Umbelliferone reduces the expression of inflammatory chemokines in HaCaT cells and DNCB/DFE-induced atopic dermatitis symptoms in mice

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ABSTRACT

Umbelliferone (UMB) is a coumarin derivative present in roots and barks of plants, such as *Angelica decursiva*, *Artemisia capillaris*, and orange. UMB has been previously reported to exhibit anti-inflammatory, anti-diabetic, and anti-cancer effects. However, the effect of UMB on atopic dermatitis (AD) remains unknown. The purpose of this study was to investigate the anti-atopic effects of UMB on 2,4-dinitrochlorobenzene (DNCB)- and house dust mite extract (*Dermatophagoides farinae* extract, DFE)-treated mice with AD-like skin lesions and on tumor necrosis factor (TNF)-α/interferon (IFN)-γ-treated HaCaT cells. In DNCB/DFE-treated mice, oral administration of UMB (20 and 40 mg/kg) for 28 days led to a significant decrease in ear thickness, spleen size and weight, serum levels of immunoglobulin E (IgE), IgG1, IgG2a, TNF-α, and interleukin 4 (IL-4), and mast cell infiltration; it also led to the suppression of pro-inflammatory cytokines and chemokines. In addition, UMB reduced the secretion of pro-inflammatory cytokines and chemokines in TNF-α/IFN-γ-treated HaCaT cells via regulation of MAPK, IkB-α/NF-κB, and STAT1 signaling pathways. Taken together, these results indicate that UMB ameliorates AD-associated symptoms and inflammation via regulation of various signaling pathways, suggesting that UMB might be a potential therapeutic agent of AD.

1. Introduction

Atopic dermatitis (AD) is a common chronic inflammatory skin disease with symptoms, such as eczema, itching, and redness [1]. AD, an allergic skin disease, can be caused by several factors, including genetic, immunological, and environmental factors [2,3]. As AD is caused by the complex interactions of various factors, its main causal factor is still unknown. Moreover, the incidence and severity of the disease is steadily increasing to date.

From an immunological point of view, AD is a skin disease that occurs and progresses due to an imbalance between abundances of T-helper (Th) 1 and Th2 cells, and is considered a Th2 cell-mediated skin disease [4]. Several inflammatory cytokines and chemokines that are secreted by Th2 cells directly affect skin cells, such as keratinocytes and activated mast cells [5,6]. Activated mast cells secrete histamine and various cytokines and chemokines to induce infiltration of immune cells into inflammatory lesions [7]. In addition, secretion of histamine promotes the production of immunoglobulin E (IgE). IgE is involved in the development of allergic diseases, and the overproduction of serum IgE is one of the characteristics of AD.

Nowadays, most AD patients use drugs, such as corticosteroids and antihistamines, to reduce inflammation and itching [8]. However, these drugs have been reported to exhibit adverse effects, such as metabolic abnormalities, virus infection, skin atrophy, and decreased cognitive function with repeated administration over a long-term [9]. Many AD patients are seeking new medicines that can relieve symptoms effectively, have no side effects, and can be used for a long time. Umbelliferone (7-hydroxycoumarin, UMB) is a derivative of coumarin found in roots and barks of many plants. To date, several pharmacological activities of UMB have been reported. Previous studies have shown that UMB exhibits anti-inflammatory [10], antioxidant [11], anti-diabetic [12], anti-cancer [13], and anti-allergic effects [14]; it also shows protective effects in liver damage [15]. However, the effect of UMB on AD remains unknown.

To the best of our knowledge, this is the first study to investigate the anti-atopic effect of UMB on 2,4-dinitrochlorobenzene (DNCB)- and house dust mite extract (*Dermatophagoides farinae* extract, DFE)-treated mice with AD-like skin lesions and on tumor necrosis factor (TNF)-α/interferon (IFN)-γ-treated keratinocytes and to investigate its underlying molecular mechanisms.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin 0.25% solution and Penicillin-streptomycin (PS) solution were purchased from Hyclone (Logan, UT, USA). *Dermatophagoides farinae extract* were purchased from PROLAGEN (Yonsei University, Korea). Umbelliferone (purity, > 98%), carboxymethylcellulose (CMC), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution, dimethyl sulfoxide (DMSO), Toluidine Blue O and Ethidium bromide(EtBr) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Total RNA isolation was performed with Ribo-Ex (Geneall Bio Co., LTD, Korea) and PrimeScript™ II 1st strand cDNA synthesis kit were purchased from Takara (Bio, Inc., USA). Cell culture dish and well plate and all plastic supports were purchased from Falcon (Beckton-Dickinson, USA). BCA protein assay kit and Nuclear and cytoplasmic extraction reagents were purchased from ThermoScientific (Waltham, MA, USA). Total serum IgE mouse ELISA kit, serum IgG$_1$ mouse ELISA kit, serum IgG$_{2a}$ mouse ELISA kit, serum TNF-α mouse ELISA kit and serum IL-4 mouse ELISA kit were obtained from BioLegend (San Diego, CA, USA). The primary antibodies β-Actin (C4), pJNK (G-7), JNK (FL), IκB-α (C-21), NFκB p65 (H-286), pSTAT1 (A-2), STAT1 p84/p91 (C-136) and secondary antibodies were purchased from Santa Cruz Biotechnology (CA, USA). The other primary antibodies pp38 (phosphor T180 + Y182), p38, pERK (pT202/pY204) + Erk2 (pT185/pY187) antibody, ERK (ERK1 + ERK2) were purchased from Abcam (Cambridge, UK).

2.2. Animals

The male BALB/c mice (6 weeks old) were purchased from SAMTAKO Bio, Korea. During the experiment, all mouse groups were provided with the same water and food ad libitum. All animals were maintained at a temperature of $22 \pm 2\,^\circ\text{C}$ and a humidity of $55 \pm 5\%$, and light/dark cycle of 12 h each. The research was carried out in accordance with the ethical regulations of the Animal Experiment Ethics Committee of Chonbuk National University (Confirmation No. CBNU 2018-081).

