



Effects of Korean red ginseng extract on the prevention of atopic dermatitis and its mechanism on early lesions in a murine model

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ABSTRACT

Ethnopharmacological relevance: Korean red ginseng (KRG) has been shown to possess various biological activities including anti-inflammatory properties.

Aim of the study: We aimed to investigate the effects and mechanism of KRG on the prevention of atopic dermatitis (AD) using a mouse model.

Materials and methods: The effect of KRG in trinitrochlorobenzene (TNCB)-treated NC/Nga mice was assessed by measuring ear thickness, transepidermal water loss (TEWL), total serum IgE, histologic changes of lesional skin, mRNA and protein expression of thymic stromal lymphopoietin (TSLP) and tumor necrosis factor (TNF)- α , immunohistochemistry for tissue interleukin (IL)-4, IL-17, and interferon (IFN)- γ .

Results: KRG significantly reduced ear thickness. Oral administration of KRG significantly prevented the increase in TEWL induced by TNCB. The serum IgE level was significantly lower in the KRG group. Histologically, lymphocyte infiltration was markedly decreased by KRG. CD1a positive (CD1a+) cells were diminished by KRG. Immunohistochemically, KRG significantly suppressed the protein expression of TSLP and TNF- α . The mRNA expression of TSLP in the lesions was significantly reduced by KRG. These results demonstrate that oral administration of KRG may inhibit the development of AD-like skin lesions in NC/Nga mice by modifying TSLP, DCs, and at least in part, the Th2 response.

Conclusion: KRG may be a potential therapeutic modality for the prevention of AD.

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1. Introduction

Atopic dermatitis (AD) is a chronic and relapsing inflammatory skin disorder (Novak and Leung, 2011). Disturbance of epidermal-barrier function and correlation to local and systemic immunologic factors in the development and severity of AD has been demonstrated by recent studies (Boguniewicz and Leung, 2011). Whether epidermal-barrier abnormalities precede immune dysregulation ('outside-in' hypothesis) (Elias et al., 2008) or immune dysregulation precedes changes in barrier function ('inside-out'

Abbreviations: KRG, Korean red ginseng; AD, atopic dermatitis; TNCB, trinitrochlorobenzene; TEWL, transepidermal water loss; TSLP, thymic stromal lymphopoietin; TNF, tumor necrosis factor; IL, interleukin; IFN, interferon; CD1a+, CD1a positive; DC, dendritic cell; Th2, type 2 helper T; LC, Langerhans cell; Th1, type 1 helper T; EPO, evening primrose oil; PFA, paraformaldehyde; ELISA, enzyme-linked immunosorbent assay; RT-PCR, reverse transcription-polymerase chain reaction; S.E.M, standard error of means; H&E, hematoxylin and eosin

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hypothesis) continues to be an unsolved issue with data supporting both hypotheses (Boguniewicz and Leung, 2011).

In the acute phase of AD, the IL-7-like cytokine, thymic stromal lymphopoietin (TSLP), triggers dendritic cell (DC)-mediated type 2 helper T (Th2) inflammatory responses. Langerhans cells (LCs) play an important role in cutaneous antigen presentation and contribute to Th2 polarization (Novak and Bieber, 2005). Inflammatory dendritic epidermal cells (IDEC) produce proinflammatory cytokines interleukin (IL)-1, IL-6, and tumor necrosis factor α (TNF- α) (Bieber, 2008). Antigen-specific Th2 cells and their cytokines such as IL-4, and IL-13 orchestrate the acute skin lesions in AD. IL-17 expression is enhanced in acute lesions. During the chronic phase, a switch to the type 1 helper T (Th1) cell-mediated responses occurs. Th1 cells mainly produce interferon (IFN)- γ , and IL-17 shows a diminished presence.

Transepidermal water loss (TEWL) is linked to the damage of the stratum corneum lipid barrier and a consecutive loss of corneocyte adhesion. Skin barrier abnormalities, including increased TEWL and decreased skin hydration, are biomarkers for the severity and itch intensity in AD (Lee and Yu, 2011).

For the management of AD, the prevention of recurrence and early treatment is important. Use of topical calcineurin inhibitors and intermittent use of topical glucocorticoids is beneficial for the

management of AD (Abramovits, 2005b). Results from several studies show the possibility that patients with AD may benefit from the traditional Oriental medicine therapy (Koo and Arain, 1999).

Korean red ginseng (KRG, the steamed root of *Panax ginseng* C.A. Meyer, family Araliaceae) is frequently taken orally as a remedy, especially in Asian countries. The major components of raw ginseng have been recently known to be ginsenosides, which contain an aglycone with dammarane skeleton (Shibata et al., 1963). KRG was further studied to have anti-allergic effects. (Bae et al., 2006; Matsuda et al., 1990; Park et al., 2003) Recently, we have demonstrated that oral administration of KRG could inhibit the inflammation of AD-like skin lesions in NC/Nga mice by suppressing LCs, Th1 related cytokines, and regulatory T (Treg) cells (Lee and Cho, 2011). To date, there have been no studies to assess the preventive effects of oral KRG administration and its mechanism on early lesions in the AD murine model. We further studied to investigate the effects and mechanism of KRG on the prevention of AD.

The aim of this study was to assess the anti-inflammatory effects of oral administration of KRG on the prevention and early events of AD-like skin lesions that were provoked by trinitrochlorobenzene (TNCB) in NC/Nga mice. In order to investigate the preventive effects of KRG, the oral administration of KRG was initiated and completed before the TNCB challenge. We aimed to determine whether KRG exerts anti-inflammatory effects on cytokines, T cells and DCs that are related to immune dysregulation in the early pathogenesis of AD. In this study, we measured ear thickness, TEWL, serum IgE levels, histologic changes, immunohistochemistry of TSLP, TNF- α , IL-4, IL-17, IFN- γ , and mRNA expression of TSLP and (TNF)- α in the lesional skin and to examine the preventive effects of KRG on the development of AD-like skin lesions in NC/Nga mice.

