Lycopus lucidus Turcz ameliorates DNCB-induced atopic dermatitis in BALB/c mice

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Abstract. Atopic dermatitis (AD) is a chronic inflammatory allergic skin disease, characterized by pruritic and eczematous skin lesions. *Lycopus lucidus* Turcz (LLT) is a perennial herb that has been reported to have various biological properties, including effects on blood circulation, as well as anti-inflammatory, antioxidant, anti-vascular inflammation and wound-healing effects. However, whether LLT improves dermatitis and the underlying mechanisms has yet to be determined. The aim of the present study was to determine whether LLT can improve 2,4-dinitrochlorobenzene (DNCB)-induced dermatitis and to verify the inhibitory effect of LLT on the expression of chemokines and pro-inflammatory cytokines in the HaCaT immortalized keratinocyte cell line. In addition, the anti-inflammatory function of LLT in RAW264.7 mouse macrophages was investigated. In the DNCB-induced AD mouse model, LLT inhibited infiltration by mast cells, eosinophils and $CD8^+$ cells in the dorsal skin tissue of AD mice, and suppressed the expression of IgE and IL-6 in serum. In addition, LLT inhibited the phosphorylation of ERK and JNK, as well as NF-κB in skin tissue. In the HaCaT cell model induced by TNF-α/IFN-γ, LLT inhibited the expression of thymus and activation-regulated chemokine, granulocyte-macrophage colony-stimulating factor, monocyte chemoattractant protein-1, TNF-α and IL-1β, whilst inhibiting the phosphorylation of NF-κB. In addition, in the lipopolysaccharide-induced RAW 264.7 cell inflammation model, LLT inhibited the expression of TNF-α and IFN-γ, the nuclear translocation of NF-κB and the phosphorylation of ERK and JNK. These results suggested that LLT may be a promising candidate for the treatment of inflammatory dermatitis.

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Introduction

Atopic dermatitis (AD) is a common chronic inflammatory skin disease (1). AD generally presents in infancy and early childhood, and its prevalence is increasing worldwide (2). Environmental, pharmacological and genetic factors may play a role in AD by disturbing the balance of the immune system (3). The main symptoms of AD include erythema, itching, eczematous lesions, excoriation, edema and thickening of the skin (4,5). In severe cases, fluid extravasation and bacterial infections may develop (5). Steroids, antihistamines and immunosuppressive agents are currently used to treat AD (6,7). However, these agents are associated with a variety of adverse effects when used in the long term or at high doses (6-8). Therefore, new, effective alternative medicines are needed for AD (7,8).

AD symptoms, such as skin thickening and cracking, are caused by T helper 1 (Th1) and T helper 2 (Th2) cells (9). The production of cytokines by Th2 cells increases the production of IgE, as well as the infiltration of eosinophils and mast cells into inflamed skin tissue (10-12). IgE is a major mediator of mast cell activation and contributes to the release of inflammatory mediators, including histamine and cytokines (13). The MAPK signaling cascade plays a key role in the differentiation of inflammatory cells (14). The transcription factor NF-κB also plays a major role in regulating several inflammatory mediators (15).

AD is induced by various chemokines and inflammatory cytokines. Thymus and activation-regulated chemokine [TARC/C-X-C motif chemokine ligand 17 (CXCL17)] is produced by dendritic cells, endothelial cells, keratinocytes and fibroblasts, and induces Th2 cell migration to the inflammatory site, as these cells express the receptor for TARC/CCL17. Therefore, TARC is an important chemokine in inflammatory skin diseases (16) and is used as a clinical biomarker to measure atopic diseases (16). Granulocyte-macrophage colony-stimulating factor (GM-CSF) is also involved in the initiation and maintenance of chronic inflammation by activating Langerhans cells (17), as well as by causing hyperproliferation (18) and apoptosis of keratinocytes (19).

In addition, high levels of GM-CSF were observed in the skin of patients with AD (20). Monocyte chemoattractant protein-1 (MCP-1) plays a key role in mast cell degranulation, histamine release from basophils and transformation of undifferentiated T cells to Th2 cells (21). In addition, scratching of itchy skin causes secretion of various pro-inflammatory cytokines, including IL-1β, TNF-α and IL-4, from keratinocytes, thereby aggravating the inflammatory reaction (11,22). According to previous reports, it is known that the expression of TNF-α/IFN-γ-induced adhesion proteins and pro-inflammatory cytokines/chemokines in keratinocytes is suppressed by inhibiting the activity of MAPKs and NF-κB (23,24).

Lycopus lucidus Turcz (LLT) is a perennial herb known as 'Taekran' in Korea (25). Traditionally, LLT has been used to treat amenorrhea, dysmenorrhea, edema, carbuncles and sores (26). Recently, LLT has been reported to have various biological properties, including effects on the blood circulation (25), anti-inflammatory (27), antioxidant (28), anti-vascular inflammation and wound-healing effects (27,29), suggesting that LLT may also affect the mechanism underlying AD.

In the present study, the thickness of the epidermis and dermis and the infiltration by mast cells and eosinophils were evaluated in mice with 2,4-dinitrochlorobenzene (DNCB)-induced AD. In addition, the expression levels of serum IgE and IL-6 and members of the NF-κB and MAPK signaling pathways were investigated. To examine the mechanism of AD inhibition, the expression of chemokines and pro-inflammatory cytokines and NF-κB/MAPK signaling pathway molecules was examined in TNF-α/IFN-γ-stimulated HaCaT cells. Furthermore, the expression of Th1 cytokines (IFN-γ and TNF-α) and NF-κB/MAPK signaling pathway-related proteins was examined in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. The results of these experiments were integrated to determine the potential applicability of LCL in the treatment of AD.

Materials and methods

Reagents. DNCB, toluidine blue, hematoxylin, eosin, human TNF-α, human IFN-γ, LPS, caffeic acid, protease inhibitor cocktail and phosphatase inhibitor cocktail were purchased from Sigma-Aldrich (Merck KGaA). Mouse IgE (cat. no. 555248), mouse IL-6 (cat. no. 555240), human GM-CSF (cat. no. 555126), human TNF-α (cat. no. 555212), human IL-1β (cat. no. 557953), human MCP-1 (cat. no. 555179), human IL-6 (cat. no. 555220) and human IL-8 (cat. no. 555244) ELISA kits were purchased from BD Biosciences. Polink-2 Plus AP rabbit kit (cat. no. D70-18) was purchased from GBI Labs. Antibodies against CD4 (cat. no. ab183685; type, monoclonal; species, anti-mouse) and CD8 (cat. no. ab209775; type, monoclonal; species, anti-mouse) were obtained from Abcam. Antibodies against phosphorylated (p)-ERK (cat. no. cs-4370; type, monoclonal, species, anti-rabbit), total (t)-ERK (cat. no. cs-4695; type, monoclonal; species, anti-rabbit), p-JNK (cat. no. cs-4668; type, monoclonal; species, anti-rabbit), t-JNK (cat. no. cs-9258; type, monoclonal; species, anti-rabbit), NF-κB (cat. no. cs-8242; type, monoclonal; species, anti-rabbit), p-NF-κB (cat. no. cs-3033; type, monoclonal; species, anti-rabbit) and IkB (cat. no. cs-4814S; type, monoclonal; species, anti-rabbit) were purchased from Cell Signaling Technology, Inc. Anti-lamin B antibody (cat. no. sc-6216; type, polyclonal; species, anti-mouse) and anti-actin antibody (cat. no. sc-8432; type, polyclonal; species, anti-mouse) were purchased from Santa Cruz Biotechnology, Inc. A SuperScript™ IV reverse transcriptase was purchased from Invitrogen (Thermo Fisher Scientific, Inc.). Taq polymerase was obtained from Kapa Biosystems (Roche Diagnostics). PCR primers were purchased from GenoTech Corp. DMEM was purchased from Welgene, Inc. Goat serum (16210064 for IHC), FBS, penicillin/streptomycin (P/S) and Dulbecco's PBS (DPBS) were purchased from Gibco (Thermo Fisher Scientific, Inc.). An aqueous non-radioactive cell proliferation assay (MTS) was purchased from Promega Corporation.

Preparation of LLT. LLT was purchased from Kyung Hee University Healthcare System (Seoul, Korea). LLT extract was prepared by decocting 500 g of the dried herb with 5 l boiling distilled water for 2 h, then filtering the mixture using filter paper. The extract was concentrated in a rotary evaporator and lyophilized. The powder (57.1 g; yield ratio, 11.4%) was stored at -20˚C until use.