2.3. Induction and treatment of AD-like skin lesions in mice

The induction of AD-like lesions by DNCB and DFE was performed based on Choi's previous research [16]. The BALB/c mice were randomly divided into five groups (n = 5 per group): (1) Normal group (vehicle treatment), (2) DNCB/DFE group (mice sensitized with DNCB/DFE and treated with water), (3) UMB 20 group (mice sensitized with DNCB/DFE and treated with 20 mg/kg UMB), (4) UMB 40 group (mice sensitized with DNCB/DFE and treated with 40 mg/kg UMB) (5) Dexa group (mice sensitized with DNCB/DFE and treated with 1 mg/kg dexamethasone). AD-like skin lesions were evoked by administering 1% DNCB and DFE (10 mg/mL). Each mouse ear was treated with 20 μL of 1% DNCB, and after 4 days, 20 μL of DFE was applied. DNCB/DFE treatment was alternatively given once a week repeatedly for 4 weeks. UMB (20 mg/kg and 40 mg/kg) and dexamethasone were orally administered every day for 4 weeks.

2.4. Measurement of ear thickness and clinical score of the AD-like symptom

The ear thickness of all mice was measured once a week for 4 weeks using a micrometer. The severity of AD score (such as edema and erythema) of ears was evaluated on the basis of Fan's criteria [17]. The criteria are shown in Table 1.

Table 1
Criteria for severity of AD dermatitis.

Item	Severity index	Score
Erythema	None	0
	Mild	1
	Moderate	2
	Severe erythema and mild scar	3
	Severe erythema and scar	4
Oedema	None	0
	Mild	1
	Moderate	2
	Severe	3
Scratching	No	0
	Yes	1

2.5. Histological observation

After completion of the above experiment, the ear skin tissue samples from each mouse were collected, fixed in 4% formaldehyde solution at room temperature, and embedded in paraffin. Each sample was serially cut to produce 6 μm-thick sections and stained with hematoxylin and eosin (H&E) and toluidine blue (TB). Histological analysis and images were taken using a microscope. The epidermal thickness and dermal thickness were analyzed by observing the portion stained with H&E at 100× magnification. TB staining was performed to evaluate the infiltration of mast cells. The number of mast cells was counted in three randomly selected sections.

2.6. Serum enzyme-linked immunosorbent assay (ELISA)

The mice were anesthetized using ethyl ether and their blood was collected. Serum samples were obtained after centrifugation at $\times 1000g$ for 10 min, and stored at $-70\,^\circ\text{C}$ until further use. The total serum levels of immunoglobulin E (IgE), immunoglobulin G (IgG$_1$), immunoglobulin G2A (IgG$_{2a}$), tumor necrosis factor-α (TNF-α), and IL-4 in the mouse were evaluated using ELISA kit, according to the manufacturer's instructions.

2.7. Cell culture and viability assay

Human keratinocyte cell line, HaCaT, was purchased from the American Type Culture Collection (ATCC, Manassas, VA). The cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin (100 units/mL), and cultured at 37 °C under 5% CO_2. The viability of cells was evaluated by MTT assay. HaCaT cells were seeded $(1 \times 10^4 \text{ cells/well})$ in a 96-well plate and exposed to various doses of UMB (0, 12.5, 25, 50, and 100 μM) for 24 h. After removal of supernatant, 100 μL MTT was added to each well and the plate was incubated for a further 4 h. Then, DMSO solution was added to dissolve the crystals and the absorbance was measured at a wavelength of 570 nm.

2.8. Quantitative real-time PCR (polymerase chain reaction)

Real-time PCR was performed to validate the expression levels of the genes in vivo and in vitro. The levels of gene expression in vivo were evaluated using ear tissues cut off after sacrificing the mice on the last day of experiment, and the levels of gene expression in vitro were examined using HaCaT cells. Total RNA was prepared using a Ribo EX reagent. cDNA synthesis was carried out, using PrimeScript™ II 1st Strand cDNA synthesis kit, according to the manufacturer's protocol. Quantitative amplification by PCR was carried out using Power SYBR Green PCR Master Mix reagent by AB StepOne system (Applied Biosystems, Foster, CA, USA). The primer sequences of TNF-a, IL-1β, IL-5, CCL2, CCL7, CCL8, CXCL8, CXCL10 and CXCL11 used in the amplification are shown in Table 2.

Table 2
Primer sequences for real-time PCR.

Gene	Forward	Reverse
mTNF-α	TAGCCAGGAGGGAGAACAGA	TTTTCTGGAGGGAGATATGG
mIL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
mIL-5	GAAAGAGACCTTTGACACAGCTG	GAACTCTTGCAGGTAATCCAGG
mCCL2	CCCAATGAGTAGGCTGGAGA	TCTGGACCCATTCCTTCTTG
mCXCL8	ACCGGAGCACTCCATAAGGC	AGGCTGCCAAGAGAGCCACG
mCXCL10	CTGAGTGGGACTCAAGGGAT	TCGTGGCAATGATCTCAACAC
mCXCL11	GGCAGAGATCGAGAAAGCTT	ATTGCCTGCATTATGAGGCG
hTNF-α	TCTCGAACCCCGAGTGACAA	TATCTCTCAGCTCCACACCA
hIL-1β	CTCTCTCACCTCTCCTACTCAC	ACACTGCCTACTTCTTGCCCC
hIL-5	GCTAGCTCTTGGAGCCT	CTTCAGTGCACAGTTGA
hCCL2	TCTCTGCCGCCCTTCTGTG	AGGTGACTGGGCATTGATTG
hCCL7	CCAAACCAGAAACCTCCAAT	ACACAGAAGTGCTGCAGAGG
hCCL8	TTCTGTGCCTGCTGCTCATG	TTGGATGTTGGTGATTCTTGTG
hCXCL8	ACCGGAGCACTCCATAAGGC	AGGCTGCCAAGAGAGCCACG
hCXCL10	TTGCTGCCTTATCTTTCTGACTC	ATGGCCTTCGATTCTGGATT
hCXCL11	GGCTGTGATATTGTGTGCTAC	GGATTTAGGCATCGTTGTCC
GAPDH	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC

TNF-α, tumor necrosis factor-α; IL-, Interleukin-; CCL-, Chemokine (C−C motif) ligand; CXCL-, C-X-C motif chemokine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

2.9. Nuclear and cytoplasmic protein extraction

HaCaT cells were pre-treated with UMB for 2 h, and then stimulated with TNF-α/IFN-γ (each 10 ng/mL) for 90 min. After the experiment, the cells were harvested, washed with 1 mL of ice-cold PBS, and centrifuged at ×500g for 5 min. Nuclear and cytoplasmic protein fractions were extracted using Extraction Reagents kit (ThermoScientific, MA, USA), according to the manufacturer's instructions.