2. Material and methods

2.1. Animals

NC/Nga female mice (5-weeks old) were purchased from Charles River Laboratories (Kanagawa, Japan).

2.2. Test drugs

TNCB was purchased from Sigma (St. Louis, MO, USA) to induce AD-like skin lesions. KRG extract was manufactured from roots of a 6-year-old fresh ginseng, *Panax ginseng* Meyer, harvested in Republic of Korea by Korea Ginseng Corporation, Seoul, Korea. Red ginseng was made by steaming fresh ginseng at 90–100 °C for 3 h and then drying at 50–80 °C. Red ginseng extract was prepared from red ginseng water extract, which was extracted at 85–90 °C for 8 h of circulating hot water three times. The water content of the pooled extract was 36% of total weight. KRG was analyzed by high-performance liquid chromatography. KRG extract contained major ginsenoside-Rb1: 8.27 mg/g, -Rb2: 3.22 mg/g, -Rc: 3.90 mg/g, -Rd: 1.09 mg/g, -Re: 2.58 mg/g, -Rf: 1.61 mg/g, -Rg1: 2.01 mg/g, -Rg2: 1.35 mg/g, -Rg3: 1.04 mg/g, and other minor ginsenosides. Cyclosporine (Cipol-N[®]) was kindly donated by Chong Kun Dang (Seoul, Korea). Evening primrose oil (EPO) (Evoprim[®]) was purchased from Dalim Biotech (Seoul, Korea). Antibodies used for immunohistochemical markers are as follows; CD1a (1:50, Thermo Scientific, Rockford, IL, USA), TSLP (Abnova, Taipei city, Taiwan), TNF- α (1:200; Abcam, Cambridge, UK), IL-4 (1:50, Biolegend), IL-17 (1:100; Abcam, Cambridge, UK), and IFN- γ (1:100; Biolegend, San Diego, CA, USA).

2.3. TNCB induced AD-like skin lesions and oral administration of drugs

Twenty-five SPF NC/Nga female mice (20 $\pm$ 5 g each) were housed at 22 °C with a 12 h light–dark cycle. The mice were placed in separate cages and assigned individual groups with ad libitum access to food and water. Mice were anesthetized with isoflurane and the hair on their back and abdomen was shaved using an electric shaver. The animal care, handling and experimental procedures were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee. The study was approved by the Institutional Review Board.

The NC/Nga mice were allocated into five groups (AD control, KRG, cyclosporine, EPO, and sham groups). TNCB was used to induce AD-like skin lesions in NC/Nga mice as previously described (Lee and Cho, 2011). The abdomens were sensitized epicutaneously by applying 150 μ l of 5% TNCB dissolved in ethanol/acetone mixture (4:1). Seven days after sensitization, the backs and dorsal surface of the ears were challenged with 190 μ l of 1% TNCB dissolved in acetone/olive oil (4:1). The KRG, cyclosporine, and EPO groups were treated with 200 mg/kg KRG, 2.5 mg/kg cyclosporine, and 50 mg/kg EPO for 5 days, respectively. The AD control group received phosphate-buffered saline (PBS) 0.2 mL, only. KRG, cyclosporine, EPO, and PBS were orally administered by gastric intubation with an animal-feeding needle. The sham group was untreated. The design of the study is summarized in Fig. 1.

2.4. Measurement of ear thickness and total clinical severity score

The severity of AD-like lesions was evaluated by scoring the clinical signs using digital photographs as in our previous study (Lee and Cho, 2011). The total clinical severity score was defined as the sum of each score grades: 0 (none), 1 (mild), 2 (moderate) and 3 (severe) for each of the four manifestations: (1) erythema/hemorrhage, (2) edema, (3) excoriation/erosion, and (4) scaling/dryness. The ear thickness was measured using a dial caliper (Kori Seiki MFG, Tokyo, Japan) at the time of 1% TNCB challenge (baseline) and 1, 3, 6, 12, 24, 48, and 72 h after the baseline. Clinical photographs of the ears and abdomens were taken with a digital camera (Canon 50D, Canon Inc., Tokyo, Japan).

2.5. Measurement of transepidermal water loss

TEWL was assessed on the dorsal skin of the NC/Nga mice using VapoMeter (Delfin Technologies, Kuopio, Finland). TEWL was measured at baseline, and 1, 3, 6, 12, 24, 48, and 72 h thereafter.

2.6. ELISA

Blood samples were collected from the retro-orbital plexus of anesthetized mice at the end of the experiment and added to tubes (Scientific Glass, Inc., Rockwood, TN, USA). Samples obtained by centrifugation were stored at –20 °C until use. The

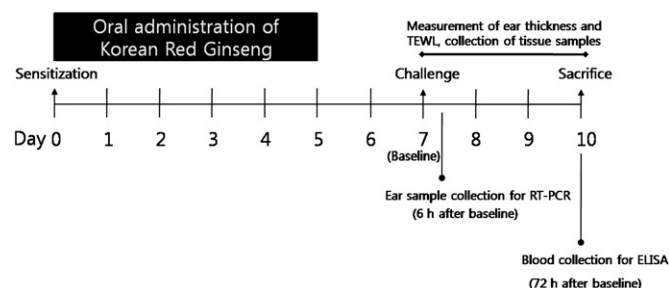


Fig. 1. Summary of the design of study.

total IgE in the blood was measured using a mouse enzyme-linked immunosorbent assay (ELISA) kit (Bethyl Laboratories, Inc., Montgomery, TX, USA) according to the manufacturer's instructions. The wells had anti-mouse IgE antibody that had been pre-adsorbed on the surface. Briefly, the blood samples (20-fold) and standards were diluted and added to wells, and they were incubated 1 h at room temperature. After washing the wells four times, 100 μ l of biotinylated detection antibody was added to each well to bind to the captured IgE, and the wells were incubated at room temperature for 1 h. After washing the plate four times, 100 μ l of streptavidin-conjugated horseradish peroxidase was added to each well. After 30 min incubation at room temperature, the plate was washed four times. The enzyme reaction was initiated by adding 100 μ l of chromogenic substrate 3,3',5,5'-tetramethylbenzidine (TMB). The plate was kept for 30 min at room temperature in a dark place. The reaction was terminated by adding 100 μ l of dilute sulfuric acid. The absorbance at 450 nm was measured with an ELISA reader (Spectra Max250, Molecular Devices, USA). All the experiments were performed in duplicate and repeated at least three times.