Animals and induction of AD-like lesions and drug treatment in mice. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of Kyung Hee University Institutional Animal Care and Use Committee [KHUASP (SE)-15-116]. A total of 32 male 6-week-old BALB/c mice (weighing 18-20 g) were obtained from Nara Biotech. Animal experiments were performed in an air-conditioned room, with a temperature of 23±2˚C, and food and water were provided *ad libitum*. After an acclimatization period of 7 days, the mice were divided into four groups (n=8 per group;) as follows: Normal (vehicle-treated), Control (DNCB-sensitized), LLT low (LLT-L; DNCB + 1 mg/kg LLT) and LLT high (LLT-H; DNCB + 5 mg/kg LLT). The mice were anesthetized with isoflurane diluted in 100% oxygen (4% induction and 2% maintenance). The dorsal skin of the mice was shaved with a clipper 1 day before the experiment. In the first sensitization, the mice (control, LLT-L and LLT-H groups) were treated with 200 µl 0.5% DNCB solution (dissolved in a 3:1 mixture of acetone and olive oil) for 3 days. The normal group was treated with PBS applied to the backs of the mice during the first sensitization period. In the second sensitization, 200 µl 1% DNCB solution was applied to the dorsal skin of the control, LLT-L and LLT-H groups (once every 3 days, 6 times in total). The normal group was treated with 200 µl 9:1 mixture of PBS and olive oil to the dorsal skin. The LLT-treated groups were challenged with 200 µl LLT in a 9:1 mixture of PBS and olive oil (LLT-L, DNCB + 1 mg/kg LLT; and LLT-H, DNCB + 5 mg/kg LLT) 2 h after the application of DNCB daily. The normal and control groups were treated with a 200 µl 9:1 mixture of PBS and olive oil (Fig. 1A). The health and behavior of the mice were monitored daily and no animals died during the experiment. The following humane endpoints were used: i) Walking uncomfortably and difficulty consuming feed or water; ii) difficulty maintaining a normal posture due to weakness; iii) decrease in weight of >20% compared with the control group of the same age; iv) coarse breathing, cyanosis, chronic discomfort or constipation; v) hematological

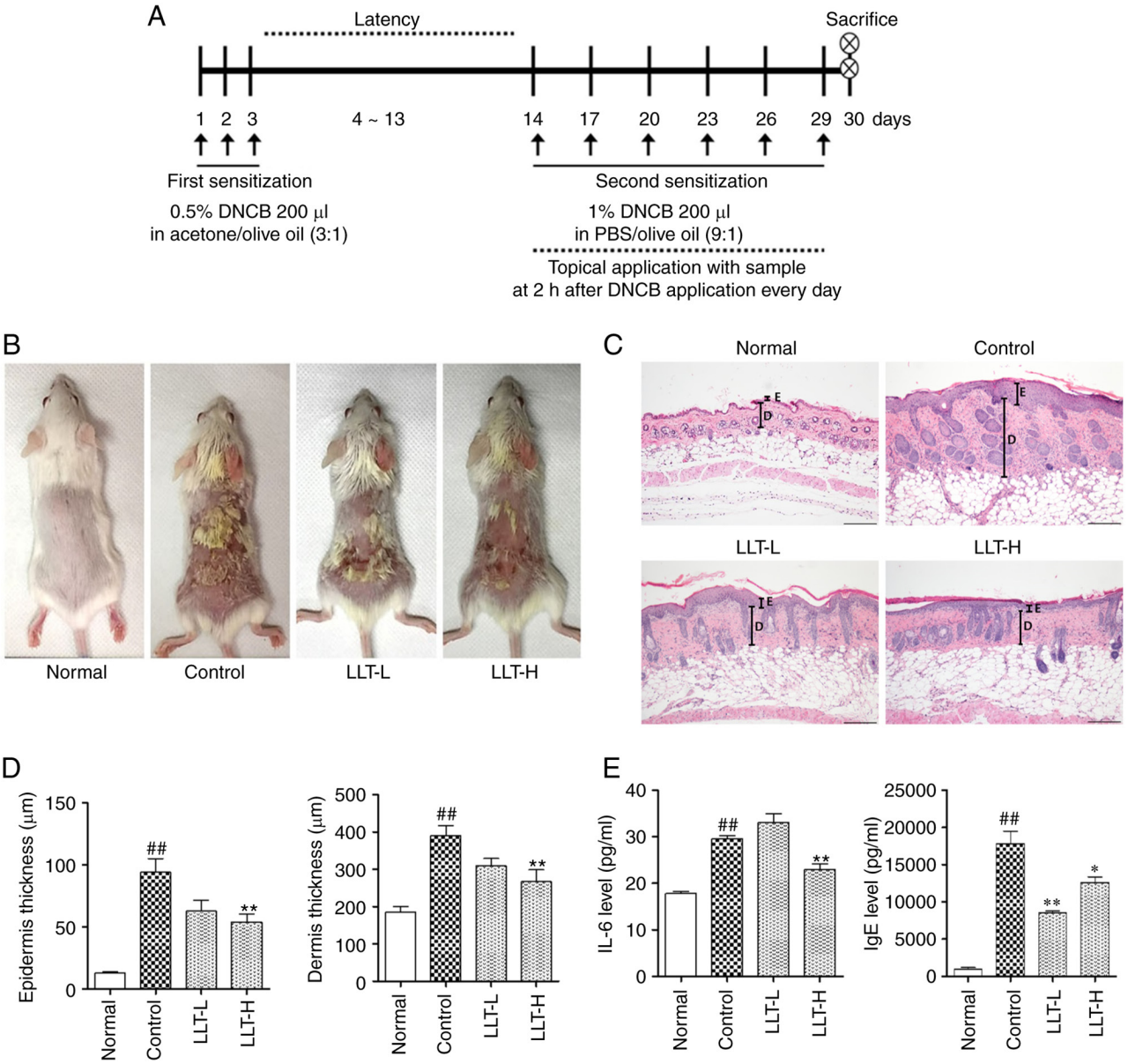

Figure 1. Effects of LLT on the histological characteristics of mice with DNCB-induced AD. (A) Schematic diagram of the experimental schedule. (B) Clinical characteristics of each treatment group on day 30. (C) Epidermal and dermal thickness was examined by H&E staining of skin sections. Magnification, x100. Scale bar, 200 μm. (D) Measurement of epidermal and dermal thickness. (E) Serum IgE and IL-6 levels were quantified by ELISA. Data represent the mean ± SEM. n=8. $^{\#\#}$P<0.01 vs. normal group; *P<0.05 and **P<0.01 vs. control group. Normal group, vehicle-treated; control group, DNCB-sensitized; LLT-L group, DNCB + 1 mg/kg LLT; LLT-H group, DNCB + 5 mg/kg LLT. AD, atopic dermatitis; LLT, *Lycopus lucidus* Turcz; DNCB, 2,4-dinitrochlorobenzene; E, epidermis; D, dermis.

or blood biochemistry parameters indicating organ function decline that compromises survival ability; vi) falling into an unconscious state and not responding to external stimuli. After the experiment was completed, the mice were deeply anesthetized intraperitoneally with 80 mg/kg pentobarbital sodium, and 800-1,000 μl blood was collected by cardiac puncture. After death was confirmed by cessation of breathing and blood circulation, a dorsal skin sample was collected.

Histological analysis. The dorsal skin was fixed in 10% neutral buffered formalin (NBF) at room temperature for 1 day, and the tissues were washed with tap water for 1 day. The tissues were embedded in paraffin and cut into 5-μm sections using a rotary microtome (Zeiss AG). The tissues were deparaffinized for 3 min, rehydrated for 3 min, and stained with hematoxylin and eosin (H&E) for 3 min or toluidine blue for 3 min. All staining reactions were performed at room temperature. The infiltration by eosinophils was examined under a light microscope (magnification, x400; 10 fields per section). The infiltration of mast cells was observed (magnification, x200; 3 fields per section). In addition, the thickness of the dermis and epidermis were also analyzed (magnification, x100; 3 fields per section). The skin thickness and inflammatory cell count were measured using ImageJ software (version 1.46; National Institutes of Health).

Immunohistochemistry (IHC) staining. The dorsal skin was fixed in 10% NBF at room temperature for 1 day, and the

tissues were washed with tap water for 1 day. The tissues were embedded in paraffin and cut into 5-μm sections using a rotary microtome (Zeiss AG). The tissues were deparaffinized and rehydrated. For epitope retrieval, the tissue was placed in sodium citrate buffer (0.1 M citric acid; 0.1 M sodium citrate) and heated using an Electric Pressure Cooker (CPC-600; Cuisinart). After the endogenous peroxidase activity was blocked using 0.3% (v/v) H_2O_2 in methanol at room temperature for 30 min, 10% goat serum (Gibco; Thermo Fisher Scientific, Inc.) in PBS was used for blocking at room temperature for 10 min. Subsequently, the tissues were incubated with primary antibodies (dilution 1:100) at 4˚C for overnight. The tissue was incubated for 1 h at room temperature with a biotinylated secondary antibody (dilution 1:100). Finally, the tissues were stained with Polink-2 Plus AP rabbit kit for 30 min at room temperature, and the nuclei were counterstained with Harris hematoxylin for 5 min at room temperature. The stained tissue was observed under a light microscope (BX51; Olympus Corporation) at a magnification of x400 (10 fields per section). The $CD4^+$ and $CD8^+$ cells were counted using ImageJ software (version 1.51j8; National Institutes of Health).

