Brevilin A inhibits IL-17A-induced inflammation in psoriasis by modulating HSP 70

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Abstract. Psoriasis, which is characterized by keratinocyte hyperproliferation, presents complex management challenges. The heat shock protein 70 (HSP 70) family, which is essential in protein folding and stress responses, also modulates inflammation, suggesting its therapeutic potential in inflammation-driven diseases. The present study aimed to explore the effects of brevilin A, a natural compound known to alleviate imiquimod-induced psoriasis, on HSP 70 expression and proinflammatory cytokine production in HaCaT cells stimulated with IL-17A. An HSP 70 inhibitor was used to determine its role in cytokine regulation, and the effect of brevilin A on skin pathology in mice was examined via immunohistochemistry and hematoxylin and eosin staining. The results revealed that brevilin A markedly decreased IL-6 and IL-8 levels after IL-17A stimulation at both 9 and 24 h in HaCaT cells, and increased HSP 70 and HSP 90 expression levels. Notably, the brevilin A-induced suppression of cytokine levels was reversed when cells were co-treated with the HSP 70 inhibitor. *In vivo*, brevilin A enhanced HSP 70 expression and reduced skin hyperproliferation. These findings suggested that brevilin A may modulate HSP 70 expression and dampen the inflammatory response induced by IL-17A, indicating its potential as an innovative treatment for psoriasis.

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Introduction

Psoriasis is a complex autoimmune skin disorder that affects ~2% of the global population, posing notable health challenges due to its chronic nature and impact on quality of life (1). In Taiwan, the prevalence of psoriasis is notably lower, affecting ~0.24% of the population; however, it remains a concern due to the debilitating symptoms it can cause (2). The clinical manifestations of psoriasis include scaling, thickened skin (acanthosis) and the formation of distinct psoriatic plaques. This condition is primarily driven by an overactive immune response, particularly involving T-helper 17 (Th17) cells, which are crucial in the proliferation of keratinocytes and the production of pro-inflammatory cytokines (3). IL-17A, secreted by Th17 cells, can induce C/CAAT-enhancer-binding proteins (C/EBPs) that are translocated into the nucleus, and can increase transcript levels of chemokines and inflammatory cytokines, such as CXCL8 and IL-6 (4). Abnormal crosstalk between keratinocytes and immune cells has been considered the main driver of skin inflammation.

Heat shock proteins (HSPs), which are critical for protein folding, assembly and intracellular trafficking, also serve a role in cellular responses to stress, such as heat, oxidative conditions or nutrient deprivation (5). When cells are exposed to high temperature, oxidative stress or energy shortage, HSP

expression is increased to rescue cell damage. Specifically targeting the regulation of HSP expression has been reported to protect against inflammatory diseases including neuro-inflammation, hepatic inflammation and psoriasis (6-8). However, the role of HSP 70 in psoriasis remains controversial; for example, HSP 70 has been shown to be released from exosomes and to activate dendritic cells to secrete proinflammatory cytokines, which may then induce overproliferation of keratinocytes (9). By contrast, a different study reported that HSP 70 reduces psoriasis-like inflammation induced by imiquimod (IMQ) in mice (8).

Brevilin A is also known to target multiple pathways, including the JAK/STAT signaling pathway, which is pivotal in immune response regulation, and has been implicated in both inflammatory disease mechanisms and oncogenesis (10,11). However, whether brevilin A inhibits skin inflammation remains to be determined. Given its broad anti-inflammatory and antiproliferative properties, the present study aimed to investigate the effects of brevilin A on the regulation of pro-inflammatory cytokines and to elucidate its underlying mechanisms in the context of psoriasis, potentially offering a novel therapeutic approach to managing this persistent skin disorder.

Materials and methods

Cell culture and treatment. The human keratinocyte HaCaT cell line (Elabscience Bionovation Inc.) was cultured in a Dulbecco's Modified Eagle's Medium (cat. no. SH30022.02; HyClone; Cytiva) supplemented with 10% fetal bovine serum (cat. no. SH30396.03; HyClone; Cytiva) and 1% penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc.). Cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2. For treatment, cells were exposed to brevilin A (CAS no. 16503-32-5; Chengdu Biopurify Phytochemicals Ltd.) at concentrations of 1.25, 2.5 and 5 μM at 37˚C for 9 or 24 h. IL-17A (Peprotech, Inc.) was applied at a concentration of 100 ng/ml to stimulate cells in co-treatment assays.

MTT assay for cell viability. Cell viability was assessed using the MTT assay (CAS no. 298-93-1; Sigma-Aldrich; Merck KGaA). Briefly, HaCaT cells were seeded at a density of $5x10^4$ cells/well in a 96-well plate, and were treated with the indicated concentrations of brevilin A (0.625-20 μM) and/or apoptozole (CAS no. 1054543-47-3; MedChemExpress) (0.009-20 μM) at 37˚C for 24 h. Each concentration of each substance was tested in triplicate wells (n=3). After 24 h of treatment, MTT solution (5 mg/ml) was added and the cells were incubated for an additional 4 h. Formazan crystals formed were dissolved in isopropanol, and the absorbance was measured at 570 nm using a microplate reader (SUNRISE; Tecan Group, Ltd).

ELISA for cytokine measurement. The levels of IL-6 (cat. no. 430504) and IL-8 (cat. no. 431504) in the supernatant were measured using ELISA kits (Biolegend, Inc.) according to the manufacturer's protocols. HaCaT cells were seeded at a density of $2x10^5$ cells/well in a 24-well plate. Samples from HaCaT cells were collected at 9 and 24 h following co-treatment with IL-17A (100 ng/ml) (12) and either brevilin A (1.25, 2.5 and 5 μM) or apoptozole (1.25, 2.5 and 5 μM). Dexamethasone (1 μg/ml; Taiwan Biotech Co., Ltd.) was used as a positive control. Each experimental condition was replicated in three wells to confirm the consistency of the results.

