

***Rhododendron album* Blume extract inhibits TNF- α /IFN- γ -induced chemokine production via blockade of NF- κ B and JAK/STAT activation in human epidermal keratinocytes**

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Abstract. *Rhododendron album* Blume (RA) has traditionally been used as an herbal medicine and is considered to have anti-inflammatory properties. It is a well-known medicine for treatment of allergic or atopic diseases. In the present study, the biological effects of an RA methanol extract (RAME) on inflammation were investigated in tumor necrosis factor- α (TNF- α)/interferon- γ (IFN- γ)-stimulated human keratinocytes. The present study aimed to investigate the potential mechanisms by which RAME inhibited TNF- α /IFN- γ -induced expression of chemokines [thymus- and activation-regulated chemokine (TARC) and macrophage-derived chemokine (MDC)] and cytokines [interleukin (IL)-6 and IL-8] through the nuclear factor- κ B (NF- κ B) pathway in human keratinocytes. The effects of RAME treatment on cell viability were investigated in TNF- α /IFN- γ -stimulated HaCaT cells. The expression of TARC, MDC, IL-6 and IL-8 was assessed using reverse transcription-quantitative polymerase chain reaction analysis or ELISA, and its effect on the inhibitory mitogen-activated protein kinase pathway was also studied using western blot analysis. TNF- α /IFN- γ induced the expression of IL-6, IL-8, TARC and MDC in a dose-dependent manner through NF- κ B and Janus kinase/signal transducers and activators of transcription (JAK/STAT) activation. Notably, treatment with RAME significantly suppressed

TNF- α /IFN- γ -induced expression of IL-6, IL-8, TARC, and MDC. In addition, RAME treatment inhibited the activation of NF- κ B and the JAK/STAT pathway in TNF- α /IFN- γ -induced HaCaT cells. These results suggest that RAME decreases the production of chemokines and pro-inflammatory cytokines by suppressing the NF- κ B and the JAK/STAT pathways. Consequently, RAME may potentially be used for treatment of atopic dermatitis.

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disorder and persistent inflammatory skin disease accompanied by eczematous lesions and severe itching. It is caused by environmental and genetic factors, including microbial infection and environmental pollutants (1-3). AD results from a combination of severe pruritus, epidermal barrier abnormalities, imbalanced immune responses and genetic predisposition (4,5). The diagnosis of AD is based on the following clinical phenotypes: Erythematous papules, eczematous skin, and severe pruritus (6,7).

Chemokines are a large group of small cytokines produced by various types of cell, and are separated into the CX3C, CXC, CC, and C subfamilies based on NH₂-terminal cysteine-motifs. On the basis of the categorization of these subfamilies, a methodical nomenclature system of the chemokine ligands has been devised in previous studies (8). The main function of chemokines is the control of inflammatory cell recruitment, including macrophages, T cells, eosinophils and the trafficking of dendritic cells (DC) at sites of inflammation and infection (9). The thymus and activation-regulated chemokines (TARC/CCL17) are CC chemokines that are constitutively expressed and produced by monocyte-derived keratinocytes and dendritic cells (10,11). Macrophage-derived chemokines (MDC/CCL22) are also the specific ligands for C-C chemokine receptor type 4 (CCR4). MDC/CCL22 is constitutively produced by epithelial cells, B cells, keratinocytes, macrophages and dendritic cells (10,12). MDC and

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TARC may serve an important function in increasing the incidence of certain skin diseases, including AD. Previous researchers have reported that serum concentrations of MDC and TARC are positively correlated with disease gravity in patients with AD patients (13). Considering all these factors, MDC and TARC may be involved in the pathogenesis of AD.

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine with multiple biological functions, including cytostatic and cytotoxic effects, differentiation and proliferation in various types of tumor cell. The antitumor effects of TNF are enhanced by interferons (IFNs) (14). The nuclear transcription factor, nuclear factor (NF)- κ B has been reported to be an anti-apoptotic factor that serves a major function in cell survival during apoptosis induced by cytokines, including TNF- α /IFN- γ , and various chemotherapeutic agents (15,16). In addition, the phosphorylation of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway has been reported to be an important anti-inflammatory factor. JAK activation stimulates cell differentiation, proliferation, cell migration and apoptosis. Kimura *et al* (14) reported that a combination of TNF- α and IFN- γ treatment resulted in an upregulated immune response in murine fibroblasts (14). In addition, this TNF- α /IFN- γ combined treatment also increased the *in vitro* and *in vivo* induction of human inflammation (17,18). Based on these observations, our group hypothesized that the synergism of atopic dermatitis and immune responses results in the downregulation of TNF- α /IFN- γ -induced cell survival mechanisms.

Rhododendron album Blume (RA) is a species of plant that belongs to the family Ericaceae. It is primarily found in humid primeval forests at high altitudes throughout western and central Java. RA is 1-2 m tall, and the leaves (5-12 cm long, and 2-3 cm wide) are stiff, with a brown, scaly underside. The corolla is widely campanulate, and is pale yellow in colour with scattered brown scales. RA is endemic to Java and is rare in the wild. However, RA is grown widely in nurseries throughout the world, particularly in the United States. Certain plants belonging to the genus *Rhododendron* have documented anti-cancer (19,20) and antioxidant functions (21). Therefore, RA may also exert anti-atopic or anti-inflammatory activity. The present study focused on the anti-inflammatory effects of RA methanol (MeOH) extract (RAME) (22). At present, there are no data on the anti-inflammatory activity of this plant. Therefore, the purpose of the present study was to evaluate the anti-inflammatory and anti-atopic dermatitis activity of RAME in HaCaT keratinocyte cells.

Materials and methods

Preparation of RA extract. The plant species was collected from Cantigi in Indonesia in 2008, and it was identified by the Center for Pharmaceutical and Medical Technology, Agency for Assessment and Application of Technology (Tangerang, Indonesia) and verified by Herbarium Bogoriense (Indonesian Institute of Sciences, Bogor, Indonesia). A voucher specimen (KRIB 0019989) has been deposited in the herbarium of the Korea Research Institute of Bioscience and Biotechnology, and also in the Center for Pharmaceutical and Medical Technology and Herbarium Bogoriense. The RA plant material was treated with MeOH and sonicated for 15 min, at

36.5 kHz using Ultrasonic Cleaning Bath (Branson Ultrasonics; Emerson Electric Co., St. Louis, MO, USA) and incubated for 2 h at room temperature. This procedure was repeated 30 times for 3 days to produce an extract. The methanol extract (360 mg) was suspended in distilled water, the same amount of n-hexane was added and mixed, and then the n-hexane soluble fraction and the water-soluble fraction were separated. This was performed three times, followed by cotton wool filtration and concentration under reduced pressure to obtain an n-hexane fraction (yield, 17.2 mg). Then, the n-hexane fraction was removed and the same amount of chloroform was added to the remaining water layer. A chloroform fraction (yield, 21.0 mg) was obtained in the same manner. The same amount of ethyl acetate was added to the remaining water layer, and an ethyl acetate fraction (yield, 14.0 mg) was obtained in the same manner. Finally, an equivalent amount of butanol was added to the water layer, and a butanol fraction (yield, 47.3 mg) was obtained in the same manner. The remaining water layer was then concentrated to obtain a water fraction (yield, 235.5 mg).