2.10. Western blot analysis

HaCaT cells were pre-treated with UMB for 2 h, stimulated with TNF-α/IFN-γ (each 10 ng/mL) for 3 h, and cultured at 37 °C under 5% CO_2. Then, the cells were harvested and total protein was extracted using cell lysis buffer. Proteins were separated by electrophoresis on an SDS-polyacrylamide gel, and transferred to PVDF membranes. Non-specific binding was blocked by treatment with 5% bovine serum albumin (BSA) dissolved in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature. The membranes were then incubated overnight in 4 °C with a 1:1000 dilution of primary antibodies. After washing 3 times with TBST buffer, the membranes were incubated with 1: 2000 dilution of horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. The membranes were analyzed using the Fusion Fx gel documentation system (Davinch-Invivo™ Imaging System, USA).

2.11. Statistical analysis

All data were presented as mean ± S.D. We performed one-way analysis of variance (ANOVA) and post tests used Tukey's Multiple Comparison test was conducted to compare the differences between the groups. All analyzes were performed using GraphPad Prism (v.5, GraphPad software., San Diego, CA) and the values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. UMB alleviates the AD-like symptoms and size variation of spleen in DNCB/DFE-treated mice

According to the experimental schedule shown in Fig. 1, we established mice model with AD-like skin lesion. We have confirmed that the ears of mice deteriorated by DNCB/DFE treatment are dose - dependent mitigated after UMB oral administration (Fig. 2A and B). In addition,

DNCB/DFE-induced increase in ear thickness was restored after oral administration of UMB (Fig. 2C). The spleen is a lymphatic organ that facilitates the functioning of immune cells and removes the cells surrounded by bacteria or antibodies. It grows larger when during infection or inflammation in the body. Therefore, we investigated the mitigation effects of AD using a natural compound, UMB. We observed that the size and weight of the spleen increased by the DNCB/DFE induction was restored in a capacity-dependent manner by the UMB (Fig. 2D and E). These results suggested that UMB ameliorated DNCB/DFE-induced AD-like skin lesion symptoms.

3.2. UMB suppresses the serum levels IgE, IgG, TNF-α, and IL-4 in DNCB/DFE-treated mice

Immunologically, AD is considered to be a Th2 cell-mediated disease [18]. AD is caused by immunoglobulin E (IgE) mediated sensitization, severe skin inflammation, and deterioration of immune response. Immunoglobulins exhibit antibody functions and are classified into five groups (IgM, IgG, IgA, IgD, and IgE) based on their structure and biological activity. IgG eliminates toxic substances or viruses in the blood and facilitates the phagocytosis or sterilization of white blood cells [19,20]. Activated B cells produce IgE under the stimulation of Th2 cytokines [21]. IgE is used for clinical evaluation and assessing the severity of allergic patients [22]. Thus, measurement of the total IgE level could be used for clinical assessment and determination of severity of AD in patients [23]. Therefore, we evaluated the serum levels of IgE, IgG_1, IgG_{2a}, TNF-α, and IL-4. Oral administration of UMB restored serum levels of IgE, IgG_1, IgG_{2a}, TNF-α, and IL-4 in dose-dependently manner elevated by DNCB/DFE (Fig. 3A–E).

3.3. UMB ameliorates histological changes in DNCB/DFE-treated mice

In AD patients, number of inflammatory cells, including eosinophil, mast cells, and lymphocytes, is significantly increased. To investigate the effect of UMB on AD, histological analysis was performed. We observed lymphocyte infiltration, epidermal and dermal thickness, and fibrosis of the dermis using H&E staining (Fig. 4A). DNCB/DFE-treated group exhibited hypertrophy and hyperkeratosis in epidermal and dermal tissue compared with normal group (Fig. 4C). However, UMB (20 and 40 mg/kg)-treated group inhibited these inflammatory changes in a dose-dependent manner. We performed TB staining to determine whether mast cells infiltrated the skin after DNCB/DFE application (Fig. 4B). The application of DNCB/DFE significantly increased mast cell infiltration in the epidermis and dermis of the ear (Fig. 4D).
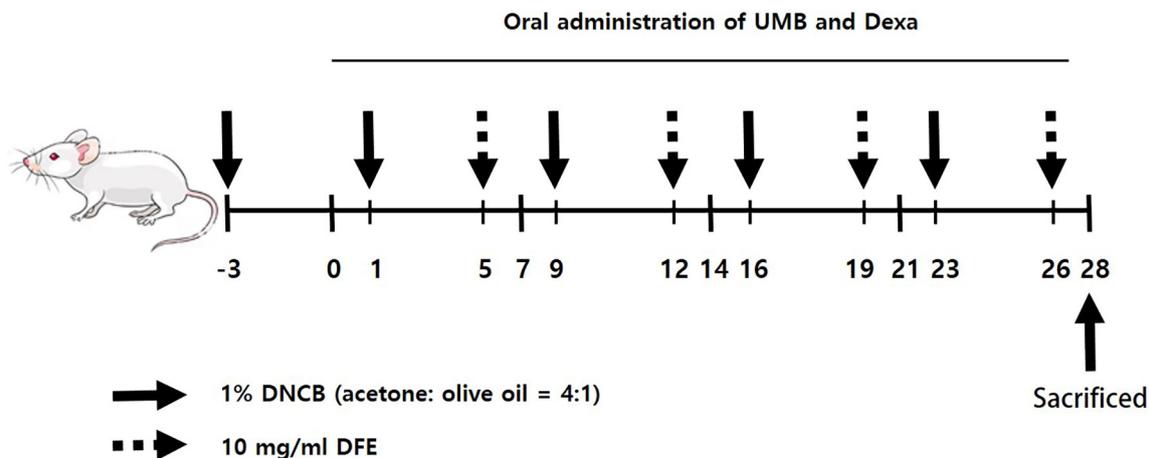
Oral administration of UMB and Dexa

→ 1% DNCB (acetone: olive oil = 4:1)