Western blot analysis. Western blot analysis was performed to assess the expression levels of HSP 70 and HSP 90 in HaCaT cells treated with specific agents. The cells were harvested and lysed using RIPA buffer (Bio Basic Inc.), which contains Tris-HCl (20 mM, pH 7.4), NaCl (150 mM), NP-40 (1%), sodium deoxycholate (0.5% w/v), SDS (0.1%), EDTA (1 mM) and EGTA (5 mM), with added protease inhibitors. After incubation on ice for 30 min, the lysates were centrifuged at 14,000 x g for 15 min at 4˚C to eliminate cell debris. The protein-containing supernatants were collected, and protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). The proteins (30 μg/lane) were then separated by SDS-PAGE on 10% gels and transferred onto PVDF membranes. These membranes were blocked with 5% non-fat milk dissolved in PBS at room temperature for 1 h and were then probed with primary antibodies for HSP 70 (1:1,000; cat. no. sc-32239; Santa Cruz Biotechnology, Inc.), HSP 90 (1:1,000; cat. no. sc-69703; Santa Cruz Biotechnology, Inc.), phosphorylated (p-)p38 (1:1,000; cat. no. 4511; Cell Signaling Technology, Inc.), p38 (1:1,000; cat. no. 8690; Cell Signaling Technology, Inc.), p-ERK (1:1,000; cat no. 4370; Cell Signaling Technology, Inc.), ERK (1:1,000; cat. no. 4695; Cell Signaling Technology, Inc.), p-JNK (1:2,000; cat. no. 4668; Cell Signaling Technology, Inc.), JNK (1:1,000; cat. no. 9252; Cell Signaling Technology, Inc.) and β-actin (loading control; 1:5,000; cat. no. 3700; Cell Signaling Technology, Inc.). Detection was performed using HRP-conjugated anti-mouse IgG (1:7,000; cat. no. 7076; Cell Signaling Technology, Inc.) or anti-rabbit IgG (1:7,000; cat. no. 7074; Cell Signaling Technology, Inc.) secondary antibodies at room temperature for 1 h, followed by enhanced chemiluminescence (MilliporeSigma). Western blots were semi-quantified using ImageJ (version 1.53e; National Institutes of Health).

In vivo psoriasis model. BALB/c female mice (n=30; age, 8 weeks; weight, 20±2 g), sourced from the National Laboratory Animal Center (Taipei, Taiwan), were maintained under specific pathogen-free conditions at a steady temperature of 23±1˚C, 50-60% relative humidity and a 12-h light/dark cycle, and had free access to food and water. The animal study protocols were approved by the Animal Care and Use Committee of Kaohsiung Veterans General Hospital (IACUC no. 2021-A039; Kaohsiung, Taiwan). The mice were allocated into six groups (n=5/group): Control, DMSO (vehicle control), brevilin A (5, 10 and 20 mg/kg) and dexamethasone (1 mg/kg) (12). All groups, with the exception of the control group, received a daily topical application of 62.5 mg 5% IMQ cream (Aldara; 3M Pharmaceuticals) on their shaved backs and right ears for 5 consecutive days. The DMSO group received daily intraperitoneal injections of 0.5% DMSO in 200 μl normal saline. The brevilin A groups were administered daily intraperitoneal injections of brevilin A at their respective doses in 200 μl normal saline, whereas the dexamethasone

group received daily injections of 1 mg/kg dexamethasone in 200 μl normal saline. On day 6, all mice were euthanized for subsequent analyses. The procedure involved placing the mice in a chamber gradually filled with CO_2 at a flow rate of 30% chamber volume/min. After the gas was introduced, the mice were observed for 3 min to ensure complete euthanasia, with death confirmed by the absence of a heartbeat, cessation of respiratory movements and pupil dilation. Following euthanasia, skin samples were harvested for histological and immunohistochemical analysis to evaluate epithelial hyperproliferation and HSP 70 expression.

Histological analysis and immunohistochemistry. The dorsal skin from each mouse was removed, fixed in 10% formalin at room temperature for 24 h, embedded in paraffin, and sectioned into 5-μm slices. These sections were subsequently stained with hematoxylin and eosin (H&E) at room temperature to examine epithelial hyperproliferation and the infiltration of immune cells. Tissue sections were deparaffinized in xylene (three changes, 5 min each) and rehydrated through a graded series of ethanol: 100% (1 min), 95% (1 min), 80% (1 min) and 70% (1 min), followed by a 1-min rinse in distilled water. Sections were then stained with hematoxylin for 5 min, washed in water for another 5 min, and counterstained with eosin Y for 5 min. Subsequently, the sections were dehydrated in 95% ethanol (10 sec), followed by four changes of 100% ethanol (10 sec for the first change and 1 min for the subsequent changes). Finally, sections were cleared in xylene (three changes, 5 min each) and mounted with a coverslip.

For immunohistochemistry (IHC), tissue sections were deparaffinized in xylene (two changes, 10 min each) and rehydrated through a graded series of ethanol: 100, 95, 80 and 70%, followed by a 5-min rinse in tap water. Antigen retrieval was performed using heat-induced epitope retrieval at 95˚C for 30 min with either citrate buffer (cat. no. CBB500; Scytek Laboratories, Inc.) for HSP 70 and IL-6, or Tris-EDTA buffer (cat. no. TES999; Scytek Laboratories, Inc.) for HSP 90. After antigen retrieval, the sections were washed in TBS-0.05% Tween 20 (TBST; cat. no. TBT999; Scytek Laboratories, Inc.) for 5 min. To block endogenous peroxidase activity, sections were incubated in Hydrogen Peroxide Block (cat. no. TA-060-HP; Epredia; Thermo Fisher Scientific, Inc.) for 10 min at room temperature, followed by two TBST washes (3 min each). Non-specific binding was blocked using either MS Blocking A and B (cat. no. D52; OriGene Technologies, Inc.) or Protein Block (cat. no. TA-060-PBQ; Epredia; Thermo Fisher Scientific, Inc.) for 10-30 min at room temperature. Primary antibody incubation was performed in a humidified chamber at room temperature for 50 min in the dark. The following primary antibodies were used: HSP 70 (1:50; cat. no. sc-32239; Santa Cruz Biotechnology, Inc.), IL-6 (1:50; cat. no. ab9324; Abcam) and HSP 90 (1:300; cat. no. 4877; Cell Signaling Technology, Inc.), diluted in antibody dilution buffer (cat. no. ADB250; Roche Tissue Diagnostics). After primary antibody incubation, the sections were washed twice in TBST (3 min each). For signal amplification, either Mouse Antibody Enhancer (cat. no. D52; OriGene Technologies, Inc.) or Rabbit Antibody Enhancer (cat. no. D39; OriGene Technologies, Inc.) was applied for 15 min, followed by another two washes with TBST (3 min each). Detection was performed using HRP-conjugated anti-mouse (cat. no. D52; OriGene Technologies, Inc.) or anti-rabbit (cat. no. D39; OriGene Technologies, Inc.) secondary antibodies, each incubated for 15 min at room temperature. After two additional TBST washes (3 min each), the signal was developed using DAB (cat. no. RE7270-K; Novolink; Leica Biosystems) for 5 min. The sections were rinsed in tap water for 5 min and then counterstained with hematoxylin (cat. no. 3801522; Leica Biosystems) for 2 min. After a final wash with tap water (5 min), the sections were dehydrated in 100% ethanol (two changes, 5 min each) and cleared in xylene (two changes, 5 min each). Finally, the slides were mounted using Surgipath Micromount mounting medium (cat. no. 3801731; Leica Biosystems).