Cell culture. The human keratinocyte HaCaT cell line (ATCC; American Type Culture Collection, Manassas, VA, USA) was cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin) at 37°C in a humidified 5% CO₂ incubator. Prior to further association studies, the growth medium was changed to serum-free medium. Cells in the control group were treated with dimethyl sulfoxide (DMSO, 0.1% per 100 μ l) alone. DMEM, FBS, penicillin, streptomycin, and PBS (pH 7.4) were purchased from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Recombinant TNF- α (10 ng/ml) was purchased from Invitrogen; Thermo Fisher Scientific, Inc. and IFN- γ (10 ng/ml) was purchased from Merck KGaA (Darmstadt, Germany). The HaCaT cells were pretreated with RAME (2.5, 5, 10, 20 μ g/ml) for 1 h and subsequently treated with TNF- α (10 ng/ml) and IFN- γ (10 ng/ml), and incubated for 24 h at 37°C. In another set of cultures, the cells were co-incubated with 5 μ M Bay11-7082, a nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor, α (I κ B- α) inhibitor, 10 μ M SB203580, a p38 inhibitor, and 10 μ M SP600125, a c-Jun N-terminal kinase (JNK) inhibitor (Calbiochem; EMD Millipore, Billerica, MA, USA) for 1 h at 37°C in a humidified 5% CO₂ incubator.

Cell viability. To confirm the effect of RAME on HaCaT cells, the reduction of MTT (Amresco, Inc., Solon, OH, USA) by viable cells was measured. The cells were plated in 96-well culture plates (SPL Life Sciences, Pocheon, Korea) at a density of 1x10⁴ cells/well, and allowed to bind for 6 h. Cells were allowed to attach for 6 h, and the culture was continued for 24 h following addition of RAME (2.5, 5, 10, 20 μ g/ml) at 37°C. The cells were cultured with 0.5 mg/ml MTT solution. After 4 h of incubation at 37°C in 5% CO₂, the supernatant was removed and DMSO was added. Absorbance was measured at 570 nm using a microplate reader (Tecan Group, Ltd., Männedorf, Switzerland). The percentage of viable cells was calculated by taking the optical density of the cells following a particular treatment and dividing that number with the optical density of untreated control cells, followed by multiplication by 100.

Interleukin (IL)-6 and IL-8 production. The culture medium was collected and the production of cytokines (IL-6 and IL-8) in the supernatant was measured using ELISA kits (IL-6; cat. no. 555220, BD Biosciences, San Jose, CA, USA; IL-8; cat. no. DY208, R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturers' protocols, as previously described (23,24).

TARC and MDC production. The culture medium of the cells was harvested, and chemokine production (TARC and MDC) in the supernatant was measured using ELISA kits (cat nos. TARC cat. no. DY364 and MDC cat. no. DY336; R&D Systems, Inc.) according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc.) for RT-qPCR analysis. cDNA was synthesized using the AMPIGENE® cDNA Synthesis kit (cat. no. ENZ-KIT106; Enzo Life Sciences, Inc., Farmingdale, NY, USA). qPCR was performed using SYBR Green PCR Master Mix (KAPA SYBR® FAST qPCR kits; Kapa Biosystems, Inc., Wilmington, MA, USA). Reactions were run in a CFX96 Real-Time PCR System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the following thermocycler conditions: Stage 1, 50°C for 2 min and 95°C for 10 min; stage 2, 95°C for 15 sec and 60°C for 1 min. Stage 2 was repeated for 40 cycles. Relative mRNA levels were calculated using the comparative threshold cycle $2^{-\Delta\Delta C_q}$ method and normalized to that of GAPDH (25). The primer sequences used in the present study are listed in Table I.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis. Briefly, the HaCaT cells were washed with cold PBS and then lysed in RIPA buffer (ELPIS Biotech, Inc., Daejeon, Korea) on ice for 20 min with a strong vortex. The cell lysate was centrifuged at 10,000 × g for 10 min at 4°C and protein concentrations were measured using the bicinchoninic acid method. Sample proteins (20 µg) were analyzed on 10% SDS-PAGE and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane, following which the PVDF membrane was blocked in 5% non-fat dry milk in Tris-buffered saline (TBS, 20 mM Tris, 0.2 M NaCl, pH 7.5) containing 0.05% Tween-20 (TBS/T) for 1 h at room temperature. The PVDF membrane was incubated with primary antibodies for overnight at 4°C. Following washing three times with TBS/T, the membranes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies, diluted 1:5,000 with 5% skim milk in TBS/T. The membranes were then visualized using SuperSignal West Pico Chemiluminescent Substrate (cat. no. 32106; Pierce; Thermo Fisher Scientific, Inc.). The primary antibodies used were as follows: anti-NF-κB p65 (cat. no. sc-8242; 1:1,000), anti-JNK (cat. no. sc-474; 1:1,000), anti-extracellular signal-regulated kinase (ERK; cat. no. sc-154; 1:1,000), anti-p38 (cat. no. sc-7149; 1:1,000), anti-p-p38 (cat. no. sc-7973; 1:1,000), anti-phosphorylated (p-) JAK1 (cat. no. sc-16773; 1:1,000), anti-JAK1 (cat. no. sc-376996; 1:1,000), anti-p-STAT1 (cat. no. sc-8394; 1:1,000), anti-STAT1 (cat. no. sc-464; 1:1,000; all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-p-ERK 1/2 (cat. no. 9101;

1:1,000), anti-p-IκB-α (cat. no. 2859; 1:1,000), anti-IκB-α (cat. no. 9242; 1:1,000), anti-NF-κB p-p65 (cat. no. 3033; 1:1,000; all from Cell Signaling Technology, Inc., Danvers, MA, USA), anti-p-JNK (cat. no. KAP-SA011; 1:1,000; Enzo Life Sciences, Inc.), and anti-β-actin (cat. no. 4967; 1:1,000; Cell Signaling Technology, Inc.). The secondary antibodies used were as follows: HRP-conjugated goat anti-rabbit IgG (cat. no. sc-2030; 1:5,000 in 5% skim milk; Santa Cruz Biotechnology, Inc.; used for detection of NF-κB p65, JNK, ERK, p38, p-p38, p-JNK, p-ERK, p-IκB-α, IκB-α, NF-κB p-p65, β-actin) and HRP-conjugated goat anti-mouse IgG (cat. no. sc-2005; 1:5,000 in 5% skim milk; Santa Cruz Biotechnology, Inc.; used for detection of JAK1, STAT1, p-JAK1, p-STAT1). Each protein was detected using an Enhanced Chemiluminescence detection system using ImageQuant LAS4000 (GE Healthcare Life Sciences, Little Chalfont, UK). Western blot bands were quantified using ImageJ software (version 1.6.0; National Institutes of Health, Bethesda, MD, USA).

