

Oleanolic acid acetate inhibits atopic dermatitis and allergic contact dermatitis in a murine model

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ABSTRACT

Atopic dermatitis (AD) and allergic contact dermatitis (ACD) are common allergic and inflammatory skin diseases caused by a combination of eczema, scratching, pruritus, and cutaneous sensitization with allergens. This paper examines whether oleanolic acid acetate (OAA) modulates AD and ACD symptoms by using an existing AD model based on the repeated local exposure of mite extract (*Dermatophagoides farinae* extract, DFE) and 2,4-dinitrochlorobenzene to the ears of BALB/c mice. In addition, the paper uses a 2,4-dinitrofluorobenzene-sensitized local lymph node assay (LLNA) for the ACD model. The oral administration of OAA over a four-week period attenuated AD symptoms in terms of decreased skin lesions, epidermal thickness, the infiltration of immune cells (CD4⁺ cells, eosinophils, and mast cells), and serum IgE, IgG2a, and histamine levels. The gene expression of Th1, Th2, Th17, and Th22 cytokines was reduced by OAA in the lymph node and ear tissue, and the LLNA verified that OAA suppressed ACD. The oral administration of OAA over a three-day period attenuated ACD symptoms in terms of ear thickness, lymphocyte proliferation, and serum IgG2a levels. The gene expression of Th1, Th2, and Th17 cytokines was reduced by OAA in the thymus and ear tissue. Finally, to define the underlying mechanism, this paper uses a TNF- α /IFN- γ -activated human keratinocyte (HaCaT) model. OAA inhibited the expression of cytokines and chemokines through the downregulation of NF- κ B and MAPKs in HaCaT cells. Taken together, the results indicate that OAA inhibited AD and ACD symptoms, suggesting that OAA may be effective in treating allergic skin disorders.

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Introduction

Atopic dermatitis (AD) is a multifactorial disease arising from complex interactions between genetic and environmental factors as well as between features of immune and skin cells (Boguniewicz and Leung, 2010; Finkelman et al., 2010; Novak et al., 2010). *Dermatophagoides farinae* is a typical environmental allergen, and the application of *D. farinae* extract (DFE) to the uninvolved skin of AD patients through

an atopy patch causes eczematous reactions in 30–50% of these patients (Leung et al., 2004). DFE-induced skin lesions display two phases: an initial phase with predominantly IL-4-producing Th2 cells and a subsequent phase after 24–48 h characterized by IFN- γ -producing Th1 cells (Leung and Bieber, 2003; Leung et al., 2004). The important role of Th1 and Th2 cytokines in skin inflammation has been demonstrated through experimental models (Novak, 2009).

Acute AD skin lesions are characterized by epidermal and dermal thickening, dermal infiltration of CD4⁺ T cells and eosinophils, and local expression of Th2 cytokines (Frohlich et al., 2009; Novak, 2009). Chronic AD skin lesions undergo tissue remodeling caused by chronic inflammation. These skin lesions are associated with epidermal hyperplasia, increased collagen deposition in the dermis. Recently, Th17 and Th22 cells have been detected in skin lesions of AD patients (Nogales et al., 2009). IL-17-producing Th17 cells are present in acute AD skin lesions (Toda et al., 2003). IL-22-producing Th22 cells are increased in skin lesions of patients with chronic AD (Fujita et al., 2011). In addition, the activation of keratinocytes is a hallmark of the pathogenesis of AD (Leung et al., 2004; Oyoshi et al., 2009).

Abbreviations: AD, atopic dermatitis; ACD, allergic contact dermatitis; OAA, oleanolic acid acetate; DFE, *Dermatophagoides farinae* extract; DNCB, 2,4-dinitrochlorobenzene; LLNA, local lymph node assay; DNFB, 2,4-dinitrofluorobenzene.

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Allergic contact dermatitis (ACD) is a chronic disease mediated by T cells caused by repeated skin exposure to contact allergens and is characterized by redness, papule, and vesicles, followed by scaling and dryness (Rose et al., 2012; Vocanson et al., 2005). Many commercial formulations for the treatment of ACD are available in gels, creams, lotions, or ointments but show limited effectiveness (Shah et al., 2012). Many studies have addressed the effects of the topical or systemic administration of corticosteroids and calcineurin inhibitors such as cyclosporine A (Cys A) and tacrolimus (FK506) (Quemeneur et al., 2003), but it is well known that the prolonged use of glucocorticoids and immune suppressors can cause various side effects.

Recently, several effective triterpenoid compounds such as ursolic acid, oleanolic acid, and butulinic acid have been introduced for the treatment of anti-allergic, anti-asthmatic, and anti-inflammatory activities (Giner-Larza et al., 2001; Lee et al., 2010). Oleanolic acid (3 β -hydroxy-olean-12-en-28-oic acid; OA) is a triterpenoid compound found in many plants. OA inhibits delayed-type hypersensitivity induced by 2,4-dinitrofluorobenzene (DNFB) in the mouse ear skin (Giner-Larza et al., 2001) and has been shown to be a remarkable inhibitor of 12-O-tetradecanoylphorbol-13-acetate-induced ornithine decarboxylase and c-Fos gene expression in the mouse skin (Oguro et al., 1998). Similarly, OA analogs are powerful inhibitors for cellular inflammatory processes such as the induction of NO synthase and cyclooxygenase 2 (Dinkova-Kostova et al., 2005). The inhibitory effect of the extract of *Vigna angularis* on 2,4-dinitrochlorobenzene (DNCB)-induced AD-like skin lesions in NC/Nga mice has been reported (Collantes et al., 2012). For incremental research, the present paper isolates OA and oleanolic acid acetate (OAA), a derivative of OA, from *V. angularis* through activity-guided fractionation. Despite the similarity in their chemical formulations (Supplementary Fig. S1), little is known about the biological effects of OAA. The paper develops a DFE- and DNCB-induced AD model (Choi et al., 2011b) and modifies the local lymph node assay (LLNA) as a stand-alone method for ACD. The purpose of this paper is to elucidate the effects of OAA on AD and ACD and define the underlying mechanism.

Materials and methods

Animals. Female BALB/c mice were purchased from SLC Inc. (Hamamatsu, Japan) and housed 5–10 mice per cage in a laminar air flow room maintained at a temperature of 22 ± 2 °C with a relative humidity of $55 \pm 5\%$ throughout the experiment. The care and treatment of the mice were in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (approval number KNU-2011-0133).

