

Original Article

Morus alba fruits attenuates atopic dermatitis symptoms and pathology *in vivo* and *in vitro* via the regulation of barrier function, immune response and pruritus

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ABSTRACT

Background: *Morus alba* fruits (MAF) belong to the *Moraceae* family, which are known to be effective in treating diabetic, autoimmune, and hormonal diseases owing to its low toxicity. MAF, as excerpted from Donguibogam, a representative Korean medical encyclopedia protected by UNESCO, has been widely used to treat lumbago, arthritis, and diabetes. Based on these effects, MAF is investigated for unidentified effects of atopic dermatitis, characterized by complex etiology of skin barrier dysfunction, inflammation, and chronic pruritus.

Methods: The antioxidant, inflammatory, and immunomodulatory properties of MAF and its bioactive compounds have been widely reported. According to an examination of 1-chloro-2,4-dinitrobenzene-induced AD-like skin lesions in NC/Nga mice, AD symptoms, such as increased dermatitis score, scratching frequency, immunoglobulin E, trans-epidermal water loss, epidermal thickness, and infiltration of mast cells, were relieved by topical MAF administration. They effectively attenuated cytokines and chemokines, such as interleukin (IL)-4, IL-5, IL-6, IL-8, IL-13, IL-17A, IL-22, IL-1 β , tumor necrosis factor- α , thymic stromal lymphopoietin (TSLP), thymic- and activation-regulated chemokine, normal T cell expression, and macrophage-derived chemokine secretion at the mRNA level in TNF- α /IFN- γ induced HaCaT (human immortalized keratinocyte) cells.

Results: Both *in vivo* and *in vitro* models, MAF increased the expression of filaggrin, involucrin, and loricrin, as well as inhibited the activation of Janus kinase 2, signal transducer and activator of transcription proteins 1, and mitogen-activated protein kinase pathways, including extracellular signal-regulated kinase, c-jun N-terminal kinase, and p38. Moreover, MAF reduced the expression of TSLP and periostin, which play important roles in skin pruritus as chronic pruritogenic factors.

Conclusion: These data indicate that MAF could be used as a potential treatment for AD-like skin lesions by regulating the inflammatory response, improving physical skin barriers, and relieving symptomatic pruritus.

Introduction

Atopic dermatitis (AD) is a prevalent inflammatory skin disorder

characterized by severe skin lesions, including redness, eczema, rash, and pruritus, and often presents with chronic relapse (Yang et al., 2020). This disease usually begins in early childhood, while adult patients

Abbreviations: AD, atopic dermatitis; DEX, dexamethasone; DNCB, 1-chloro-2,4-dinitrobenzene; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; H&E, hematoxylin and eosin; HAS 1, hyaluronic acid synthase 1; IFN, interferon; IgE, immunoglobulin E; IHC, immunohistochemical; IL, interleukin; JAK, Janus kinase; MAF, *Morus alba* fruits; MAPK, mitogen-activated protein kinase; MKK, mitogen-activated protein kinase kinase; STAT, signal transducer and activator of transcription; TEWL, transepidermal water loss; Th, T helper; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin.

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commonly experience chronic torment with allergic manifestations for decades (Margolis et al., 2014). Therefore, research on new treatments has been ongoing for long periods. AD has an intricate etiology, with three crucial factors in a vicious circle: various immunologic and inflammatory disorders, skin barrier dysfunction, and pruritus. In particular, as an innate immune mechanism, T cells manifest T helper (Th) 2 polarization in the early stages of AD and then increase the combination of Th1-, Th17-, and Th22-driven inflammation in the chronic stages (Li et al., 2020; Maintz and Novak, 2011). In addition to T cell-mediated immune responses, IgE-mediated hypersensitivity, with the expression of high-affinity IgE receptors (FcεRI), is also crucial for initiating immune responses as well as activating mast cells and dendritic cells (DCs) (Furie et al., 2017). These activated cells and their subsets secrete cytokines and chemokines at each stage, including interleukin (IL)-4, IL-5, IL-6, and IL-13 by Th2 cells and interferon (IFN)- γ , tumor necrosis factor (TNF), and IL-1 β by Th1 cells (Furie et al., 2017; Werfel et al., 2016). As an innate immune system, the epidermal barrier regulates transcutaneous water loss, dermal penetration of external allergens, and microbial infections. It consists of cross-linked protein layers with epidermal molecules, such as filaggrin, loricrin, and involucrin (Rerknimitr et al., 2017; Tsakok et al., 2019). These molecules, particularly filaggrin, influence cell differentiation, pro-inflammatory cytokine expression, and skin barrier function by controlling trans-epidermal water loss (TEWL) and preserving SC hydration. The unusual differentiation of these epidermal molecules has been observed in patients (Tsakok et al., 2019; Yang et al., 2020).

The major symptoms of chronic AD include skin lesions, pruritus, induced rubbing and scratching, and aggravated itch-scratch cycle (Frazier and Bhardwaj, 2020). Specific IgE-induced histamine release in mast cells and Th2-secreted cytokines are rapidly involved by stimulating activated inflammatory pruritogens, specifically thymic stromal lymphopoietin (TSLP), IL-31, IL-4, IL-13, and IL-17 (Brunner et al., 2018; McAleer et al., 2021; Noda et al., 2015). These pruritogens induce the accumulation of periostin in the dermis of skin lesions, thus acting as a new biomarker for AD (Yamaguchi, 2014). Furthermore, loss-of-function mutations in the filaggrin gene and degraded skin barrier integrity also exacerbate recurring allergic inflammation and intolerable pruritus (Palmer et al., 2006). Therefore, studies are underway to discover novel anti-atopic and antipruritic strategies for breaking this cycle.

Topical steroidal therapy is the first-choice therapy for cutaneous AD lesions. Currently, corticosteroids are approved for the treatment of AD; these agents alleviate inflammation, bacterial surface colonization, and pruritus. However, chronic steroid use can cause critical adverse effects, such as atrophoderma, vascular injury, telangiectasia, acne, and contact hypersensitivity responses (Chun and Lehman, 2020; Hajar et al., 2015). Their severity depends on the type and intensity of skin lesions as well as the patient's age, making it more difficult to use for AD treatment (Jeziorkowska et al., 2015). Recently, there has been increasing attention on finding alternative natural agents, including traditional medicines and medicinal herbs.

Morus species are medicinal herbs belonging to the *Moraceae* family, of which all parts of the plant, including the leaves, root bark, stems, and fruits remain viable for therapy. Although they grow from one body, their potencies, properties, and phytochemical compounds vary slightly in each part (Rodrigues et al., 2019). In previous experiments (Ariyo et al., 2020; de Padua Lucio et al., 2018; Lim et al., 2013; Umeyama et al., 2021), most parts of *Morus alba* L. were found to be particularly effective in relieving inflammation; among them, the Mori cortex was found to have anti-atopic properties *in vitro* and *in vivo* (Lim et al., 2014). However, the study of *Morus alba* fruits (MAF), known as white mulberry, has not been undertaken in AD. Furthermore, collagenase inhibition as well as anti-wrinkle effects were shown in a sample containing MAF (Ghimeray et al., 2015); when used for sole treatment, it was found to be effective in treating skin wounds (Woo et al., 2017). In the ancient Korean medical book, Donguibogam, it was described as having

outstanding effects on lumbago, arthritis, diabetes, lack of essence, senile constipation, and senescence (Heo). Many scientific studies have shown that MAF has antioxidant, neuroprotective, immunomodulatory, antitumor, antihyperglycemic, anti-cholesterol, anti-obesity, and hepatoprotective properties (Deng et al., 2020; He et al., 2018; Ping-Ping et al., 2020; Yu-Hao et al., 2021). Among the abundant bioactive compounds of MAF, rutin (Choi and Kim, 2013), chlorogenic acid (Tsang et al., 2016), linoleic acid (Tang et al., 2020), palmitic acid, and oleic acid (Earlia et al., 2019) were effective against atopic dermatitis (Chen et al., 2021). Thus, based on the above reasons, we evaluated the effects of MAF as a therapeutic candidate for AD management by using TNF- α /IFN- γ -stimulated HaCaT keratinocytes and 1-chloro-2,4-dinitrobenzene (DNCB)-treated NC/Nga murine model.

Material and methods

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Life Technologies, Inc. (Grand Island, NY, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), toluidine blue O, and dimethyl sulfoxide were purchased from Millipore (Billerica, MA, USA). Recombinant human tumor necrosis factor (TNF)- α and human IFN- γ were purchased from Bio-Techne Ltd. (Abingdon, OX, UK). Primary antibodies against phospho-JAK2 (cat. no. 3776), p-STAT1 (cat. no. 9167), p-ERK (cat. no. 9101), ERK cellular signal-regulated kinase (ERK; cat. no. 9102), p-JNK (cat. no. 9251), JNK (cat. no. 9252), p-p38 (cat. no. 9211), p38 (cat. no. 9212), p-MKK7 (cat. no. 4171), MKK7 (cat. no. 4172), and p-MKK3/6 (cat. no. 12280) were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). Primary antibodies against periostin (cat. no. sc-398631), JAK2 (cat. no. sc-390539), STAT1 (cat. no. sc-271661), filaggrin (cat. no. sc-66192), involucrin (cat. no. sc-21748), p-MEK1/2 (cat. no. sc-81503), MEK1/2 (cat. no. sc-81504), MKK3/6 (cat. no. sc-13069), β -actin (cat. no. sc-81178), and peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). The primary antibody against loricrin (cat. NBP1-33610) was purchased from Novus Biologicals LLC (Newcastle, UK). The primary antibody against TSLP (cat. ab188766) were purchased from Abcam (Cambridge, UK). Enzyme-linked immunosorbent assay (ELISA) kits for TNF- α were obtained from R&D Systems Inc. (Minneapolis, MN, USA). The IgE ELISA kit was obtained from Enzo Life Sciences Inc. (Farmingdale, NY, USA). Filaggrin, loricrin, involucrin, HAS1, TNF- α , TSLP, IL-4, IL-6, IL-13, IL-5, IL-22, IL-8, IL-17A, IL-1 β , MDC, RANTES, TARC, and GAPDH oligonucleotide primers were purchased from the Bioneer Corporation (Daejeon, South Korea).