Luciferase assay. HaCaT cells were transfected with 0.1 µg pGL4.32 (luc2P/NF-κB-RE/Hygro, Promega Corporation, Madison, WI, USA) plasmids, using Lipofectamine 2000 transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Then, 20 h after transfection, the cells were stimulated with TNF-α/IFN-γ (10 ng/ml) for 24 h at 37°C, harvested, and then assessed for luciferase activity using the ONE-Glo™ luciferase reporter assay system (Promega Corporation) according to the manufacturer's protocol. Normalization was performed by comparison with *Renilla* luciferase activity.

Immunocytochemistry (ICC). HaCaT cells were cultured in Lab-Tek™ chamber slides (Thermo Fisher Scientific, Inc.) and immobilized in ethanol at 4°C for 15 min. Slides were washed three times with PBS and blocked with 2% (w/v) bovine serum albumin (BSA100, Bovogen Biologicals Pty Ltd., Melbourne, Australia) in PBS for an additional 1 h at room temperature. Slides were then incubated with anti-NF-κB p65 subunit (cat. no. sc-8242; rabbit polyclonal IgG; 1:200, Santa Cruz Biotechnology) and anti-STAT1 (cat. no. sc-464; mouse monoclonal IgG; 1:100, Santa Cruz Biotechnology, Inc.) antibodies for 24 h at 4°C. Following washing with PBS three times to remove excess primary antibody, the slides were further incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG (cat. no. A-11034; 1:1,000; Invitrogen; Thermo Fisher Scientific, Inc.) or Texas Red conjugated goat anti-mouse IgG secondary antibodies (cat. no. A-11003; 1:1,000; Invitrogen; Thermo Fisher Scientific, Inc.) for 2 h at room temperature, washed with PBS, and stained with Gold Antifade reagent containing DAPI (Invitrogen; Thermo Fisher Scientific, Inc.) for 5 min prior to the position and quantification of ProLong nuclei. Subsequently, the slides were coverslipped and visualized using confocal laser scanning microscopy (LSM 510 m; Carl Zeiss AG, Oberkochen, Germany). All samples were quantified from the images obtained, which were taken under the same exposure conditions.

Statistical analysis. The data represent the mean ± standard error of the mean. Statistical differences among groups were

Table I. Primer sequences.

Gene	Primer sequences ^a (5'-3')	Fragment size (bp)
TARC		222
Forward	CACGCAGCTCGAGGGACCAATGTG	
Reverse	TCAAGACCTCTCAAGGCTTTGCAGG	
MDC		362
Forward	AGGACAGAGCATGGCTCGCCTACAGA	
Reverse	TAATGGCAGGGAGGTAGGGCTCCTGA	
IL-6		124
Forward	GACAGCCACTCACCTCTTCA	
Reverse	AGTGCCTCTTTGCTGCTTTC	
IL-8		299
Forward	ATGACTTCCAAGCTGGCCGTGGCT	
Reverse	TTATGAATTCTCAGCCCTCTTCAAAAA	
β -actin		250
Forward	CATGTACGTTGCTATCCAGGC	
Reverse	CTCCTTAATGTCACGCACGAT	

^aPrimer sequences are human. TARC, thymus- and activation-regulated chemokine; MDC, macrophage-derived chemokine; IL, interleukin.

determined by one-way analysis of variance with repeated measures followed by Student-Newman-Keuls testing in SPSS 14.0 software (SPSS, Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Cell viability. The effect of RAME on cell viability was confirmed using HaCaT cells and an MTT assay. As presented in Fig. 1, RAME did not result in a significant cytotoxic effect, failing to affect cell viability even at a relatively high concentration of 20 $\mu\text{g/ml}$ for a treatment period of 24 h. Therefore, RAME was experimentally confirmed to not show toxicity, even when HaCaT cells were treated with 20 $\mu\text{g/ml}$. In addition, the effect of RAME and solvent fraction (n-hexane, chloroform, ethyl acetate, butanol, water layer) on cell viability was confirmed using HaCaT cells and MTT assays. RAME and solvent fraction did not affect cell viability, even at a relatively high concentration of 20 $\mu\text{g/ml}$ for a 24 h treatment period (data not shown).

Treatment with RAME inhibits TNF- α /IFN- γ -induced TARC and MDC expression in HaCaT cells. Next, the effect of suppressing TARC and MDC production by RAME in HaCaT cells stimulated with TNF- α /IFN- γ was investigated using ELISA and RT-qPCR (Fig. 2). TARC and MDC were significantly increased in the group treated with TNF- α /IFN- γ compared with the untreated group. In addition, when HaCaT cells were treated with TNF- α /IFN- γ following RAME treatment, TARC and MDC mRNA expression decreased in a concentration-dependent manner, compared with the group treated with TNF- α /IFN- γ . Furthermore, RAME treatment inhibited the expression of TARC and MDC protein in the HaCaT cells stimulated with TNF- α /IFN- γ .

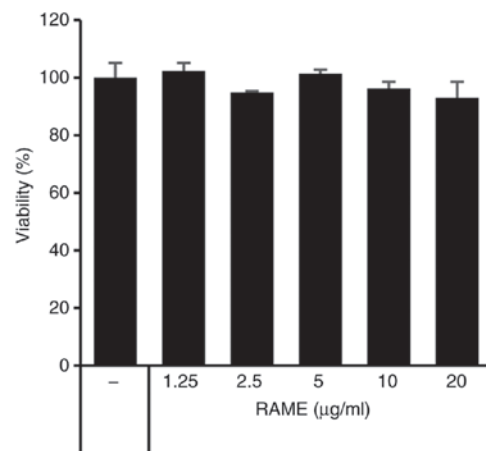


Figure 1. Cytotoxicity of RAME treatment in HaCaT cells. Cells were seeded in 96-well plates and treated with RAME (2.5, 5, 10, 20 $\mu\text{g/ml}$) for 24 h. Cell viability was assessed using the MTT assay. RAME, *Rhododendron album* Blume methanol extract.

Treatment with RAME inhibits TNF- α /IFN- γ -induced IL-8 and IL-6 expression in HaCaT cells. Next, the effect of inhibiting the production of IL-6 and IL-8 through RAME treatment was investigated in HaCaT cells stimulated with TNF- α /IFN- γ using ELISA and RT-qPCR (Fig. 3). The results revealed that IL-6 and IL-8 were significantly increased in the group treated with TNF- α /IFN- γ compared with the untreated group. In addition, when HaCaT cells were treated with TNF- α /IFN- γ following RAME treatment, IL-6 and IL-8 mRNA expression decreased in a concentration-dependent manner compared with the group treated with TNF- α /IFN- γ . Furthermore, RAME treatment inhibited the expression of IL-6 and IL-8 protein in the HaCaT cells stimulated with TNF- α /IFN- γ .