Preparation of the extract and the purification of compounds from *V. angularis*. *V. angularis* was purchased from an herbal medicine store in Jeongeup, Korea. The authenticity of the plants was verified (Y. H. Kim, College of Pharmacy at Chungnam National University). *V. angularis* sample (10 kg) was dried, pulverized to a fine powder, and extracted twice with 95% EtOH at 70 °C. The EtOH extract (120 l) was then passed through a 0.45 μ m filter and vaporized in a rotary evaporator to yield 100 g of residues. In addition to evaporation, the EtOH extract solution was further extracted to give four fractions: distilled water, hexane, EtOAc, and BuOH extracts. For the isolation of compounds, the EtOAc extract was further chromatographed on a silica gel (230–400 mesh, 1 kg, Merck) by using a step gradient of a hexane–EtOAc solvent system (100:1, 80:1, 60:1, 40:1, 20:1, 10:1, and 1:1; each 1 l, v/v) to yield 5 fractions (H1–H5) by TLC profiles. The recrystallization of H-3 in MeOH yielded compound 1: OAA.

Reagents and cell cultures. All materials and reagents were purchased from Sigma (St. Louis, MO) unless otherwise stated. OAA was dissolved in N,N-dimethylacetamide/Cremophor/phosphate-buffered

saline (PBS; 1:1:8 solution), and DFE was dissolved in PBS containing 0.5% Tween 20. DNCB (1%) was dissolved in an acetone/olive oil (1:3) solution. A human keratinocyte cell line, namely HaCaT, was maintained in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin G and 100 μ g/ml streptomycin) at 37 °C in 5% CO₂. DMEM, antibiotics, and trypsin-EDTA were obtained from Invitrogen (Grand Island, NY). Human TNF- α and IFN- γ were purchased from R&D Systems (Minneapolis, MN).

Induction of atopic dermatitis in the mouse ear. The induction of AD by using DFE and DNCB was performed based on previous research (Choi et al., 2011b). Female BALB/c mice (6 weeks) were divided into six groups, and the surfaces of both ear lobes were stripped five times with surgical tape (Nichiban, Tokyo, Japan). After stripping, 20 μ l of DNCB (1%) was painted on each ear, followed by 20 μ l of DFE (10 mg/ml) after 4 days. The treatment of DFE/DNCB was repeated once a week alternatively for 4 weeks. Two weeks after the first induction, tail bleeding was performed to check the serum IgE level. After confirming the atopic condition by the IgE level, OAA (2, 10, or 50 mg/kg) was orally administered until the end of the 4-week induction period. Ear thickness and clinical scores were measured 24 h after DFE or DNCB application with a dial thickness gauge (Kori Seiki MFG, Co., Japan).

On day 28, blood samples were collected by an orbital puncture. The plasma was stored at -70 °C for further analysis. After blood collection, ears were removed and used for a histopathological analysis. Serum IgE and IgG2a levels were measured using an ELISA kit (BD Biosciences, Oxford, UK) according to the manufacturer's instructions. The DFE-specific IgE level was indicated by the O.D. value.

Histological and immunohistochemical analysis. The ears were fixed with 10% formaldehyde and embedded in paraffin. Then 5 μ m sections were stained with hematoxylin and eosin (H&E). Infiltrated lymphocytes, the thickening of the epidermis, and fibrosis in the dermis were observed using a microscope. For the measurement of mast cell infiltration, skin sections were stained with toluidine blue, and the number of mast cells at five sites chosen at random was counted. Eosinophils and CD4⁺ T cells were counted blindly in 10 high-power fields at 400 $\times$ magnification. Dermal thickness was analyzed in H&E-stained sections viewed under 100 $\times$ magnification and measured in five randomly selected fields for each sample.

Histamine assay. Histamine content was measured through the o-phthalaldehyde spectrofluorometric procedure based on previous research (Bae et al., 2011). The blood from the mice was centrifuged at 400 g for 10 min, and the serum was withdrawn to measure histamine content. Fluorescent intensity was measured using 355 nm excitation and 450 nm filters and the fluorescence spectrometer LS-50B (Perkin-Elmer, Norwalk, CT).

FACS sorting. At the end of the experiment, mice were euthanized, and lymph node CD4⁺ T cells were isolated from each mouse. Separated auricular lymph nodes from each mouse were ground using 70 μ m nylon cell strainers (Falcon, Bedford, MA). Cells were washed with PBS, fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 antibodies (Santa Cruz Biotech, Santa Cruz, CA) were added to samples, and incubated at 4 °C for 30 min. The FACS sorting of lymph node single cells was performed using a FACSAria (BD Biosciences, Oxford, UK).

Real-time polymerase chain reaction. A quantitative real-time polymerase chain reaction (PCR) assay was carried out using the Thermal Cycler Dice TP850 (Takarabio Inc., Shiga, Japan) according to the manufacturer's protocol. Briefly, 2 μ l of cDNA (100 ng), 1 μ l of sense and antisense primer solution (0.4 μ M), 12.5 μ l of SYBR Premix Ex Taq (Takarabio Inc., Shiga, Japan), and 9.5 μ l of dH₂O were mixed together

to obtain a final 25 μ l reaction mixture in each reaction tube. The conditions for the PCR were similar to those in Choi et al. (2011b) except for the primer of IFN- γ (F 5'-TCA AGT GGC ATA GAT GTG GAA GAA-3', R 5'-TGG CTC TGC AGG ATT TTC ATG-3'), TSLP (F 5'-AGG CTA CCC TGA AAC TGA G-3', R 5'-GGA GAT TGC ATG AAG GAA TAC C-3'), IL-17 (F 5'-TCC CTC TGT GAT CTG GGA AG-3', R 5'-CTC GAC CCT GAA AGT GAA GG-3'), IL-22 (F 5'-TTG AGG TGT CCA ACT TCC AGC A-3', R 5'-AGC CGG ACG TCT GTG TTG TTA-3'). The normalization and quantification of mRNA expression was performed using the TP850 software supplied by the manufacturer.

Induction of allergic contact dermatitis in the mouse ear. The murine local lymph node assay (LLNA) is recognized by the U.S. Interagency coordinating Committee on the Validation of Alternative Methods (ICCVAM) as a stand-alone method for assessing allergic contact hypersensitivity (1999). The induction of ACD by using 0.15% 2,4-DNFB was performed as described previously (Lee et al., 2011). Female BALB/c mice (8 weeks) were assigned randomly to dose and control groups for each group ($n = 5$). A 25 μ l aliquot of the test solution or solvent only (4:1, acetone:olive oil, AOO) was applied daily to the dorsum of both ears of each mouse for three consecutive days (days 1–3). OAA (2, 10, or 50 mg/kg) was orally administered 2 h before DNFB treatment on days 1–3. The mice were allowed to rest on days 4 and 5. On day 4, 24 h after the last treatment, ear thickness was measured. For the LLNA, ^{3} H-methyl thymidine (^{3} H-TdR, 20 μ Ci/animal) was injected via the tail vein into all test and control mice. At 5 h after the injection, the mice were euthanized, and the auricular lymph nodes were placed in PBS. Single-cell suspensions of the lymph node in 5 ml of PBS were prepared by passing them through sterile 70 μ m nylon cell strainers (Falcon). The lymph node cell suspension was washed twice with PBS, and the cell pellet was incubated in 5 ml of 5% trichloroacetic acid at 4 °C for 18 h. Each cell pellet was resuspended in 1 ml of trichloroacetic acid and transferred to 5 ml of scintillation fluid. The incorporation of ^{3} H-TdR was measured with a liquid scintillation counter as disintegrations per minute for each mouse.