Sample preparation

MAF was purchased from Nanum Herb (Gyeongbuk, Korea). Dried, ground MAFs (200 g) were extracted using 2l 30% EtOH for 4 h. The extracts were filtered; the filtrate was evaporated in a rotary vacuum evaporator at 40 °C; and the residue was freeze-dried in a vacuum using a freeze dryer. Finally, the yield of the dried extract from the starting crude material was approximately 14% and was stored at -20 °C for bioassays. To prepare the sample for the *in vitro* experiment, the resultant residue from the drying process was dissolved in distilled water.

Analysis using high performance liquid chromatography (HPLC)

MAF extract was analyzed by HPLC to identify the component profile. The extract was precisely quantified to 10 mg, dissolved in 1 ml of MeOH and filtered to prepare an extract sample. The HPLC and Triart C18 ExRS column (250 $\times$ 4.6 mm I.D., S-5 μ m, 12nm, YMC, Kyoto, Japan), consisting of pump (1525 Binary HPLC pump, Waters, MA, USA)

and PDA detector (996 Photodiode array detector, waters, MA, USA), were connected and analyzed. As mobile solvent systems, A channel: 0.1% formic acid distilling water and B channel: ACN were used. The slope profile proceeded as follows: 0–40 min, 10–100% B linear. The flow rate was maintained at 1.0 ml/min during the analysis. 20 µl of MAF extract solution at a concentration of 10 mg/ml was injected, and rutin (Cayman chemical, Ann Arbor, MI, USA) of various concentrations (0.005–0.08 mg/ml) was injected in the same volume. The detection wavelength was adjusted to 254 nm. A linear calibration curve was calculated by plotting the log of the injected concentration versus the log of the peak area for each assay. Rutin content of MAF extract was assessed using the HPLC-DAD method. Rutin peak area values obtained by repeatedly injecting MAF extract at a concentration of 10 mg/ml three times were obtained by substituting a linear regression model. Mean value of three determinations $\pm$ S.D.

AD animal model

Male NC/Nga mice (20–26 g body weight, 6–7 weeks old) were obtained from the Dae Han Bio Link Co. (Daejeon, Korea). The animals were kept under the following standard conditions: temperature of 22–25 °C, humidity of 40%–60%, a 12/12 h light/dark cycle, and with free access to water and food. After 1 week of acclimatization and hair loss, the mice were randomly assigned to one of five groups ($n = 7$ per group): (1) baseline-applied normal group (normal control, NC), (2) DNCB-sensitized group (DNCB), (3) DNCB-sensitized and dexamethasone (DEX)-challenged group (positive control), and (4–5) DNCB-sensitized and topically applied with 50 or 100 mg/kg of MAF group. The concentrations of MAF were determined based on previous experiments (Jung et al., 2021; Ullah et al., 2016). To induce AD-like skin lesions, 1% DNCB was dissolved in an acetone and olive oil mixture (3:1 v/v), and then topically applied to the shaved dorsal skin (150 µl) twice in the first week. Next, 0.35% DNCB dissolved in an acetone and olive oil mixture (3:1 v/v) was applied to the dorsal skin (150 µl) on the same area thrice per week for 4 weeks. After the first week of challenge for AD-like symptom induction, the mice were topically treated with DEX (5 mg/kg admixed in Vaseline) or MAF extract (50 or 100 mg/kg admixed in Vaseline) 4 h after DNCB treatment, with a dose interval of once per day. An equal volume of Vaseline (vehicle) was used in the control group. The mice were sacrificed at the end of the experiment; blood and skin samples were subsequently collected. The experimental scheme is shown in Fig. 2A. The experiment date was from 2021.2.22 to 2021.3.26, a total of 5 weeks period. All animal procedures were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of Sangji University (IACUC animal approval protocol no. 2021-5).

Evaluation of dermatitis severity and scratching behavior

The severity of clinical dermatitis was evaluated once per week according to the SCORAD index. The severity of edema, scarring/dryness, erythema/hemorrhage, and excoriation/erosion was scored as follows: 0, none; 1, mild (<20%); 2, moderate (20%–60%); and 3, severe (>60%). The individual scores were summed to obtain the dermatitis scores. At the end of the animal experiment, the duration of body scratching with their hind paws was measured by recording a video of the mice for 10 mins for symptom quantification. The dermatitis score and scratching behavior were then measured by two independent observers and determined as the average of the two measurements.

Measurement of TEWL

On the last day of the experiment, TEWL was assessed on the dorsal skin of NC/Nga mice using a GPskin Barrier Light (Gpskin, Seoul, Republic of Korea). To measure the TEWL value ($\text{g/m}^2/\text{h}$), the probe was

placed at the center of the shaved dorsum area at a temperature of 24 °C and humidity of 50%–55%. In proceeding with the sample administration on the previous day, an interval of 24 hs between topical application and TEWL measurement was kept (at least 12 hs required). All mice had an acclimatization time for 30–40 mins before TEWL measurement. Statistical values were expressed in terms of fold change as compared to the control group.

Enzyme-linked immunosorbent assay (ELISA) for cytokine analysis

Mouse serum and cell culture supernatants were prepared. The serum was obtained from the blood by centrifugation at $1,700 \times g$ for 30 mins and stored at -70 °C until analysis. Serum levels of IgE were measured using a mouse IgE ELISA kit (BD OptEIA™, BD Science, CA, USA) according to the manufacturer's protocol. Culture media were obtained approximately 24 h after treatment with MAF and stored at -70 °C. The expression levels of TNF- α and TSLP were assessed using ELISA kits (BD OptEIA™, BD Science, CA, USA) according to the manufacturer's protocol.

Histological analysis

After euthanasia and dorsal area isolation, the samples were fixed in 10% buffered formalin and embedded in paraffin. They were then sectioned into 4-µm slices and stained with hematoxylin-eosin (H&E) and toluidine blue to detect epidermal thickness and inflammatory cell infiltration, respectively. For immunohistochemical (IHC) staining, the slides were deparaffinized with xylene, rehydrated in ethanol, and hydrated with water. Endogenous peroxidase activity was blocked using 0.6% H_2O_2 in 50% MeOH. Then, the slides were treated with 0.3% Triton in phosphate-buffered solution for permeabilization and pre-blocking with 10% normal goat serum for 1 h, followed by overnight incubation with a specific antibody at 4 °C. The sections were washed and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The activity was visualized with 3, 3'-diaminobenzidine chromogen and counterstained with H&E. Pathological changes in all the stained skin sections were observed using a DM IL LED microscope (Leica Microsystems, Wetzlar, Germany) and photographed using a DFC295 camera (Leica Microsystems). Digital images were captured from each slide and analyzed using Leica Application Suite (Leica Microsystems). For immunofluorescence (IF) staining, the slides were deparaffinized, antigen-retrieved, permeabilized, and blocked by non-specific binding with 5% normal goat serum (NGS). After blocking, the sections were incubated with primary antibodies overnight at 4 °C, while the secondary antibodies were incubated the next day. Antigens were then detected using a mouse periostin antibody (cat. no. sc-398631). Alexa Fluor 594 (1:100 dilution; Molecular Probes) was used as a secondary antibody. Slides were counterstained with 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI; Life Technologies, CA, USA). The conjugated proteins were detected using an optical microscope (ECLIPSE Ni-U, Nikon, Tokyo, Japan). For analysis of periostin, the dermal area of the skin and the positive fluorescence area were selected using the ImageJ program. The percentage of positive area per total epidermal area was calculated as follows: % of positive staining intensity = (expression area/total dermal area) $\times$ 100.

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis

Total RNA was isolated from HaCaT cells using an Easy Blue kit (Intron Biotechnology, Inc., Seoul, Korea) according to the manufacturer's protocol. Next, it was quantified using an Epoch microvolume spectrophotometer system (BioTek Instruments, Inc., Winooski, VT, USA). Then, cDNA was obtained using isolated total RNA (2 µg), d(T)16 primers, and avian myeloblastosis virus reverse transcriptase. Relative gene expression was measured by RT-qPCR (7500 Real-Time PCR

Table 1
List of primer sequences for humans.