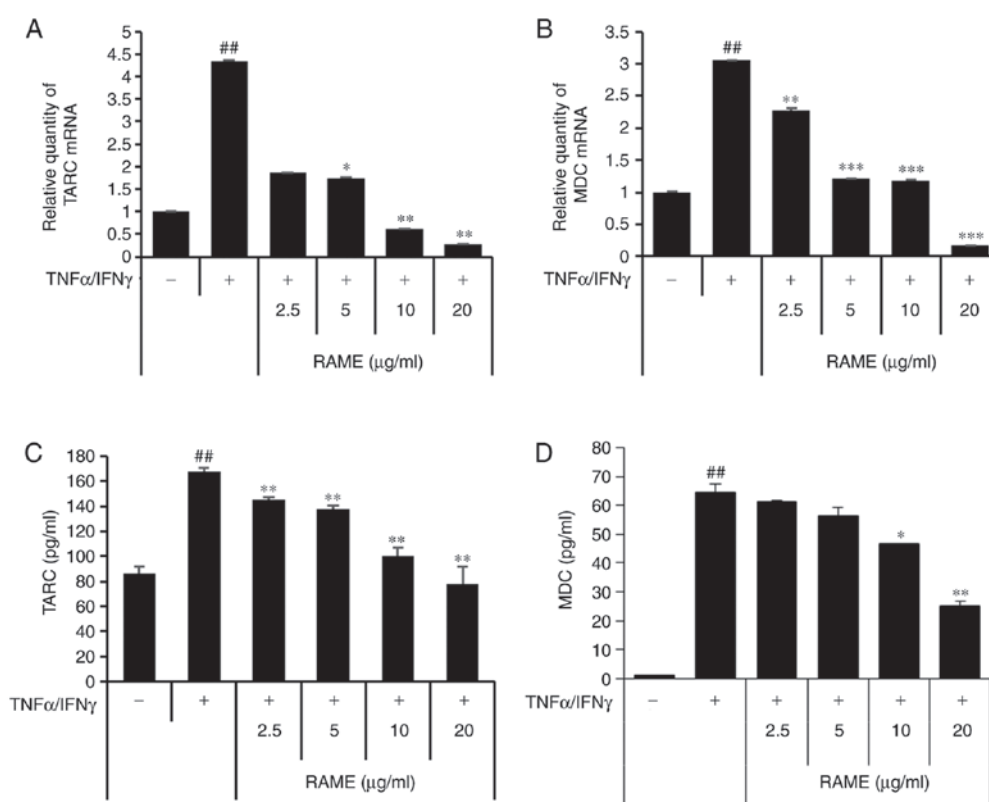


Figure 2. RAME affects the expression of chemokines in HaCaT cells. HaCaT keratinocytes were pretreated with RAME (2.5, 5.0, 10.0 or 20.0 μg/ml) and stimulated with TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) for 24 h. (A) TARC and (B) MDC mRNA expression in HaCaT cells. (C) TARC and (D) MDC levels were measured using culture supernatants of cells treated with RAME and TNF-α/IFN-γ for 24 h. Data are presented as the mean ± standard error of the mean of 3 samples. ##P<0.01 vs. the negative control; *P<0.05, **P<0.01 and ***P<0.001 vs. TNF-α/IFN-γ stimulated cells. RAME, *Rhododendron album* Blume methanol extract; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; TARC, thymus- and activation-regulated chemokine; MDC, macrophage-derived chemokine.

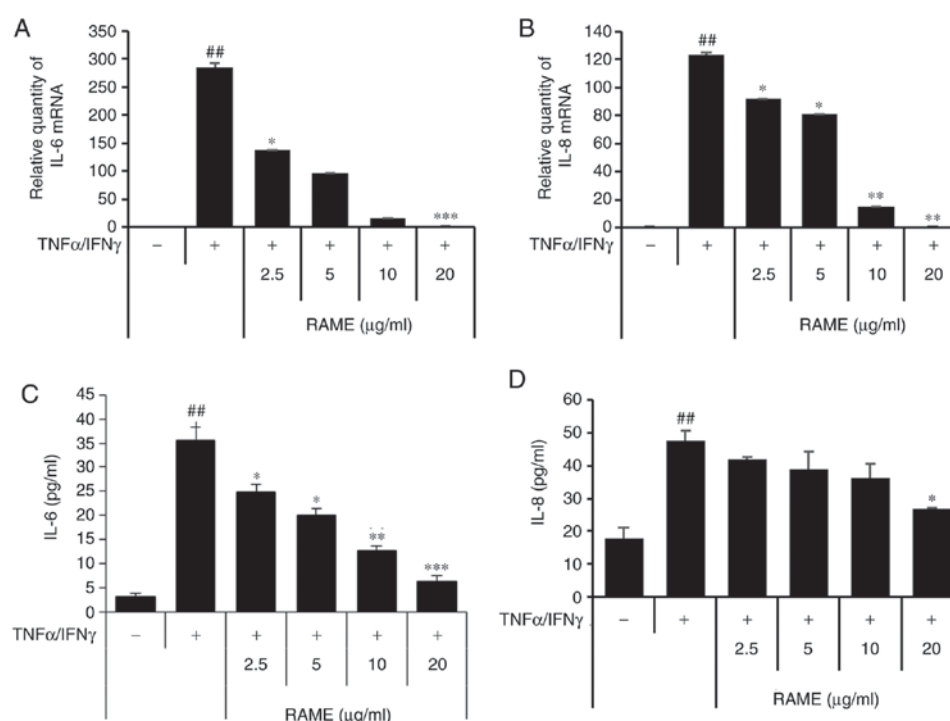


Figure 3. Effect of RAME on the expression of cytokines and intracellular adhesion molecules from HaCaT cells. HaCaT cells pretreated with RAME (2.5, 5.0, 10.0 or 20.0 μg/ml) and then induced with TNF-α (10 ng/ml) and IFN-γ (10 ng/ml) for 24 h. (A) IL-8 and (B) IL-6 mRNA expression in HaCaT cells. (C) IL-6 and (D) IL-8 protein expression was measured using culture supernatants of cells treated with RAME and TNF-α/IFN-γ for 24 h. A group of bars represents the average of three independent experiments. Data are presented as the mean ± standard error of the mean of 3 samples. ##P<0.01 vs. the negative control; *P<0.05, **P<0.01 and ***P<0.001 vs. TNF-α/IFN-γ stimulated cells. RAME, *Rhododendron album* Blume methanol extract; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; IL, interleukin.