Cell culture. HaCaT cells were purchased from the CLS Cell Lines Service GmbH (cat. no. CLS 300493). HaCaT cells were cultured in DMEM with 10% FBS and 1% P/S. RAW 264.7 cells were obtained from the Korean Cell Line Bank (Korean Cell Line Research Foundation; cat. no. KCLB 40071). RAW 264.7 cells were cultured in DMEM with 10% FBS and 1% P/S. Both cell lines were cultured in a cell incubator at 37˚C in a humidified atmosphere of 5% CO_2 in air.

Ex vivo ELISA. To prepare serum, blood collected from the mice was separated by centrifugation at 14,310 x g and 4˚C for 10 min. The concentrations of IgE and IL-6 in the serum were detected using ELISA kits *in vitro*. The HaCaT cells were seeded in a 6-well plate at a density of $1x10^6$ cells/well. After 24 h, the cells were pretreated with LLT at concentrations of 1, 10 and 100 μg/ml for 1 h and stimulated with TNF-α/IFN-γ (10 ng/ml) for 24 h in a CO_2 incubator maintained at 37˚C. The culture medium was not changed for pretreatment and stimulant treatment. The concentrations of various pro-inflammatory cytokines and chemokines (GM-CSF, TNF-α, IL-1β, MCP-1, IL-6 and IL-8) in the culture medium were also detected using ELISA kits. All experiments were carried out according to the manufacturer's protocol.

Western blot analysis

Ex vivo. To extract nuclear fraction, the frozen skin tissue was homogenized with NE-PER nuclear and cytoplasmic extraction reagent (cat. no. 78835, Thermo Fisher Scientific Inc.). To extract whole protein, the frozen skin tissue was homogenized with lysis buffer (20 mM HEPES, pH 7.5; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 0.1 M NaCl; 0.2 mM DTT; 0.5 mM Na_3VO_4). Proteins were obtained after centrifugation at 58,440 x g for 30 min at 4˚C. *In vitro*. HaCaT cells were seeded in a 6-well plate, with a density of $1x10^6$ cells/well. After 24 h, the cells were pretreated with LLT at concentrations of 1, 10 and 100 μg/ml for 1 h and stimulated with TNF-α/IFN-γ (10 ng/ml) for 5 min (nuclear and cytoplasmic protein) and 30 min (whole protein) in a CO_2 incubator maintained at 37˚C. The culture medium was not changed for pretreatment and stimulant treatment. RAW 264.7 cells were seeded in a 6-well plate, with a density of $1x10^6$ cells/well. After 24 h, the cells were pretreated with LLT at concentrations of 1, 10 and 100 μg/ml for 1 h and stimulated with LPS (1 μg/ml) for 5 min (nuclear and cytoplasmic protein) and 30 min (total protein) in a CO_2 incubator maintained at 37˚C. The culture medium was not changed for pretreatment and stimulant treatment. When extracting the nuclear and cytoplasmic protein, the HaCaT and RAW 264.7 cells were washed with PBS and lysed with nuclear and cytoplasmic extraction reagents. To extract the whole protein, the cells were washed with DPBS and proteins were extracted using RIPA buffer (50 mM Tris-Cl, 150 nM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail and phosphatase inhibitor cocktail) for total protein lysis.

The same method was used for *ex vivo* and *in vitro* experiments after protein extraction. The protein concentration was determined using a BCA assay. The protein samples (30 μg) were separated by SDS-PAGE on 10% gels, then transferred to a nitrocellulose membrane. The membrane was blocked with 5% skimmed milk for 1 h at room temperature. After blocking, the membrane was incubated overnight with primary antibodies against t-ERK (dilution 1:1,000), p-ERK (dilution 1:1,000), t-JNK (dilution 1:1,000), p-JNK (dilution 1:1,000), t-p38 (dilution 1:1,000), p-p38 (dilution 1:1,000), NF-κB (dilution 1:1,000), p-NF-κB (dilution 1:1,000), lamin B (dilution 1:1,000), IκB (dilution 1:1,000) and actin (dilution 1:1,000) at 4˚C. The membrane was then incubated with secondary antibodies (dilution 1:10,000) for 1 h at room temperature. The protein bands were visualized using an enhanced chemiluminescence (cat. no. RPN2106; Cytiva) detection reagent and semi-quantified using ImageJ software (version 1.51j8, National Institutes of Health).

Cell viability assay. The MTS assay was used to evaluate the cytotoxicity of LLT in HaCaT and RAW 264.7 cells. The cells were seeded in a 96-well plate at a density of $5x10^4$ cells/well. After 24 h, LLT (1, 10 or 100 μg/ml) was added to the medium for 24 h in a CO_2 incubator maintained at 37˚C. In addition, in order to confirm the toxicity of LLT in HaCaT cells stimulated with TNF-α/IFN-γ, the cells were seeded in a 96-well plate at a density of $5x10^4$ cells/well. After 24 h, LLT (1, 10 or 100 μg/ml) and TNF-α/IFN-γ (10 ng/ml) was added to the medium for 24 h in a CO_2 incubator maintained at 37˚C. After each reaction was completed, a volume of 20 μl MTS solution was then added to each well for 2 h, and then the optical density was measured using a microplate reader at a wavelength of 562 nm.

Reverse transcription PCR (RT-PCR) analysis. HaCaT cells were seeded in a 6-well plate at a density of $1x10^6$ cells/well. After 24 h, the cells were pretreated with LLT at concentrations of 1, 10 and 100 μg/ml for 1 h at 37˚C CO_2 incubator and stimulated with TNF-α/IFN-γ (10 ng/ml) for 24 h in a CO_2 incubator maintained at 37˚C. The culture medium was not changed prior to pretreatment and stimulation. RAW 264.7 cells were seeded in a 6-well plate at a density of $2x10^6$ cells/well. After 24 h, the cells were co-treated with LLT at concentrations of 1, 10 and 100 μg/ml and LPS (1 μg/ml) for 6 h in a CO_2 incubator maintained at 37˚C.

Table I. Primer sets for reverse transcription PCR.

Primer name	Primer sequence, 5'→3'	Gene name	Number of cycles	Annealing temperature,˚C	Genbank accession no.
h-TARC F	ACTGCTCCAGGGATGCCATCGTTTTT	*CCL17*	44	57.5	NM_002987.3
h-TARC R	ACAAGGGGATGGGATCTCCCTCACTG				
h-GAPDH F	CGTCTAGAAAAACCTGCCAA	*GAPDH*	30	50	NM_001256799.3
h-GAPDH R	TGAAGTCAAAGGAGACCACC				
m-TNF-α F	GCAGAAGAGGCACTCCCCCA	*Tnf*	30	58	NM_001278601.1
m-TNF-α R	GATCCATGCCGTTGGCCAGG				
m-IFN-γ F	CTCAAGTGGCATAGATGT	*Ifng*	38	57	NM_008337.4
m-IFN-γ R	GAGATAATCTGGCTCTGCAGGATT				
m-GAPDH F	AACTTTGGCATTGTGGAAGG	*GAPDH*	30	58	NM_008084.3
m-GAPDH R	ACACATTGGGGGTAGGAACA				

h-, human; m-, mouse; TARC, thymus and activation-regulated chemokine; CCL17, C-C motif chemokine ligand 17; F, forward; R, reverse.

Total RNA was extracted using RNAiso Plus (cat. no. 9108; Takara Bio, Inc.), according to the manufacturer's protocol, and the mass of RNA (2 μg) was equalized after measuring the concentration of the samples with a NanoDrop (Thermo Fisher Scientific, Inc.) at 250-260 nm. cDNA was prepared from total RNA using a reverse transcription kit, then amplified using a Taq polymerase and target primers. The PCR thermocycling conditions were: 30-44 cycles of 1 min at 94˚C (denaturation), 1 min at 50-58˚C (annealing) and 1 min at 72˚C (extension)]. The sequences of the primers and specific annealing temperatures are listed in Table I. The amplified samples were separated on a 1.2% agarose gel, stained with SYBR green (Invitrogen; Thermo Fisher Scientific, Inc.). The stained agarose gel was captured using NαBI (NeoScience). Semi-quantification of the bands was performed using ImageJ software (version 1.51j8; National Institutes of Health). The expression of each target gene was quantified using GAPDH.