⇢ 10 mg/ml DFE

Fig. 1. Experimental design of AD-like skin lesions mice model. BALB/c mice were randomly divided into five groups (n = 5 per group). AD-like skin lesions were evoked by administering 1% DNCB and DFE (10 mg/ml). 20 μL of 1% DNCB was applied on each ear, and after 4 days 20 μL of DFE was applied. DNCB/DFE was alternatively treated once a week repeatedly for 4 weeks. UMB (20 mg/kg and 40 mg/kg) and dexamethasone were orally administered every day for 4 weeks.
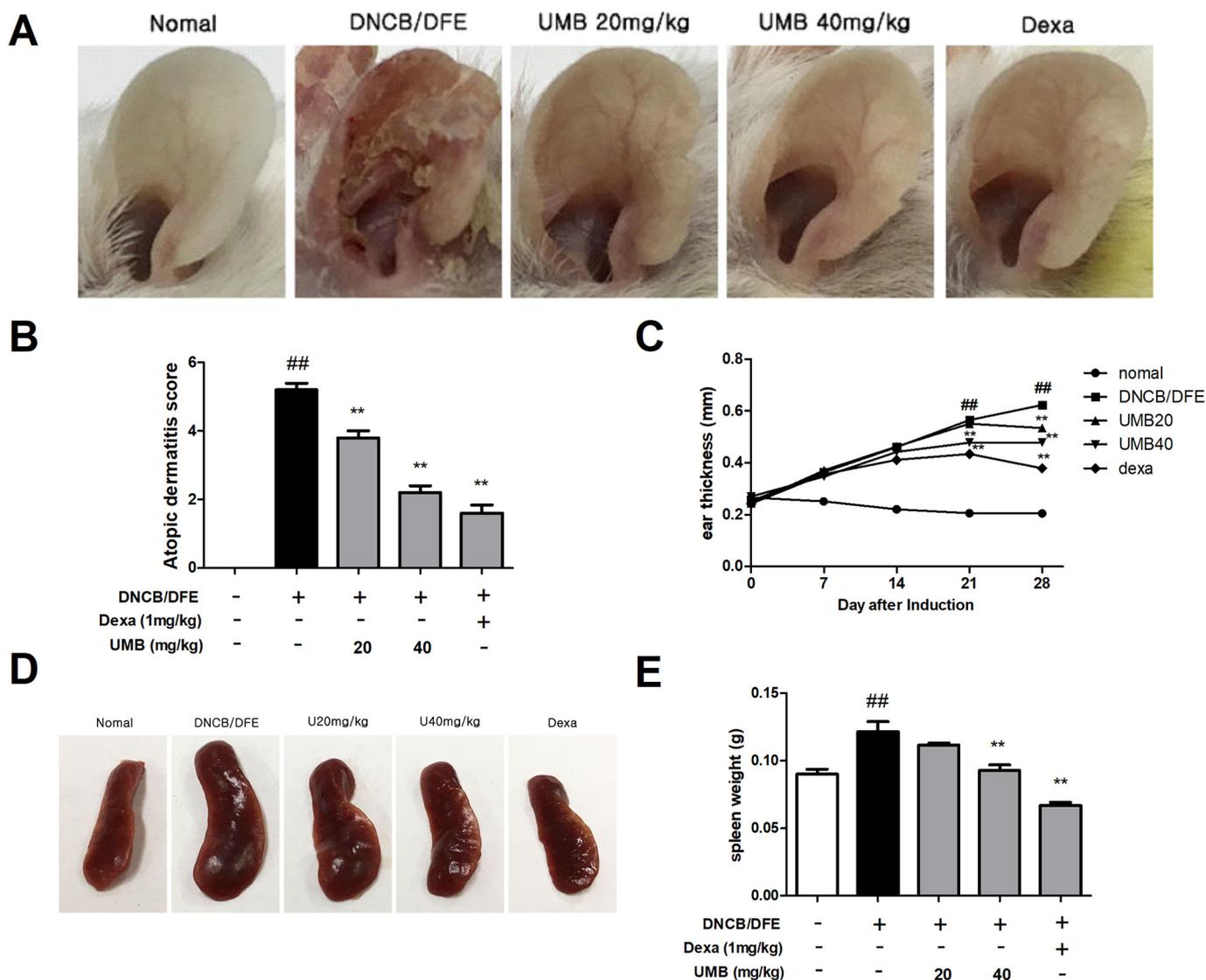
Fig. 2. Effect of UMB treatment on AD-like skin symptoms and size variation of spleen in DNCB/DFE-treated mice. (A) Therapeutic effect of UMB on DNCB/DFE-induced AD-like skin symptoms. Measurement of (B) ear thickness and (C) DNCB/DFE-induced AD score in mice. (D) Visual evaluation of spleen tissue. (E) Measurement of spleen weight. Data are shown as mean ± SD of the three independent experiments. ##$p < 0.01$ versus normal group, and *$p < 0.05$, and **$p < 0.01$ versus DNCB/DFE treated group.
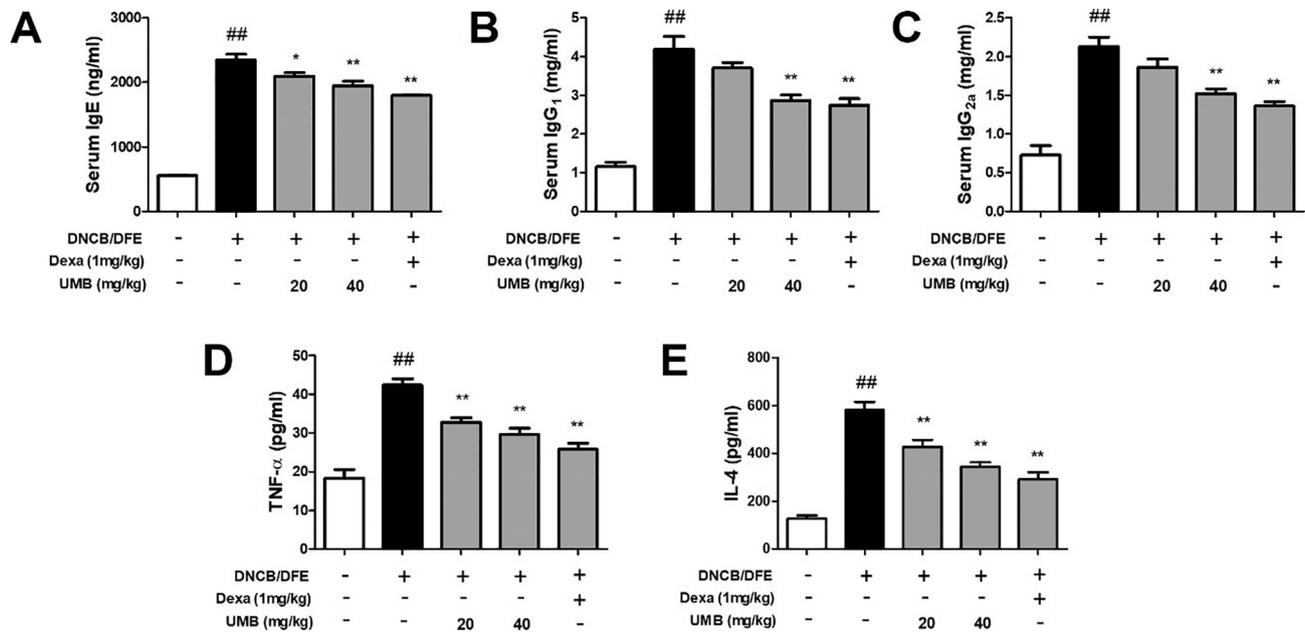
Fig. 3. Effect of UMB on serum levels in DNCB/DFE-treated mice. After 28 days, blood samples of all mice were collected by cardiac puncture. (A) Total serum levels of IgE, (B) serum IgG$_1$, (C) serum IgG$_{2a}$, (D) serum TNF-α, and (E) serum IL-4 were measured by ELISA. Data are shown as mean ± SD of the three independent experiments. ##$p < 0.01$ versus normal group, and *$p < 0.05$, and **$p < 0.01$ versus DNCB/DFE treated group.
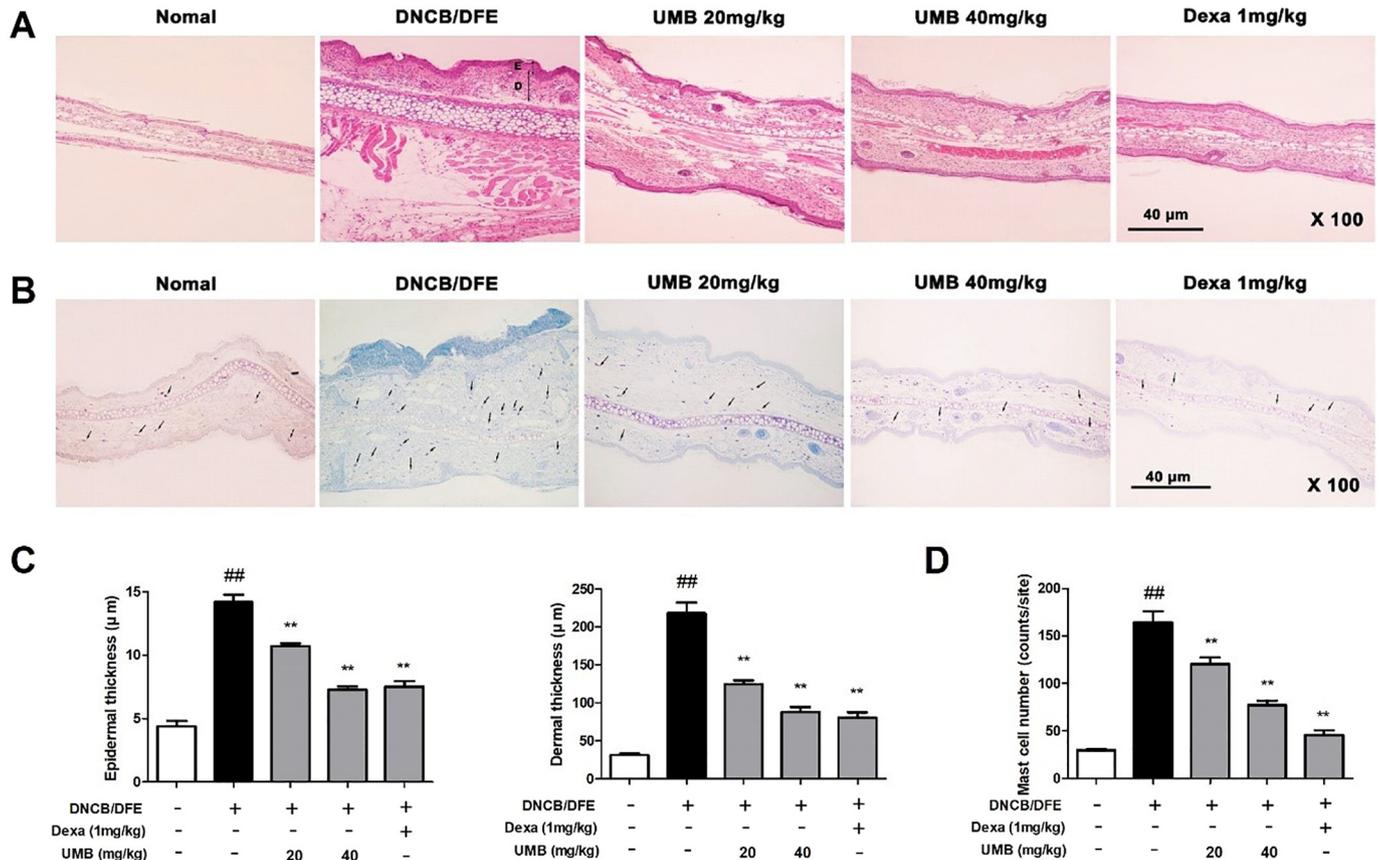