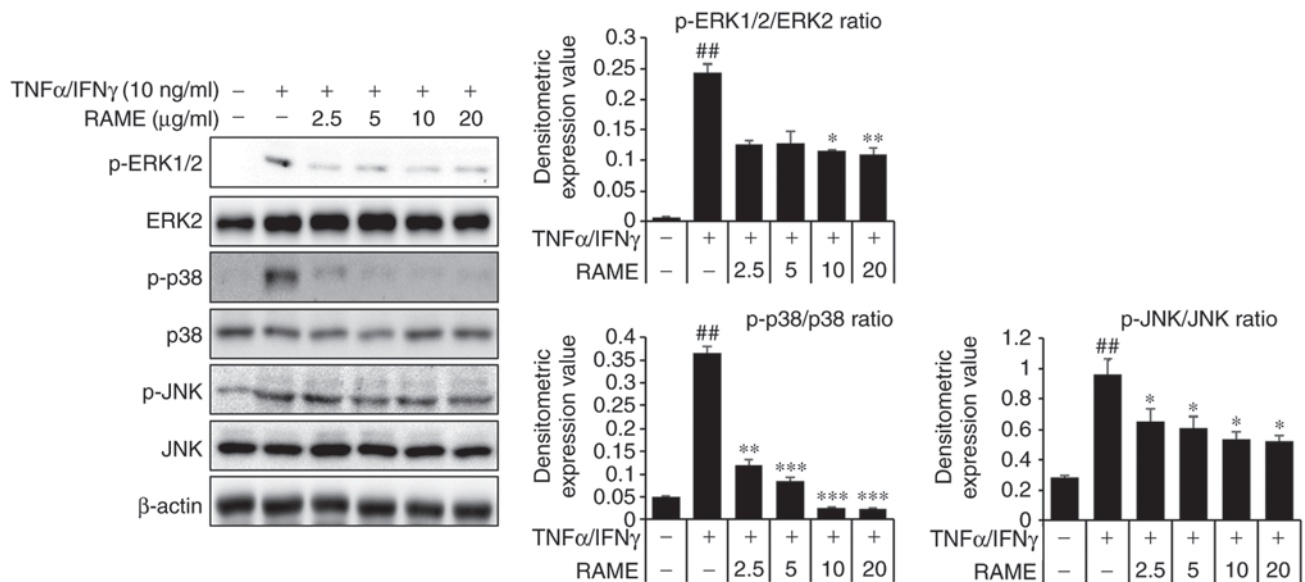


Figure 4. Effect of RAME treatment on TNF- α /IFN- γ induced MAPKs in HaCaT cells. Cells were pretreated with 2.5, 5.0, 10.0, and 20.0 μ g/ml RAME for 1 h and then exposed to TNF- α and IFN- γ (each 10 ng/ml) for 2 h. Cell extracts were prepared and MAPK activation was analyzed by western blotting, using specific antibodies. ## P <0.01 vs. the negative control; * P <0.05, ** P <0.01 and *** P <0.001 vs. TNF- α /IFN- γ stimulated cells. RAME, *Rhododendron album* Blume methanol extract; TNF- α , tumor necrosis factor- α ; IFN- γ , interferon- γ ; MAPK, mitogen-activated protein kinase; p-, phosphorylated; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

Treatment with RAME inhibits the activation of mitogen activated protein kinases (MAPKs) in HaCaT cells. TNF- α /IFN- γ activate JNK, ERK and p38 expression (24). Therefore, the effect of RAME on JNK, ERK and p38 protein expression in HaCaT cells was investigated (Fig. 4). HaCaT cells were treated with TNF- α /IFN- γ and RAME was added after 2 h. Phosphorylation changes to JNK, ERK, and p38, which are MAPKs that are known to be important in the atopy pathway, were confirmed. The results revealed an increase in the phosphorylation of JNK, ERK, and p38 in HaCaT cells stimulated for 1 h with TNF- α /IFN- γ , compared with unstimulated HaCaT cells. Furthermore, this experiment confirmed that when the cells were pretreated with RAME, the phosphorylation of JNK, ERK, and p38 was markedly suppressed as compared with the group treated with TNF- α /IFN- γ alone. Consequently, RAME exerted an inhibitory effect on the expression of p-JNK, p-ERK, and p-p38 following TNF- α /IFN- γ stimulation.

RAME inhibited the activation of NF- κ B in HaCaT cells. NF- κ B signaling is critical in skin inflammatory AD responses induced by MDC and TARC (26). To examine whether RAME downregulated TNF- α /IFN- γ -mediated NF- κ B activation in the keratinocyte cells, the expression of associated proteins was examined by western blot analysis using specific antibodies, ICC, and luciferase assays. TNF- α /IFN- γ significantly increased the phosphorylation of NF- κ B p65 at 1 h. RAME decreased the phosphorylation of NF- κ B p65 in TNF- α /IFN- γ -induced HaCaT cells (Fig. 5A). TNF- α /IFN- γ -induced nuclear translocation of p65 was inhibited by treatment with RAME at 10 μ g/ml (Fig. 5B). Furthermore, the inhibitory effect of RAME on TNF- α /IFN- γ -stimulated nuclear translocation of NF- κ B p65 was also observed during ICC analysis (Fig. 5C). These results revealed that RAME inhibited the activation of NF- κ B in HaCaT cells.

RAME and Bay11-7082 inhibited the activation of NF- κ B in HaCaT cells. The p65 subunit is an important element of activated NF- κ B. Activation of NF- κ B in TNF- α /IFN- γ induced HaCaT cells was studied by measuring the phosphorylation of NF- κ B subunit p65 by western blot analysis. Pretreatment with RAME and Bay11-7082 decreased the levels of p-I κ B- α and p-p65 compared with the TNF- α /IFN- γ treatment only, while the TNF- α /IFN- γ treatment group increased the levels of p-I κ B- α and p-p65 compared with the negative control (Fig. 6A). Stimulation of HaCaT cells with TNF- α /IFN- γ induced nuclear translocation of p65 NF- κ B and degradation of I κ B- α . NF- κ B-dependent gene reporter analysis was used to confirm the inhibitory effect of RAME and Bay11-7082 on NF- κ B activation. RAME and Bay11-7082 suppressed TNF- α /IFN- γ -stimulated nuclear translocation of p65 NF- κ B (Fig. 6B). As presented in Fig. 6C, the TNF- α /IFN- γ stimulated NF- κ B promoter activity in HaCaT cells was significantly reduced by RAME and Bay11-7082 inhibitors. In addition, stimulation of HaCaT cells with TNF- α /IFN- γ induced the expression of chemokines and pro-inflammatory cytokines. RAME and Bay11-7082 treatment suppressed TNF- α /IFN- γ -stimulated expression of TARC, MDC, IL-6, and IL-8 (Fig. 7A-D). As presented in Fig. 7, the expression of TNF- α /IFN- γ stimulated chemokines (TARC and MDC) and pro-inflammatory cytokines (IL-6 and IL-8) in HaCaT cells was significantly decreased by treatment with RAME and Bay11-7082 inhibitors.