Images of the H&E- and IHC-stained sections were captured using an APEXVIEW APX100 Digital Imaging System (Olympus Corporation) and a light microscope, and were analyzed with ImageJ software (version 1.5.3; National Institutes of Health). For HSP 70, HSP 90 and IL-6 staining analysis, tissue samples were stained, and 3-5 regions of the same size were randomly selected from each sample for semi-quantification. The average staining intensity for each group was calculated, and statistical differences between groups were analyzed using appropriate statistical methods. This approach ensured that the quantification was unbiased, reproducible and reflective of the staining patterns across the samples.

Statistical analysis. Data were analyzed using GraphPad Prism software (version: 6.01, Dotmatics). Differences between groups were evaluated using one-way ANOVA followed by Tukey's post hoc test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Brevilin A inhibits IL-17A-induced IL-6 and IL-8 levels. To evaluate the cytotoxicity and anti-inflammatory effects of brevilin A on HaCaT cells, an MTT assay and ELISA were performed. The results demonstrated that brevilin A exhibited cytotoxic effects at higher concentrations, since 10 and 20 μM significantly reduced cell viability compared with that in the control group, whereas lower concentrations (0.625, 1.25, 2.5, and 5 μM) had no significant impact on viability (Fig. 1A). Additionally, brevilin A effectively suppressed IL-17A-induced pro-inflammatory cytokine production in HaCaT cells in a dose-dependent manner. At both 9 and 24 h, brevilin A significantly reduced the levels of IL-6 and IL-8, with the effects being comparable to or exceeding those of dexamethasone (12), a positive control (Fig. 1B). These findings indicated the potential of brevilin A as an anti-inflammatory agent capable of modulating keratinocyte responses in inflammatory conditions.

Brevilin A increases HSP 70 and HSP 90 expression in keratinocytes. HSPs regulate inflammatory responses in autoimmune diseases and are upregulated during inflammation (13). However, the specific function of HSPs in skin inflammation remains ambiguous. In the current study, the influence of brevilin A on the expression levels of HSP 70 and HSP 90 in keratinocytes was examined. Initially, HaCaT cells

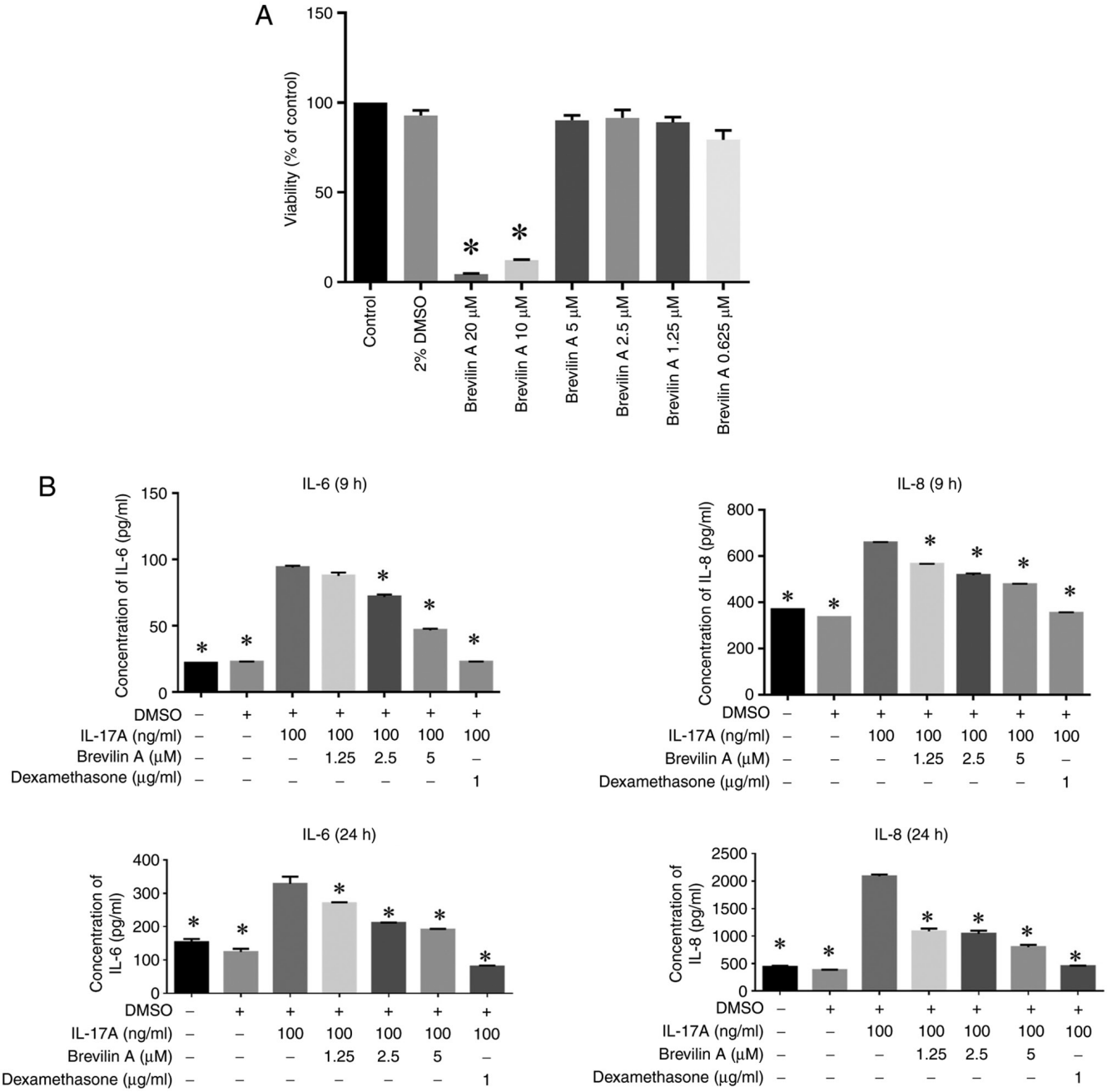

Figure 1. Effects of brevilin A on cell viability and IL-17A induced IL-6 and IL-8 production in HaCaT cells. (A) HaCaT cells were treated with different doses of brevilin A (0.625, 1.25, 2.5, 5, 10 and 20 μM) or 2% DMSO for 24 h. Cell viability was measured by MTT assay. Results are presented as a percentage of control (untreated) cells. Data are expressed as mean ± SEM (n=3). *P<0.05 vs. control. (B) Concentrations of IL-6 and IL-8 in the supernatant were determined using ELISA kits. HaCaT cells were seeded at a density of $2x10^5$ cells/well in a 24-well plate and were co-treated with IL-17A (100 ng/ml) and different concentrations of brevilin A (1.25, 2.5 and 5 μM) or dexamethasone (1 μg/ml). Samples were collected after 9 and 24 h of treatment. The concentrations of IL-6 and IL-8 were quantified, and results are presented as the mean ± SEM (n=3). *P<0.05 vs. IL-17A-treated group.