Reverse transcriptase-polymerase chain reaction. HaCaT cells were pretreated with OAA for 1 h and then stimulated using TNF- α (10 ng/ml) and IFN- γ (10 ng/ml) for 6 h. Total cellular RNA was isolated from cells (2×10^5 cells/24-well plates) as described previously (Kim et al., 2007). cDNA was synthesized from 2 μ g of total RNA by using the Maxime RT-Pre Mix Kit (iNtRON Biotechnology, Daejeon, Korea). The respective primers were chosen using the Primer3 program (Whitehead Institute, Cambridge, MA), and all primers were obtained from Genotech (Daejeon, Korea). The conditions for the reverse transcription and PCR steps were similar with our previous research (Bae et al., 2011) except for the primers of TARC (F 5'-ACT GCT CCA GGG ATG CCA TCG TTT TT-3', R 5'-ACA AGG GGA TGG GAT CTC CCT CAC TG-3'). The product was separated through electrophoresis on a 1.5% agarose gel and visualized through staining with ethidium bromide. The gels were photographed using a Kodak DC 290 digital camera.

Nuclear protein extraction. HaCaT cells were pretreated with OAA for 1 h and then stimulated with TNF- α (10 ng/ml) and IFN- γ (10 ng/ml) for 20 min. After stimulation, cells (2×10^6 /6-well plate) were washed in 1 ml of ice-cold PBS, centrifuged at 1200 g for 5 min, resuspended in 400 μ l of ice-cold hypotonic buffer (10 mM HEPES/KOH, 2 mM MgCl₂, 0.1 mM EDTA, 10 mM KCl, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 10 min, vortexed, and centrifuged at 15,000 g for 30 s. After washing, pelleted nuclei were resuspended in 50 μ l of ice-cold saline buffer (50 mM HEPES/KOH, 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.5 mM PMSF, pH 7.9), left on ice for 20 min, vortexed, and centrifuged at 15,000 g for 5 min at 4 °C, and the supernatant was gathered.

Western blot. Samples for the Western blot were prepared as described previously (Lee et al., 2008). Briefly, cells (2×10^6 cells/6-well plate) were incubated for 20 min for MAPKs and 2 h for NF- κ B then rinsed twice with ice-cold PBS, and total cell lysates were gathered in 200 μ l of lysis buffer. The lysates were spun in a microcentrifuge for 20 min at 4 °C, and the supernatant was collected. Proteins were electrophoresed using 8–12% SDS-PAGE and then transferred to nitrocellulose membranes. The membranes were stained with reversible Ponceau S to ascertain the equal loading of samples in the gel. Immunodetection was performed using an enhanced chemiluminescence detection kit (Amersham, Piscataway, NJ).

Statistical analysis. Statistical analyses were performed using Prism 5 (GraphPad Software, San Diego, CA), and treatment effects were analyzed using a one-way ANOVA, followed by Dunnett's test. Here $P < 0.05$ was used to indicate significant differences.

Results

Effects of oleanolic acid acetate on atopic dermatitis in mice

For the validation of the effect of OAA on AD, a DFE/DNCB-induced AD model was used. Fig. 1A shows the experimental procedure. During the induction period, there was no difference in the tendency of ear swelling until 2 weeks. After 2 weeks of AD induction, the oral administration of OAA showed improvements in ear thickness (Fig. 1B). The administration of OAA induced no change in body weight (Supplementary Fig. S2).

A microscopic analysis of the ear skin showed many alterations. In comparison with the case of AD mice, OAA reduced DFE/DNCB-induced epidermal and dermal thickness in a dose-independent manner and the infiltration of eosinophils and CD4⁺ T cells (Figs. 2 and 3A–C). The infiltration of mast cells, an important group of effector cells and a source of histamine in AD, was examined. Histamine is a representative symptom-inducing substance in AD (Choi et al., 2011a,b). OAA inhibited the infiltration of mast cells and serum histamine (Figs. 2 and 3D–E).

IgE production is associated with a predominant Th2 cellular response. By contrast, IgG2a is associated with the Th1 response. For the differentiation of the role of OAA in the Th1 or Th2 response, serum levels of IgE (total and DFE-specific) and IgG2a were examined. Noteworthy is that OAA reduced the levels of total IgE, DFE-specific IgE, and IgG2a in a dose-dependent manner (Fig. 4).

T cell polarization in AD is biphasic. In the acute phase, there is a predominantly Th2 response, whereas the chronic phase is characterized by the Th1 response (Bieber, 2010; Brandt and Sivaprasad, 2011; Oyoshi et al., 2009). CD4⁺ T cells can be divided according to their effector cytokines: Th1 (TNF- α , IFN- γ), Th2 (IL-4, IL-5, IL-31), Treg (TGF- β , IL-10), Th17 (IL-17), and a new subset Th22 (IL-22) (Duhon et al., 2009; Trifari et al., 2009). In addition, thymic stromal lymphopoietin (TSLP) produced by skin keratinocytes has been found to be a key player in AD pathogenesis (Jiang et al., 2012). For a better understanding of the role of OAA in AD, various cytokines were measured using a real-time PCR. All cytokines were induced in AD, and OAA reduced all cytokines and TSLP (Fig. 5A) and chemokines (Supplementary Fig. S3) in mice ear tissue in a dose-dependent manner. Because the lymph node is a crucial organ in AD pathogenesis, CD4⁺ T cells were isolated from the auricular lymph node, and the gene expression of representative cytokines was measured for T-cell subsets. OAA inhibited the expression of IFN- γ , IL-4, IL-17, and IL-22 from lymph node CD4⁺ cells (Fig. 5B). Taken together, OAA down-regulated all Th subtypes and TSLP responses in DFE/DNCB-induced AD.