RAME and inhibitors inhibit the expression of chemokines and cytokines in TNF- α /IFN- γ -stimulated HaCaT cells. The translocation of NF- κ B into the nucleus was reduced in RAME-treated HaCaT cells (Fig. 5). The MAPKs may be activated by TNF- α /IFN- γ , a key stimulator of the skin inflammation response in keratinocytes, as well as multiple other types of cell. As presented in Fig. 4, RAME markedly

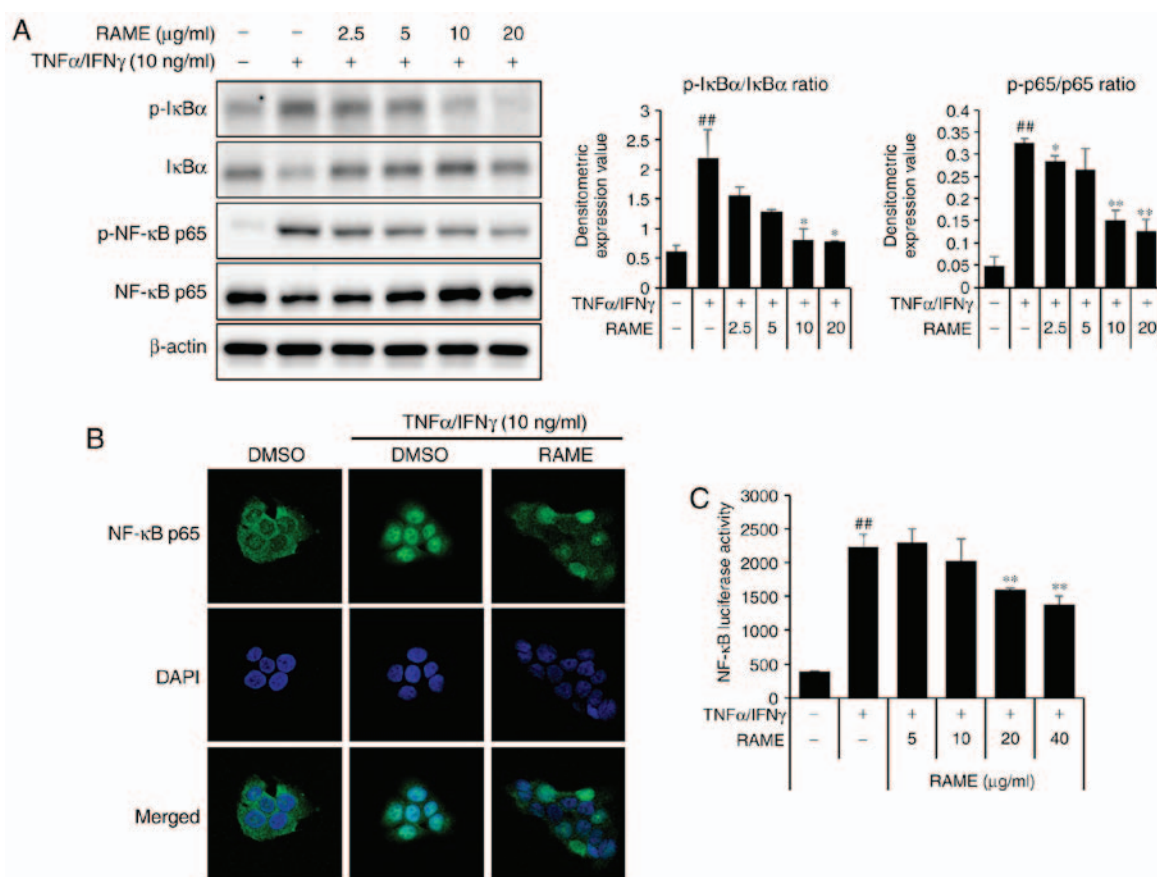


Figure 5. Effect of RAME treatment on TNF- α /IFN- γ induced NF- κ B activation in HaCaT cells. (A) The activation of p65 and I κ B- α was analyzed using western blotting. (B) Cell localization of p65 was determined by immunocytochemistry (x400 magnification). (C) HaCaT cells were transfected with the expression vector luciferase (Luc) reporter plasmid (0.1 μ g). Then, 24 h after transfection, HaCaT cells were treated with RAME for 1 h and the luciferase activity was measured. Data are presented as the mean \pm standard error of the mean of three samples. ^{##}P<0.01 vs. the negative control; ^{*}P<0.05 and ^{**}P<0.01 vs. TNF- α /IFN- γ stimulated cells. RAME, *Rhododendron album* Blume methanol extract; TNF- α , tumor necrosis factor- α ; IFN- γ , interferon- γ ; p-, phosphorylated; I κ B- α , nuclear factor of κ light polypeptide gene enhancer in B-cells.

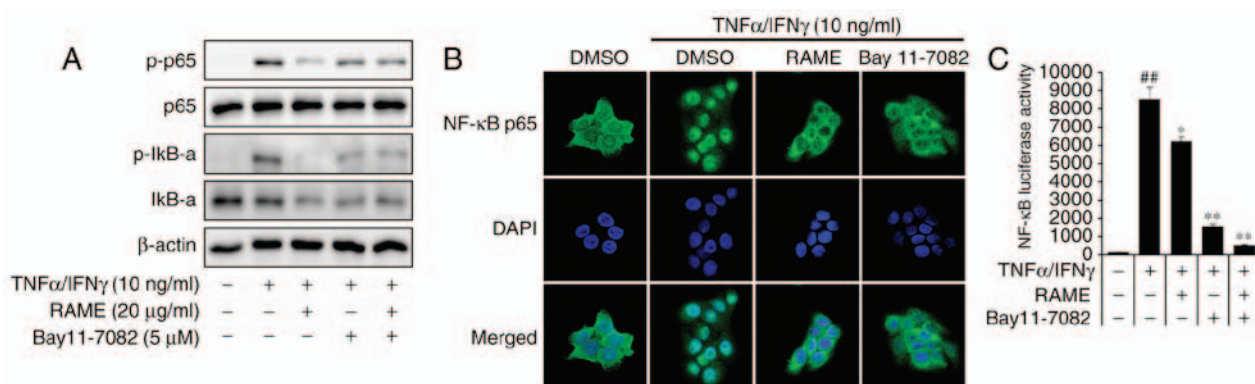


Figure 6. Effect of RAME and Bay11-7082 on TNF- α /IFN- γ induced NF- κ B activation in HaCaT cells. (A) The phosphorylation of p65 and I κ B- α was analyzed using western blotting. (B) Intracellular NF- κ B staining from TNF- α /IFN- γ -stimulated HaCaT cells pretreated with RAME and Bay11-7082. DAPI staining was used to visualize the nuclei of the corresponding cells (x400 magnification). (C) Analysis of NF- κ B p65 luciferase reporter activity in HaCaT cells pretreated with increasing concentrations of Bay11-7082 prior to TNF- α /IFN- γ stimulation for 30 min. Data are presented as the mean \pm standard error of the mean of three samples. ^{##}P<0.01 vs. the negative control; ^{*}P<0.05 and ^{**}P<0.01 vs. TNF- α /IFN- γ stimulated cells. RAME, *Rhododendron album* Blume methanol extract; TNF- α , tumor necrosis factor- α ; IFN- γ , interferon- γ ; NF- κ B, nuclear factor- κ B; p-, phosphorylated; I κ B- α , nuclear factor of κ light polypeptide gene enhancer in B-cells.