Gene	Sequence (Forward)	Sequence (Reverse)
Filaggrin	AGTGTCTAAACCGGATTCA	GGCACTGAAAGGCAAAAGG
Involucrin	GCTCTGGGTTTCTGCTTTC	GCAAGAATGTGAGCAACAGC
Loricrin	TCAATGGCTTCTCCAGGTA	AGGAGTTGGAGGTGTTTTC
TSLP	AGGGCTGGTGTTAACTTACG	AGACGGTGTGTTGAACCTCG
TARC	AAGCTGAAGACGTGGTACCA	TCTTGTGTTGGGGTCCGAA
RANTES	TCGCTGTCATCCTCATTGCT	AGCACTTGCCACTGGTGTAG
MDC	GTGAAGCCCCAAATTTGCC	GCCAAGACCACACCAATTGC
IL-1 β	CCACAGACCTTCCAGGAGAATG	GTGCAGTTCAGTGATCGTACAGG
IL-4	CCGTAACAGACATCTTGTGTCG	GAGTGTCTTCTCATGTGTGGCT
IL-5	GGAATAGGCACACTGGAGAGTC	CTCTCCGTCTTCTTCTCCACAC
IL-6	CTGACCCAACCACAAATGCC	TGCTACATTTGCCGAAGAGC
IL-8	GAGAGTGATTGAGAGTGGACAC	CACAACCCTCTGCACCCAGTTT
IL-13	ACGGTCATTGCTCTCACITGCC	CTGTCAGGTTGATGCTCCATACC
IL-17A	CGGACTGTGATGGTCAACCTGA	GCACITTTGCCTCCAGATCACA
IL-22	AGCCTATATCACCAACCGC	TCTCCCAATGAGACGAACG
TNF- α	CTCTTCTGCCTGCTGCACITTTG	ATGGGCTACAGGCTTGTCACTC
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCTGTGTGCTGATGCCAA

System, Applied Biosystems, Foster City, CA, USA) with SYBR premix Ex Taq. The synthesized cDNA was 200 base pairs in size. Expression data were calculated based on cycle threshold (Ct) values using the $\Delta\Delta Ct$ method of quantification. The results are expressed as the optical density ratio relative to GAPDH. The primer sequences are listed in Table 1.

Cell culture and sample treatment

HaCaT keratinocytes were provided by Prof. Kyung-Tae Lee (Kyung Hee University). The cells were cultured in DMEM supplemented with 10 % FBS, penicillin (100 U/ml), and streptomycin (100 μ g/ml), and stored in an incubator at 37 °C with 5% CO₂. Then, HaCaT keratinocytes were seeded at a density of 1×10^5 cell/well and then incubated for 24 h at 37 °C in humidified air with 5% CO₂. Next, the cells were treated with MAF at 125, 250, and 500 μ g/ml for 1 h prior to stimulation with 10 ng/ml TNF- α /IFN- γ for the indicated time.

Cell viability assay

Cell viability was determined using a colorimetric MTT assay. HaCaT keratinocytes were plated at a density of 5×10^4 cells/well in 96-well plates and allowed to attach for 24 h. Then, the cells were treated in a medium containing various concentrations of MAF and incubated for 24 h. The next day, the cells were treated with 50 μ l of MTT (5 mg/ml) for 4 h. The formazan precipitate was dissolved in DMSO; the absorbance was measured at 540 nm using an Epoch microplate spectrometer (BioTek, Winooski, VT, USA).

Western blot analysis

Protein extracts from the dorsal skin and HaCaT cell lysates were prepared using the PRO-PREP™ protein extraction solution (Intron Biotechnology, Seoul, Korea) and homogenized at 4 °C. Cell debris was removed via microcentrifugation at $11,000 \times g$ for 30 mins at 4 °C, followed by rapid freezing of the supernatant. Protein concentration was measured using Bio-Rad protein assay reagent (Bio-Rad Laboratories,

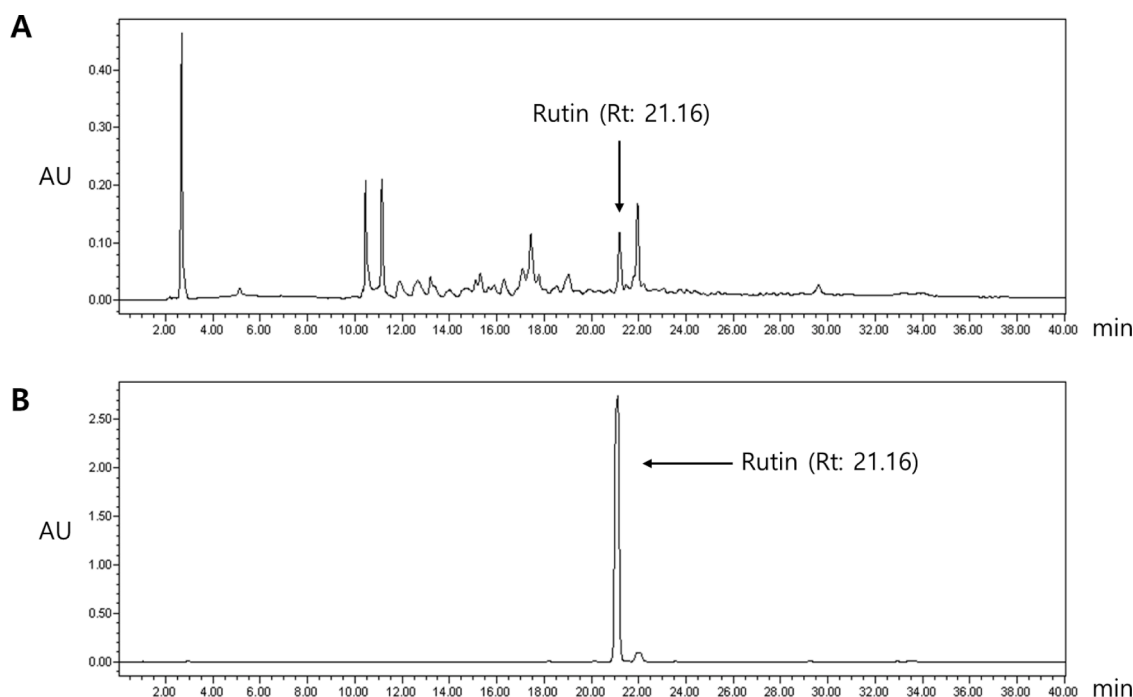


Fig. 1. HPLC chromatogram of MAF extract (A) and rutin (B) as a standard compound.

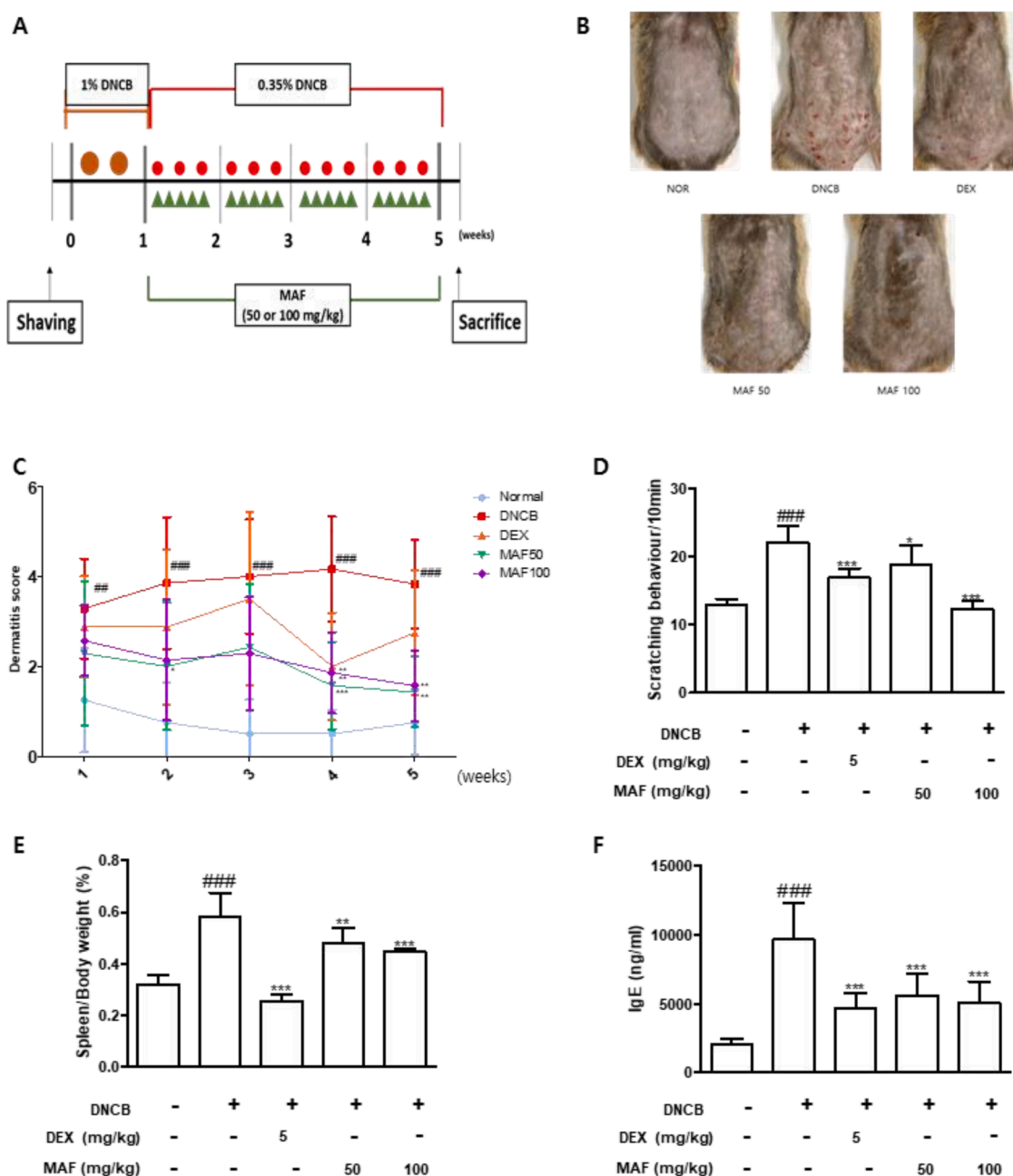


Fig. 2. Animal experimental scheme and effects of MAF on the clinical features of DNCB-induced AD skin in NC/Nga mice. (A) Schematic diagram of the animal experiment schedule. (B) Photographic images of dorsal skin lesions were taken on the last day of the experiment. (C) Dermatitis score was evaluated once a week for 5 weeks and defined as the sum of scores for each symptom. (D) Scratching frequency and (E) the weight of spleen were measured at the end of experiment. (F) Serum IgE levels were measured by ELISA. Data shown represent mean $\pm$ standard deviation (SD) of triplicate independent experiments. ## $p < 0.01$, ### $p < 0.001$ vs. the control group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. DNCB-induced AD group.