High performance liquid chromatography (HPLC) analysis. HPLC analysis was carried out on the Waters 2695 system with a 2996 dual λ absorbance detector (Waters Corporation). The system was equipped with the XBridge™ C18 column (250x4.6 mm; 5 mm; Waters Corporation). The mobile phase consisted of acetonitrile (solvent A) and 1% acetic acid (solvent B) at a flow rate of 1.0 ml/min. The injection volume of the extract was 10 μl. The elution phase consisted of 0-40 min of 10-40% solvent A and 90-60% of solvent B. Caffeic acid (also known as 3,4-Dihydroxybenzeneacrylic acid; cat. no. C0625; Sigma-Aldrich; Merck KGaA), an indicator component of LLT, was used as a standard. The elution was monitored at 368 nm.

Statistical analysis. Data are presented as the mean ± SEM. All experiments were repeated at least three times. Statistical analysis was performed using GraphPad Prism Software (version 5.01; GraphPad Software, Inc.). One-way ANOVA was used to evaluate the treatment effect, followed by Tukey's multiple-comparisons test. P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of LLT on the skin and serum of DNCB-induced AD mice. To evaluate the therapeutic efficacy of LLT in the DNCB-induced AD mouse model, DNCB was applied to the dorsal skin of Balb/c mice. As shown Fig. 1B, it was confirmed that the control group exhibited erythema, edema and eczematous skin lesions on the dorsal skin, and LLT treatment improved these atopic-like symptoms. To evaluate the effect of LLT on histological characteristics, the thickness of the epidermis and dermis of the dorsal skin was measured in H&E-stained sections (Fig. 1C). The thickness of the epidermis and dermis was increased in the DNCB-induced control group compared with the normal group (P<0.01). The LLT-H group exhibited significantly reduced epidermal and dermal thickness compared with the control group (P<0.01) (Fig. 1D). To assess the clinical symptoms of AD, the serum levels of IgE and IL-6 were measured using ELISA (Fig. 1E). The serum IL-6 levels were significantly increased in the control group compared with the normal group (P<0.01). The serum IL-6 levels were significantly reduced in the LLT-H groups compared with the control group (P<0.01). The serum IgE levels were significantly increased in the control group compared with the normal group (P<0.01). The serum IgE levels were significantly reduced in the LLT-L and LLT-H groups compared with the control group (P<0.01 and P<0.05, respectively).

Effects of LLT on histological changes in mice with DNCB-induced AD. To examine eosinophil and mast cell infiltration in the dermis, sections of dorsal skin were stained with H&E and toluidine blue (Fig. 2A and B). Eosinophil infiltration significantly increased in the control group compared with the normal group (P<0.01); however, application of LLT significantly inhibited eosinophil infiltration (P<0.01; Fig. 2E). In addition, mast cell infiltration was significantly increased in the control group compared with the normal group (P<0.01), and application of LLT significantly inhibited mast cell infiltration (P<0.01; Fig. 2F). The effects of LLT application on $CD4^+$ and $CD8^+$ cell infiltration induced by DNCB was

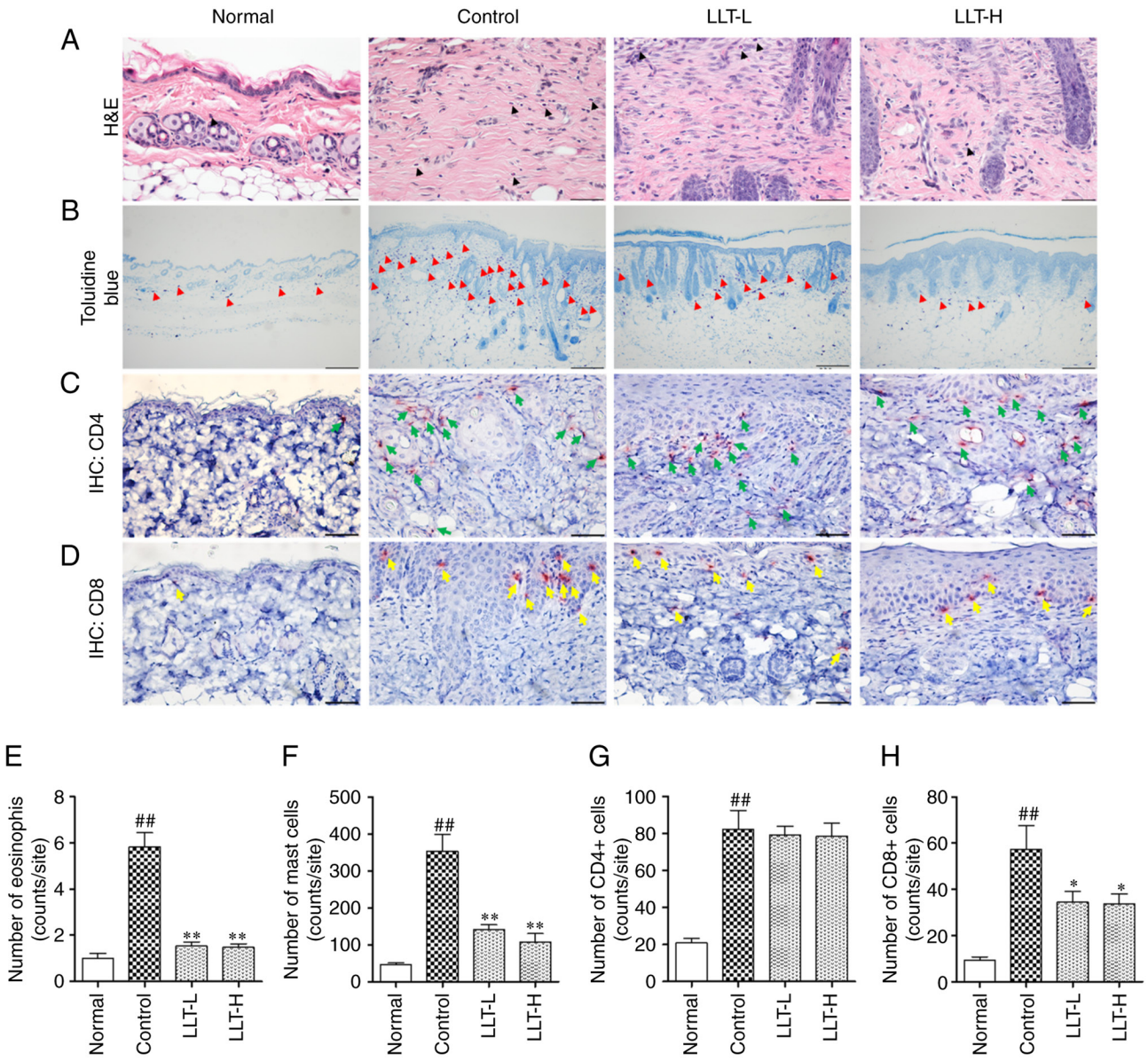

Figure 2. Effect of LLT on immune cell infiltration and the numbers of $CD4^+$ and $CD8^+$ cells in mice with DNCB-induced AD. Infiltration by (A) eosinophils (black arrow; magnification, x400; scale bar, 50 μm) and (B) mast cells (red arrow; magnification, x100; scale bar, 200 μm) in dermal lesions was examined by H&E and toluidine blue staining of skin sections. (C) $CD4^+$ (green arrow) and (D) $CD8^+$ (yellow arrow) cells were examined by IHC. Magnification, x400. Scale bar, 50 μm. The number of (E) eosinophil cells, (F) mast cells, (G) $CD4^+$ cells and (H) $CD8^+$ cells was counted using ImageJ software. Data represent the mean ± SEM. n=8. $^{\#\#}$P<0.01 vs. normal grou; *P<0.05 and **P<0.01 vs. control group. Normal group, vehicle-treated; control group, DNCB-sensitized; LLT-L group, DNCB + 1 mg/kg LLT; LLT-H group, DNCB + 5 mg/kg LLT. AD, atopic dermatitis; DNCB, 2,4-dinitrochlorobenzene; LLT, *Lycopus lucidus* Turcz; H&E, hematoxylin and eosin; IHC, immunohistochemistry.

demonstrated by IHC staining (Fig. 2C and D). The infiltration by $CD4^+$ cells increased in the control group compared with the normal group (P<0.01). However, application of LLT did not significantly affect $CD4^+$ cell infiltration (Fig. 2G). In addition, the infiltration by $CD8^+$ cells were increased in the control group compared with the normal group (P<0.01), and application of LLT-H significantly inhibited $CD8^+$ cell infiltration (P<0.05; Fig. 2H).

Effect of LLT on the translocation and of NF-κB and phosphorylation of ERK and JNK in dorsal skin tissue of AD mice. To investigate the anti-inflammatory role of LLT, proteins from dorsal skin tissue were extracted and the translocation of NF-κB and phosphorylation of ERK and JNK were measured by western blotting (Fig. 3A). The expression of NF-κB was significantly increased in the control group compared with the normal group. In addition, application of LLT significantly inhibited the expression of NF-κB in a concentration-dependent manner (P<0.01; Fig. 3B). DNCB treatment in Balb/c mice induced phosphorylation of ERK and JNK in the dorsal skin tissue, and LLT application significantly inhibited this phosphorylation (P<0.01; Fig. 3C and D).