Fig. 4. Effects of UMB on the histology of ear lesions in DNCB/DFE-treated mice. Ear sections were stained with (A) hematoxylin-eosin (scale bar = 40 μM) and (B) toluidine blue (scale bar = 40 μM). The images were observed at 100× magnification. (C) Measurement of epidermal and dermal thickness. (D) Measurement of number of mast cells (arrowheads). Data are shown as mean ± SD of the three independent experiments. ##$p < 0.01$ versus normal group, and *$p < 0.05$, and **$p < 0.01$ versus DNCB/DFE treated group.
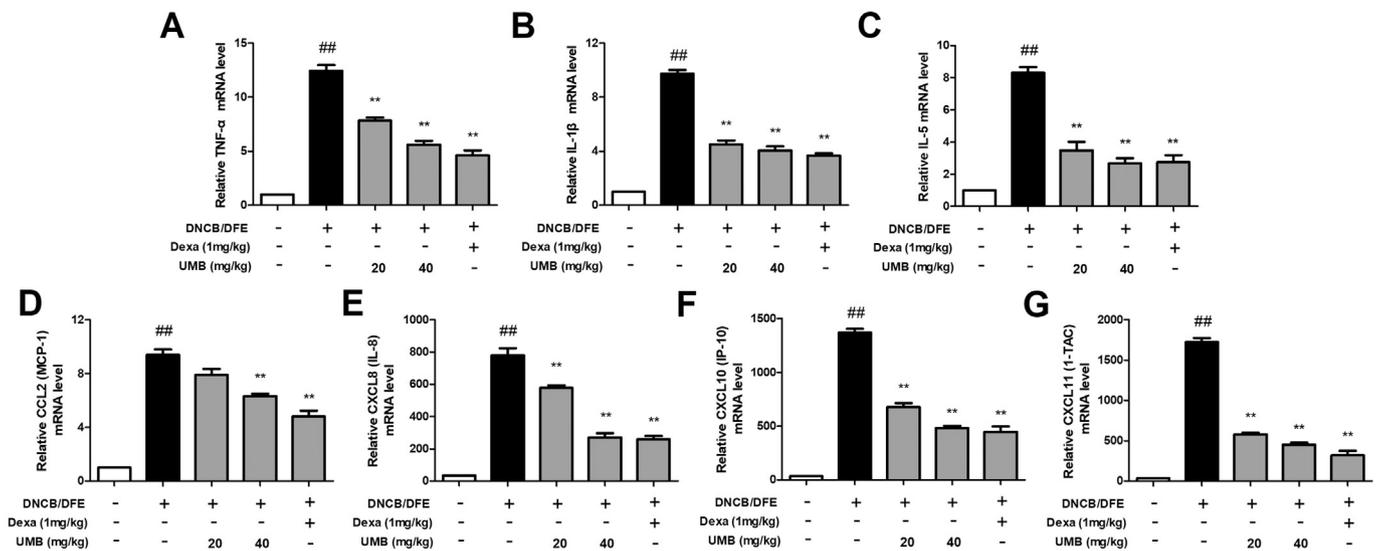
Fig. 5. Effects of UMB on the mRNA expression levels of inflammatory cytokines and chemokines in DNCB/DFE-treated mice ear tissues. Ear tissues of all mice were cut on day 28. The level of mRNA expression levels of (A) TNF-α, (B) IL-1β, (C) IL-5, (D) CCL2, (E) CXCL8, (F) CXCL10 and (G) CXCL11 was analyzed using real-time PCR. Data are shown as mean ± SD of the three independent experiments. $\#\#p < 0.01$ versus normal group, and $*p < 0.05$, and $**p < 0.01$ versus DNCB/DFE treated group.