Inc. CA, USA) according to the manufacturer's protocol. Cellular proteins from the treated and untreated cell extracts were electrophoreted onto a polyvinylidene fluoride membrane following separation by 8–12% SDS-PAGE. The membrane was incubated for 30 mins with a blocking solution (2.5 or 5% skim milk) at room temperature, followed by overnight incubation with the primary antibodies (1:1,000) at 4 °C. After three washings with T/TBS, the blots were incubated with horseradish peroxidase-conjugated secondary antibody (1:2000) for 2 h at room temperature. The blots were further washed for three times with T/TBS and then visualized using an enhanced chemiluminescence system (GE Healthcare, WI, USA). Densitometric analysis was performed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The intensity of the individual bands on the western blot analysis

was measured using ImageJ gel analyzer tools and was subsequently normalized to β -actin as well as to its total protein. The graphs represent normalized values as fold changes over the control condition.

Statistical analysis

Data are expressed as the mean $\pm$ standard deviation of triplicate experiments. Comparisons among groups were performed using one-way analysis of variance (ANOVA), followed by Dunnett's post-hoc test using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). A p -value of <0.05 was considered as a statistically significant difference.

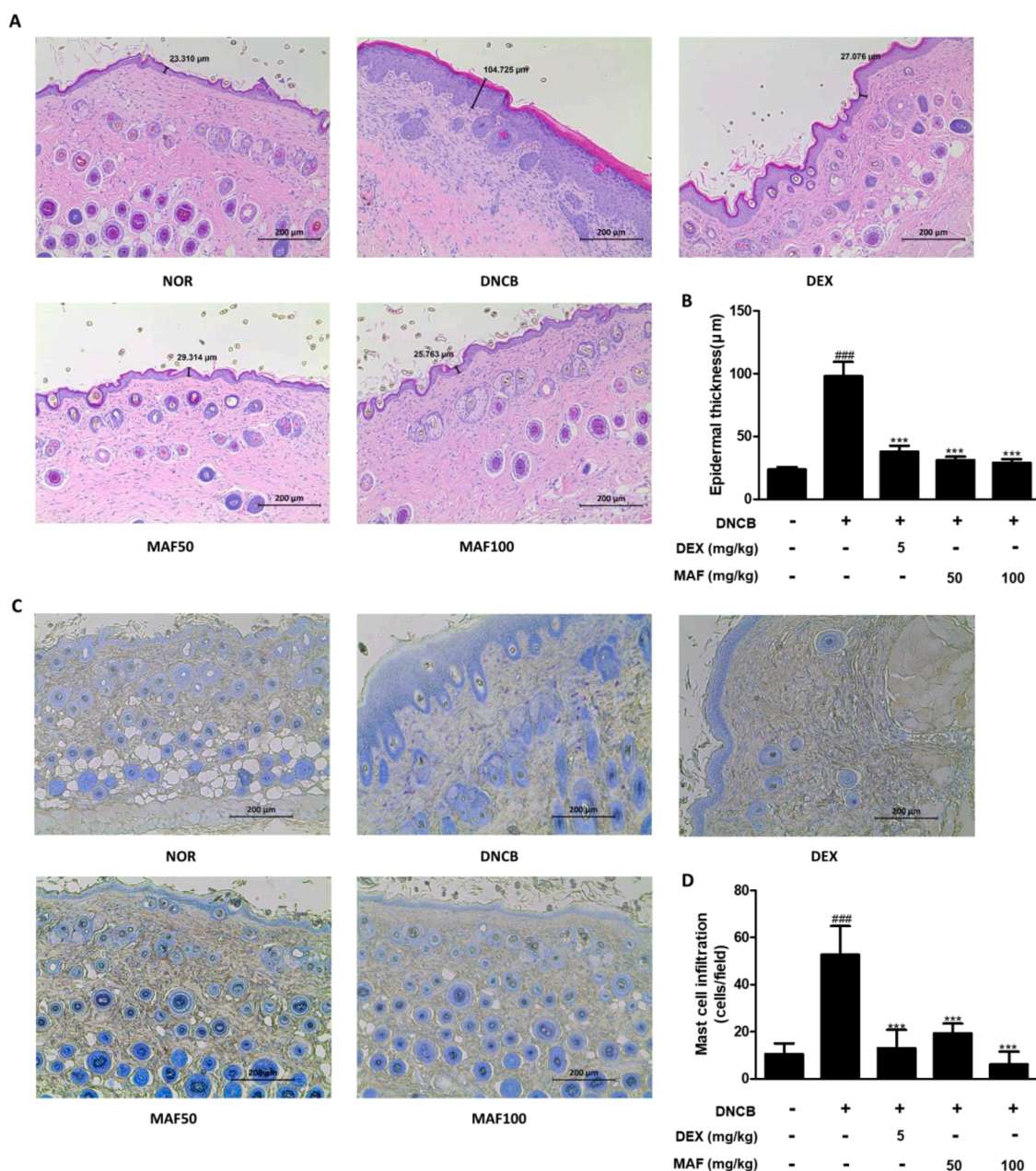


Fig. 3. Effects of MAF on the histological features and infiltration of mast cells in DNCB-induced AD skin in NC/Nga mice. (A) The slides of sectioned tissues were stained with hematoxylin and eosin (scale bar = 200 μm). (B) Epidermal thickness in H&E stained sections was measured under a microscope. (C) The slides of sectioned tissues were stained with toluidine blue to identify mast cells. (scale bar = 200 μm). (D) Mast cells per field of a dorsal skin lesion were counted with a microscope. Data shown represent mean $\pm$ standard deviation (SD) of triplicate independent experiments. ### p < 0.001 vs. the control group; *** p < 0.001 vs. DNCB-induced AD group.

Results

HPLC analysis of MAF extracts

We have confirmed the HPLC chromatogram of MAF and the standard compound rutin. Data from the HPLC analysis of MAF extract and rutin were obtained in the form of chromatograms by monitoring detector responses at 254 nm. As shown in Fig. 1, it was confirmed that the peak of rutin appeared at 21.16 min (Fig. 1B), and the MAF extract also showed a peak at the same retention time, 21.16 min. In addition, we also checked the content evaluation of rutin in the MAF extract. The content values of rutin are showed as 1.1805 ± 0.0215 mg/g (0.12 ± 0.00 %) in the sample of MAF extract. Therefore, this result suggested that MAF extract contained rutin as a one of the major component.

MAF has a protective effect against DNCB-induced AD mice

To assess the therapeutic potential of MAF in AD-like cutaneous conditions, we induced inflammation in the dorsal skin of NC/Nga mice by applying DNCB with or without MAF or DEX treatment. As representative images of the dorsal skin in the five groups (Fig. 2B), repeated DNCB application exacerbated prominent AD-like skin lesions, including edema, scarring, dryness, erythema, excoriation, and erosion; MAF treatment restored these symptoms. MAF attenuated dermatitis severity in the MAF- and DEX-treated groups as compared to that in the DNCB group (Fig. 2C). The indicator of antipruritic effect, that is, the frequency of scratching, increased in the DNCB-treated group as compared to the normal group; MAF administration decreased the scratching frequency (Fig. 2D). To determine whether MAF could influence immune

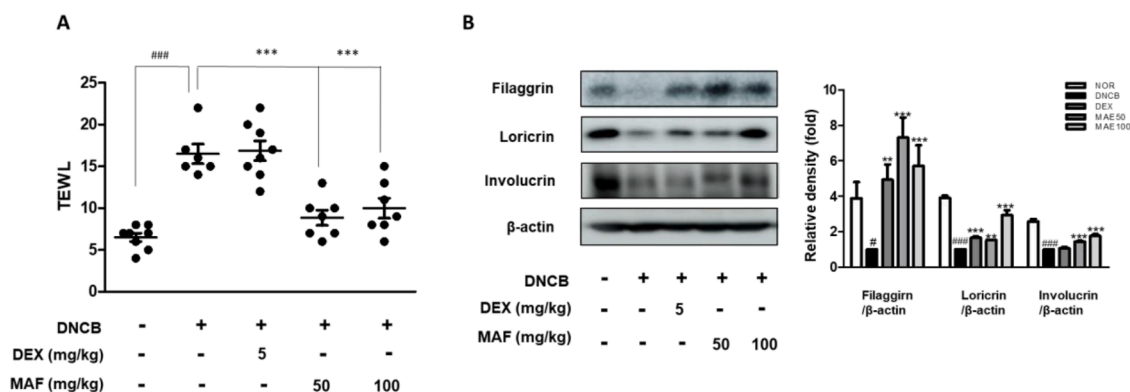


Fig. 4. Effects of MAF on the skin barrier dysfunction in DNCB-induced AD skin in NC/Nga mice (A) The level of TEWL was measured at the end of the experiment. (B) Total proteins were prepared and western blotted for filaggrin (34 kDa), loricrin (26 kDa), and involucrin (68 kDa). The protein levels were quantified by band density in proportion to β -actin as an internal control. Data shown represent mean $\pm$ standard deviation (SD) of triplicate independent experiments. # $p < 0.05$, ### $p < 0.001$ vs. the control group; ** $p < 0.01$, and *** $p < 0.001$ vs. DNCB-induced AD group.

responses, spleen weight and serum IgE levels were measured. The DNCB-treated group exhibited splenomegaly, which is one of the features of systemic immune disorders. However, splenomegaly in the MAF-treated group gradually decreased in a dose-dependent manner (Fig. 2E). The DNCB group also showed significantly increased serum

IgE levels, which is the principal feature of AD, whereas the MAF groups showed decreased IgE levels (Fig. 2F). Taken together, these results suggest that MAF treatment alleviated the clinical symptoms of AD *in vivo*.