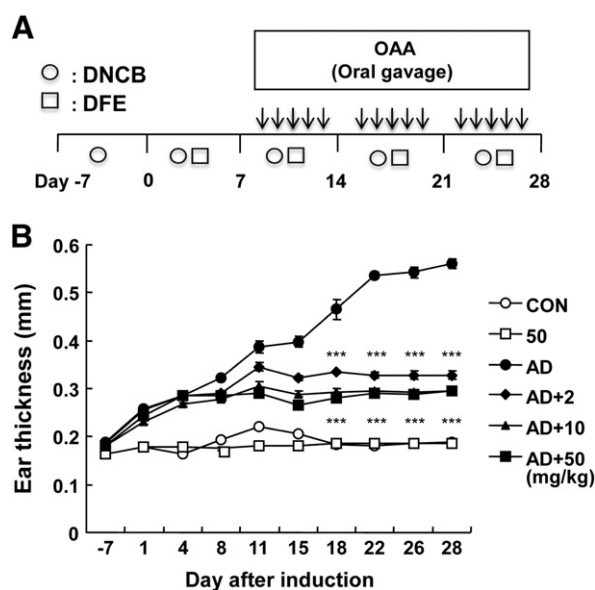


Fig. 1. Experimental design and ear thickness in AD mice. (A) Experimental design for the induction of AD. 7 days after the first boost with 1% DNBCB (20 μ l/ear), DFE (10 mg/ml, 20 μ l/ear) and DNBCB were alternatively applied to both ears once a week for 4 weeks. After 1 week of induction, OAA (2, 10, or 50 mg/kg BW) was orally administered every day for 3 weeks ($n = 5$). (B) Effects of OAA on ear thickness. Ear thickness was measured 24 h after DFE or DNBCB application with a dial thickness gauge. Data are presented as mean $\pm$ SD of five determinations. *** $P < 0.001$ ** $P < 0.01$; * $P < 0.05$ significantly lower than AD. BW, body weight.

Effects of oleanolic acid acetate on allergic contact dermatitis in mice

The effect of OAA on ACD was tested using the LLNA, a stand-alone method for examining ACD (Lee et al., 2011). In the LLNA, ear swelling and lymphocyte proliferation in draining lymph nodes were used as a measure of the immune response to an applied allergen (Anderson et al., 2011). The LLNA was induced by DNFB, a contact dermatitis sensitizer. Fig. 6A shows the schematic experimental procedure for DNFB-sensitized ACD. Cyclosporin A (Cys A), a clinically used medication for the systemic therapy of psoriasis or AD, was used as a positive control. Despite no changes in body weight in all groups (Supplementary Fig. S4), there were significant changes in ear thickness (Fig. 6B). From day 3, DNFB-sensitized mice showed

an increase in ear thickness. In comparison with the case of DNFB-sensitized mice, the oral administration of OAA reduced the thickness of the DNFB-induced ear.

The dermal application of 0.15% DNFB to mouse ears increased the number of auricular draining lymph node cells (Komutarin et al., 2004). DNFB-sensitized mice showed a high level of lymphocyte proliferation with the uptake of ^{3} H-thymidine (Fig. 6C). OAA reduced lymphocyte proliferation in auricular draining lymph nodes in a dose-dependent manner.

Contact hypersensitivity is considered a cell-mediated immunity response. Th1 and Th2 cells specifically induce antigen-specific B cells to secrete IgG2a and IgG1, respectively (Jin et al., 2009). Therefore, the serum IgG2a level was examined as an indirect measure of the systemic Th1 response to DNFB sensitization. There was a significant increase in the serum IgG2a level through repeated challenge, and this increase was inhibited by OAA (Fig. 6D).

The effects of OAA on cytokines related to ACD were examined. The expression of cytokines related to Th1 (TNF- α , IFN- γ) and Th17 (IL-17) in the ear and thymus of mice was determined by a real-time PCR. DNFB caused a significant increase in the expression of TNF- α , IFN- γ and IL-17 in the ear and thymus. OAA reduced all Th1 and Th17 cytokines (Figs. 7A–B). However, DNFB, a strong contact sensitizer, has been shown to induce mixed Th1/Th2 responses (Adenuga et al., 2012). Therefore, we also measured the typical Th2 cytokines (IL-4, IL-10). Noteworthy is that DNFB-induced IL-4 and IL-10 levels were slightly but not significantly reduced by OAA. These results suggest that OAA had a greater controlling effect on the immune reaction for Th1 and Th17 but not for Th2.

Effects of oleanolic acid acetate on the activation of keratinocytes

After the analysis of inhibitory effects of OAA on AD and ACD, a keratinocyte model was used to clarify the biological function and molecular mechanism of OAA. Keratinocytes have been shown to have similar effects on the immune response during AD development (Guttman-Yassky et al., 2011). Microbial products, physical injury, or inflammatory cytokines induce the production of TSLP by human keratinocytes (He and Geha, 2010; Ziegler and Artis, 2010). Therefore, TNF- α /IFN- γ -induced CCL17/TARC and CCL22/MDC in keratinocytes are considered as the most important mediators of AD development (Vestergaard et al., 2000). HaCaT cells, a human keratinocyte cell line, were pretreated with OAA for 1 h and then stimulated with TNF- α /IFN- γ for 6 h. OAA (0.1, 1, 10 μ M) inhibited the TNF- α /

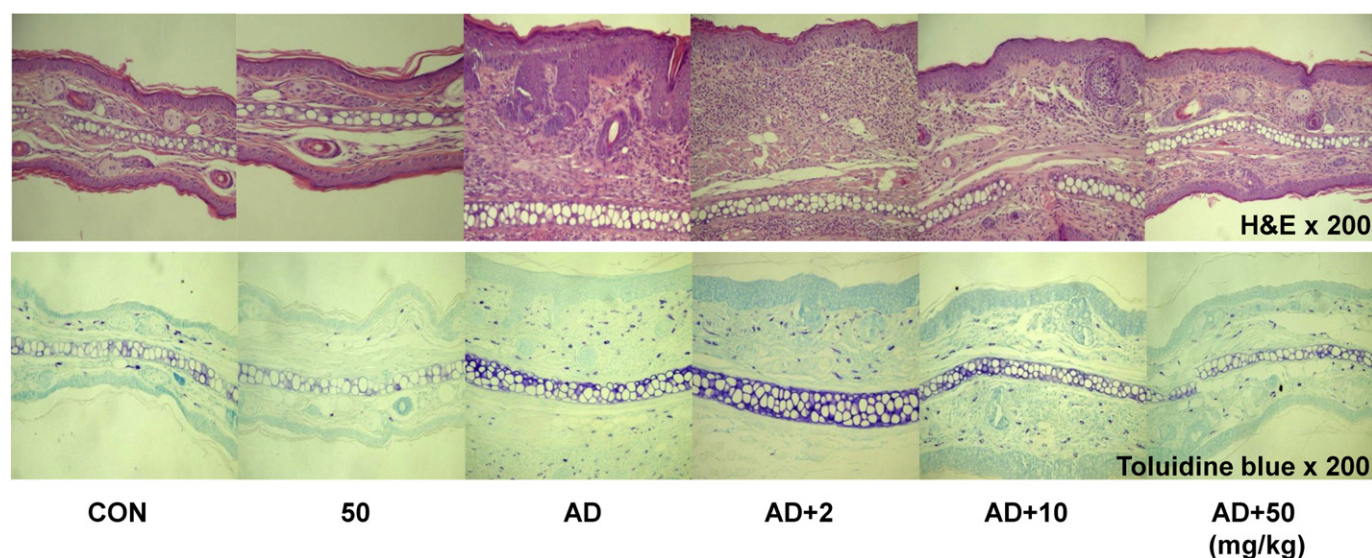


Fig. 2. Histological analysis of AD mice. Representative photomicrographs of ear sections were stained with hematoxylin and eosin or toluidine blue (original magnification: $\times 200$).