However, UMB (20 and 40 mg/kg)-treated group exhibited decrease in mast cells infiltration in a dose-dependent manner.

3.4. UMB decreases the levels of pro-inflammatory cytokines and chemokines in DNCB/DFE-treated mice ear tissue

To investigate the inflammatory effect of UMB in mice ear tissues, we evaluated the mRNA expression levels of pro-inflammatory cytokines and chemokines using real-time PCR. As shown in Fig. 5, the expression levels of pro-inflammatory cytokines and chemokines were significantly increased in the DNCB/DFE-treated group. However, the oral administration of UMB significantly inhibited the expression levels of TNF-a, IL-1β, IL-5, CCL2, CXCL8, CXCL10, and CXCL11.

3.5. UMB decreases the levels of pro-inflammatory cytokines and chemokines in TNF-α/IFN-γ-treated keratinocytes

Keratinocytes play an important role in immune response related to allergies, AD, and other skin diseases [24]. Therefore, we explored the biological activities and underlying mechanisms of UMB using the TNF-α/IFN-γ-treated keratinocytes. The UMB cytotoxicity was validated using MTT assay after culturing the cells with various concentrations (0 to 100 μM) of UMB for 24 h. As shown in Fig. 6A, none of the concentrations of UMB showed any significant cytotoxicity. To evaluate the inflammatory effect of UMB on the cells, we evaluated the mRNA expression levels of pro-inflammatory cytokines and chemokines using real-time PCR in TNF-α/IFN-γ-treated HaCaT cells. The mRNA expression levels of various pro-inflammatory cytokines and chemokines were elevated in TNF-α/IFN-γ-treated HaCaT cells. However, pre-treatment of UMB significantly inhibited the mRNA expression levels of the pro-inflammatory cytokines (TNFα, IL-1β and IL-5) and chemokines (CCL2, CCL7, CCL8, CXCL8, CXCL10 and CXCL11) in TNF-α/IFN-γ-treated HaCaT cells (Fig. 6B–J).

3.6. UMB regulates the MAPK, IkB-α/NF-kB, and STAT1 signaling pathways in TNF-α/IFN-γ-treated keratinocytes

Since phosphorylation of MAPKs (p38, ERK, and JNK) is known to induce generation of pro-inflammatory cytokine, we investigated the effect of UMB on MAPK signaling pathways [25]. As shown in Fig. 7A and B, UMB (12.5 to 100 μM) significantly decreased the

phosphorylation of MAPKs (p38, ERK, and JNK) in TNF-α/IFN-γ-treated HaCaT cells in a dose-dependent manner. The NF-κB and STAT1 signaling pathways have been reported to be involved in the production of pro-inflammatory cytokines and chemokines in TNF-α/IFN-γ-treated HaCaT cells [26]. TNF-α/IFN-γ treatment in HaCaT cells increased IκB-α degradation and NF-kB nuclear translocation. However, pre-treatment with UMB significantly suppressed IκB-α degradation and nuclear translocation of NF-kB in a dose-dependent manner (Fig. 7C and D). In addition, we observed that UMB decreased STAT1 phosphorylation in a dose-dependent manner (Fig. 7E and F). These results suggested that UMB significantly regulated MAPK, NF-κB, and STAT1 signaling pathways, and thus, inhibited the production of pro-inflammatory cytokines and chemokines.

4. Discussion

UMB is a derivative of coumarin and has been known to exhibit various effects, such as anti-inflammatory, anti-oxidant, and anti-allergic. In this study, we investigated the effect of UMB on AD - like skin inflammation using in vivo and in vitro models.

Repeated application of DNCB and DFE (house dust mite extracts) to the ear of mice induces AD-like skin lesions [27]. We induced AD-like skin lesions on ear of mice, and orally administered them UMB and dexamethasone every day for 28 days. We observed a consistent increase in ear thickness in mice with AD-like skin lesions, which was restored to normal levels in the group orally administered with UMB (20 and 40 mg/kg). The mitigation of AD symptoms by UMB was visually observable, and the score of AD was significantly decreased in UMB-treated group compared to the DNCB/DFE-treated group.