were co-treated with IL-17A (100 ng/ml) and 5 μM brevilin A. The results showed that HSP 70 and HSP 90 experssion levels were significantly upregulated after co-culture with IL-17A and 5 μM brevilin A for 6 and 9 h (Fig. 2A-C). Subsequently, to explore dose-dependent effects, HaCaT cells were treated with different doses of brevilin A (1.25, 2.5 and 5 μM) for 9 h. The results showed that the expression levels of HSP 70 and HSP 90 were significantly increased after stimulation with 5 μM brevilin A (Fig. 2D and E). The current study also investigated whether brevilin A alone induces HSP 70 and HSP 90 expression. HaCaT cells were treated with different doses of brevilin A for 9 h, and it was revealed that brevilin A (5 μM) alone was sufficient to significantly elevate HSP 70 and HSP 90 levels (Fig. 2F and G).

To further confirm that brevilin A has the ability to counteract IL-17A-induced inflammatory cytokine production, the effects of IL-17A, brevilin A and their combination were examined on IL-6 and IL-8 levels. The results demonstrated that IL-17A significantly induced the production of IL-6 and IL-8, while brevilin A alone did not increase IL-6 or IL-8 levels. In the IL-17A and brevilin A co-treatment group, the levels of IL-6 and IL-8 were significantly reduced compared

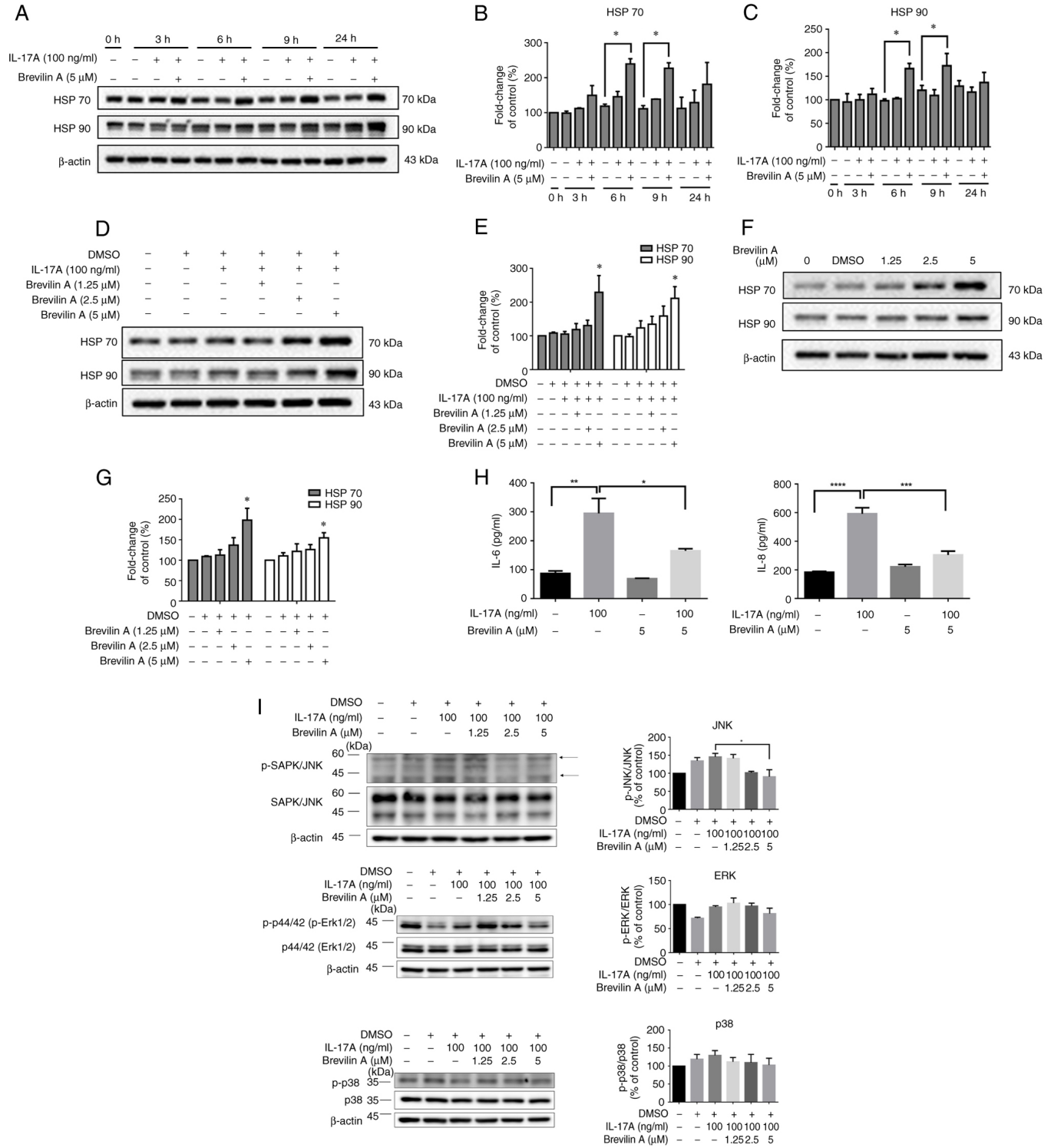

Figure 2. Brevilin A increases HSP 70 and HSP 90 expression in HaCaT cells. (A) HaCaT cells were treated with IL-17A alone (100 ng/ml), or were co-treated with IL-17A and brevilin A (5 μM) for different durations (0, 3, 6, 9, and 24 h). Expression levels of HSP 70, HSP 90 and β-actin (loading control) were detected by western blotting. Semi-quantitative analysis of (B) HSP 70 and (C) HSP 90 expression levels. The intensity of HSP 70/HSP 90 bands was normalized to β-actin and expressed as fold change relative to control (untreated) cells. (D) Western blot analysis of the expression levels of HSP 70 and HSP 90 in HaCaT cells treated with IL-17A (100 ng/ml) and various concentrations of brevilin A (1.25, 2.5 and 5 μM) for 9 h. β-actin was used as a loading control. (E) Semi-quantitative analysis of HSP 70 and HSP 90 band intensity was performed using ImageJ. The intensity of bands was normalized to β-actin and expressed as fold change relative to control (untreated) cells. (F) Western blot analysis of the expression levels of HSP 70 and HSP 90 in HaCaT cells treated with various concentrations of brevilin A (1.25, 2.5 and 5 μM) for 24 h. β-actin was used as a loading control. (G) Semiquantitative analysis of HSP 70 and HSP 90 band intensity was performed using ImageJ. The intensity of bands was normalized to β-actin and expressed as fold change relative to control (untreated) cells. (H) HaCaT cells were treated with IL-17A (100 ng/ml) alone, brevilin A (5 μM) alone, or a combination of IL-17A and brevilin A for 9 h. The secretion of IL-6 and IL-8 was measured by ELISA. (I) Brevilin A modulates the MAPK signaling pathway in IL-17A-treated cells. Western blot analysis was performed to assess the phosphorylation levels of SAPK/JNK, ERK and p38 MAPK after treatment with IL-17A (100 ng/ml) and different concentrations of brevilin A (1.25, 2.5 and 5 μM) for 9 h. Total SAPK/JNK, ERK and p38 protein levels were used as controls, and β-actin served as the loading control. Semi-quantification of p-SAPK/JNK, p-ERK and p-p38 was normalized to total protein levels and presented as a percentage of the control group. Data are presented as the mean ± SEM (n=3). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. control or as indicated. HSP, heat shock protein; p-, phosphorylated.