2.7. Tissue samples

At baseline and 1, 3, 6, 12, 24, 48, 72 h thereafter, 4 mm punch biopsies were performed from the backs of the mice. At 6 h after the baseline, one half of the ears were excised for RT-PCR to investigate TSLP and TNF- α . At 72 h after baseline, all the remaining ears were excised and fixed in 4% paraformaldehyde (PFA) for histologic examination.

2.8. Histologic examination

At the end of the experiment, the ears were excised for histological observation. Ear samples were fixed with 4% PFA, embedded in paraffin and then 5- μ m thin sections were made on probe-on-plus slides (Fisher Scientific, Pittsburg, USA). The deparaffinized skin sections were stained with H&E. Other sections were stained for immunohistochemical markers using monoclonal antibodies to CD1a (1:50; Thermo Scientific, Rockford, IL, USA), TSLP (Abnova, Taipei city, Taiwan), TNF- α (1:200; Abcam, Cambridge, UK), IL-4 (1:50; Biolegend), IL-17 (1:100; Abcam, Cambridge, UK), and IFN- γ (1:100; Biolegend, San Diego, CA, USA).

Immunohistochemical analysis was performed with a high temperature antigen unmasking technique. The sections were heated in the unmasking solution (citrate buffer, pH 6.0), washed, and then were incubated with primary monoclonal antibodies at room temperature for 60 min. This procedure was followed by incubation with the secondary antibodies (EnVision Detection System and MOM Kit, DAKO, Gostrup, Denmark). These reaction products were developed with the use of diaminobenzidine solution as a chromogen. After the chromogen reactions, the sections were rinsed and then counterstained with hematoxylin. After rinsing, the sections were dehydrated and covered with permount (Crystal Mount, Biomed, Foster City, CA, USA). They were examined by light microscopy to assess the histological changes. A 'blind' observer evaluated the serial sections. For quantitative analysis, the cells were counted in three independent microscopic fields ($\times 400$).

2.9. RT-PCR

RNA was reverse transcribed into cDNA using High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). The primers of TSLP (forward: 5'-ACT GCAACTTCACGTCAATTACG-3', reverse: 5'-TTGCTCGAAGTTAGCCCTTT-3') and TNF- α (forward: 5'-CAGGCGGTGCCTATGTCTC-3', reverse: 5'-CGATCACCCGAAGTTCAGTAG-3') were designed by Bionics (Seoul, Korea). The expression of

mRNA transcripts of TSLP, TNF- α and housekeeping gene GAPDH were determined by real-time RT-PCR. The real-time PCR analysis was performed on an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems). Reactions were set up in a total volume of 20 μ l using 0.5 μ l of cDNA and 10 μ l of FastStart Universal SYBR Green Master (Roche, Mannheim, Germany). For amplification, the samples were heated to 50 $^{\circ}$ C for 2 min, 95 $^{\circ}$ C for 10 min and cycled 40 times with the following condition: 95 $^{\circ}$ C for 30 s, 60 $^{\circ}$ C for 30 s and 72 $^{\circ}$ C for 30 s. Using standards, the amount of TSLP and TNF- α cDNA was determined and normalized by the amount of GAPDH cDNA.

2.10. Statistical analysis

The statistical analysis of the differences in the means for each experimental group of animals was carried out using the ANOVA test. SPSSv10.0 (SPSS Inc., Chicago, IL, USA) was used for all calculations. All the values are expressed as means $\pm$ standard error of means (S.E.M.). Differences were considered significant for $p < 0.05$.

3. Results

3.1. Oral administration of KRG significantly prevented the increase in ear thickness induced by TNCB in NC/Nga mice

The total clinical severity scores of the AD control group were significantly increased compared to the sham group (Fig. 2A). The clinical signs and symptoms of the ear after TNCB application began with itching and edema. The clinical signs were ameliorated by KRG and cyclosporine at 72 h compared to the AD control group (Fig. 2B). The ear thickness significantly increased with time in the TNCB-applied groups. The AD control group showed a significant increase in ear thickness compared to the sham group at 72 h after baseline ($p < 0.001$). The ear thickness of the AD control group was 0.63 ± 0.04 mm at 72 h after baseline. KRG ($p < 0.001$), cyclosporine ($p < 0.001$), and EPO ($p < 0.05$) groups significantly inhibited the increase in ear thickness. Compared to the AD control group, KRG (0.42 ± 0.03 mm), cyclosporine (0.42 ± 0.03 mm), and EPO (0.53 ± 0.03 mm) decreased the ear thickness by 33.3%, 33.3%, 16% respectively (Fig. 2C). Overall, KRG, EPO, and cyclosporine reduced ear thickness significantly, and EPO showed results to a lesser extent than KRG and cyclosporine. KRG and cyclosporine showed statistically similar values in ear thickness.

3.2. Oral administration of KRG significantly prevented the increase in TEWL induced by TNCB in NC/Nga mice

TEWL was measured to access the functional state of the epidermal barrier. The AD control group showed an increase in TEWL compared to the sham group at 1, 3, 6, 12, 24, 48, 72 h after baseline. 6 h after TNCB application, KRG ($p < 0.001$) and cyclosporine ($p < 0.001$) showed less TEWL values compared to the AD control group. EPO did not show a significant effect on TEWL (Fig. 3).

3.3. KRG significantly prevented the increase in blood IgE level in the TNCB-applied NC/Nga mice

The IgE levels of the AD control group at the end of experiment were significantly increased ($p < 0.001$), as compared with those of the sham group. KRG ($p < 0.001$) and EPO ($p < 0.001$) both prevented the increase in blood IgE levels induced by TNCB application. Cyclosporine did not show an effect on blood IgE. There was no significant difference between the serum IgE levels of KRG and EPO (Fig. 4).

