1β, IL-6, TSLP and TNF-α were measured by the ELISA method. (b) IL-1β, IL-6, TSLP and TNF-α mRNA levels were measured by RT-PCR after stimulation for 6 h. (c) Cell viability was evaluated by the MTT assay. $^{\#}P < 0.05$; significantly different from the non-stimulated cells, $^{*}P < 0.05$; significantly different from PMA + A23187-stimulated cells. PMACI: PMA + A23187; BS: bamboo salt; CS: *Citrus sunki*; DEX: dexamethasone; ELISA: enzyme-linked immunosorbent assay; IL: interleukin-1β; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NG: naringin; TSLP: thymic stromal lymphopoietin; TNF-α: tumor necrosis factor-alpha

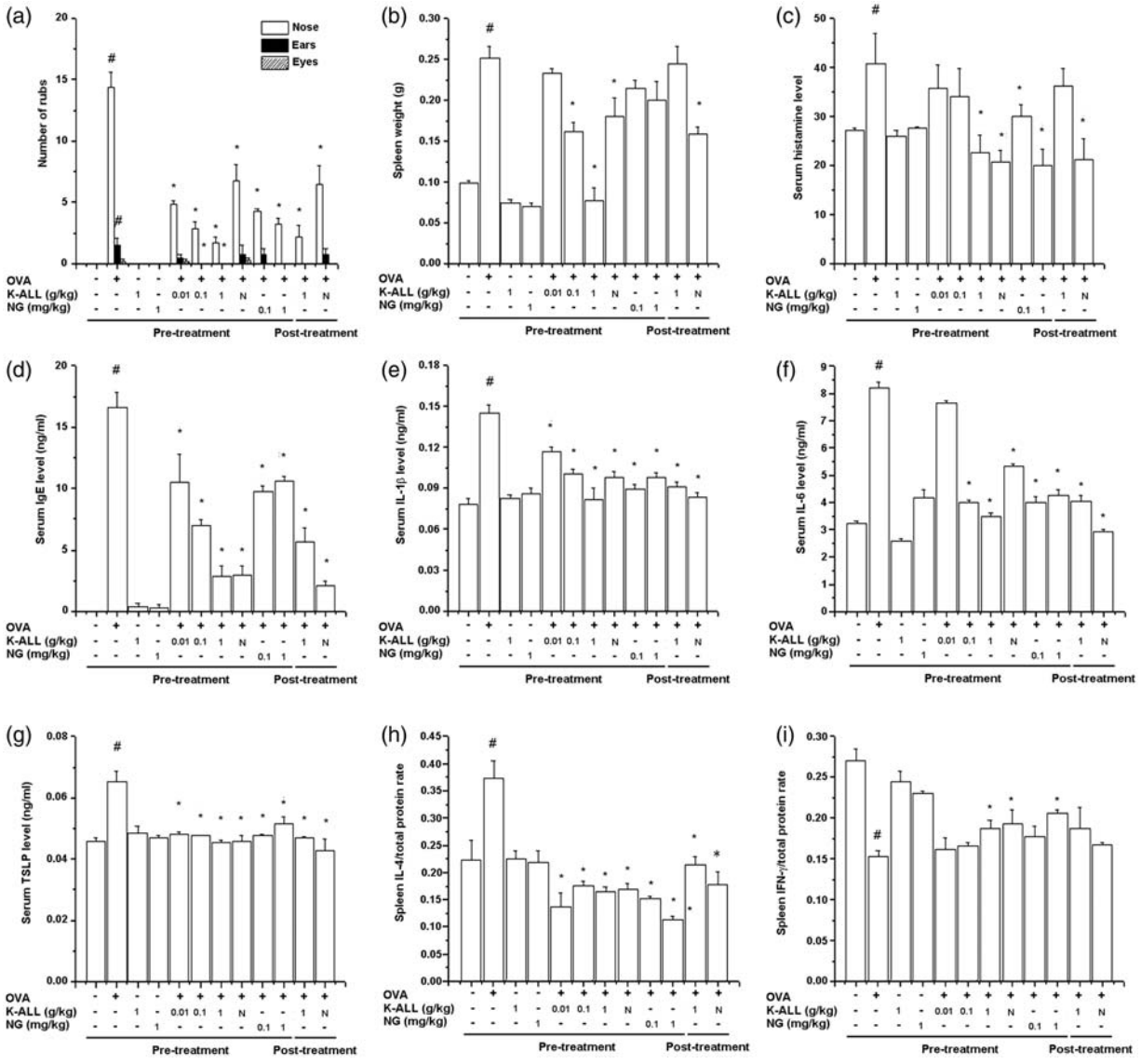

Figure 5 Effects of K-ALL on the clinical symptoms, IL-1β and TSLP levels and IL-4/IFN-γ levels in AR mice. Mice were sensitized on days 1, 5 and 14 by i.p. injection of 100 μg OVA and challenged with 1.5 mg OVA. Mice received K-ALL (0.01, 0.1 or 1 g/kg), naringin (0.1 or 1 mg/kg) before (pretreatment)/after (posttreatment) intranasal OVA challenge for 10 days by oral or nasal administration (N, direct nasal inhalation of K-ALL 0.2 mg). (a) The number of nasal rubs 10 min after the OVA intranasal provocation. (b) Spleen weight. (c) Serum isolated from blood was assayed for histamine. (d) Serum IgE, (e) IL-1β, (f) IL-6 and (g) TSLP levels were measured by the ELISA method. (h) IL-4 and (i) IFN-γ levels were measured by the ELISA method in the spleen. $^{\#}P < 0.05$; significantly different from OVA-unsensitized mice, $^{*}P < 0.05$; significantly different from OVA-sensitized mice. $n = 5$. AR: allergic rhinitis; BS: bamboo salt; CS: *Citrus sunki*; DEX: dexamethasone; ELISA: enzyme-linked immunosorbent assay; IL-1β: interleukin-1β; NG: naringin; OVA: ovalbumin; TNF-α: tumor necrosis factor-alpha; TSLP: thymic stromal lymphopoietin