Effect of LLT on the expression of pro-inflammatory cytokines and chemokines and the NF-κB signaling following TNF-α/IFN-γ stimulation in HaCaT cells. Prior to the *in vitro* experiment, an MTS assay was conducted to measure the toxicity of LLT in HaCaT cells. After treatment for 24 h, it

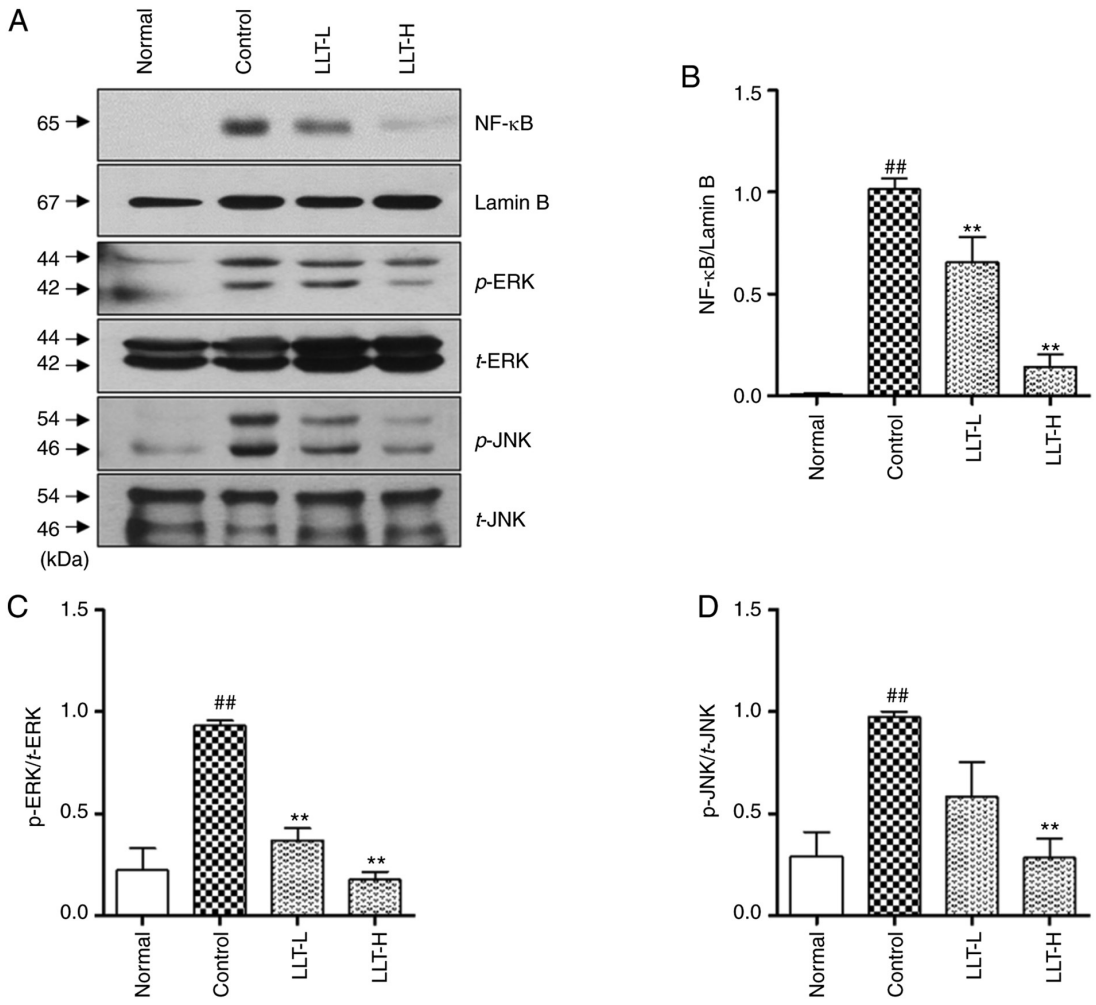

Figure 3. Effects of LLT on NF-κB and MAPK protein expression in BALB/c mice with DNCB-induced AD. (A) NF-κB, p-ERK1/2 and p-JNK expression levels were examined by western blotting. (B) NF-κB expression level was normalized to lamin B, (C) p-ERK expression level was normalized to t-ERK and (D) p-JNK expression level was normalized to t-JNK using ImageJ software. Data represent the mean ± SEM (n=8). $^{\#\#}$P<0.01 vs. normal group and **P<0.01 vs. control group. Normal group, vehicle-treated; control group, DNCB-sensitized; LLT-L group, DNCB + 1 mg/kg LLT; LLT-H group, DNCB + 5 mg/kg LLT. DNCB, 2,4-dinitrochlorobenzene; LLT, *Lycopus lucidus* Turcz; AD, atopic dermatitis; p-, phosphorylated; t-, total; LPS, lipopolysaccharide.

was observed that LLT did not significantly affect the viability of HaCaT cells (Fig. 4A). LLT and TNF-α/IFN-γ stimulation were then used to treat HaCaT cells, and cytotoxicity was also measured. No significant change in cell viability was observed (Fig. 4B). To investigate the effect of LLT on the production of pro-inflammatory cytokines and chemokines in AD, their mRNA expression levels were measured using RT-PCR and their levels in the culture medium using ELISA. As shown in Fig. 4C and CD, the mRNA expression of TARC significantly increased following treatment with TNF-α/IFN-γ in HaCaT cells (P<0.01). Moreover, the addition of 100 μg/ml LLT significantly reduced the expression of TARC (P<0.01). As shown Fig. 4E-H, the expression of GM-CSF, MCP-1, TNF-α and IL-1β in the cell medium significantly increased following treatment with TNF-α/IFN-γ (P<0.01), and 100 μg/ml LLT significantly inhibited this effect (P<0.01, P<0.05, P<0.01 and P<0.01, respectively).

The effect of LLT on the translocation and phosphorylation of NF-κB, which regulates the expression of pro-inflammatory cytokines and chemokines, was investigated. Treatment with TNF-α/IFN-γ induced the expression and phosphorylation of NF-κB in the nuclear protein fraction (P<0.01) and degradation of IκB in the cytoplasmic protein fraction (P<0.01). LLT inhibited the effects of TNF-α/IFN-γ (P<0.01) (Fig. 4I). Following standardization with lamin B, LLT inhibited the expression (10 and 100 μg/ml, both P<0.05; Fig. 4J) and phosphorylation of NF-κB (1, 10 and 100 μg/ml P<0.01, P<0.01 and P<0.05, respectively; Fig. 4K). In addition, the ratio of p-NF-κB/NF-κB was significantly increased through TNF-α/IFN-γ stimulation (P<0.01), LLT inhibited the expression (1, 10 and 100 μg/ml, P<0.01, P<0.01 and P<0.05, respectively; Fig. 4L). It also inhibited the degradation of IκB (100 μg/ml, P<0.05; Fig. 4M).

Effects of LLT on the expression of inflammatory cytokines, MAPKs and NF-κB in LPS-stimulated RAW 264.7 cells. The potential cytotoxicity of LLT was measured in RAW 264.7 cells using an MTS assay. As shown in Fig. 5A, none of the concentrations of LLT (1, 10 and 100 μg/ml) affected the viability of the RAW 264.7 cells. The inhibitory effects of LLT on the mRNA expression of inflammatory cytokines were confirmed in LPS-stimulated RAW 264.7 cells (Fig. 5B). The expression of TNF-α and IFN-γ was increased in the LPS-stimulated