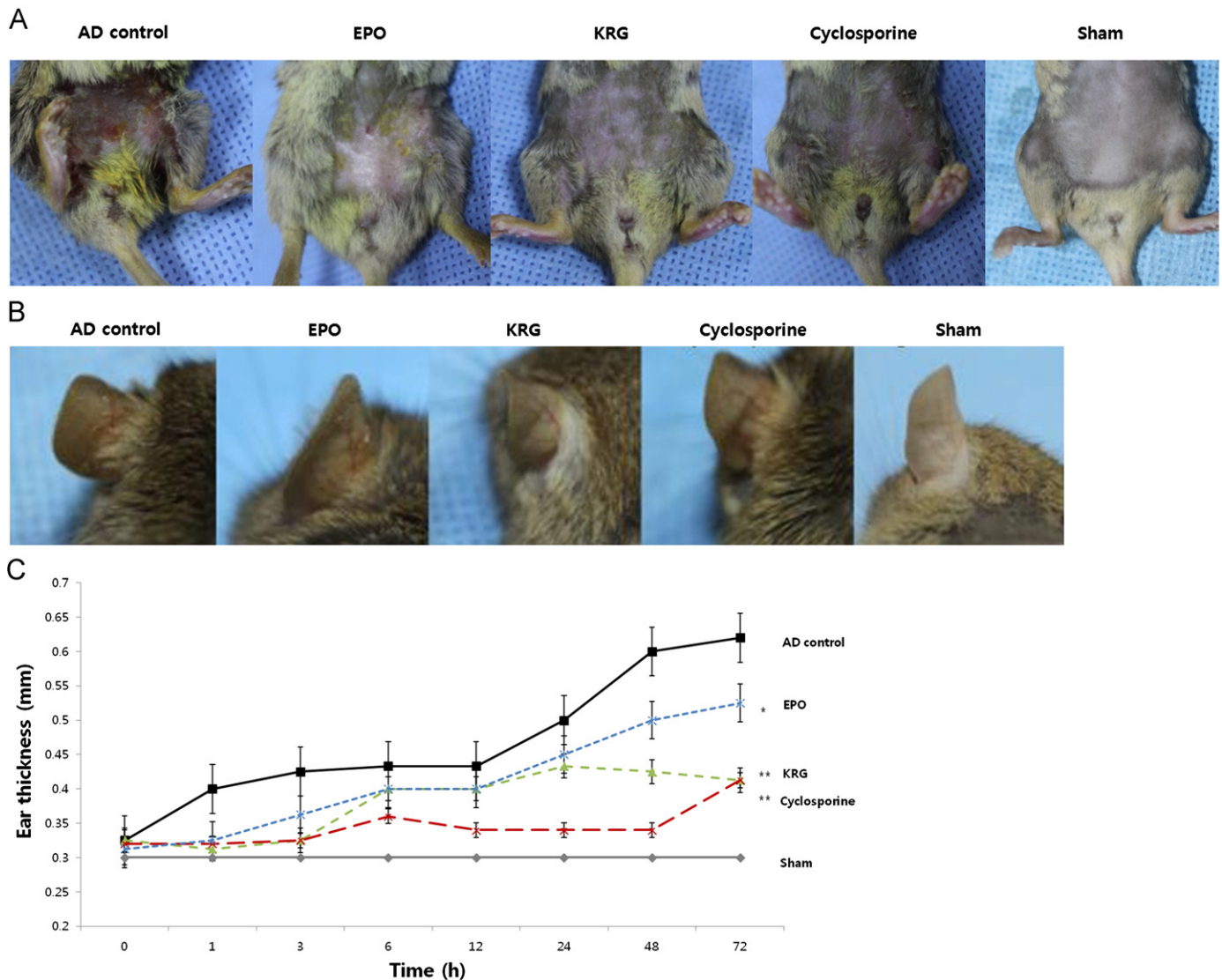


Fig. 2. Oral administration of KRG significantly prevented the increase in ear thickness induced by TNCB in NC/Nga mice. (A) Clinical photographs of the TNCB-sensitized abdomens. Photographs were taken 72 h after baseline. The total clinical severity scores of the representative NC/Nga mice in the figure are as follows; 12 points for AD control, 6 points for EPO, 2 points for KRG, 2 points for cyclosporine, and 0 for sham group. (B) Clinical outcome after oral KRG administration. Photographs were taken 72 h after TNCB challenge on the ears of NC/Nga mice. (C) The change in ear thickness by oral administration of KRG. Data are shown as mean $\pm$ S.E.M. of the changes in the ear thickness of mice in each group ($n=5$ mice per group). * $p < 0.05$, ** $p < 0.001$ compared with the AD control group. ("KRG" for Korean red ginseng; "EPO" for evening primrose oil).

3.4. Oral administration of KRG significantly prevented lymphocyte infiltration in the skin

As shown in Fig. 5a, lymphocyte infiltration in the upper dermis was noted in the sections of the AD control group at 72 h after TNCB application (419 ± 4.04). In contrast, the KRG group showed significantly diminished lymphocyte infiltration in the upper dermis (157.67 ± 0.55 , $p < 0.001$). The cyclosporine group showed decreased lymphocyte infiltration as well (89.33 ± 0.88 , $p < 0.001$). No significant difference was noticed between the AD control group and the EPO group (422.33 ± 4.91). The sham group did not show any specific signs of inflammation (15 ± 0.95).

3.5. Oral administration of KRG markedly diminishes the over-expression of DCs in AD-like skin lesions

We further investigated the effect of KRG on the infiltration of CD1a+ DCs. As shown in Fig. 5B and C, TNCB challenge significantly increased CD1a+ cells in the skin at 3 h after application

(31.67 ± 1.40). CD1a+ cells were expressed significantly less in the KRG group (13.00 ± 1.79) as compared to the AD control. Cyclosporine suppressed the expression of CD1a+ cells as well (13.00 ± 0.79), but a difference between KRG and cyclosporine groups were not exhibited. EPO did not show significant changes in the number of CD1a+ cells in the AD-like skin lesions (30.67 ± 1.12).