levels in the spleen. The IL-4 levels in the AR mice were significantly increased compared to normal mice but significantly decreased in mice administered with K-ALL or NG administered mice (Figure 5h, $P < 0.05$). In contrast, IFN-γ levels were significantly increased by K-ALL or NG administration (Figure 5i, $P < 0.05$).

K-ALL reduces inflammation-related proteins in the AR animal model

To evaluate the regulatory effects of K-ALL or NG administration on inflammation-related protein levels, we analysed the levels of several inflammation-related proteins (IL-1β, IL-6, TSLP, MIP-2, ICAM-1 and COX-2) in the OVA-induced AR mice. The levels of IL-1β, IL-6, TSLP, MIP-2, ICAM-1 and COX-2 in the OVA-induced AR mice were significantly higher than those in the nasal mucosa tissues of the normal mice (Figures 6a to c, $P < 0.05$). All of these increased protein levels were significantly reduced by K-ALL or NG administration (Figures 6a to c, $P < 0.05$).

K-ALL reduces eosinophil and mast-cell infiltration in the AR animal model

The respective numbers of inflammatory cells (eosinophils and mast cells) in the nasal mucosa of AR mice were significantly higher than those in normal mice. In the K-ALL (pre or postadministration) or NG-administered mice, the eosinophil and mast-cell infiltration increased by OVA significantly decreased (Figure 7, $P < 0.05$).