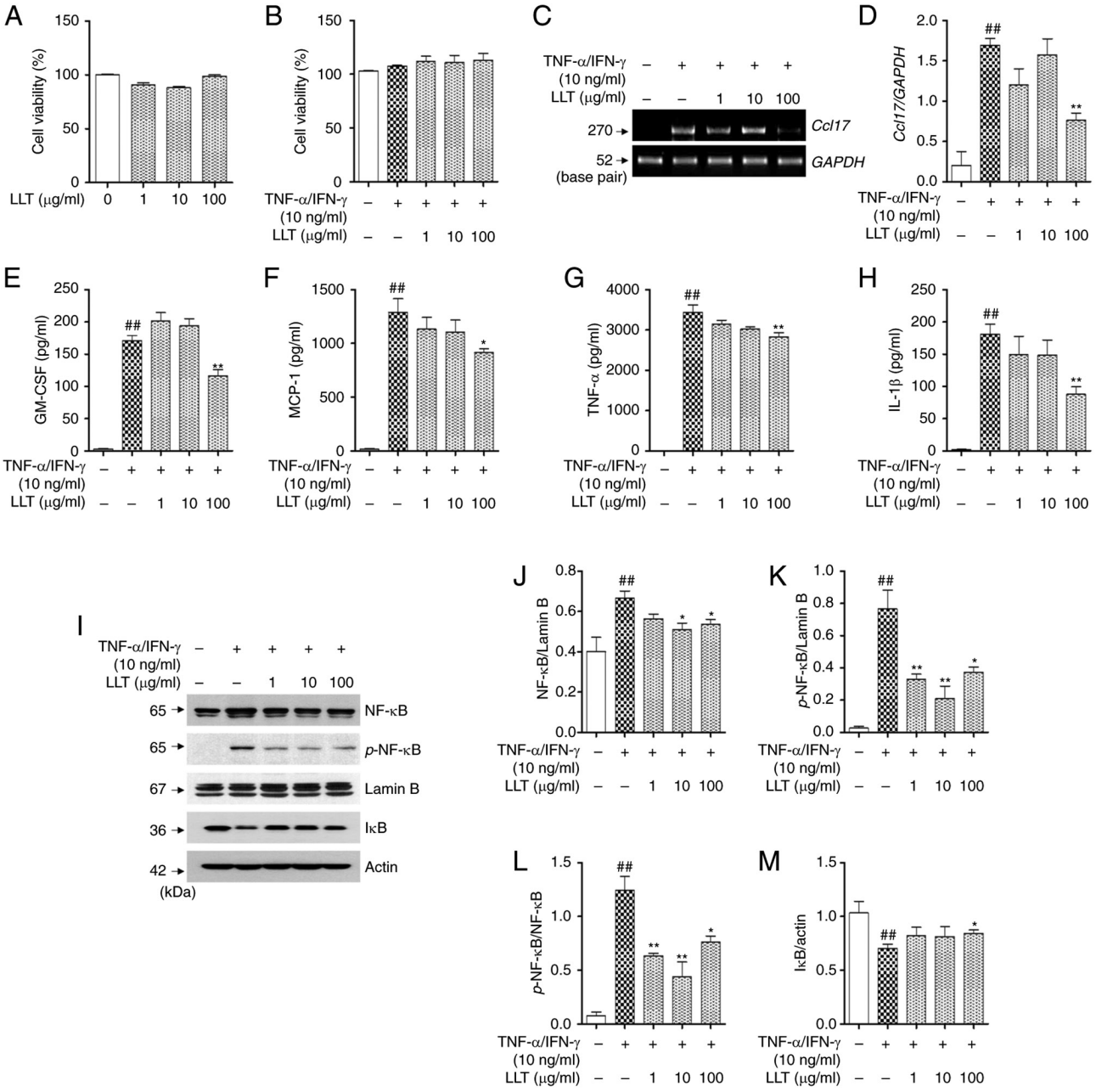

Figure 4. Effects of LLT on the expression of chemokines, pro-inflammatory cytokines and NF-κB signaling in stimulated HaCaT cells. (A) LLT cytotoxicity and (B) LLT + TNF-α/IFN-γ cytotoxicity were determined using MTS assays. (C) Reverse transcription-PCR was performed to verify the effect of LLT on TARC mRNA expression. (D) TARC expression was normalized to GAPDH and quantified using ImageJ software. The effects of LLT on the expression of (E) GM-CSF, (F) MCP-1, (G) TNF-α and (H) IL-1β were analyzed by ELISA. (I) Western blotting was performed to verify the effect of LLT on the NF-κB signaling. (J) NF-κB and (K) p-NF-κB expression was normalized to lamin B and (L) NF-κB. (M) IκB expression was normalized to actin. Each band was quantified using ImageJ software. Data represent the mean ± SEM of three independent experiments. $^{\#\#}$P<0.01 vs. non-treated HaCaT cells. *P<0.05 and **P<0.01 vs. TNF-α/IFN-γ stimulated HaCaT cells. LLT, *Lycopus lucidus* Turcz; TARC, thymus and activation-regulated chemokine; p-, phosphorylated; GM-CSF, granulocyte-macrophage colony-stimulating factor; MCP-1, monocyte chemoattractant protein-1.

control group compared with that in the normal group (P<0.01 and P<0.05). TNF-α expression was significantly downregulated in the LLT-treated groups (10 and 100 μg/ml; P<0.01 and P<0.05, respectively; Fig. 5C). LLT at concentrations of 1, 10 and 100 μg/ml significantly reduced the levels of IFN-γ in RAW 264.7 cells (1, 10 and 100 μg/ml P<0.05, P<0.05 and P<0.01, respectively; Fig. 5D).

To determine the effect of LLT on the NF-κB pathway in RAW 264.7 cells, the effect of LLT on the expression and phosphorylation of NF-κB and degradation of IκB was investigated (Fig. 5E). Treatment with LPS induced the expression (P<0.05) and phosphorylation of NF-κB (P<0.01) in the nuclear protein and degradation of IκB in the cytoplasmic protein (P<0.05). The expression and phosphorylation of NF-κB in the LLT-treated groups was reduced compared with the control, but the difference was not statistically significant (Fig. 5F and G). In addition, the ratio of p-NF-κB/NF-κB was significantly increased through LPS stimulation (P<0.01), but no significant change was observed with LLT treatment (Fig. 5H). Although LLT appeared to inhibit the degradation