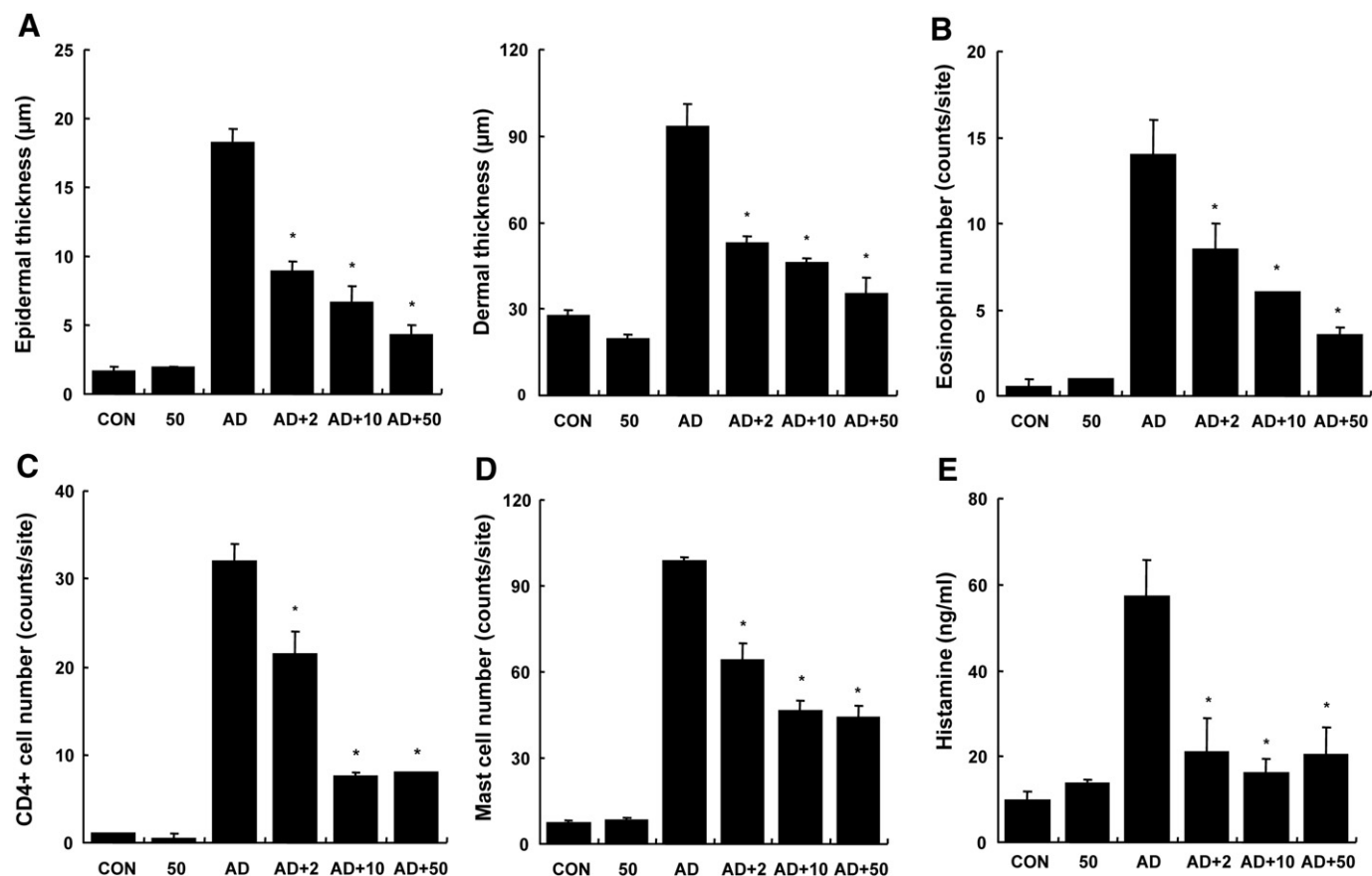


Fig. 3. Epidermal and dermal thickness, the infiltration of immune cells, and serum histamine in AD mice. (A) Epidermal and dermal thickness. Numbers of eosinophils (B), CD4⁺ T cells (C), and mast cells (D) infiltrating the dermis. (E) Serum histamine levels were measured on day 28. Data are presented as mean $\pm$ SD ($n = 5$). * $P < 0.05$ significantly lower than AD.

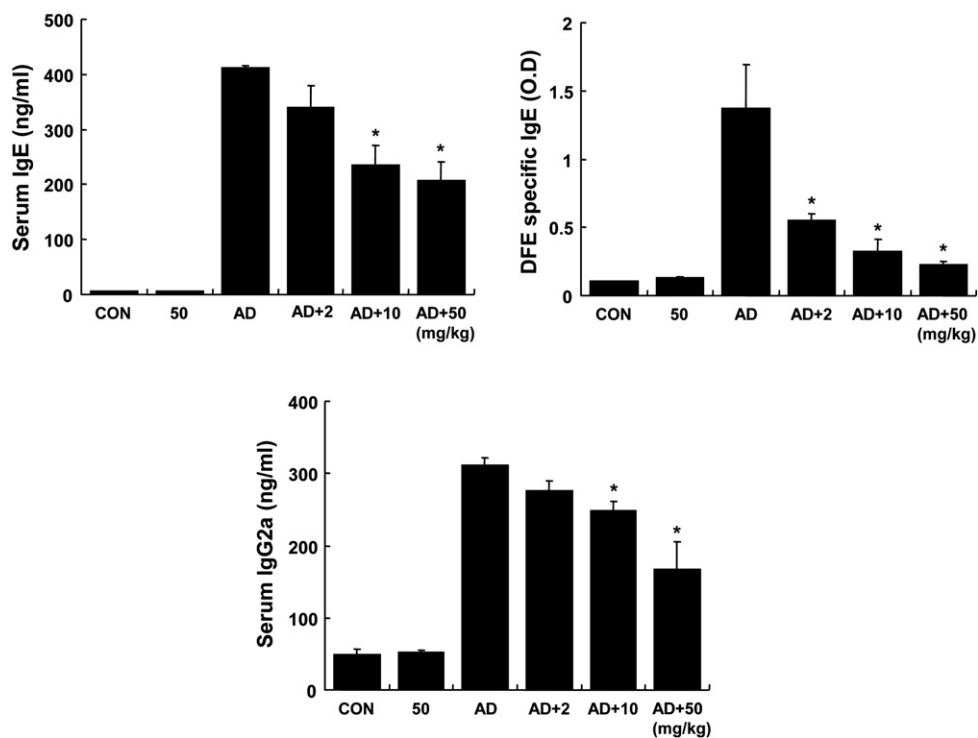


Fig. 4. Serum IgE and IgG2a levels in AD mice. Blood samples of various groups—the vehicle, OAA (50 mg/kg BW) only, DFE/DNCB plus the vehicle, and DFE/DNCB plus orally administered OAA (2, 10, or 50 mg/kg BW)—were collected by an orbital puncture on day 28. Data are presented as mean $\pm$ SD ($n = 5$). * $P < 0.05$ significantly lower than AD.