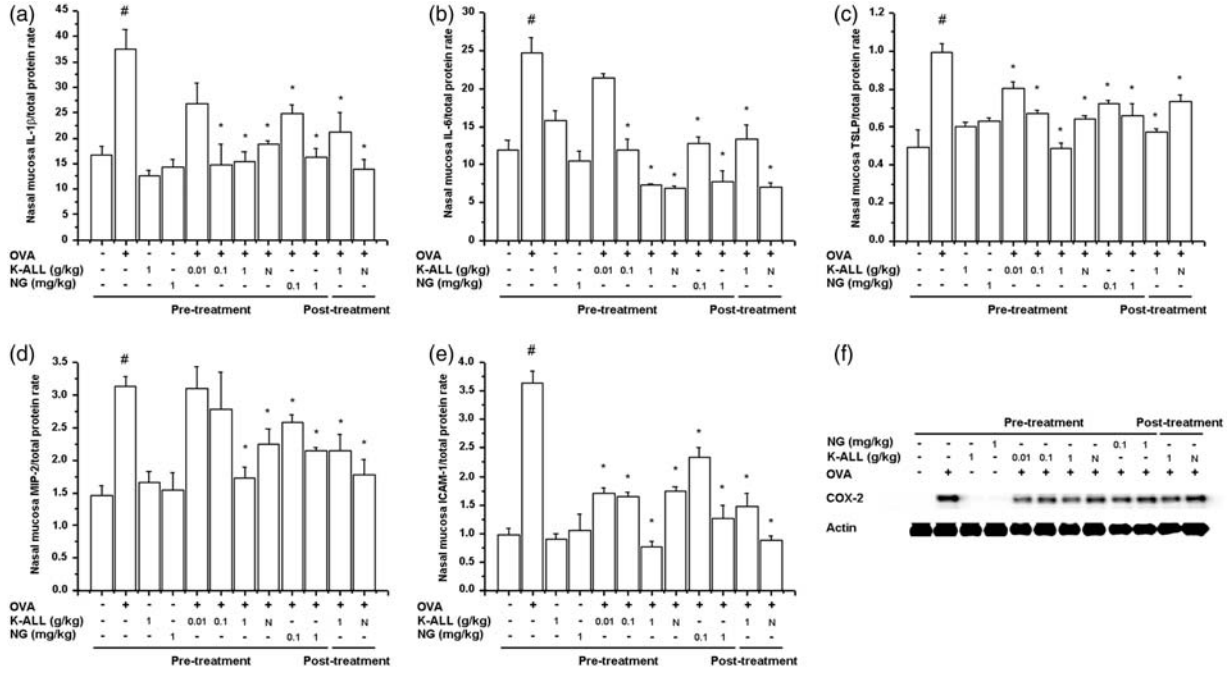

Figure 6 Effects of K-ALL on the inflammation-related proteins in the nasal mucosa of AR mice. (a–e) IL-1β, IL-6, TSLP, MIP-2 and ICAM-1 were measured by the ELISA method in nasal mucosa tissue. (f) COX-2 protein expression was evaluated by Western blot analysis. All parameters measured in the tissue homogenate are presented as a ratio to total protein levels in the tissue. N, direct nasal inhalation of K-ALL (0.2 mg). $^{\#}P < 0.05$; significantly different from OVA-unsensitized mice, $^{*}P < 0.05$; significantly different from OVA-sensitized mice. $n = 5$. AR: allergic rhinitis; IL-1β: interleukin-1β; NG: naringin; OVA: ovalbumin; TSLP: thymic stromal lymphopoietin

Discussion

Mast cells play a pivotal role in AR by producing histamine, proteases, cytokines and growth factors. In mice, mast cells are divided into the connective tissue mast cell (CTMC, localized in the skin and peritoneal cavity) and mucosal mast-cell subtypes. CTMCs contain large amounts of histamine.[21] Histamine is especially important as its interaction with histamine receptor 1 results in nasal obstruction, rhinorrhea and sneezing.[22–24] In addition, mast cell-specific proteases secreted by degranulation of mast cells induce the production of the chemoattractants IL-8, ICAM-1 and IL-1β on epithelial cells in the inflammation process.[25] Furthermore, proinflammatory cytokines, such as IL-1β, IL-6, TSLP and TNF-α, are released within the first hours of allergen challenge. Numerous reports have established that the stimulation of mast cells with compound 48/80 or IgE initiates the activation of signal transduction pathways leading to degranulation.[26,27] RPMCs (CTMCs type) are a good experimental model for the study of compound 48/80-induced histamine release.[28] RPMCs stimulated by compound 48/80 release a range of mediators, such as histamine and some cytokines.[28] Compound 48/80 has been reported to increase the permeability of the lipid bilayer membrane by causing a perturbation of the membrane. This report indicates that the membrane permeability increase may be an essential trigger for the release of mediators from mast cells.[29] DSCG prevents the release of the mediators of type I allergic reaction as a mast-cell stabilizer. The drug does not inhibit the binding of IgE to mast cells or the interaction between cell-bound IgE and specific antigen; instead, DSCG suppresses the release of histamine.[30] In this study, we showed that K-ALL significantly inhibited the compound 48/80-induced histamine release and systemic anaphylaxis. Thus, we presuppose that K-ALL might act on the lipid bilayer membrane affecting the prevention of the perturbation induced by compound 48/80 and regulate the degranulation of mast cells by stabilizing membrane fluidity.