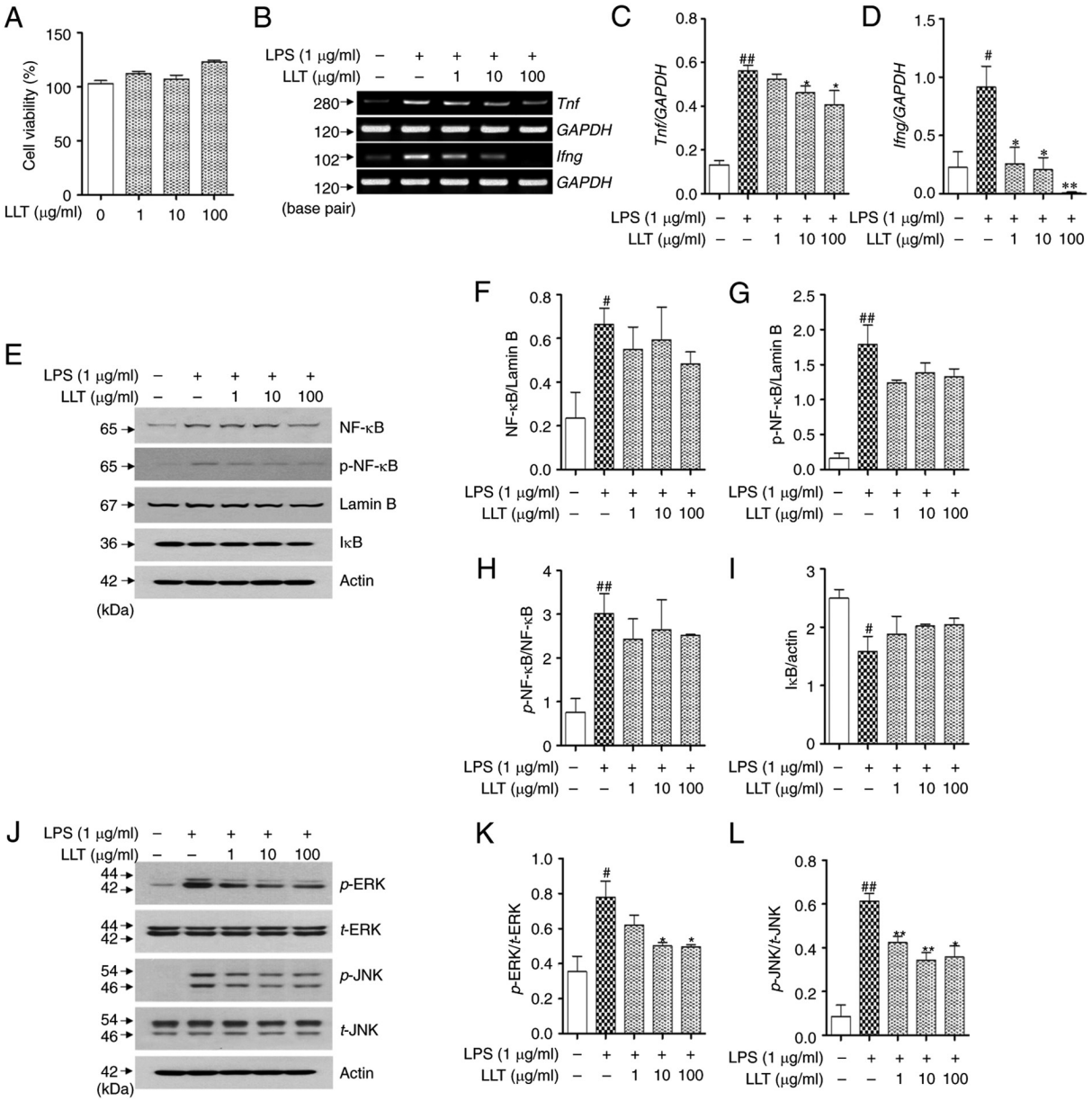

Figure 5. Effects of LLT on TNF-α, IFN-γ, NF-κB signaling and MAPK expression in LPS-stimulated RAW 264.7 cells. (A) LLT cytotoxicity was determined by MTS assay. (B) Reverse-transcription PCR was performed to verify the effect of LLT on TNF-α and IFN-γ mRNA expression. The expression of (C) TNF-α and (D) IFN-γ was normalized to GAPDH and quantified using ImageJ software. (E) Western blotting was performed to verify the effect of LLT on the expression of NF-κB signaling in RAW 264.7 cells stimulated with LPS. (F) NF-κB and (G) p-NF-κB expression was normalized to lamin B and (H) NF-κB. (I) IκB expression was normalized to actin. (J) The effect of LLT on ERK and JNK phosphorylation was verified by western blotting. (K and L) p-ERK and p-JNK levels were normalized to t-ERK and t-JNK, respectively, and quantified using ImageJ software. Data represent the mean ± SEM of three independent experiments. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. non-treated RAW 264.7 cells. *P<0.05 and **P<0.01 vs. LPS-stimulated RAW 264.7 cells. LLT, *Lycopus lucidus* Turcz; LPS, lipopolysaccharide; p-, phosphorylated; t-, total.

of IκB following LPS treatment, the difference was not significant (Fig. 5I).

To determine the effect of LLT on the MAPK pathways, the protein levels of the MAPKs ERK and JNK were assessed using western blot analysis (Fig. 5J). The levels of p-ERK and p-JNK were significantly increased in the control group compared with those in the normal group (P<0.01 and P<0.05, respectively). The phosphorylation of ERK and JNK was significantly reduced in the LLT treatment groups compared with the control group (P<0.05 and P<0.01, respectively). The level of p-ERK was significantly decreased in the LLT-treated groups compared with the control group (10 and 100 μg/ml; both P<0.05; Fig. 5K). Similar to the results of p-ERK expression, the level of p-JNK was decreased in the LLT-treated groups compared with the control group (1, 10 and 100 μg/ml P<0.01, P<0.01 and P<0.05, respectively; Fig. 5L).

HPLC analysis. Caffeic acid has been used as a standard marker for LLT in various studies (30-32). As shown Fig. 6, the retention time of the caffeic acid was 10.62 min (Fig. 6A). The chromatographic peak of the LLT was 10.62 min at a wavelength of 254 nm (Fig. 6B). Since the peaks of LLT and caffeic acid were detected at the same time period, the LLT

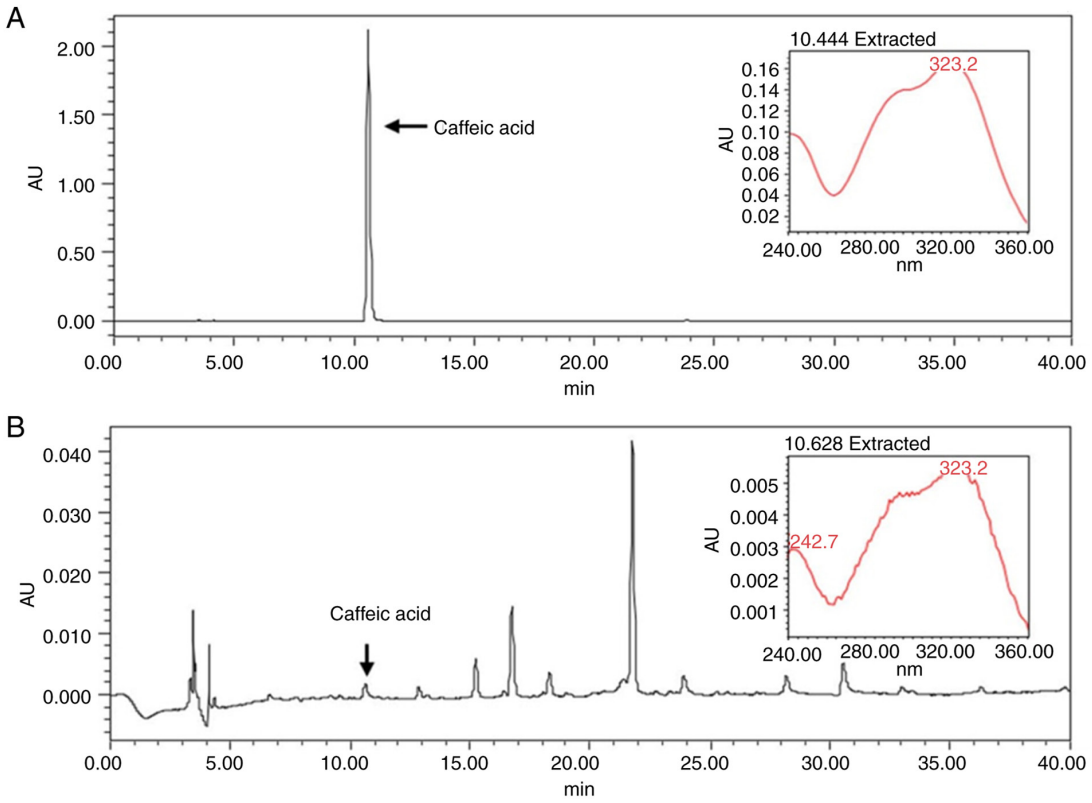

Figure 6. Representative high-performance liquid chromatograms of (A) caffeic acid and (B) LLT detected at 368 nm. LLT, *Lycopus lucidus* Turcz; AU, arbitrary units.

used in this experiment was proved to be the same as that used in other studies.

Discussion

To the best of the authors' knowledge, the present study is the first to confirm the beneficial effect of LLT on DNCB-induced AD and its effect on the expression of cytokines and chemokines in HaCaT and RAW 264.7 cells. It was demonstrated that LLT inhibited DNCB-induced hyperkeratosis in the epidermis and dermis, and infiltration by eosinophils, mast cells and $CD8^+$ cells. LLT also inhibited the expression of IgE and IL-6 in the serum, and the phosphorylation of MAPK and NF-κB in the skin tissues. In addition, LLT inhibited the expression of inflammatory chemokines and cytokines in HaCaT and RAW 264.7 cells. Taken together, these findings confirmed that LLT has the potential of becoming a new alternative AD treatment. Balb/c mice, in which AD was induced through DNCB stimulation, are characterized by a Th2-dominated immune response and are widely used in the field of Immunology (33). In addition, DNCB was found to promote the expression of various cytokines (34) and chemokines (35) that induce AD. HaCaT cells are a naturally immortalized line of human keratinocytes. These cells are widely used in skin biology and differentiation studies. RAW 264.7 cells are macrophages derived from Abelson leukemia virus-infected mice, and they are used in various studies to verify the effects on the inflammatory response (36). LPS is a gram-negative bacterium, which increases various inflammatory cytokines and a large amount of nitric oxide (NO) to cause tissue damage, edema and inflammatory response (37). Therefore, the LPS-induced RAW 264.7 cell model has been used in various studies to examine the mechanism of AD (38,39).

Skin thickening is a notable clinical symptom of AD (40,41). Continuous allergic and inflammatory responses may result in hardened and thickened skin (42). In the present study, LLT inhibited the thickening of the epidermis and dermis, indicating that LLT exerts an inhibitory effect on hyperkeratosis and alleviates skin thickening, which is one of the main symptoms of AD. The cytokines secreted by Th1 and Th2 cells promote the development of AD (43). Th2-cell allergic inflammation predominates in the acute stage of AD, resulting in increased IL-6 and IgE expression, whereas Th1-cell inflammation predominates in the chronic stage of AD, resulting in increased expression of IFN-γ and TNF-α (5,44,45). Th2 cells activate B cells, which produce IgE (46). IgE binds to the IgE receptor, FcεRI, and initiates the activation of mast cells, leading to the release of histamine and other mediators of inflammation (47,48). In the present study, the levels of IgE and IL-6 were significantly increased in the serum of mice with DNCB-induced AD, whereas LLT decreased the production of IgE and IL-6.