with in the IL-17A treatment group (Fig. 2H). These findings indicated that brevilin A may exert a protective effect by preventing keratinocytes from producing inflammatory cytokines in response to IL-17A stimulation.

To further explore the molecular mechanisms underlying the effects of brevilin A on IL-17A-induced responses, the activation of the MAPK signaling pathway was examined; this pathway serves a critical role in inflammation and stress responses. Co-treatment with IL-17A and brevilin A (1.25, 2.5 and 5 μM) for 9 h reduced the phosphorylation of JNK in a dose-dependent manner compared with in the IL-17A treatment group, whereas the phosphorylation of ERK and p38 remained largely unaffected (Fig. 2I). Brevilin A did not significantly affect p-ERK or p-p38 levels, suggesting that its regulatory effect is selective for the JNK pathway. These results indicated that brevilin A may selectively inhibit IL-17A-induced JNK phosphorylation without affecting ERK or p38, highlighting its potential role in modulating inflammatory signaling through the JNK pathway. These findings suggested that brevilin A specifically suppresses the IL-17A-induced activation of the JNK pathway, which may contribute to its effects on HSP 70 and HSP 90 expression and its anti-inflammatory properties. All of the experiments were performed in triplicate to ensure the reliability and reproducibility of the results. These findings highlight the capability of brevilin A to induce HSP 70 and HSP 90 expression independently and in conjunction with IL-17A in keratinocytes, suggesting a potential protective or regulatory role in inflammatory responses within the skin.

Brevilin A increases HSP 70 expression in keratinocytes and alleviates inflammation in a mouse model of IMQ-induced psoriasis. To further investigate the effects of brevilin A on inducing HSP 70 expression, an animal model of IMQ-induced psoriasis was generated. Briefly, 8-week-old BALB/c mice were randomly divided into six groups (n=5/group): Control (untreated), DMSO (vehicle control), brevilin A (5, 10 and 20 mg/kg) and dexamethasone (1 mg/kg) groups. BALB/c mice were first treated with different doses (5, 10 or 20 mg/kg) of brevilin A for 5 days. Epithelial cell hyperproliferation, and HSP 70, HSP 90 and IL-6 expression were then detected using H&E staining and IHC. Representative images of the skin on the backs of the mice showed that brevilin A treatment reduced erythema and scaling compared with that in the vehicle control group (Fig. 3A). H&E staining revealed decreased epithelial cell hyperproliferation and immune cell infiltration in the brevilin A-treated groups and the dexamethasone-treated group compared with that in the vehicle control group, suggesting a reduction of skin inflammatory responses (Fig. 3B). Furthermore, IHC demonstrated that brevilin A treatment led to a dose-dependent increase in HSP 70 expression in epithelial cells, with the highest expression observed in the 20 mg/kg group (Fig. 3C and F). In addition, in the dexamethasone treatment group, the expression of HSP 70 was significantly increased compared with that in the vehicle control group. By contrast, IHC staining of IL-6 showed a significant reduction in its expression in the brevilin A-treated groups and the dexamethasone-treated group, indicating its role in mitigating inflammatory responses (Fig. 3D and F). HSP 90 expression was moderately upregulated in brevilin A-treated groups in a dose-dependent manner, with the highest levels at 20 mg/kg (Fig. 3E and F). Furthermore, dexamethasone treatment also increased HSP 90 expression conpared with that in the vehicle control group. These results collectively indicated that brevilin A may exert protective effects against psoriasis by alleviating inflammation and upregulating HSP 70 expression in epithelial cells.

An HSP 70 inhibitor reverses the effects of brevilin A on IL-17A-induced proinflammatory cytokine production. To elucidate the underlying mechanisms regarding the effects of brevilin A on reducing IL-17A-induced IL-6 and IL-8 production through increasing HSP 70 expression, the present study assessed the effects of an HSP 70-specific inhibitor, apoptozole. Initially, it was revealed that reatment with 20 μM apoptozole for 24 h significantly reduced the viability of HaCaT cells; therefore, 1.25, 2.5 and 5 μM apoptozole were used in subsequent experiments (Fig. 4). Next, the current study analyzed HaCaT cells co-treated with different doses of apoptozole, 100 ng/ml IL-17A and 5 μM brevilin A for 9 and 24 h; the results showed that co-treating cells with apoptozole and IL-17A did not increase the levels of IL-6 or IL-8 compared with those in the IL-17A treatment group; however, after co-treating cells with apoptozole (2.5 and 5 μM), IL-17A and brevilin A, the levels of IL-6 and IL-8 were significantly higher in a dose-dependent manner compared with those in the IL-17A + brevilin A group (Fig. 5). To determine whether the HSP 70-specific inhibitor could reverse the anti-inflammatory effects of brevilin A on cytokine expression, HaCaT cells were co-treated with 5 μM apoptozole and different doses of brevilin A for 9 and 24 h. The results showed that 5 μM brevilin A significantly reduced IL-17A-induced IL-6 and IL-8 production compared with that in the IL-17A-group, whereas the 5 μM brevilin A + 5 μM apoptozole group showed a reversal of this reduction, restoring IL-6 levels at 24 h and IL-8 levels at 9 h (Fig. 6). These data indicated that HSP 70 may serve a major role in the anti-inflammatory effects of brevilin A treatment.