Cross-linking of IgE-bound FcεRIs by a multivalent antigen on mast cells induces the release of biologically active mediators; the preformed mediators stored in the cytoplasmic granules include histamine and newly synthesized mediators such as leukotrienes and cytokines.[26,27] Han *et al.*[31] reported that inflammatory cytokines such as IL-1β, IL-4, IL-5, IL-6, IL-13, TNF-α and VEGF were significantly increased in serum after PCA.[31] The spectrum of cytokines produced by HMC-1 cells with PMA plus A23187 stimulation supports the well-recognized role of mast cells' immediate hypersensitivity. Our results showed that K-ALL inhibits IgE-mediated PCA. Inflammatory cytokine production in serum after PCA and activated HMC-1 cells was also reduced. It is conceivable that K-ALL inhibits the inflammatory reactions, probably through its interference with the degranulation system.

Increased numbers of mast cells have been demonstrated in a variety of autoimmune and inflammatory conditions.[20,26] The number of mast cells in the nasal mucosa of the AR mice is significantly higher than those in OVA-unsensitized mice.[32] Activated mast cells release inflammatory mediators such as histamine, serotonin, leukotrienes and various cytokines such as IL-1β, IL-6 and TNF-α.[33]

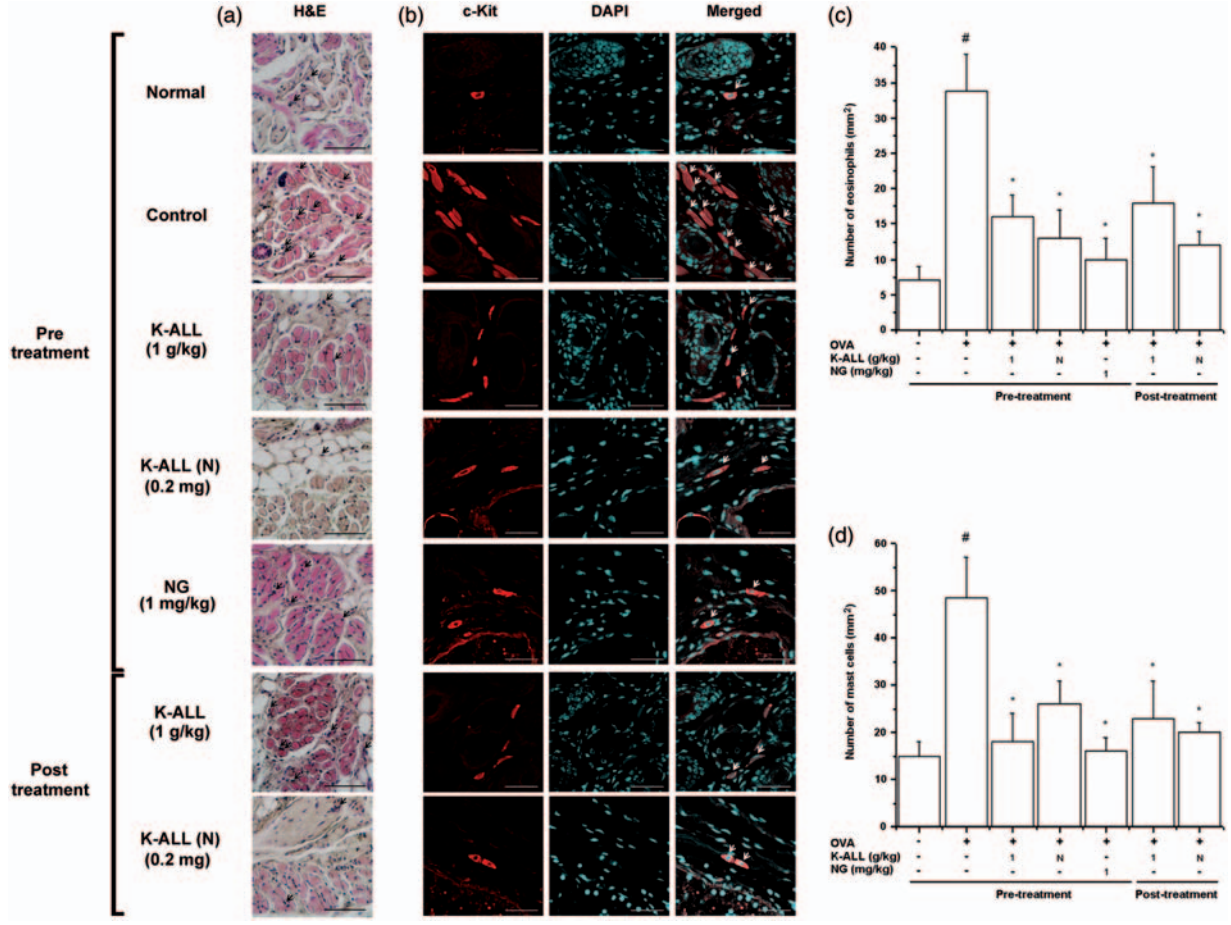

Figure 7 Effect of K-ALL on the eosinophil and mast cell infiltration in AR nasal mucosa tissue. (a) Nasal mucosa tissue was stained with H&E for eosinophils (black arrow). (b) Mast cells were stained by a primary anti-c-Kit for 1 h and then incubated with secondary TRITC-conjugated IgG for 1 h (white arrow). (c, d) Eosinophils and mast cells on five randomly selected tissue sections per mouse were counted. The absolute number of cells counted are shown as the mean ± SEM. $^{\#}P < 0.05$; significantly different from OVA-unsensitized mice, $^{*}P < 0.05$; significantly different from OVA-sensitized mice. $n = 5$. Normal: OVA-unsensitization; Control: OVA-sensitization; K-ALL (N): nasal inhalation of K-ALL (0.2 mg); (original magnification × 400 for H&E, scale bar = 100 μm; × 600 for mast cell, scale bar = 50 μm). AR: allergic rhinitis; H&E: hematoxylin and eosin; NG: naringin; OVA: ovalbumin. (A colour version of this figure is available in the online journal)