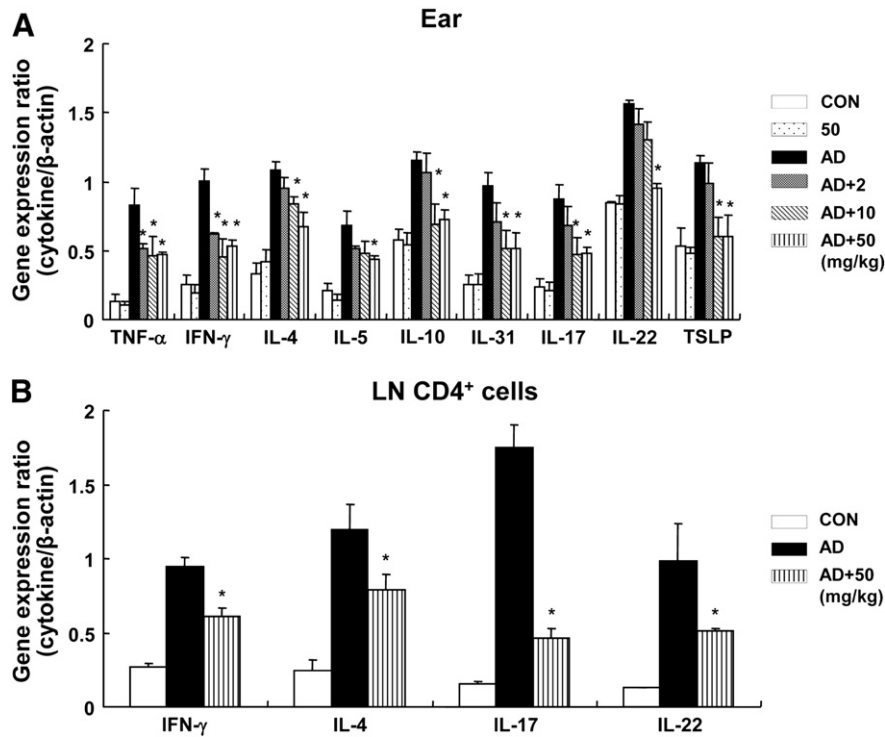


Fig. 5. Expression of cytokines in the ear and lymph node CD4⁺ T cells of AD mice. (A) Ears were excised, and total RNA was isolated. (B) Lymph node CD4⁺ T cells were isolated from each mouse by FACSaria. The quantitative real-time PCR was performed as described in the [Materials and methods](#) section. Data are presented as mean $\pm$ SD ($n = 5$). * $P < 0.05$ significantly lower than AD.

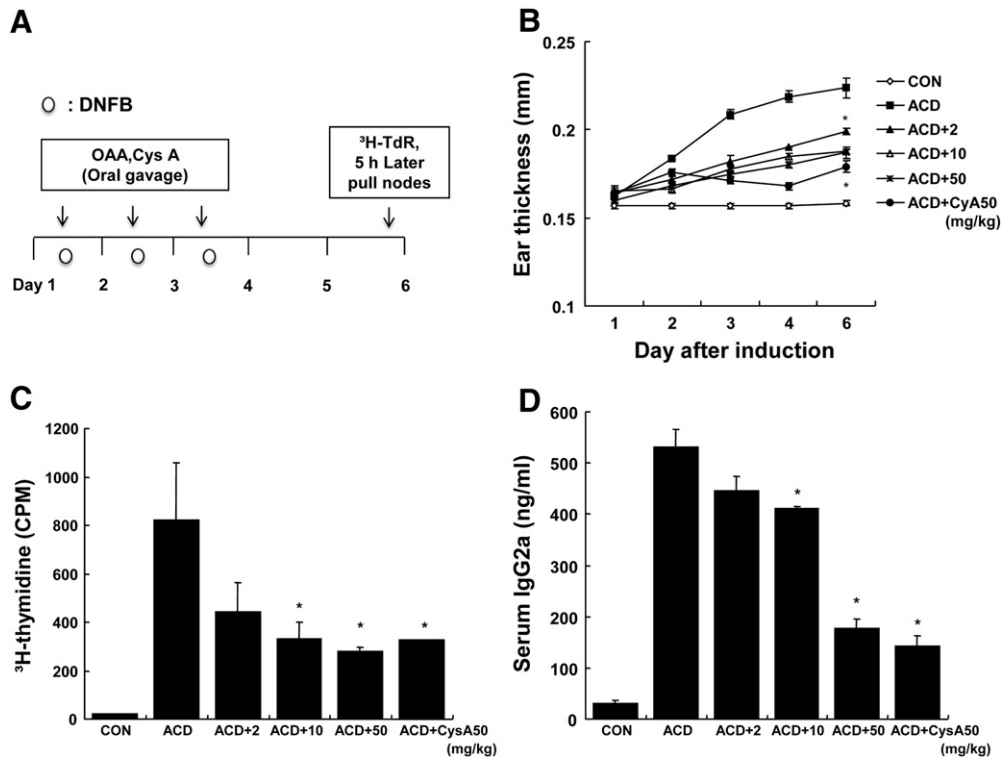


Fig. 6. Effects of OAA in ACD mice. (A) The experimental design for the sensitization of ACD. Mice ($n = 5$) were treated with the test article, the vehicle (acetone/olive oil), a positive control (0.15% DNFB, 25 μ l/ear), OAA (2, 10, or 50 mg/kg BW), and Cys A (50 mg/kg BW). OAA and Cys A were orally administered at a dose of 2–50 mg/kg BW 2 h before DNFB. Mice were euthanized on day 6, and auricular lymph nodes were removed. (B) Effects of OAA on ear thickness in ACD mice. Ear thickness was measured 24 h after DNFB application with a dial thickness gauge. (C) Mice were injected intravenously with 20 μ Ci ³H-thymidine in 250 μ l PBS on day 6. After 5 h, lymph nodes were excised. Lymph node cells were lysed and incubated overnight in 5% trichloroacetic acid, and cpm was measured by liquid scintillation spectrophotometry. (D) Serum IgG2a levels were measured on day 6. Data are presented as mean $\pm$ SD ($n = 5$). * $P < 0.05$ significantly lower than ACD. BW, body weight. Cys A, cyclosporin A.