We found that spleen size and weight were increased in mice with DNCB/DFE-induced AD-like skin lesions; however, the size and the weight of the spleen decreased with oral administration of UMB in a dose-dependent manner.

In AD patients, Th2 mediated responses are more prominent in acute phase and Th1 mediated responses are more prominent in chronic AD disease [28]. Th2 cells mainly secrete IL-4 and IL-5, and these cytokines stimulate B cells, which, in turn, secrete IgE. AD patients are characterized by IgE overproduction, which, in turn, affects IgG_1 and IgG_{2a} levels in the blood. In addition, mast cells, activated by various cytokines and IgE, produce inflammatory cytokines, such as TNF-α, IL-1β, and IL-4 [29].
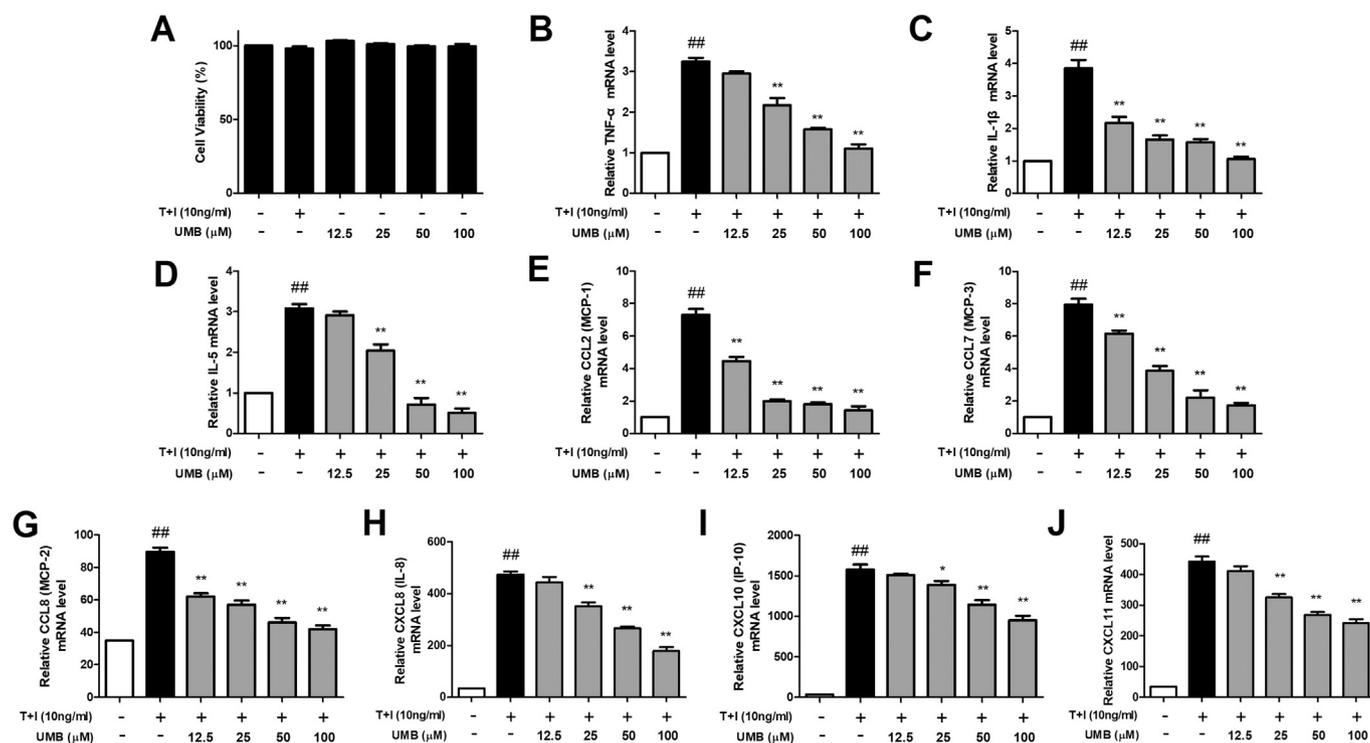
Fig. 6. Effects of UMB on the cell viability, and mRNA expression levels of inflammatory cytokines and chemokines in TNF-α/IFN-γ-treated HaCaT cells. (A) Cell viability was measured using the MTT assay. The mRNA expression levels of (B) TNF-α, (C) IL-1β, (D) IL-5, (E) CCL2, (F) CCL7, (G) CCL8, (H) CXCL8, (I) CXCL10, and (J) CXCL11 were determined using real-time PCR. Data are shown as mean ± SD of the three independent experiments. $\#\#p < 0.01$ versus normal group, and $*p < 0.05$, and $**p < 0.01$ versus DNCB/DFE treated group.

Chemokines are signal peptides involved in several inflammatory skin diseases, including AD [30]. They are classified into four main subfamilies: CXC, CC, CX3C, and XC, and are selectively expressed on the target cell surface. Previous studies have shown that CCL- chemokines exhibit a crucial effect on allergic inflammation by recruiting immune cells, such as eosinophils, basophils, mononuclear cells, and Th2 cells [26]. CCL2 and CCL7 recruit mono-nuclear cells in damaged tissue or inflammatory areas produced by infection [31–33]. In addition, CCL8 is involved in allergic reactions and activates several immune cells, including monocytes, T cells and NK cells [34,35]. During AD, the infiltration of Th2 cells into the inflammatory skin lesions is associated with high expression levels of chemokines [36], such as CCL2, CXCL8, CXCL10, and CXCL11 [37,38]. Among them, CXCL8, also known as IL8, is one of the most widely studied chemokines and an important inflammatory mediator that attracts neutrophils [39].

Through these mechanisms, inflammatory cytokines and chemokines promote more severe inflammatory responses, causes itching, and lead to intense damage in the skin which results in inflammatory lesions of AD.

To determine the effect of UMB on the immune response, we first measured the serum levels of IgE, IgG$_1$, IgG$_{2a}$, TNF-α, and IL-4 of mice. The DNCB/DFE-treated groups exhibited significantly increased serum levels of IgE, IgG$_1$, IgG$_{2a}$, TNF-a, and IL-4 compared to normal groups, and oral administration of UMB (20 and 40 mg/kg) reduced their levels in a dose-dependent manner.

Histologic analysis showed that macrophages, eosinophils and mast cells were found in the ear lesions of AD-like mice, as seen in human atopic dermatitis. In addition, epithelial and dermal hypertrophy and keratosis were induced, but oral administration of UMB significantly decreased them.