decreased the expression levels of p-p38, p-ERK, and p-JNK in TNF- α /IFN- γ -induced HaCaT cells compared with untreated cells. These data suggested that RAME suppresses the inflammatory response via partial regulation of NF- κ B and MAPK

signaling. To assess the function of NF- κ B and MAPKs in the production of TNF- α /IFN- γ -induced inflammatory mediators, the effects of selective NF- κ B and MAPK inhibitors on TNF- α /IFN- γ -induced chemokine and cytokine production

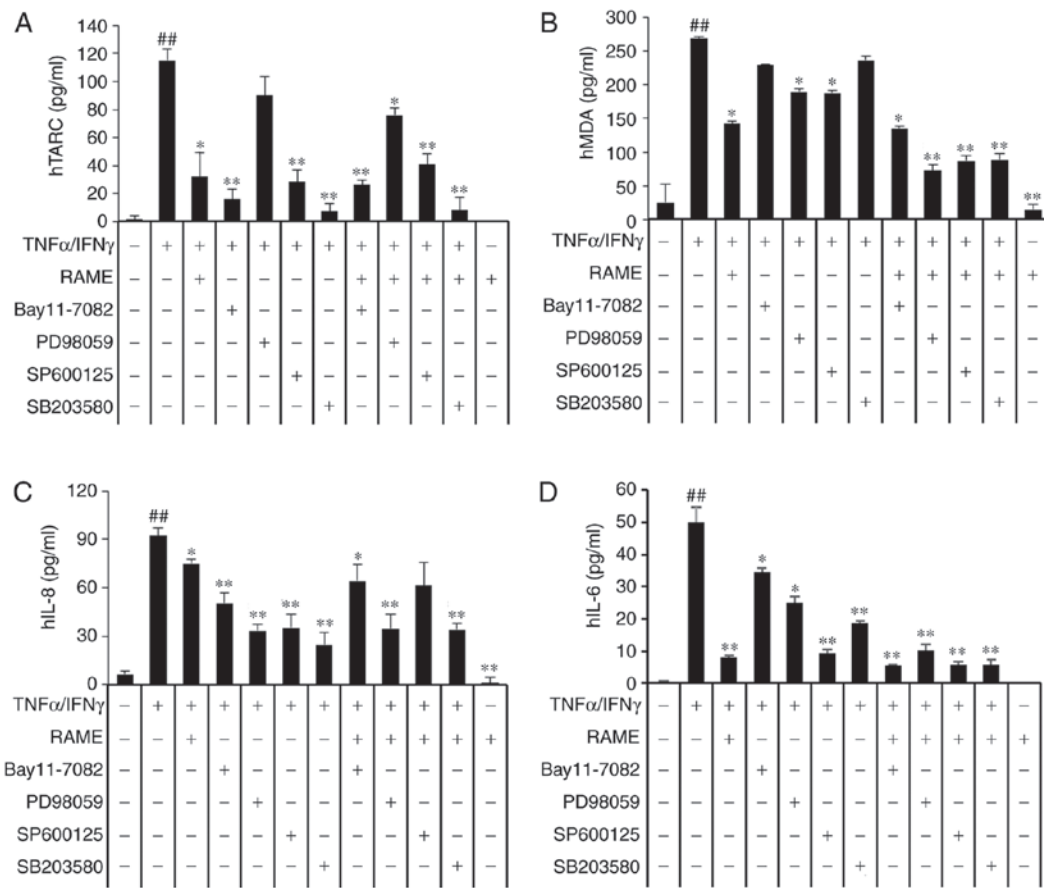


Figure 7. Effects of mitogen-activated protein kinase inhibitors on the expression of chemokines and pro-inflammatory cytokines. HaCaT cells were pretreated with 20 μ g/ml RAME, and 5 μ M Bay11-7082, 10 μ M SB203580, 10 μ M PD98059 and 10 μ M SP600125 for 1 h followed by incubation with 10 ng/ml TNF- α /IFN- γ for 18 h. (A) TARC (B) MDC (C) IL-8 and (D) IL-6 levels were measured by ELISA. Data are presented as the mean \pm standard error of the mean (n=3). ##P<0.01 vs. the normal control group, *P<0.05 and **P<0.01 vs the TNF- α /IFN- γ stimulated cells. RAME, *Rhododendron album* Blume methanol extract; TNF- α , tumor necrosis factor- α ; IFN- γ , interferon- γ ; TARC, thymus- and activation-regulated chemokine; MDC, macrophage-derived chemokine; IL, interleukin.

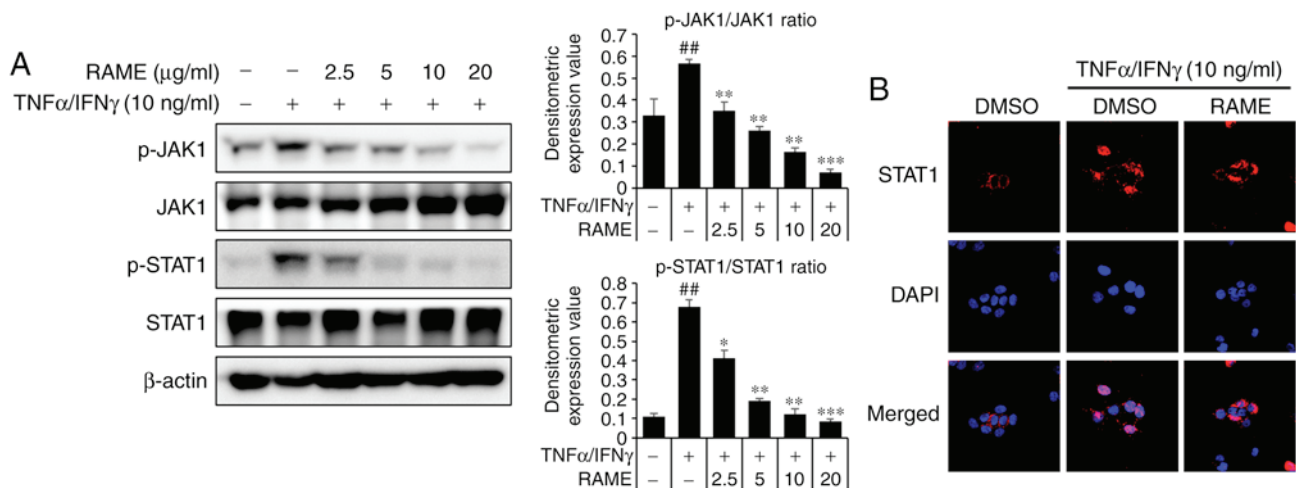


Figure 8. Effect of RAME on TNF- α /IFN- γ induced NF- κ B activation in HaCaT cells. (A) The phosphorylation of JAK1, JAK2 and STAT1 was analyzed using western blotting. (B) Cell localization of STAT1 was determined by immunocytochemistry. Using DAPI (blue), the nuclei were visualized and observed at x400 magnification. Data are presented as the mean \pm standard error of the mean of three samples. ##P<0.01 vs. the negative control; *P<0.05, **P<0.01 and ***P<0.001 vs. TNF- α /IFN- γ stimulated cells. RAME, *Rhododendron album* Blume methanol extract; TNF- α , tumor necrosis factor- α ; IFN- γ , interferon- γ ; NF- κ B, nuclear factor- κ B; JAK, Janus kinase; STAT, signal transducers and activators of transcription; p-, phosphorylated.

were investigated. ELISA analysis revealed that Bay11-7082 (I κ B- α inhibitor) SP600125 (JNK inhibitor), PD98059 (ERK

inhibitor) and SB203580 (p38 inhibitor) markedly inhibited TARC, MDC, IL-6, and IL-8 expression, respectively.