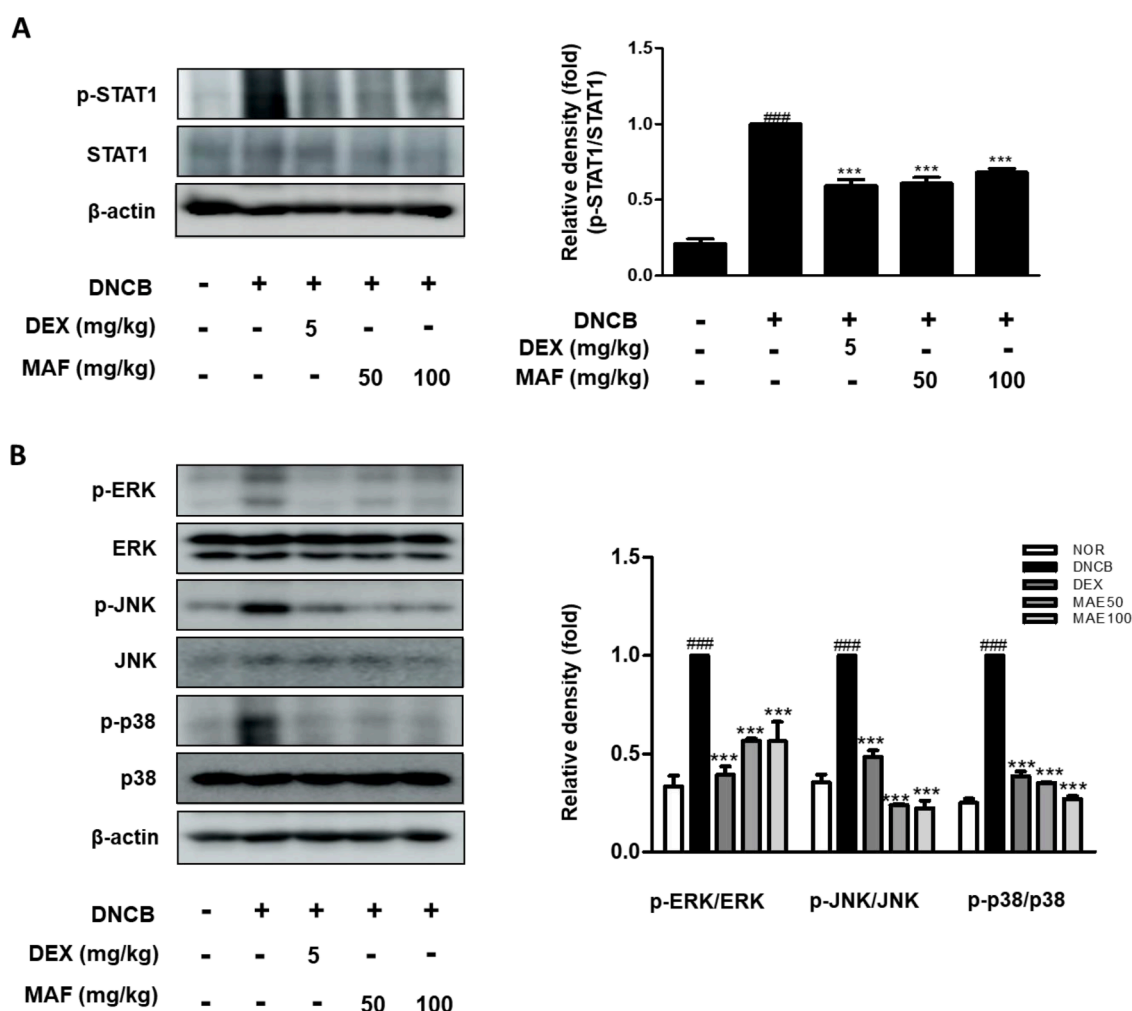


Fig. 5. Effects of MAF on STAT1 and MAPKs pathway activation in DNCB-induced AD skin in NC/Nga mice. Total proteins were prepared from mouse cutaneous tissue and immunoblotted for (A) p-STAT1, STAT1, (B) p-ERK, ERK, p-JNK, JNK, p-p38, and p38 using each specific antibody. The protein levels were quantified by band density in proportion to the expression of total form. Data shown represent mean $\pm$ standard deviation (SD) of triplicate independent experiments. ### $p < 0.001$ vs. the control group; *** $p < 0.001$ vs. DNCB-induced AD group.

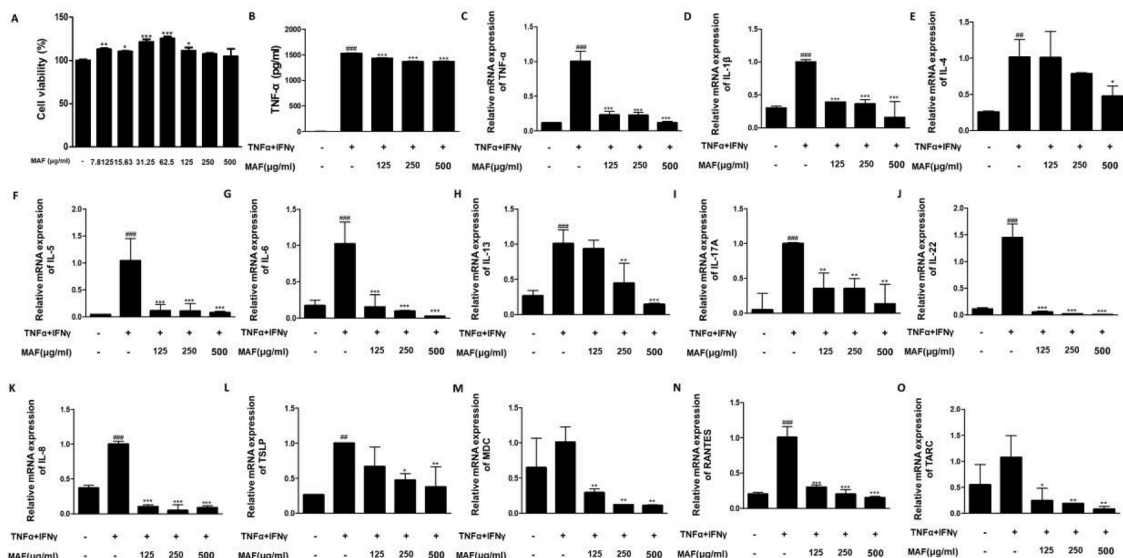


Fig. 6. Effects of MAF on cell viability in HaCaT keratinocytes & AD-related cytokines in TNF- α /IFN- γ -stimulated HaCaT keratinocytes. (A) HaCaT keratinocytes were incubated with various concentrations of MAF for 24 h, and cell viability was measured by MTT assay. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. MAF non-treated group. HaCaT keratinocytes were pre-treated with MAF for 1 h and then stimulated with TNF- α /IFN- γ (10 ng/ml) for 24 h. (B) The level of TNF- α in cell culture supernatants was measured using an ELISA kit. After the pre-treatment of MAF for 1 h, HaCaT keratinocytes were stimulated by TNF- α /IFN- γ (10 ng/ml) and the cells were further incubated for 6 h. The mRNA level of (C) TNF- α , (D) IL-1 β , (E) IL-4, (F) IL-5, (G) IL-6, (H) IL-13, (I) IL-17A, (J) IL-22, (K) IL-8, (L) TSLP, (M) MDC, (N) RANTES, and (O) TARC were determined by qRT-PCR. The results were expressed as the optical density ratio relative to GAPDH. All data shown represent mean $\pm$ standard deviation (SD) of triplicate independent experiments. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ vs. the control group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. TNF- α /IFN- γ -treated group.