IFN- γ -induced gene expression of TNF- α , IL-1 β , IL-6 and TARC in HaCaT cells using RT-PCR analysis (Fig. 8A) and confirmed by real time-PCR (Supplementary Fig. S5). The concentration of OAA and Cys A used in this experiment did not affect the cell viability of HaCaT cells (Supplementary Fig. S6).

TNF- α /IFN- γ -induced pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6) and chemokine (TARC) are regulated by MAPKs and NF- κ B (Kim et al., 2011). For the identification of the mechanism underlying the inhibitory effect of OAA on cytokine expression, the effects of OAA on TNF- α /IFN- γ -induced MAPKs and NF- κ B were examined. HaCaT cells were stimulated with TNF- α /IFN- γ for 20 min with or without OAA. As shown in Fig. 8B, TNF- α /IFN- γ treatment activated all three types of MAPKs and NF- κ B. OAA (10 μ M) reduced the TNF- α /IFN- γ -induced phosphorylation of ERK, p38, and JNK and the nuclear translocation of p65 NF- κ B after the degradation of I κ B α . These results imply that OAA suppressed TNF- α /IFN- γ -induced cytokines and chemokines through the reduction of MAPKs and NF- κ B in keratinocytes.

Discussion

The results indicate that OAA inhibited DFE/DNCB-induced AD and DNFB-sensitized ACD symptoms. A new AD model was developed by alternatively repeating the application of DFE/DNCB (Choi et al., 2011b). The results for the morphological and clinical features of the AD model show increases in ear and epidermal thickening as well as in the dermal infiltration of CD4⁺ T cells, eosinophils, and mast cells and are consistent with chronic AD skin lesions. OAA mitigated typical AD symptoms and significantly reduced the number of dermal mast cells and the release of serum histamine. Inflammatory mediators and histamine derived from mast cells contributed to itching and inflammation in AD. Serum histamine levels have been found to be significantly higher in AD patients (Ohsawa and Hirasawa, 2012), and therefore this model of DFE/DNCB-induced chronic AD is an important tool for examining the role of histamine in AD.

The infiltration of CD4⁺ cells and eosinophils, and the upregulation of Th2 cytokines are typical features of AD lesions. The role of Th2 cytokines in AD symptoms is well understood. The levels of Th2 cytokines (IL-4, IL-5, IL-13, and IL-10) are elevated in the skin of AD patients, and IL-5 and IL-13 levels are further increased in patients with elevated IgE levels (Jeong et al., 2003). IL-31 produced by Th2 cells has been reported to induce severe itching and dermatitis in mice and has recently been found to promote pruritus in AD patients (Guttman-Yassky et al., 2011). Keratinocyte-derived TSLP can enhance the differentiation of Th2 cells (Leung et al., 2004). In addition, it is known that IL-4 and IFN- γ drive the production of IgE and IgG2a, respectively (Jin et al., 2009). In our AD model, OAA markedly inhibited the expression of Th1 (IFN- γ), and Th2 (IL-4, IL-5, IL-10, and IL-31) cytokines, TSLP, and

serum levels of IgE and IgG2a. These results imply that OAA suppressed both Th1 and Th2 responses.

IL-17 is a pro-inflammatory cytokine capable of causing tissue damage, and IL-22 is involved in epidermal remodeling and can lead to the hyperplasia of keratinocytes in the epidermis (Eyerich et al., 2009). Increased IL-17 levels have been shown in patients with acute AD, whereas IL-22 and IFN- γ levels are upregulated in those with chronic AD (Fujita et al., 2011). In this paper, OAA markedly inhibited DFE/DNCB-induced Th17 and Th22 cytokines. In addition, OAA reduced the chemokine level in ear tissue, clearly mitigating AD symptoms (Supplementary Fig. S3). These results imply that OAA reduced both acute and chronic AD symptoms.

ACD is a highly prevalent and potentially chronic disease mediated by T cells caused by repeated skin exposure to contact allergens. ACD derives from animal models referred to as contact hypersensitivity. Such models induce skin inflammation by applying haptens on the skin (Rose et al., 2012). Haptens can directly stimulate keratinocytes present in the epidermis of the skin to release inflammatory mediators (Anderson et al., 2011). The present paper verifies the beneficial effects of OAA on ACD by focusing on the immune response of a contact allergen using the LLNA. The LLNA assesses the sensitization potential of a substance by monitoring the induced proliferation response of lymphocytes in draining lymph nodes (Adenuga et al., 2012). A DNFB-sensitized ACD model is characterized by significant skin swelling throughout the experiment. The results indicate that OAA markedly inhibited ear thickness and the proliferation of lymph node cells. In addition, the oral application of OAA prevented ACD by reducing IgG2a and Th1/Th17 cytokines (TNF- α , IFN- γ , and IL-17).

The increased IgG2a response in ACD mice may be due to the absence of a regulatory effect of IFN- γ on IgG2a-producing B cells. According to the results, OAA had a greater controlling effect on Th1 and Th17 than on Th2. Noteworthy is that the inhibitory effect of OAA on lymphocyte proliferation and Th1/Th17 cytokines was greater than that of cyclosporin A, a clinically used medication for psoriasis or AD. These results suggest that OAA can be a useful treatment option for ACD and that it may help mitigate Th1 and Th17 responses in ACD patients.

Keratinocytes from AD patients produce a unique profile of chemokines and cytokines following some mechanical stimulation. TNF- α and IFN- γ can synergistically induce important cytokines for AD symptoms in keratinocytes, and this experimental model has been widely used for mimicking AD symptoms in vitro (Leung and Bieber, 2003; Leung et al., 2004; Vestergaard et al., 2000). The present paper examines the role of OAA in TNF- α /IFN- γ -stimulated HaCaT cells. The results indicate that OAA suppressed TNF- α /IFN- γ -induced TARC, IL-6, TNF- α and IL-1 β , and verify the inhibitory effect of OAA in the AD model.

The role of NF- κ B, particularly in cytokine production in allergic inflammation, has been characterized (Blackwell et al., 1997). The

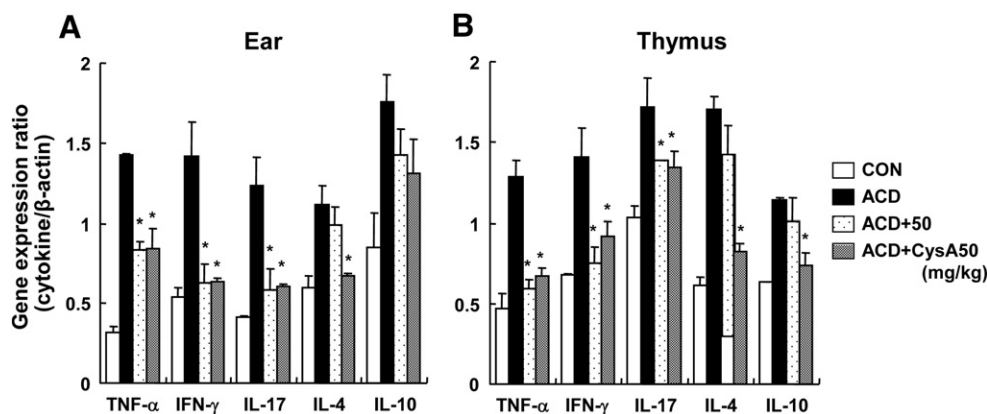


Fig. 7. Expression of cytokines in the ear and thymus of ACD mice. The ear (A) and thymus (B) were excised from each mouse. The quantitative real-time PCR was performed as described in the Materials and methods section. Data are presented as mean $\pm$ SD ($n = 5$). * $P < 0.05$ significantly lower than ACD.