Discussion

HSP 70 is known to serve a role in suppressing disease progression and exerting immunomodulatory effects (14); however, the role of HSP70 in psoriasis treatment remains controversial, as it can either suppress inflammation by inhibiting pro-inflammatory cytokines, or exacerbate the disease by promoting immune activation and keratinocyte survival, depending on its localization and function. The present study demonstrated that brevilin A reduced IL-17A-induced IL-6 and IL-8 levels in HaCaT cells, and the expression levels of HSP 70 and HSP 90 were upregulated in the brevilin A-treated group. In the *in vivo* experiment, brevilin A reduced epithelial cell hyperproliferation and immune cell infiltration, and increased HSP 70 expression. The mechanistic experiment showed that an HSP 70-specific inhibitor, apoptozole, reversed the effects of brevilin A on reducing IL-17A-induced IL-6 and IL-8 levels in HaCaT cells. Taken together, these results suggested that brevlin A may inhibit IL-17A-induced proinflammatory cytokine production, acting through HSP 70 uprgulation.

In the pathogenesis of psoriasis, proinflammatory cytokines have an important role in disease progression and

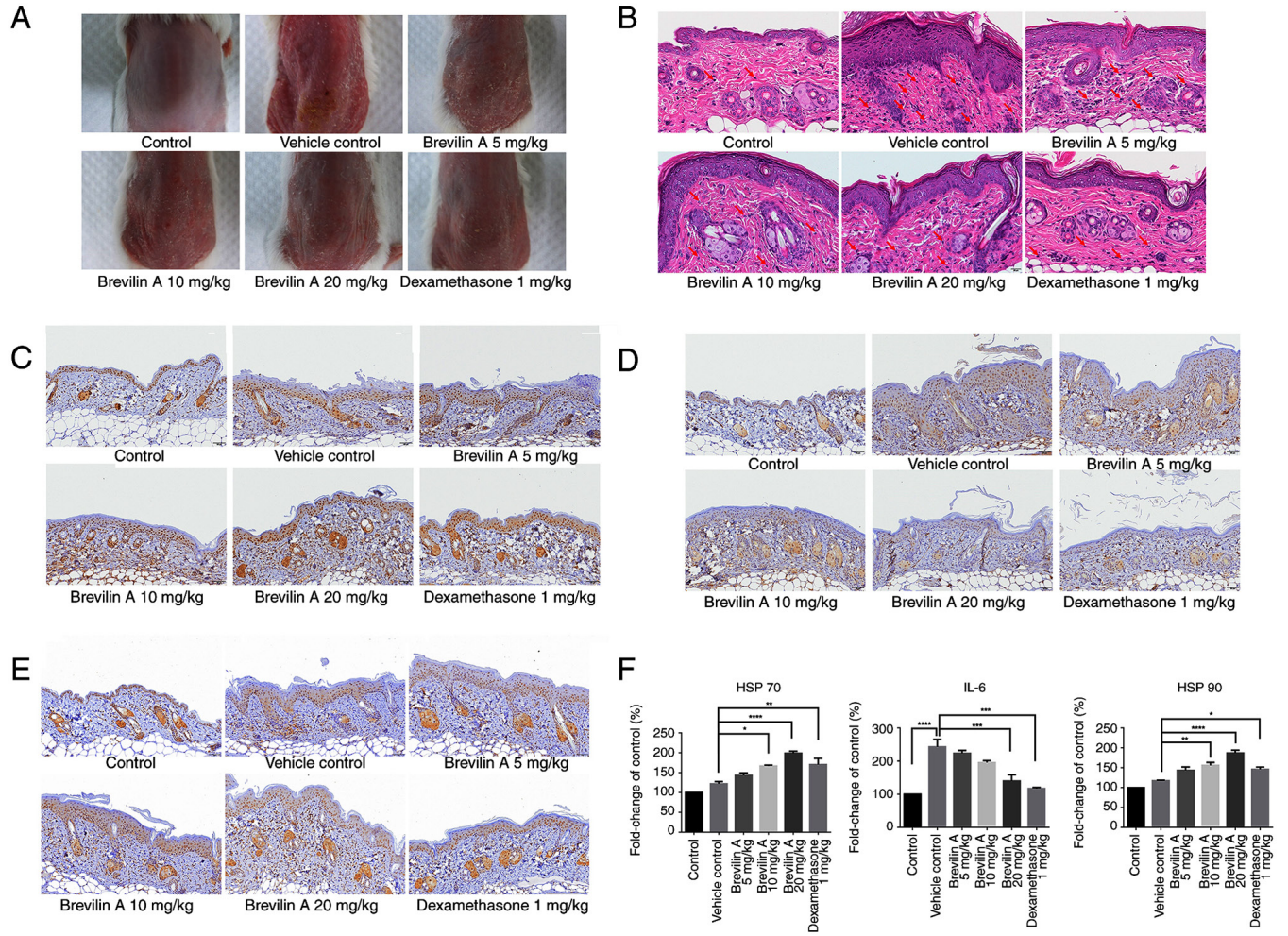

Figure 3. Brevilin A reduces IMQ-induced skin inflammation, decreases IL-6 expression and enhances HSP 70 expression in a mouse model of psoriasis. Briefly, 8-week-old BALB/c mice (n=5/group) with IMQ-induced psoriasis were treated with brevilin A (5, 10 and 20 mg/kg) or dexamethasone (1 mg/kg) by intraperitoneal injection for 5 consecutive days. (A) Representative images of the skin on the backs of mice showed improvement in erythema and scaling in brevilin A- and dexamethasone-treated groups compared with the vehicle control. (B) Skin pathology was assessed using hematoxylin and eosin staining (x400 magnification). Representative images show the degree of epithelial hyperproliferation and immune cell infiltration, with immune cells indicated by red arrows. Scale bar=20 μm. (C) IHC staining of HSP 70 showed increased expression in brevilin A-treated groups, with the highest expression observed at 20 mg/kg. Scale bar=50 μm; x200 magnification. (D) IHC staining of IL-6 revealed reduced expression in brevilin A-treated groups, suggesting its anti-inflammatory effects. Scale bar=50 μm; x200 magnification. (E) IHC staining of HSP 90 showed increased expression in brevilin A-treated groups, with the highest expression observed at 20 mg/kg. Scale bar=50 μm; x200 magnification. (F) Semi-quantification of HSP 70, IL-6 and HSP 90 staining intensity are presented as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001. IHC, immunohistochemistry; IMQ, imiquimod

comorbidities. For example, IL-6 is a major cytokine that induces the differentiation of naïve T cells into Th17 cells, and its expression is upregulated in the serum and skin lesions of patients with psoriasis (15). Another cytokine, IL-8, which increases the infiltration of polymorphonuclear cells, and induces keratinocyte proliferation and angiogenesis, is also involved in skin inflammation (16). The levels of both of these cytokine were increased in keratinocytes after IL-17A stimulation in the present study. By contrast, brevilin A reduced IL-17A-induced production of IL-6 and IL-8. Previous studies have shown that brevilin A, a sesquiterpene lactone isolated from *Centipeda minima*, exhibits anti-inflammatory and anticancer properties (10,11,17-19). Brevilin A has also been reported to inhibit NF-κB activation, which is a crucial pathway in the inflammatory response (11). Additionally, brevilin A has been shown to suppress the production of other proinflammatory cytokines, such as PGE2 and IL-1β, in the cartilage tissue of an osteoarthritis mouse model (20). These data suggested that brevilin A may be a promising drug for the treatment of psoriasis and other IL-17A-induced inflammatory diseases.