MAF reduces the thickness of epidermis and infiltration of mast cells in DNCB-induced AD mice

AD-like cutaneous lesions are characterized by epidermal hyperplasia and hyperkeratosis. In our study, H&E staining revealed the presence of hyperkeratosis. Furthermore, the DNCB-treated group displayed significant hyperkeratosis than the control group, whereas MAF-treated groups showed an apparent decline. In the high-concentration group, the degree was similar to that in the normal group (Fig. 3A and B). Thus, to investigate inflammatory cell infiltration into the skin, we counted the number of mast cells in the dorsal skin tissues by staining with toluidine blue. The DNCB-treated group showed an accumulation of mast cells, whereas the MAF-treated group showed significantly reduced mast cell infiltration. In particular, the high-concentration group (MAF 100 mg/kg) showed a significantly greater effect than the DEX-treated group (Fig. 3C and D).

MAF restores skin barrier function in DNCB-induced AD mice

To investigate the recovery effect of MAF on skin barrier function, we measured TEWL, an indicator of skin barrier function and hydration, as well as skin barrier proteins in the dorsal skin. On the last day of the experiment, the TEWL was estimated before euthanasia. The DNCB-treated group showed an increase in the TEWL score, whereas the MAF-treated groups showed an effective decrease (Fig. 4A). The expression levels of skin barrier proteins, including filaggrin, loricrin, and involucrin, were remarkably decreased in the DNCB-treated group as compared to those in the normal group. On the other hand, these protein levels were increased in the MAF-treated groups as compared to the DNCB group (Fig. 4B).

MAF restrains the expression of STAT1 and MAPK pathways in DNCB-induced AD mice

Allergic inflammation is mediated by activated signal transducers and activators of transcription (STAT) and mitogen-activated protein

kinase (MAPK) signaling pathways. To further investigate the mechanisms underlying the regulation of STATs and MAPKs, we examined the protein levels of STAT1, ERK, JNK, and p38 using western blot analysis. As shown in Fig. 5, DNCB induced the phosphorylation of STAT1 in the skin of DNCB-induced AD mice; in contrast, this was attenuated by MAF (Fig. 5A). Likewise, DNCB induced the phosphorylation of ERK, JNK, and p-38, whereas these were attenuated by MAF (Fig. 5B).

MAF has no cytotoxicity effect and modulated the production of inflammatory cytokines and chemokines in TNF- α /IFN- γ -stimulated HaCaT keratinocytes

To determine the cytotoxicity of MAF treatment in HaCaT cells, the cells were treated with several concentrations of MAF extract (7.81 to 500 μ g/ml) for 24 h. After the MTT assay, significant changes were not observed in the cell viability of HaCaT cells; therefore, the succeeding experiments were conducted using the highest to the third concentrations (500, 250, and 125 μ g/ml) (Fig. 6A). To further investigate the effects of MAF on Th cell polarization and inflammation types regarding AD skin phenotypes, inflammatory cytokines and chemokine-related genes were analyzed using RT-qPCR in TNF- α /IFN- γ -stimulated HaCaT keratinocytes. TNF- α cytokine levels were measured in the culture supernatant by ELISA; these were significantly increased in TNF- α /IFN- γ -stimulated HaCaT cells. On the contrary, treatment with MAF inhibited TNF- α production (Fig. 6B). The mRNA expression of Th1-mediated cytokines TNF- α and IL-1 β ; Th2-mediated cytokines IL-6, IL-4, IL-13, and IL-5; Th17-mediated cytokine IL-17A; Th22-mediated cytokine IL-22; innate immunity-related cytokines TSLP and IL-8 (CXCL8); and T cell-attracting chemokines MDC (CCL22), RANTES (CCL5), and TARC (CCL17) were also measured. As a result, most cytokines and chemokines were significantly increased by stimulation with TNF- α /IFN in HaCaT cells as compared to the control group, except for MDC and TARC. On the other hand, most of their expression was significantly inhibited by MAF in a dose-dependent manner, except for IL-4 (Fig. 6C-O). Overall, MAF mitigated the inflammatory responses by regulating inflammatory cytokines in TNF- α /IFN- γ -stimulated HaCaT cells.

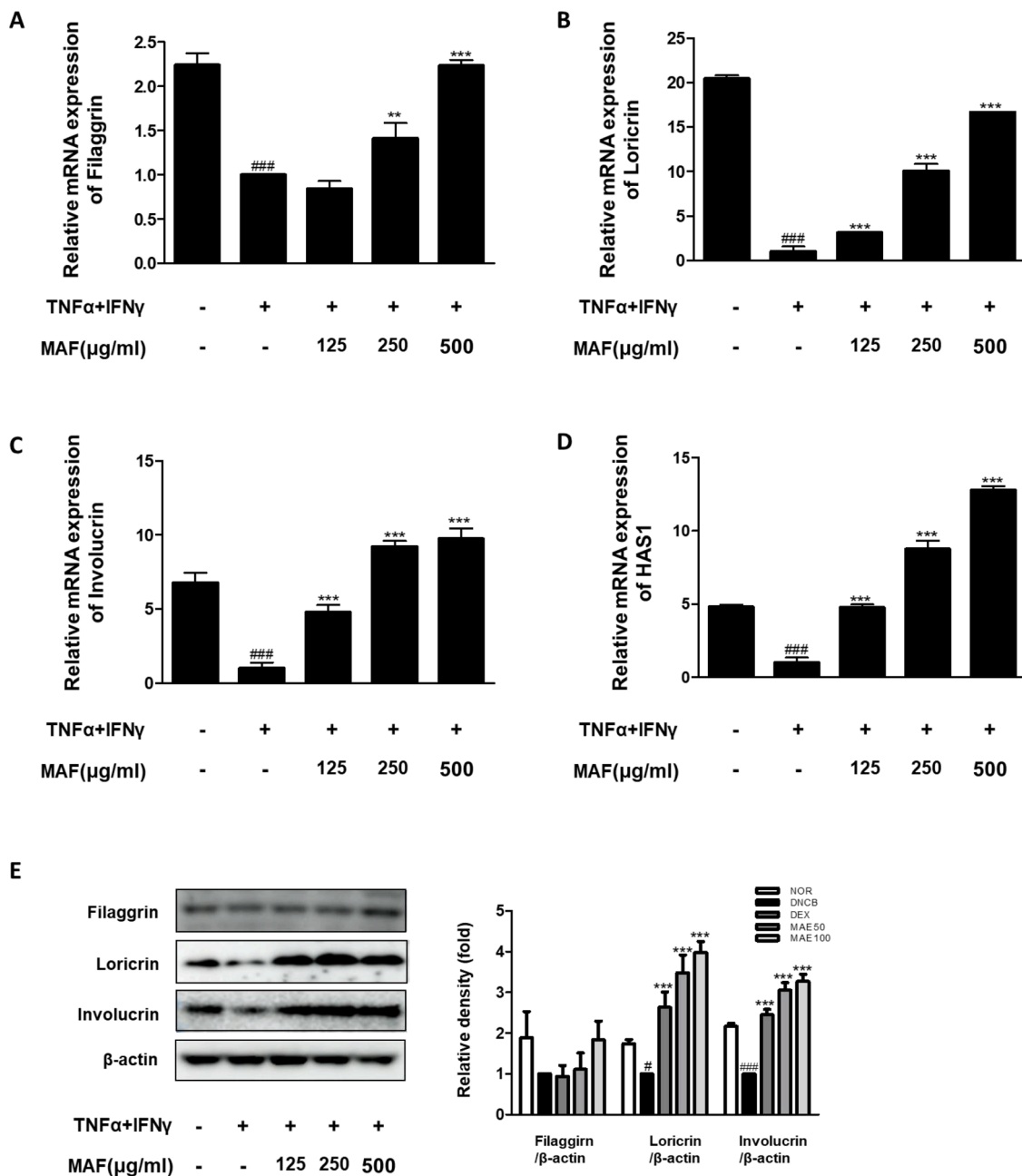


Fig. 7. Effects of MAF on the skin barrier dysfunction in TNF- α /IFN- γ -stimulated HaCaT keratinocytes. Total RNA prepared from HaCaT keratinocytes, and gene expression of (A) filaggrin, (B) loricrin, (C) involucrin, and (D) hyaluronic acid synthase 1 (HAS1) were determined by RT-qPCR. The results were expressed as the optical density ratio relative to GAPDH. (E) Total proteins were prepared and western blotted for filaggrin (34 kDa), loricrin (26 kDa), and involucrin (68 kDa). The protein levels were quantified by band density in proportion to β -actin as an internal control. Data shown represent mean $\pm$ standard deviation (SD) of triplicate independent experiments. # $p < 0.05$, ### $p < 0.001$ vs. the control group; ** $p < 0.01$, and *** $p < 0.001$ vs. TNF- α /IFN- γ -treated group.

MAF induces the production of the skin barrier function related factors in TNF- α /IFN- γ -stimulated HaCaT keratinocytes

In vitro experiments of skin barrier protein gene expression, including filaggrin, loricrin, involucrin, and hyaluronic acid synthase (HAS) 1, were measured by RT-qPCR. The expression of filaggrin, loricrin, involucrin, and HAS 1 was remarkably decreased in the TNF- α /IFN- γ -stimulated group as compared to that in the normal group. Overall, the MAF-treated groups showed significantly increased production of filaggrin, loricrin, involucrin, and HAS 1 in a dose-dependent manner (Fig. 7A-D). Furthermore, immunoblotting showed that the expression levels of filaggrin, loricrin, and involucrin were decreased in the TNF- α /IFN- γ -stimulated group as compared to those in the normal

group. However, these proteins were increased in the MAF-treated groups. In particular, the expression of loricrin and involucrin, was highly significant (Fig. 7E).