3.6. Oral administration of KRG markedly diminishes the over-expression of TNF- α and TSLP in the AD-like skin lesions

As shown in Fig. 5D, E and F, TNF- α (20.20 ± 1.42) and TSLP (49.20 ± 2.21) were strongly stained in the skin lesions of the AD control group at 24 h and 48 h after TNCB challenge compared to the sham group, respectively. The expression of TNF- α and TSLP was decreased in the AD-like skin lesions in the KRG (TNF- α : 0.93 ± 0.23 , TSLP: 13.46 ± 0.83) and cyclosporine group (TNF- α : 0.87 ± 0.22 , TSLP: 11.00 ± 1.90). The EPO group did not show a significant change compared to the AD control group (TNF- α : 16.67 ± 1.64 , TSLP: 46.47 ± 2.30). On the other hand there was little difference in the expression of IL-4, IL-17, and IFN- γ between the AD control

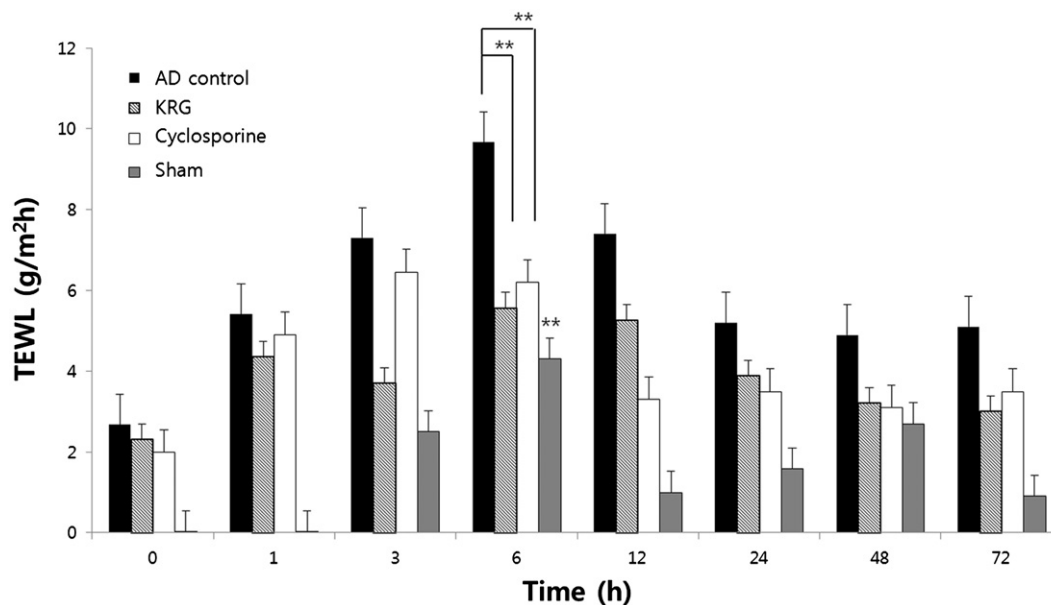


Fig. 3. Oral administration of KRG significantly prevented the increase in TEWL induced by TNCB in NC/Nga mice. Change in TEWL by oral administration of KRG. Data are shown as mean $\pm$ S.E.M. of the changes in TEWL of mice in each group. ($n=5$ mice per group). * $p < 0.05$, ** $p < 0.001$ compared with the AD control group. ("KRG" for Korean red ginseng).

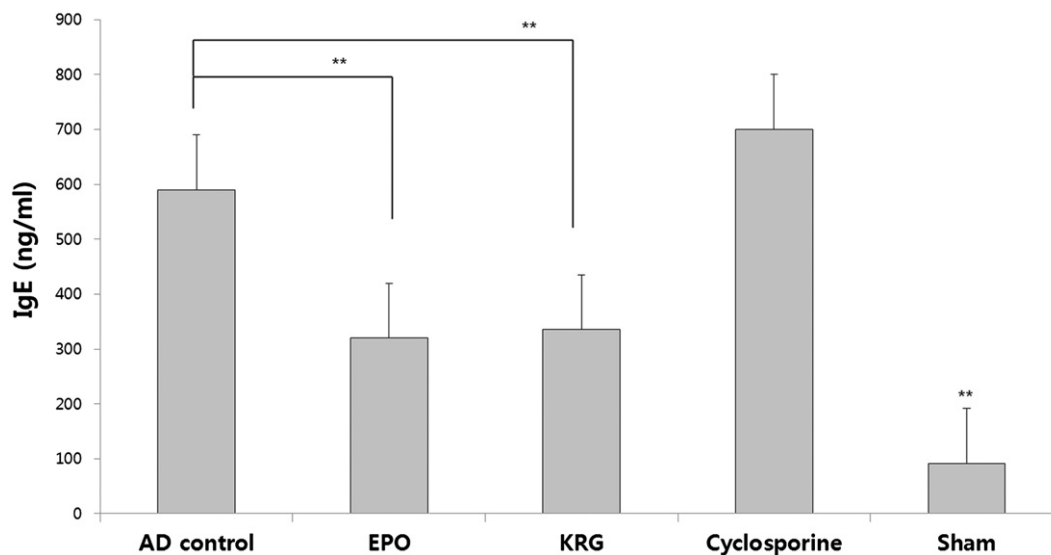


Fig. 4. Oral administration of KRG significantly reduced the level of IgE. At 72 h after TNCB challenge, blood samples were collected from the retro-orbital plexus and IgE was quantified by ELISA. Each experimental group consisted of five mice. All values are expressed as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.001$ compared with the AD control. ("KRG" for Korean red ginseng; "EPO" for evening primrose oil).

group and KRG group. The expression of IL-4, IL-17, and IFN- γ in the cyclosporine and EPO groups did not show a significant difference compared to the AD control group.