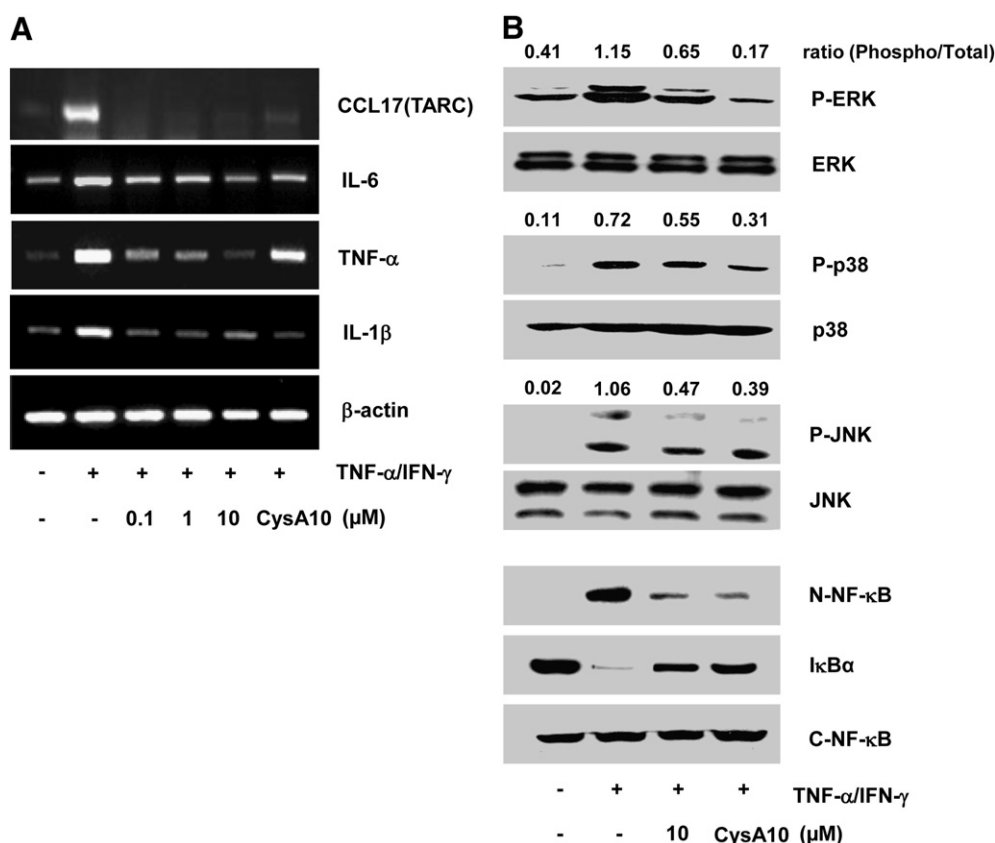


Fig. 8. Effects of OAA on TNF- α /IFN- γ -stimulated HaCaT cells. Cells were pretreated with OAA (0.1–10 μ M) and Cys A (10 μ M) for 1 h before being stimulated with TNF- α (10 ng/ml) and IFN- γ (10 ng/ml). (A) Expression levels of TNF- α , IL-1 β , IL-6, and CCL17 (TARC) were determined by the RT-PCR after 6 h of stimulation. (B) Cells were stimulated with TNF- α /IFN- γ during 20 min. The phosphorylation of MAPKs, the degradation of I κ B and the nuclear translocation of NF- κ B were analyzed by the Western blot. The experiment shown is representative of three independent experiments. C-NF- κ B, cytoplasmic NF- κ B. N-NF- κ B, nucleus NF- κ B. Cys A, cyclosporin A.

activation of the NF- κ B signaling cascade results in the degradation of I κ B through phosphorylation. In this paper, OAA inhibited the degradation of I κ B and the nuclear translocation of NF- κ B. MAPKs represent an important signaling pathway in the immune response and cross talk with NF- κ B and regulate inflammation (Arbabi and Maier, 2002). It is known that TNF- α /IFN- γ -stimulated keratinocytes activate all three types of MAPKs: ERK, JNK, and p38 (Kim et al., 2011). Previous research has reported that p38, not ERK or JNK, is involved in the TNF- α /IFN- γ -stimulated expression of TARC in HaCaT cells (Nakayama et al., 2004; Qi et al., 2011). However, the results of this paper indicate that OAA markedly inhibited the activation of all three types of MAPKs in HaCaT cells. These results provide evidence of the inhibitory effect of OAA on the expression of pro-inflammatory cytokines and TARC mediated by the blocking of MAPKs and NF- κ B pathways in HaCaT cells.

It has been reported that triterpenoid compounds such as oleanolic acid (OA), kaurenoic acid, and α -spinasterol prevent acute and chronic skin inflammation (Boller et al., 2010). OA is present in more than 120 plant species and is known to inhibit leukotriene synthesis, improve the recovery of the epidermal barrier function, and prevent the degranulation of mast cells (Dai et al., 1989; Giner-Larza et al., 2001; Lee et al., 2006). Based on the known anti-allergic and anti-inflammatory effects of OA, this paper examines the role of OAA, a derivative triterpenoid of OA, in AD and ACD models. In sum, the results demonstrate that the oral administration of OAA, a triterpenoid compound, suppressed the AD and ACD symptoms in mouse models. OAA inhibited important cytokines in AD and ACD by reducing NF- κ B and MAPKs (ERK, p38, and JNK) in HaCaT cells. However, the clinical relevance of the results for OAA to AD and ACD remains unclear. This suggests that well-designed clinical trials are required to address this issue. It should be noted that we cannot confirm at this stage that the efficacy of OAA is exclusively

via immune regulation. In addition, we cannot discriminate whether OAA modulates the induction phase or elicitation phase of ACD. The results nevertheless suggest that OAA may be an effective treatment option for allergic skin disorders. More specifically, OAA may help prevent AD and be used as an effective pharmacological agent.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.taap.2013.03.001>.

Conflict of interest statement

The authors have no conflicts to disclose.

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