AD is associated with high IgE levels and infiltration by inflammatory cells, such as mast cells and eosinophils (49). The majority of patients with AD are pathologically characterized by infiltration of the skin lesions by eosinophils and mast cells (50,51). The activation of mast cells has been associated with allergic inflammation and the expression of various inflammatory mediators, including histamine, which induces AD-like skin lesions (52,53). Eosinophils are cells of

the immune system, which, together with mast cells, act as mediators of allergic reactions (13). Mast cells and eosinophils are recruited and activated in the inflamed dermis, where they play a major role in aggravating allergic skin conditions (54). Research has shown that these cells modulate pruritus in AD and induce allergic inflammation (55,56). Increased numbers of eosinophils and mast cells are a characteristic feature of AD in both humans and DNCB-induced AD mice (57-59). To examine the effects of LLT on the infiltration of eosinophils and mast cells, the present study performed H&E and toluidine blue staining of dorsal skin tissues obtained from DNCB-induced AD mice. It was observed that LLT decreased the number of eosinophils and mast cells in the skin of AD mice, indicating that LLT may inhibit the infiltration of the dermis by mast cells and eosinophils by suppressing IgE and the release of pro-inflammatory cytokines.

$CD4^+$ T cells (Th cells) induce humoral immunity by acting on specific B lymphocytes to promote responses to antigens (60,61). Humoral immunity is an antigen-antibody reaction caused by the activation of B cells and T cells by $CD4^+$ T cells and the mechanism is as follows. The antigen-memory $CD4^+$ T cells induce the differentiation of B cells. Differentiated B cells meet with T cells to form antibodies in T cells and these antibodies circulate in body fluids and cause antigen/antibody reactions (62). In a normal allergic environment, Th2 cells induce the production of Th2 cytokines responsible for the expression of allergen-specific IgE (63). $CD8^+$ cells (cytotoxic/suppressor T cells) release toxic substances, such as perforin, granzyme and granulicin, that can directly target antigen-bearing cells, and when stimulated, Th1 cells are activated to induce the expression of inflammatory cytokines such as TNF-α (64); therefore, the numbers of $CD4^+$ and $CD8^+$ cells are important indicators for measuring the immune function (65). In the present study, LLT reduced the number of $CD8^+$ cells in the skin tissue with DNCB-induced AD, but did not significantly affect the number of $CD4^+$ cells. Because $CD4^+$ cells are not under the control of LLT in the present study, the reason why the expression of IgE mediated by Th2 cytokines is suppressed may be related to the suppression of IL-6 expression. A previous study has shown that the expression of IL-6 upregulates the expression of IgE (66). From this study, the IgE expression inhibitory effect of LLT is hypothesized to be suppressed through IL-6, not through $CD4^+$ cells. In addition, the expression of IgE induces the degradation of the mast cells to express various inflammatory cytokines. In this study, the invasion of the mast cells in the tissue was inhibited, as evidenced by toluidine blue staining. Therefore, following the results of these studies, it was demonstrated that the infiltration of mast cells was induced by the expression of IgE and that LLT inhibited them. Combining the experimental results of serum and tissue analysis, these results indicated that the inhibition of the inflammatory response by LLT in AD is mediated through inhibition of $CD8^+$ cell, but not $CD4^+$ cell infiltration.

The activation of NF-κB is a key step in the progression of inflammatory skin conditions (15,67,68). Therefore, the inhibition of the NF-κB expression may represent a target for the treatment of inflammatory diseases, including AD (69). The activation of MAPK signaling plays a major role in NF-κB activation (70,71). The MAPK (ERK/1/2 and JNK) signaling pathway plays an important role in the proliferation, degranulation and activation of diverse immune cells (72). In the present study, LLT inhibited the expression of NF-κB in the dorsal skin tissues obtained from mice with DNCB-induced AD. Furthermore, LLT inhibited the expression and phosphorylation of NF-κB in HaCaT cells induced by TNF-α/IFN-γ in nuclear protein and it also inhibited the degradation of IκB in the cytoplasmic protein. In addition, LLT treatment appeared to show a positive effect on the NF-κB pathway in RAW 264.7 cells stimulated with LPS, although the difference was not significant. These results indicated that the effect of LLT may be mediated through inhibition of expression and phosphorylation of NF-κB, a target in AD treatment. In addition, LLT significantly inhibited the phosphorylation of ERK and JNK in tissues and phosphorylation of LPS-induced MAPK pathway-related proteins in RAW 264.7 cells.

MAPK and NF-κB signaling induce AD through the expression of various pro-inflammatory cytokines and chemokines (73). TARC is a representative Th2 chemokine that acts on the C-C chemokine receptor 4 expressed on T cells to induce the migration and invasion of Th2 cells in inflammatory lesions (74). TARC is produced by a variety of cells, including endothelial cells, dendritic cells and keratinocytes. In AD, TARC induces integrin-dependent adhesion and passage of T cells through the blood vessel wall, acting in the first stage of T-cell recruitment to the lesion (75). The expression of TARC is high in the basal layer of the epidermis in skin lesions (74), and serum TARC levels are markedly elevated in patients with AD, which is directly proportional to the severity of the symptoms (76) and may be used as an index of AD severity. In the present study, LLT was shown to inhibit TARC expression in HaCaT cells stimulated with TNF-α/IFN-γ. GM-CSF is a cytokine secreted by lymphocytes, and its expression is increased in chronic AD, which is known to inhibit apoptosis and promote survival of lymphocytes, causing chronic inflammation of skin lesions (77). MCP-1 is a member of the CC family of chemokines and it serves as a chemotactic factor for monocytes. MCP-1 has been demonstrated to affect the migration and invasion of monocytes, macrophages, memory T cells and natural killer cells, and an increase in MCP-1 has been observed in studies on patients with chronic and idiopathic urticaria, a chronic inflammatory skin disease (78). In the present study, LLT inhibited GM-CSF and MCP-1 expression in HaCaT cells stimulated by TNF-α/IFN-γ.

When HaCaT cells are exposed to stress, cytokines, such as TNF-α and IL-1β, activate several inflammation-related cell signaling pathways (79). TNF-α is a pro-inflammatory cytokine that is involved in the initiation of inflammatory reactions. TNF-α regulates the lipid barrier function of the skin, either by acting alone or in combination with Th2 cytokines, and is also involved in the proliferation of thymic stromal lymphopoietin (80). IL-1β is mainly secreted by activated mononuclear phagocytic cells (81). As a lymphocyte-activating factor, IL-1β promotes the activation and proliferation of $CD4^+$ T lymphocytes and secretion of IFN-γ, and activates vascular endothelial cells to cause leukocyte adhesion (82). Therefore, IL-1β plays an important role in the inflammatory process and is involved in type I hypersensitivity reactions in allergic diseases (11). In the present study, LLT inhibited the expression of TNF-α and IL-1β in stimulated HaCaT cells, and

suppressed the expression of TNF-α and IFN-γ in stimulated RAW 264.7 cells. Collectively, these experimental results indicate that LLT may suppress the expression of chemokines and inflammatory cytokines by inhibiting the expression of MAPKs and NF-κB signaling. Finally, LLT may improve AD by increasing the thickness of the skin barrier and inhibiting infiltration by eosinophils and mast cells.

This study has the following limitations. First, in an *in vitro* assay, it was verified the NF-κB pathway in HaCaT cells and RAW 264.7 cells. LLT showed a significant inhibitory effect on the expression and phosphorylation of NF-κB in HaCaT cells, and showed a positive effect compared to the induced group, although the difference was not significant in RAW 264.7 cells. However, in the study of NF-κB in animal tissues, the expression of NF-κB was only measured in the nuclear protein fraction. As a result, the role of the NF-κB pathway in AD remains unconfirmed. In future studies, additional research on NF-κB phosphorylation or IκB degradation may provide further insight into the ability of LLT to inhibit AD. Moreover, this study verified the anti-atopic effect of LLT, a herbal medicine, but it is unclear which ingredients in LLT play an anti-atopic role. In previous study, LLT was shown to possess various active ingredients such as schizotenuin A, 3-O-(caffeoyl)-rosmarinic acid, rosmarinic acid ethyl ester, apigenin, acacetin, acacetin-7-O-b-D-glucuronopyranoside, luteolin, scutellarin and hispidulinc (83). Among these ingredients, rosmarinic acid improves the scoring atopic dermatitis (SCORAD) index (a standardized method for the severity of atopic dermatitis in the European atopic dermatitis task force consensus report) (84) and skin moisture index in clinical trials (85), luteolin was effective in treating skin diseases by inhibiting the expression of pro-inflammatory mediators in the NF-κB pathway (86). In addition, hispidulin inhibited AD by regulating the expression of IL-1β, IL-6, IL-8 and chemokine C-C (87). However, the pharmacological effects of the remaining ingredients on atopic diseases have not yet been verified. In the future, directly separating the components contained in LLT, may further confirm and improve our understanding of the pharmacological effect of LLT in each atopic disease.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HSJ designed the experiments. EYK, GYM and SH performed the *in vivo* experiments. GYM, JHK, MK and EJK performed the *in vitro* experiments. YS, HSJ and JHP performed the data analysis. GYM wrote the manuscript. GYM and HSJ confirm the authenticity of the raw data in this manuscript. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were performed with the approval of The Institutional Animal Care and Use Committee of Kyung Hee University [approval no. KHUASP (SE)-15-116].

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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