In mice with AD-like skin lesions, DNCB/DFE treatment significantly increased the levels of pro-inflammatory cytokines (TNF-a, IL-1β, and IL-5) and chemokines (CCL2, CXCL8, CXCL10, and CXCL11); however, oral administration of UMB (20 to 40 mg/kg) significantly

downregulated the expression of pro-inflammatory cytokines and chemokines.

Human keratinocytes, HaCaT cells, are one of the cell lines used to mimic AD-like inflammatory symptoms in vitro in response to inflammatory stimulations, such as TNF-α and IFN-γ. HaCaT cells release pro-inflammatory cytokines, chemokines, and proteases after TNF-α/IFN-γ stimulation and contribute to inflammation and immune responses.

We observed that the expression levels of pro-inflammatory cytokines (TNF-a, IL-1β, and IL-5) and chemokines (CCL2, CCL7, CCL8, CXCL8, CXCL10, and CXCL11) were significantly elevated in HaCaT cells stimulated with TNF-α/IFN-γ. However, the expression levels of pro-inflammatory cytokines and chemokines were significantly suppressed by pretreatment of UMB (12.5 to 100 μM).

In addition, we investigated relevant mechanisms of action of UMB in AD. We observed that UMB inhibited the phosphorylation of p38, ERK, and JNK in TNF-α/IFN-γ-treated HaCaT cells. Moreover, we found that UMB suppressed IkB degradation, nuclear translocation of NF-κB, and phosphorylation of STAT1 in a dose-dependent manner.

These results indicated that UMB inhibits the inflammatory response in HaCaT cells by suppressing the expression of pro-inflammatory cytokines and chemokines via regulation of MAPKs, IkB-α/NF-κB, and STAT1 signaling pathways.

In conclusion, our study shows that UMB exhibits significant anti-inflammatory effect on AD, and thus, has the potential to be used as a safe and effective remedy for AD.

Declaration of competing interest

The authors have no competing interests to declare.
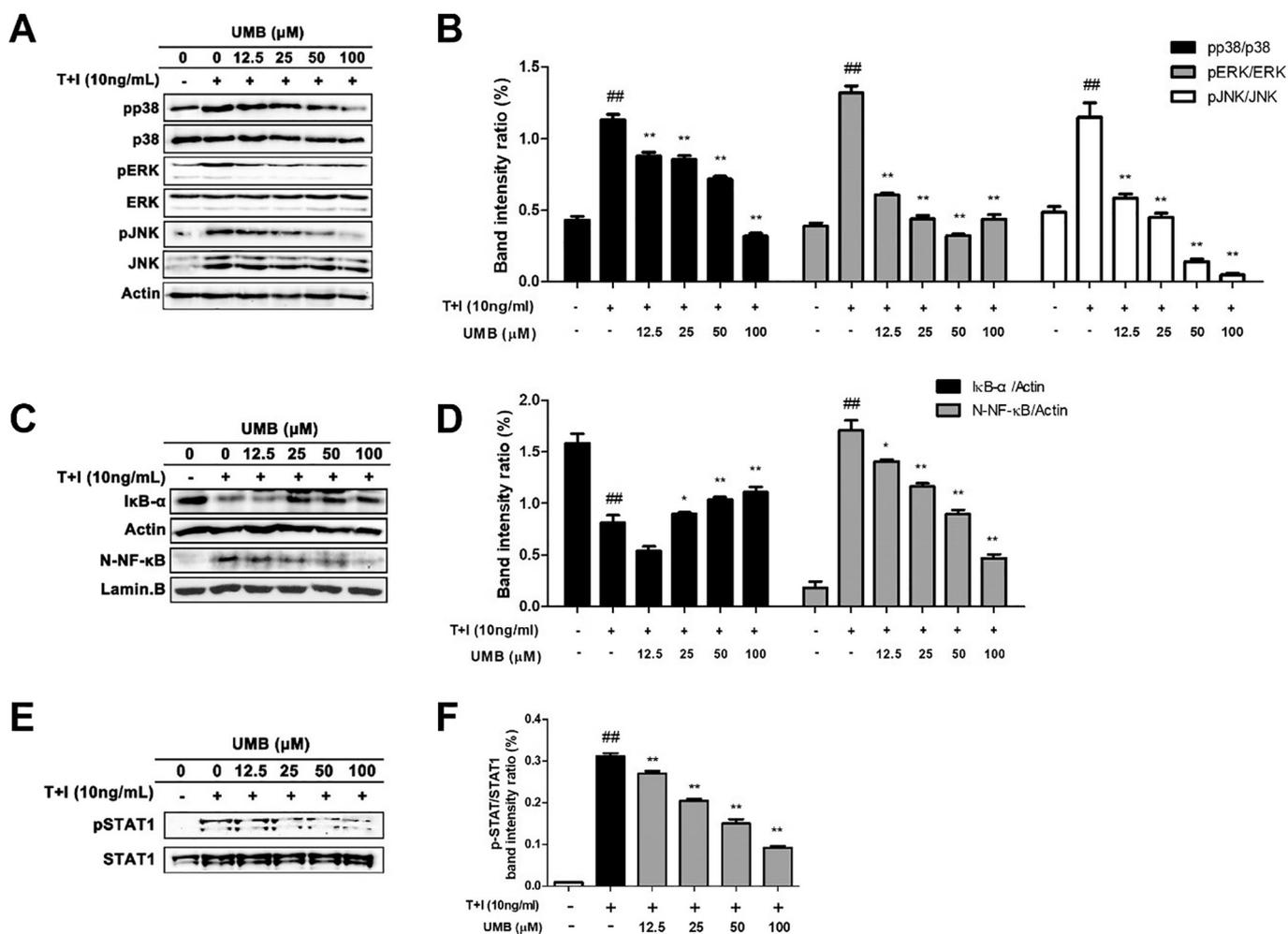
Acknowledgements

Fig. 7. Effects of UMB on the MAPK, IkB-α/NF-kB, and STAT1 signaling pathways in TNF-α/IFN-γ-treated HaCaT cells. Protein levels of (A) MAPK (p38, ERK, and JNK), (B) IκB-α and nuclear NF-kB, and (C) STAT1 were analyzed using western blot. Western blot bands were quantified using Gel QuantNET system and normalized to levels of Lamin B and β-actin. Data are shown as mean ± SD of the three independent experiments. $\#\#p < 0.01$ versus normal group, and $*p < 0.05$, and $**p < 0.01$ versus DNCB/DFE treated group.

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