3.7. Oral administration of KRG significantly down-regulated the mRNA expression of TSLP

To further investigate the effect of oral KRG administration on the cytokines related to the early events in AD, mRNA levels of TSLP and TNF- α were quantitatively measured by real-time RT-PCR. Compared to the sham group, mRNA expression of TSLP ($p < 0.001$) was significantly increased in the AD control group at 6 h after TNCB application. The mRNA expression of TSLP was significantly down-regulated in the KRG ($p < 0.001$), EPO ($p < 0.001$) and cyclosporine ($p < 0.001$) groups. The mRNA

expression of TSLP did not differ significantly between the KRG and cyclosporine groups (Fig. 6A). The AD control group showed a trend in higher TNF- α expression than the sham group. KRG, cyclosporine, and to a lesser extent, EPO showed a lower trend in the mRNA levels of TNF- α than the AD control group (Fig. 6B). However, the difference in mRNA levels of TNF- α was not statistically significant among the treatment groups.

4. Discussion

We demonstrated for the first time that preceding oral KRG administration markedly prevents AD-like skin lesions in TNCB-induced NC/Nga mice. Clinically, prevention of an increase in ear thickness was exhibited by oral KRG administration. Skin

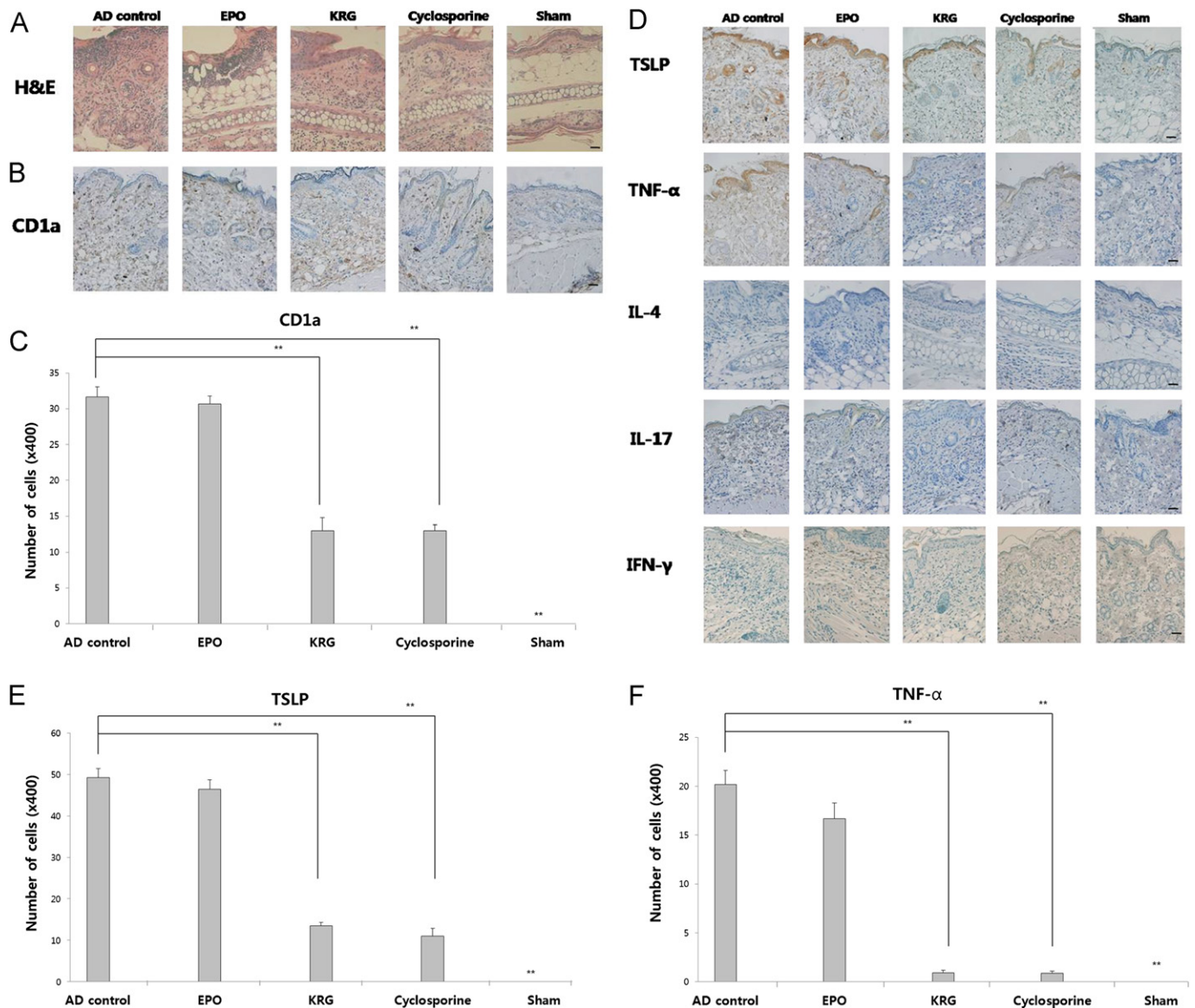


Fig. 5. Oral administration of KRG significantly reduced leukocyte infiltration, CD1a+ cells, and expression of TSLP and TNF- α . (A) Histology of the AD skin lesions orally treated with KRG. At 72 h after TNCB challenge, the ear tissues were prepared for hematoxylin and eosin (H&E) staining. Sections were observed at an original magnification of $\times 400$ scale bar = 200 μ m. (B) Oral administration of KRG reduced CD1a+ cell infiltration in the skin provoked by TNCB. Sections were observed at an original magnification of $\times 400$ scale bar = 200 μ m. (C) The number of CD1a+ cells at $\times 400$ magnification. All values are expressed as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.001$ compared with the AD control. (D) The effect of oral KRG on the expression of TSLP, TNF- α , IL-4, IL-17 and IFN- γ on the AD-like skin lesions. Oral administration of KRG markedly diminished the over-expression of TSLP and TNF- α on AD-like skin lesions at 48 h and 24 h after TNCB challenge, respectively. There was little difference in the IL-4, IL-17 and IFN- γ expression between the positive control group and the KRG group. Sections were observed at an original magnification of $\times 400$. A representative picture from three independent fields is shown scale bar = 200 μ m. (E) The number of TSLP+ cells at $\times 400$ magnification. (F) The number of TNF- α + cells at $\times 400$ magnification. All values are expressed as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.001$ compared with the AD control. ("KRG" for Korean red ginseng; "EPO" for evening primrose oil).