HSPs are cellular chaperones that respond to stress and intracellular protein misfolding. Notably, the role of HSPs in anti-inflammatory responses and immunomodulation has previously been reported (14,21). Among the HSP superfamily, HSP 70 is crucial due to its widespread influences in protein folding, protection against stress and modulating immune responses. HSP 70 regulates classical intracellular signaling pathways related to the expression of proinflammatory cytokines, such as MAPK and NF-κB. By inhibiting the binding of NF-κB to inflammation-related genes, and inhibiting IκB-α phosphorylation, HSP 70 is able to downregulate the levels of proinflammatory cytokines (22). The results of the present study, alongside supporting evidence from the literature (23), highlight the modulatory role of HSP 70 in the inflammatory response driven by IL-17-mediated pathways. HSP 70 appears

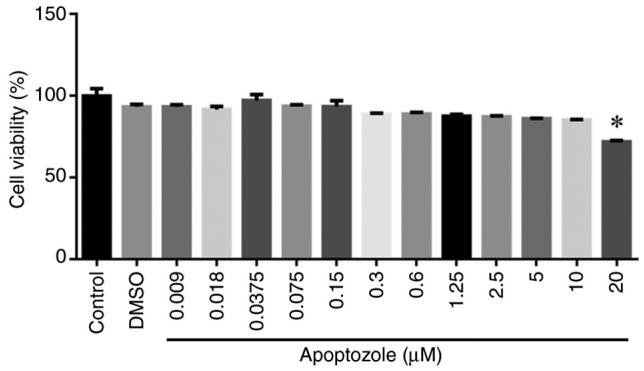

Figure 4. Effect of apoptozole on HaCaT cell viability. Cell viability was determined using the MTT assay. HaCaT cells were seeded at a density of $5x10^4$ cells/well in a 96-well plate and were treated with different concentrations of apoptozole for 24 h. Each concentration was tested in triplicate wells (n=3). Data are expressed as the mean ± SEM. *P<0.05 vs. control.

to regulate the levels of the key pro-inflammatory cytokines IL-6 and IL-8 through its influence on critical signaling pathways, such as NF-κB and MAPK. Notably, HSP 70 has been shown to inhibit NF-κB activation under specific conditions, leading to a suppression of IL-6 and IL-8 production (24); however, under cellular stress conditions, HSP 70 may stabilize certain signaling intermediates, which can enhance cytokine expression (25). These observations suggest that HSP 70 predominantly modulates the levels of IL-6 and IL-8, rather than being directly influenced by these cytokines. This finding underscores the importance of HSP 70 as a pivotal regulator within the IL-17-driven inflammatory axis and provides valuable insights into its potential as a therapeutic target for chronic inflammatory diseases. Nevertheless, further research is warranted to elucidate the precise mechanisms underlying these interactions in disease-specific contexts, such as psoriasis and autoimmune disorders.

HSP 70 has also been shown to have roles in apoptosis regulation, where it inhibits apoptosis by binding to apoptosis-regulating proteins, such as Apaf-1 and AIF (26). HSP 70 also serves a role in cellular signaling by modulating the JNK and p38 MAPK pathways, contributing to its cytoprotective functions under stress conditions (27). Moreover, HSP 70 is involved in antigen presentation, enhancing the immune response by facilitating the maturation of dendritic cells and improving the presentation of antigenic peptides on MHC class I molecules (28). Furthermore, HSP 70 activates STAT3 and ERK signaling pathways in myeloid-derived suppressor cells (MDSCs) to increase IL-10 expression and suppress immune responses (14).

The present study focused on HSP 70 due to its crucial roles in modulating immune responses and regulating proinflammatory cytokine production, which are key pathways in psoriasis pathogenesis. While HSP 90 also serves important roles in protein folding and stabilizing various regulatory proteins (29), its anti-inflammatory functions are less direct compared with HSP 70. The present study showed that brevilin A can induce HSP 70 expression, which may be associated with reduced inflammatory responses in psoriasis. This finding provided a strong basis for focusing on HSP 70 to understand the therapeutic effects of brevilin A. Future research could explore the potential synergistic effects of targeting both HSP 70 and HSP 90; however, the present study highlighted the critical role of HSP 70 in the effects of brevilin A, offering insights into its potential as a treatment for psoriasis and other IL-17A-induced inflammatory diseases.

In the present study, dynamic, time-dependent variations in the expression of HSP 70 and HSP 90 were observed following treatment with IL-17A and brevilin A. Specifically, western blotting results showed significant upregulation at the 6- and 9-h time points, whereas the 24-h data did not achieve statistical significance. This discrepancy may be attributed to peak expression periods occurring at earlier time points, reflecting a transient response. By 24 h, cellular regulatory mechanisms may have modulated the expression levels, leading to variability between samples. Notably, the current study did not observe significant downregulation of HSP 70 or HSP 90 at the 24-h mark compared with in the control and IL-17-treated groups. These findings align with the known dynamic nature of HSP expression, which can vary based on treatment duration, stress intensity, and cellular context (30). The significant induction of HSP 70 and HSP 90 at earlier time points underscores their role in the cellular response to IL-17A and brevilin A treatment, even as their expression levels fluctuate over time.

The present study adopted the approach of simultaneous model creation and drug administration to better simulate a clinical treatment scenario. This approach reflects real-world therapeutic interventions, where treatment typically begins at the early onset of disease or cellular stress, rather than as a preventive measure. The aim was to evaluate the therapeutic effects of the drug in modulating disease progression or the inflammatory response during the early stages, which aligns more closely with clinical practice. While a pretreatment approach might be valuable for studying preventive effects, it does not accurately represent the timing of most clinical interventions. For future investigations, comparing pretreatment and simultaneous administration strategies could provide further insights into the influence of treatment timing on drug efficacy.

IL-17A induces inflammatory cytokines as a result of activating several intracellular signaling pathways, such as NF-κB, MAPK or C/EBPs (31). In the present study, it was observed that brevilin A reduced the levels of IL-17A-induced proinflammatory cytokines. Furthermore, HSP 70 is known to regulate NF-κB and MAPK signaling pathways (32); therefore, in the current mechanistic study, a specific HSP 70 inhibitor, apoptozole, which blocks the interaction between HSP 70 and related intracellular proteins (33), was used to identify the possible mechanism underlying the effects of brevilin A on reducing IL-17A-induced inflammatory responses in HaCaT cells. The results demonstrated that apoptozole reversed the effects of brevilin A on IL-6 and IL-8 levels, indicating that HSP 70 may serve a major role in brevilin A-regulated signaling pathways. However, the expression levels of NF-κB- and MAPK-related protein in the apoptozole- and brevilin A-co-treated group remain to be clarified in future studies.