MAF inhibits the activations of JAK2/STAT1 and MAPK/MAPKK pathways in TNF- α /IFN- γ -stimulated HaCaT keratinocytes

The mechanisms underlying the regulation of STATs and MAPKs *in vitro* were examined using western blot analysis, similar to those performed in the animal experiments. Protein expression levels of STAT1 and JAK2, its upstream factor, were measured. The results showed that the levels of phosphorylated STAT1 and JAK2 were increased by stimulation with TNF- α /IFN- γ but reduced by treatment with MAF in HaCaT

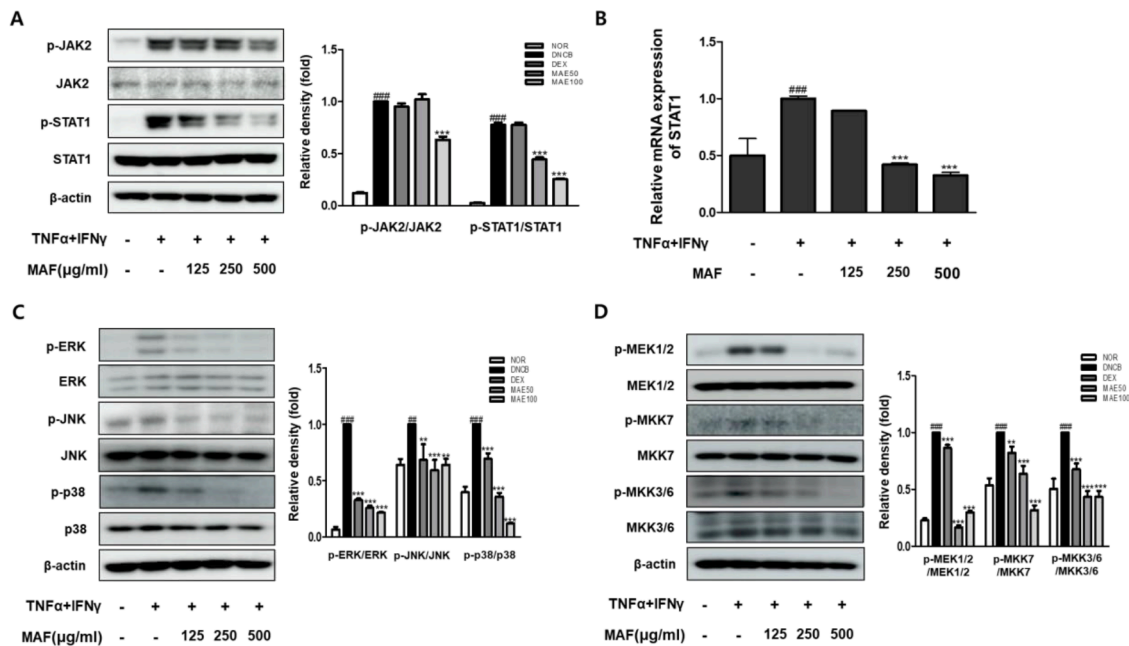


Fig. 8. Effects of MAF on JAK2/STAT1 and MAPK/MAPKK pathways activation in TNF- α /IFN- γ -stimulated HaCaT keratinocytes. Total proteins were prepared from HaCaT keratinocytes and western blotted for (A) p-JAK2, JAK2, p-STAT1, STAT1, (C) MAPKs (p-ERK, ERK, p-JNK, JNK, p-p38, and p38) and (D) MAPKKs (p-MEK1/2, MEK1/2, p-MKK7, MKK7, p-MKK3/6, and MKK3/6) using each specific antibody. B-actin was used as the internal control. The protein levels were quantified by band density in proportion to each total form. (B) Total RNA prepared from HaCaT keratinocytes, and gene expression of STAT1 was determined by RT-qPCR. The results were expressed as the optical density ratio relative to GAPDH. Data shown represent mean $\pm$ standard deviation (SD) of triplicate independent experiments. ## $p < 0.01$, ### $p < 0.001$ vs. the control group; ** $p < 0.01$, and *** $p < 0.001$ vs. TNF- α /IFN- γ -treated group.

cells (Fig. 8A). To further investigate the regulation of STAT1, its mRNA expression was estimated. As a result, activated mRNA expression of STAT1 by stimulation with TNF- α /IFN- γ was decreased by MAF treatment (Fig. 8B). As shown in Fig. 8C, stimulation with TNF- α /IFN- γ induced phosphorylation of ERK, JNK, and p38; however, all concentrations of MAF significantly weakened their expression in HaCaT keratinocytes. For in-depth *in vitro* models, we measured the protein levels of MEK1/2, MKK7, and MKK3/6 as MAPK upstream factors. Treatment with MAF significantly suppressed the phosphorylation of MAPKs *in vitro*, in comparison with that in the TNF- α /IFN- γ -stimulated group (Fig. 8D). These data indicated that MAF treatment moderated the immune response by regulating the phosphorylation of JAK2/STAT1 and MAPK/MAPKK pathways.

MAF inhibits TSLP/periostin *in vitro* and *in vivo*

TSLP-periostin reciprocal activation plays a critical role in pruritus in AD (Mishra et al., 2020). As shown in Fig. 9A and E, the activation levels of TSLP and periostin were increased by treatment with TNF- α /IFN- γ or DNCB; however, in both groups treated with MAF, the activation levels of TSLP and periostin were degraded. TSLP stimulation activates multiple STAT proteins, including STAT1, JAK1, and JAK2. Furthermore, TSLP provokes Th2 response by mediating IL-4 and IL-13 in skin fibroblasts (Kuwatsuka and Murota, 2019) and their subsequent response to periostin. This stimulation by periostin causes repetition of the STAT-TSLP-periostin loop (Furue, 2020). As described above, previous experimental results revealed that all related factors were effective upon MAF application. Thus, we evaluated the protein expression of periostin, which is abundant in the skin of AD patients (Arima et al., 2015). Moreover, IHC analysis of periostin showed significantly increased expression in the DNCB-treated group as compared to the normal group. In contrast, the DEX- and MAF-treated groups showed reduced expression, which presented as a light brown color (Fig. 9B). Consistent with previous results, the same tendency of periostin expression was observed in IF analysis (Fig. 9C). We quantified the percentage of periostin

expression per total dermal area (Fig. 9D). Results indicated that MAF suppresses the production of TSLP and periostin related pro-inflammatory cytokines.

Discussion

The skin is the most extensive and outermost organ of the human body. It provides dual external-internal protection by keratinization, in which epidermal cells in the basal epidermis, mostly keratinocytes, undergo cytodifferentiation and maturity to form the flattened SC cells. As the outermost part of the epidermis, SC prevents skin dehydration by reinforcing the skin's mechanical strength with cross-linked proteins, such as flaggrin, loricrin, and involucrin, which are connected to the lipid-rich extracellular matrix (Bhattacharya et al., 2019; van Smeden et al., 2014). However, this study revealed that keratinocytes expressed high levels of cytokines, chemokines, and pruritogenic factors in AD skin lesions in a mouse model and in AD patients.

Immune dysfunction, a prominent pathogenesis of AD, induces incremental expression of IgE levels, T cells, and mediated immune cell cytokines in keratinocytes, dendritic cells, mast cells, basophils, eosinophils, and lymphocytes (Otsuka and Kabashima, 2015; Yang et al., 2020). In this study, *in vivo* or *in vitro* models showed that MAF reduced the expression of serum IgE (Fig. 2F), Th-mediated cytokines, and chemokines (Fig. 6) via the inhibition of STAT1 and MAPKs pathways (Figs. 5 and 8). In the incipient stage, IL-4 and IL-13 initiate the Th2 immune response and maintain themselves by recruiting more Th2 cells. As chronic AD progresses, the more Th1 and Th17 cells are activated, as well as with steady activation of Th2 and Th22 cells (Renert-Yuval and Guttman-Yassky, 2020; Werfel et al., 2016). TNF- α is a Th1 cytokine that regulates various immune cell responses, including cell proliferation, differentiation, and apoptosis. IL-1 β acts as a TSLP inducer that induces Th2 responses involved in both adaptive and innate immunity (Furue et al., 2017; Yang et al., 2020). Furthermore, Th17 activation produces IL-17A, IL-17F, IL-22, IL-26, GM-CSF, and inter alia. IL-17A signifies skin inflammation by directly promoting keratinocyte proliferation as well as