hydration was preserved by KRG administration, as shown by TEWL levels. Histologically, oral KRG administration led to prevention in infiltration of lymphocytes in the dermis. DCs are key cells in the development of AD. TNCB application resulted in CD1a+ DC infiltration of the skin in our study. The extensive CD1a+ cell infiltration was largely suppressed by oral KRG administration. Furthermore, KRG suppressed the over-expression of cytokines TSLP and TNF- α in the AD-like skin lesions in NC/Nga mice. Considering the effect of KRG on AD-like lesions in this study, KRG has a potential as an adjuvant oral agent for the prevention and treatment of early lesions in AD.

We recently demonstrated that oral KRG could exhibit beneficial effects on AD-like skin lesions in NC/Nga mice treated with

TNCB by suppressing Langerhans cells, Th1 related cytokines, and Treg cells (Lee and Cho, 2011). The total clinical severity score evaluating itching, erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness was significantly reduced by KRG. Topical application of KRG and ginsenosides Rh2 and Rg3 have shown efficacy in other recent studies as well (Kim et al., 2011; Park et al., 2010; Sohn et al., 2011). Topical application of KRG and ginsenosides Rh2 and Rg3 resulted in improved AD-like skin lesions, and led to a decrease in the mRNA expression of IL-4 (Kim et al., 2011; Park et al., 2010; Sohn et al., 2011). NC/Nga mice spontaneously develop AD-like lesions under conventional conditions. In a preliminary study, we examined the effects of oral KRG administration on NC/Nga without being treated with TNCB. Compared to the untreated control group, harmful or beneficial

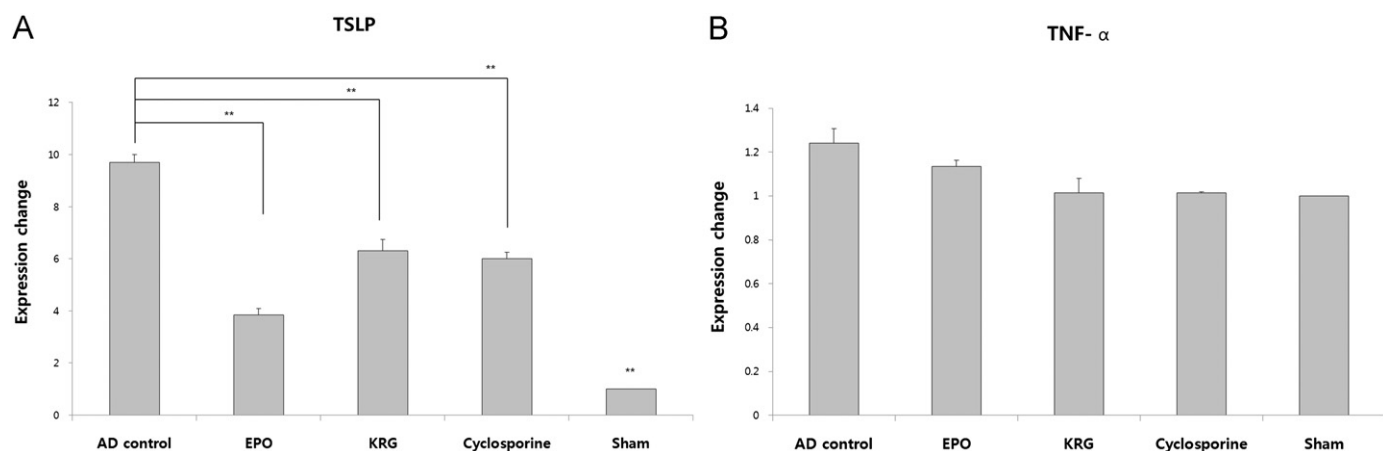


Fig. 6. Effect of oral KRG treatment on the mRNA expression of TSLP on the AD-like skin lesions. Oral administration of KRG significantly down-regulated the mRNA over-expression of TSLP on the AD skin lesions. Each experimental group consisted of five mice. All values are expressed as mean $\pm$ S.E.M. * $p < 0.05$, ** $p < 0.001$ compared with the AD control (KRG for Korean red ginseng; "EPO" for evening primrose oil).

effects of KRG were not noted and not assessable, as both groups did not develop AD-like lesions either. Late onset and low incidence of AD-like lesions are known to be disadvantages of NC/Nga mice not treated with sensitizers such as TNCB.

For the prevention and maintenance of AD, intermittent use of topical glucocorticoids and use of topical calcineurin inhibitors are the only approved modalities other than skin and environmental control. Topical calcineurin inhibitors are approved for the prevention of recurrence and early treatment in AD. Although cyclosporine has not been approved by the FDA for the treatment of AD, it is approved in Europe for the short-term treatment of severe AD that cannot be controlled with topical therapy (Amor et al., 2010). Both topical calcineurin inhibitors and oral cyclosporine interact with calcineurin, thereby suppressing the activation of T cells (Yamashita et al., 2010). To compare the effects of AD under the same method of oral administration, this study was designed to compare cyclosporine with KRG. EPO is a plant oil extract containing gamma linolenic acid (GLA), an essential fatty acid. EPO emerged as a possible alternative treatment in AD. However, many studies have evaluated the efficacy of oral GLA supplementation for AD, with conflicting evidence of benefit (Williams, 2003; Williams and Grindlay, 2008). We investigated the efficacy of KRG and compared it to cyclosporine and EPO on AD lesions in our study. Clinically, preceding oral administration of KRG and cyclosporine resulted in statistically similar ear thickness levels at 72 h after TNCB challenge. Oral administration of KRG and cyclosporine may have a sustained effect on AD lesions. Overall, EPO reduced IgE levels and the mRNA expression of TSLP, but did not demonstrate an effect on ear thickness, TEWL nor the infiltration of inflammatory cells. Cyclosporine showed beneficial effects on ear thickness, TEWL, infiltration of inflammatory cells, and the mRNA and protein expression of cytokines, but did not affect IgE levels. In comparison, KRG prevented AD-like skin lesions by modifying both local and systemic inflammation, demonstrated by the changes in ear thickness, TEWL, infiltration of inflammatory cells, mRNA and protein expression of cytokines, and IgE levels.