TSLP also plays a role in allergen-driven models of airway inflammation. In the OVA-induced model of mouse asthma, TSLP expression increases in response to antigen challenge and correlates with inflammatory cell infiltrate.[34] In the present study, K-ALL (pre or posttreatment) and NG reduced clinical symptoms and IL-1β, IL-6, TSLP and TNF-α level in the AR *in vivo* and *in vitro* models. Thus, we hypothesize that K-ALL can be helpful in preventing and treating inflammatory diseases including AR.

Traditional medicines have many benefits, few side effects and display low cytotoxicity. Over the past decades, many researchers have been studying the potential treatment of AR with traditional medicines. Many researchers have reported antiallergic and anti-inflammatory effects of traditional medicines; however, preventative and curative therapies for AR are lacking because traditional medicines are very effective *in vitro* but have a small effect in clinical trials. In previous studies, we investigated the antiallergic effects of various traditional medicines. As part of our continuing search for biologically active antiallergic and anti-inflammatory agents from traditional medicines, we found the most effective medicine, K-ALL. Our results suggest that K-ALL possesses preventive and curative effects in allergic diseases. Further work will address this possibility in human model systems.

Author contributions: KHM, JHJ and OHA designed and conducted research; OHA analysed the data; KMJ extracted the main compound (naringin); STY performed the histamine assay; KHM, JHJ and OHA wrote the paper. All authors read and approved the final manuscript.

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