Furthermore, treatment with RAME and inhibitors were demonstrated to markedly inhibit TNF- α /IFN- γ -mediated expression of TARC (Fig. 7A), MDC (Fig. 7B), IL-8 (Fig. 7C), and IL-6 (Fig. 7D). These results suggested that RAME-mediated inactivation of NF- κ B p65, JNK, ERK, and p38 MAPK may, at least in part, be responsible for the suppression of TARC, MDC, IL-8 and IL-6 in HaCaT cells. In addition, TNF- α /IFN- γ stimulation induced an increase in the activation of NF- κ B and MAPKs, while treatment with RAME inhibited this effect.

Treatment with RAME inhibits the activation of JAK/STAT in HaCaT cells. The JAK/STAT pathway is important to the immune system. To investigate whether RAME treatment resulted in the downregulation of TNF- α /IFN- γ -mediated JAK/STAT activation in HaCaT cells, western blot analysis and immunocytochemistry using specific antibodies were utilized (Fig. 8). RAME pre-treatment 1 h prior to TNF- α /IFN- γ exposure in the HaCaT cells. TNF- α /IFN- γ increased the phosphorylation of JAK/STAT signaling for 1 h. As a result, RAME was revealed to inhibit the phosphorylation of the JAK/STAT pathway in TNF- α /IFN- γ -stimulated HaCaT cells (Fig. 8A). In addition, RAME treatment was revealed to decrease translocation of STAT1 in HaCaT cells by immunocytochemistry (Fig. 8B). These results revealed that RAME inhibited the activation of JAK/STAT in HaCaT cells.

Discussion

Traditional medicine using natural herbs may help to prevent and treat several immune-related diseases, including AD, atopic inflammation and allergy (27). The present study revealed that RAME treatment reduced the production of various inflammatory and allergy-mediated chemokines and cytokines in TNF- α /IFN- γ -induced HaCaT cells, through inactivation of NF- κ B and MAPKs.

Regulation of cytokine production is central to the pathogenesis of allergic disorders. Cho *et al* (27) observed increased levels of IL-6, IL-8, IL-10, IL-23 and TGF- β expression in patients with AD compared with healthy individuals. In the present study, RAME decreased IL-8 and IL-6 gene expression in HaCaT cells (Fig. 3A-D). These data suggested that RAME exerted anti-inflammatory effects by suppressing the production of inflammatory chemokines and cytokines. In addition, chemokines and their receptors serve an important function in AD by regulating the onset and worsening of inflammation in response to allergens (28,29). Giuliani *et al* and Chen *et al* (30,31) have demonstrated that serum levels of MDC and TARC are increased in AD, and that this increase is positively correlated with the illness severity in AD. To the best of our knowledge, the present study is the first to demonstrate that RAME inhibits TNF- α /IFN- γ -induced TARC and MDC expression in HaCaT keratinocyte cells (Fig. 2A-D).

NF- κ B represents part of an important pro-inflammatory signaling pathway. NF- κ B regulates the expression of several asthma, inflammation, allergy and immune-associated genes through the degradation of I κ B (30-32). Therefore, RAME may have an anti-allergic effect based on a decrease in activated NF- κ B levels (Fig. 5A-C). The I κ B- α inhibitor Bay11-7082 (33), which prevents the phosphorylation and activation of p65, was

used because specific inhibitor constructs that reduce p65 and I κ B- α levels in HaCaT cells were not identified (Fig. 6A-C). The effect of the NF- κ B specific inhibitor Bay11-7082 was investigated and revealed to significantly inhibit the activation of NF- κ B. This result seems to be due to the mechanisms underlying the anti-inflammatory effects of RAME, which are caused by the suppression of NF- κ B transcriptional activity. The MAPK cascade is one of the signaling pathways involved in immune responses (24,34,35). The MAPK signaling pathway regulates multiple cellular processes, including gene expression, cell death and survival, cell motility and cell proliferation (36,37). MAPKs include three major subfamilies, namely p42/p44 ERKs, p38 MAPKs, and JNKs. The inhibition of MAPKs has been reported to reduce the synthesis of pro-inflammatory cytokines and their intracellular signaling pathways, and inhibit the activation of NF- κ B (38,39). TNF- α /IFN- γ stimulation of HaCaT cells was confirmed to activate three major MAPK modules, including ERK, JNK and p38. Treatment with RAME, the p38 MAPK inhibitor SB203580, the ERK inhibitor PD98059 and the JNK inhibitor SP600125 reduced TNF- α /IFN- γ -activated expression of pro-inflammatory cytokine (IL-6 and IL-8) and chemokines (TARC and MDC) to baseline values. Furthermore, these inhibitors and RAME reduced pro-inflammatory cytokine (IL-6 and IL-8) and chemokine (TARC and MDC) expression by ~50% (Fig. 7). These results suggested that the inhibition of JNK, ERK and p38 MAPK by RAME treatment reduces the production of pro-inflammatory cytokines and chemokines in HaCaT cells (Fig. 4). The fact that RAME reduces TNF- α /IFN- γ -induced MAPK, MDC, and TARC mRNA expression again suggested that RAME has anti-allergic and anti-inflammatory effects.

In mammals, the JAK/STAT pathway is the main signaling mechanism for a wide array of growth factors and cytokines. JAK/STAT signaling is one of a number of pleiotropic cascades used to transduce signals for development in animals and humans. The results of the present study suggested that the RAME may have an anti-allergic effect based on the decrease in activated JAK/STAT (Fig. 8A and B).

In conclusion, the present study confirmed that RAME inhibits the expression of AD-associated chemokines and cytokines in TNF- α /IFN- γ -induced keratinocytes. These effects were considered to be associated with the suppression of NF- κ B activation. These experimental results provide a scientific basis for the use of RAME to treat AD. Additional experiments to test the *in vitro* effects of RAME on AD are currently in progress in our laboratory.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

JWP analyzed the data and wrote the manuscript. HSL and YRL conducted the *in vitro* experiments. JHP, OKK, JHK, IP and PY prepared the *Rhododendron album* Blume, and analyzed and edited the manuscript. SC, SRO and KSA designed the study and edited the manuscript. All authors critically revised the article and have consented to the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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