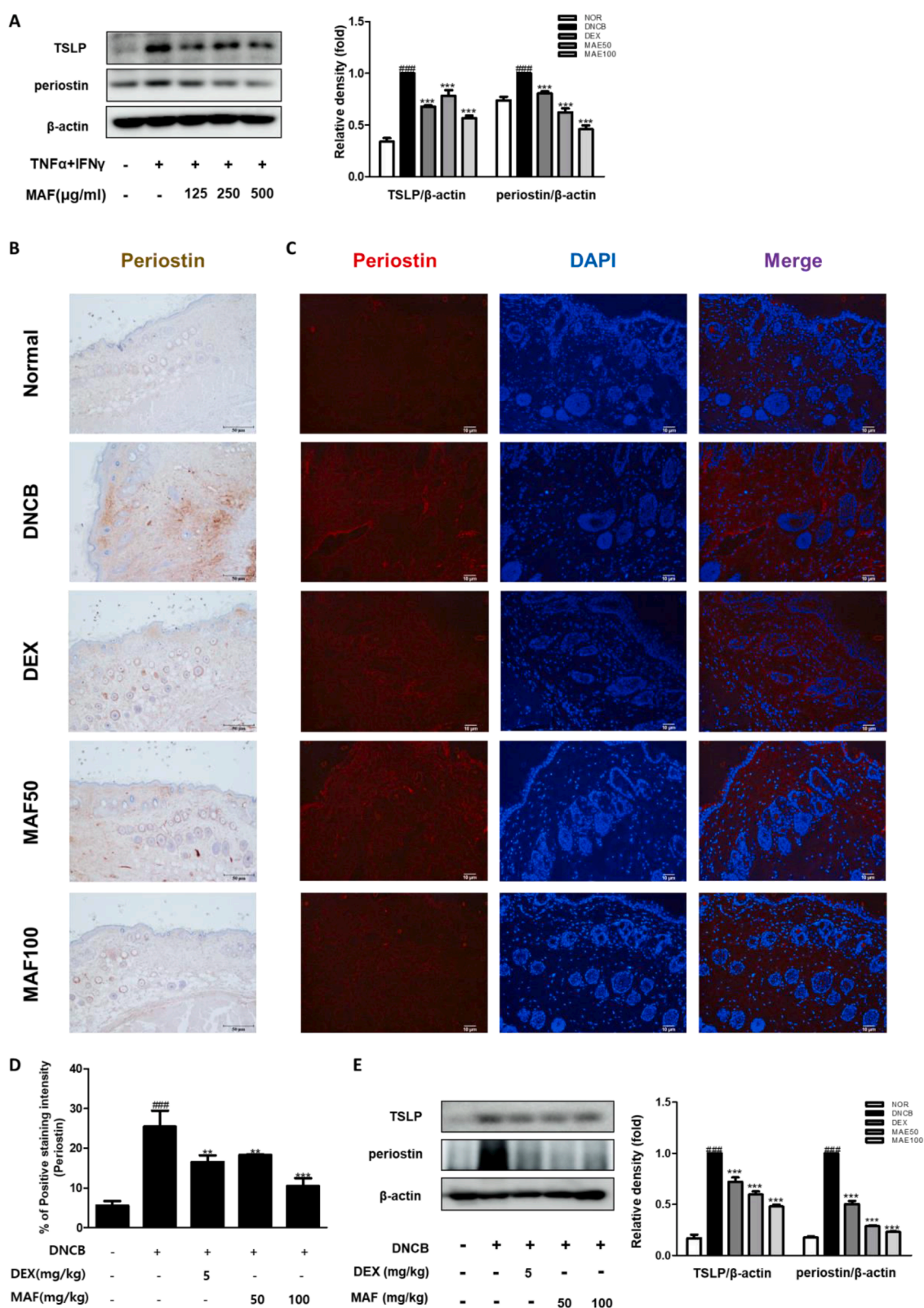


Fig. 9. Effects of MAF on TSLP/periostin activation *in vitro* and *in vivo*. (A, E) Total proteins were prepared from HaCaT keratinocytes or mouse cutaneous tissue, and then immunoblotted for TSLP and periostin using each specific antibody. The protein levels were quantified by band density in proportion to β -actin as an internal control. (B) Representative histologic section findings of dorsal tissue were immunostained with periostin antibody by immunohistochemical staining (scale bar = 50 μ m). (C) The slides of dorsal sections were stained with antibody specific for periostin (labelled with Alexa Fluor 594, red), and DAPI (labelled with Alexa Fluor 488, blue) for immunofluorescence staining (scale bar = 10 μ m). (D) As result of IF staining, percentage of periostin positive staining area per total skin area was calculated using Image J. In the dermal layer, MAF application groups showed lower immunostaining intensity of periostin than the DNCB-treated group. Two slides for each mouse were analyzed. Data shown represent mean $\pm$ standard deviation (SD) of triplicate independent experiments. ### $p < 0.001$ vs. the control group; *** $p < 0.001$ vs. TNF- α /IFN- γ -treated group or DNCB-induced AD group.

the induction of IgG, IgE, and IL-4. IL-22, which is expressed by Th17 and Th22 cells, also promotes keratinocyte proliferation but inhibits their differentiation in the chronic stage (Furue et al., 2017; Rener-t-Yuval and Guttman-Yassky, 2020; Sugaya, 2020). These cytokines are known to be partially mediated by the MAPK pathway, including IL-4, IL-5, and IL-13. They are often stimulated by the release of intracellular Ca^{2+} , which provokes mast cell degranulation and FcεRI-IgE cross-linking (Lorz et al., 2020).

Skin barrier abnormalities in AD are associated with Th-cytokine cascades and downregulated expression of barrier-related proteins. IL-4, IL-13, IL-22, IL-31, and TNF-α induce the downregulation of ceramide synthesis and barrier function, thereby reducing the expression of involucrin, loricrin, and filaggrin in keratinocytes (Furue, 2020; Schwartz et al., 2019). In this study, the expression levels of all the factors mentioned above were increased by MAF treatment *in vivo* (Fig. 4) and *in vitro* (Fig. 7). Filaggrin, a skin barrier protein composed of intermediate filaments, is related to the innate immune system; its gene mutation reinforces the outer epidermal barrier by controlling cell differentiation, moisturization, and sensitivity to allergic inflammation. Loricrin and involucrin construct and strengthen the cornified envelope with high structural homology in granular layers (Cabanillas and Novak, 2016; Drislane and Irvine, 2020; Furue, 2020). Additionally, hyaluronic acid (HA) is synthesized into the extracellular matrix by hyaluronic acid synthases (HAS); the balance of HA is important for the proliferation, differentiation, and migration of keratinocytes, which then sustains the epidermal barrier. In normal keratinocytes, HAS1 plays a central role in the mass production of HA (Malaisse et al., 2014; Park et al., 2020).

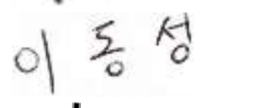
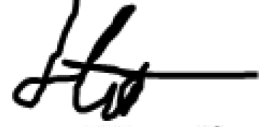
Pruritus is the fundamental cause of repeated scratching and thus aggravates skin breaches, chronic inflammation, and AD severity, with may further progress into itchier and more eczematous lesions (Steinhoff et al., 2006). TSLP, IL-13, and IL-31, which are major pruritogenic cytokines in chronic AD, are overexpressed in various epithelial cells in the skin lesions of AD patients and in murine models (Barr et al., 2019; Steinhoff et al., 2006). Emerging as a novel chronic biomarker for AD, periostin, which is a fasciclin extracellular matrix protein, is produced during fibroblast-stimulated IL-4 and IL-13 differentiation as well as keratinocyte Th2 cytokines and TSLP proliferation, with the iterative loop between periostin and TSLP (Garcovich et al., 2021). In the present study, MAF mitigated the scratching behavior (Fig. 2D), mast cell infiltration (Fig. 3C and D), and TSLP and periostin production (Figure 9) by inhibiting STAT1 expression *in vivo* (Fig. 5A) and *in vitro* (Fig. 8A and B). In previous studies, periostin accumulation in the dermis was observed in 257 adult AD patients (Kou et al., 2014) as well as in BALB/c and C57BL/6 mouse models with house dust mite extract (HDM) stimulus (Izuhara et al., 2019; Yamaguchi, 2014). These pruritus-inducing cytokines are known to be partially mediated via JAK/STAT pathways, thus enabling their candidacy as a promising target of broad-spectrum drugs (Dong and Dong, 2018; Yosipovitch et al., 2018). In addition, restraining periostin function could be a potential target for diagnostic or therapeutic agents by directly acting on local inflammation (Izuhara et al., 2019).

In previous studies, the AD effect of Mori cortex was assessed in TNF-α/IFN-γ-stimulated HaCaT cells and in HDM-induced NC/Nga mice. Mori cortex was effective in inhibiting TARC production *in vitro* and inhibiting IgE and histamine levels *in vivo* (Lim et al., 2014). Based on this previous experiment, the anti-AD effect of MAF, which is more accessible and has similar efficacy, was evaluated. For a more in-depth study, additional cytokines and chemokines, including TARC (Fig. 6), and pruritogenic factors (Fig. 9), including mast cell infiltration (Fig. 3C and D) and IgE (Fig. 2F), were identified; all of which were alleviated by MAF treatment. Although the mechanism has been confirmed, further studies using genetic models are necessary to confirm its precise effects and mechanisms. Further research on the sensory nerve is needed to identify the antipruritic effect of MAF through periostin inhibition. Notwithstanding additional questions, this study is deemed significant,

as it proposes a potential AD treatment for MAF. In conclusion, MAF alleviates the complex interaction of AD with immunological irregularity, skin barrier disruption, and pruritus. This study on the anti-atopic effect of MAF may be regarded meaningful and supports the new properties of the *Morus* species; it suggests its potential as an herbal therapeutic agent that may serve as an alternative to existing medicines.

Author agreement

All authors have seen and approved the final version of the manuscript being submitted. This article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

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CRediT authorship contribution statement

Hye-Min Kim: Investigation, Visualization, Writing – original draft, Writing – review & editing. **Yun-Mi Kang:** Methodology, Visualization, Writing – review & editing. **Bo-Ram Jin:** Writing – review & editing, Validation. **Hwan Lee:** Investigation. **Dong-Sung Lee:** Validation, Data curation. **Minho Lee:** Validation, Funding acquisition. **Hyo-Jin An:** Conceptualization, Supervision.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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