Classically, the balance between the Th1 and Th2 response has been known to be important for appropriate immune functioning in AD (Abramovits, 2005a). The discovery of Th17 lineage and Treg cells has shifted the historical concept further (Yamanaka and Mizutani, 2011). Delicate regulation of the balance among Th1, Th2, Th17/22 and Treg cells is required for the control of AD. In addition, cross-talk between aberrant immune responses and barrier defects with decreased cornification, decreased expression

of terminal differentiation proteins, and decreased lipids are noted in AD skin lesions (Guttman-Yassky et al., 2011).

Skin barrier abnormalities, including increased TEWL and decreased skin hydration, are markers for the severity and itch intensity in AD. Recent studies demonstrate that TEWL correlates well with disease activity in AD (Angelova-Fischer et al., 2005). TEWL is a consistent, reproducible and reliable measure for the assessment of severity of AD (Sugarman et al., 2003). In our study, TEWL was increased in the AD control group compared to the sham group. Elevated TEWL was reduced in the KRG and cyclosporine groups. By assessing TEWL, we are able to speculate that KRG may modulate the disease activity in AD.

The production of proinflammatory cytokines, such as TNF- α , by epidermal cells has been suggested as one of the key events mediating the initiation of AD (Homey et al., 2006). Proinflammatory cytokines produced at the initiation stage of AD induce the expression of chemokines and adhesion molecules which affect the recruitment, proliferation and survival of leukocytes within the skin (Homey et al., 2006). In our study, oral KRG administration markedly suppressed TNCB-induced elevation of TNF- α expression in the skin lesions of NC/Nga mice which may have directed leukocyte recruitment. Oral cyclosporine administration significantly suppressed TNF- α expression in the skin lesions as well. mRNA levels of TNF- α at 6 h after TNCB challenge in the AD control group showed a higher trend than the sham group. KRG, cyclosporine, and to a lesser extent, EPO showed a lower trend in the mRNA levels of TNF- α than the AD control group. However, the difference among the groups was not statistically significant. We speculate that mRNA expression yields a bigger difference at a different time point. As we studied mRNA expression at 6 h after TNCB challenge, we plan to further investigate mRNA expression closely at different time points in our future study.

TSLP is regarded as an important factor in AD, capable of enhancing the Th2 polarizing properties of DCs (Novak, 2012). TSLP has been called a 'master switch for allergic inflammation' and has shown to exhibit effects on key cells involved in cutaneous inflammation, including mast cells, basophils, and eosinophils (Liu, 2006). Quantitative RT-PCR results revealed that oral KRG and cyclosporine administration significantly down-regulated mRNA over-expression of TSLP on the AD-like skin lesions. Immunohistochemical analysis of skin lesions also showed that oral KRG and cyclosporine administration markedly diminished the over-expression of TSLP on the AD-like skin lesions. From these results, we suggest that oral administration

of KRG and cyclosporine may inhibit very early inflammatory responses in AD involving TNF- α and TSLP. In the case of EPO, mRNA expression of TSLP was down-regulated; however, protein expression of TSLP in the AD-like lesions was not suppressed. The question of whether and how changes in mRNA correspond to actual changes in protein expression has been dealt within limited studies. The mRNA-protein expression shows an overall positive correlation, but discordance may result from technological or biological factors (Guo et al., 2008).

Antigen-presenting cells (APCs) are pivotal in the pathophysiology of AD. DCs play a key role in the recognition, phagocytosis, and transmission of information between the environment and the immune systems. TSLP-stimulated DCs amplify the Th2 immune response, and inflammatory DC subtypes are recruited and predominate in the epidermis and dermis of patients with AD (Novak, 2012). In this study, extensive CD1a+ cell infiltration following TNCB application was largely suppressed by KRG administration. Cyclosporine is known to have effects on the migration, but not the differentiation and maturation of DCs (Ehser et al., 2008). No significant difference was observed in the immunohistochemistry of IL-4, IL-17 and IFN- γ . These results suggest that the favorable effects of KRG may have been mediated by modifying TSLP, DCs, and at least in part, the Th2 response. The therapeutic effect may not have been sufficient enough to suppress the inflammation in progress, as shown by the results of IL-4, IL-17 and IFN- γ .

Th2 polarization further results in eosinophil recruitment and augments the production of IgE by B cells. Although blood IgE is not essential to diagnose AD, its elevation in AD defines the phenotype of extrinsic AD (elevated IgE) rather than intrinsic AD (normal IgE). Serum IgE levels are elevated in approximately 70–80% of AD patients. IgE hyperproduction is known to cause both acute and chronic AD-like skin symptoms: The severity of AD was highly correlated with blood IgE levels in one study (Ogawa et al., 1971). Itch intensity in AD patients has been reported to be closely related to IgE (Lee et al., 2006). In our study, total IgE levels were markedly increased by TNCB application. KRG and EPO significantly modulated the total IgE levels, while cyclosporine did not. This finding is in line with previous studies, in which the efficacy of cyclosporine on AD was not associated with levels of IgE (Caproni et al., 2000). Cyclosporine was even associated with increased blood IgE in a subset of AD patients (Hijnen et al., 2007).

In conclusion, we demonstrated the preventive efficacies and immune modulation of early events in AD by oral KRG administration. Our results demonstrate that oral administration of KRG could inhibit the development of the AD-like skin lesions in NC/Nga mice both systemically and in lesional skin by suppressing TSLP, DCs, and the Th2 response, in part. Taken together, it is suggested that oral administration of KRG may be a novel approach to the prevention and treatment of early lesions in AD.

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