The present study demonstrated that brevilin A reduced IL-17A-induced IL-6 and IL-8 levels in HaCaT cells through upregulating the HSP 70 expression. However, there are some limitations in this study. First, despite evidence that brevilin A ameliorated psoriasis in an IMQ-induced murine model, *in vivo*

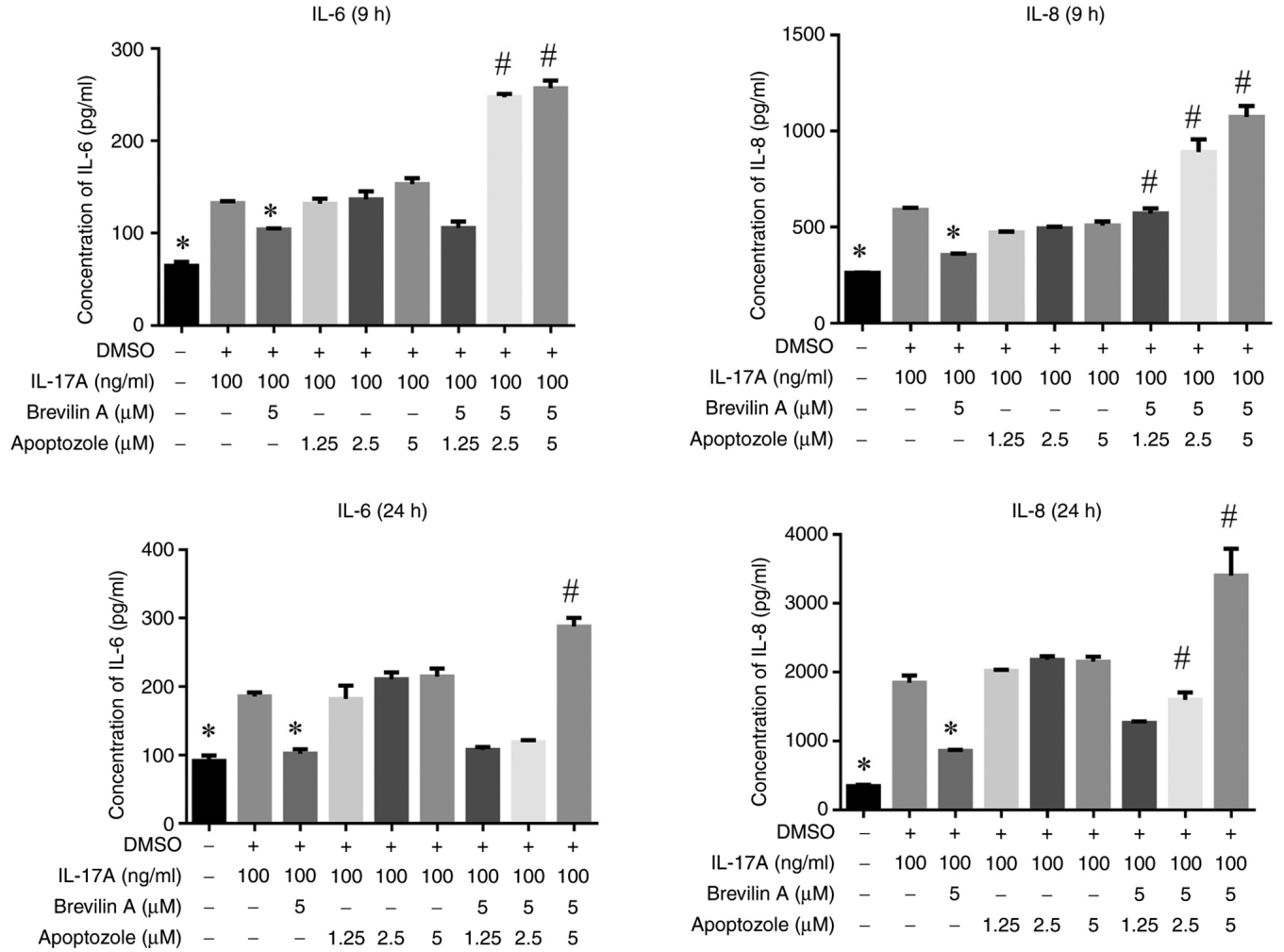

Figure 5. Apoptozole neutralizes the effects of brevilin A on decreasing IL-17A-induced IL-6 and IL-8 levels. HaCaT cells were seeded at a density of $2x10^5$ cells/well in a 24-well plate, and were co-treated with IL-17A (100 ng/ml), either brevilin A (5 μM) and different concentrations of apoptozole (1.25, 2.5 and 5 μM) for 9 and 24 h. Levels of IL-6 and IL-8 were measured by ELISA. Data are presented as the mean ± SEM (n=3). *P<0.05 vs. IL-17A-treated group; #P<0.05 vs. IL-17A + brevilin A co-treated group.

data on the reversal of brevilin A treatment effects by HSP 70 inhibitors were lacking. Second, HSP 70 could have interacted with numerous intracellular proteins to inhibit inflammatory responses, wheras the present study only demonstrated that HSP 70 expression was upregulated after brevilin A treatment. Other proteins that are also regulated by HSP 70 remain to be investigated in the future. Third, the protein expression levels of IL-8 could not be assessed in skin tissues from brevilin A-treated mice. The present study focused on HSP 70 due to its well-established roles in anti-inflammatory and cytoprotective mechanisms, which are directly relevant to the therapeutic effects of brevilin A on psoriasis. HSP 70 has been shown to modulate inflammatory responses and enhance cellular protection under stress conditions, making it a key target in the context of psoriasis pathophysiology. While HSP 90 is another important member of the HSP family, its primary functions are more closely associated with protein stabilization and signal transduction, which were beyond the scope of this investigation. The present findings on the upregulation of HSP 70 further support its potential as a critical mediator in the anti-inflammatory effects of brevilin A. Future studies could explore the role of HSP 90 in psoriasis to provide a more comprehensive understanding of HSPs in this disease model.

In conclusion, the present study demonstrated that brevilin A reduced IL-17A-induced IL-6 and IL-8 levels through upregulating HSP 70 expression. The results indicated that brevilin A not only regulates immune cell activation, but can also inhibit proinflammatory cytokine-induced inflammatory responses in keratinocytes. These results suggested that brevilin A may be a promising compound that could be applied to treat psoriasis or other inflammatory skin disorders.

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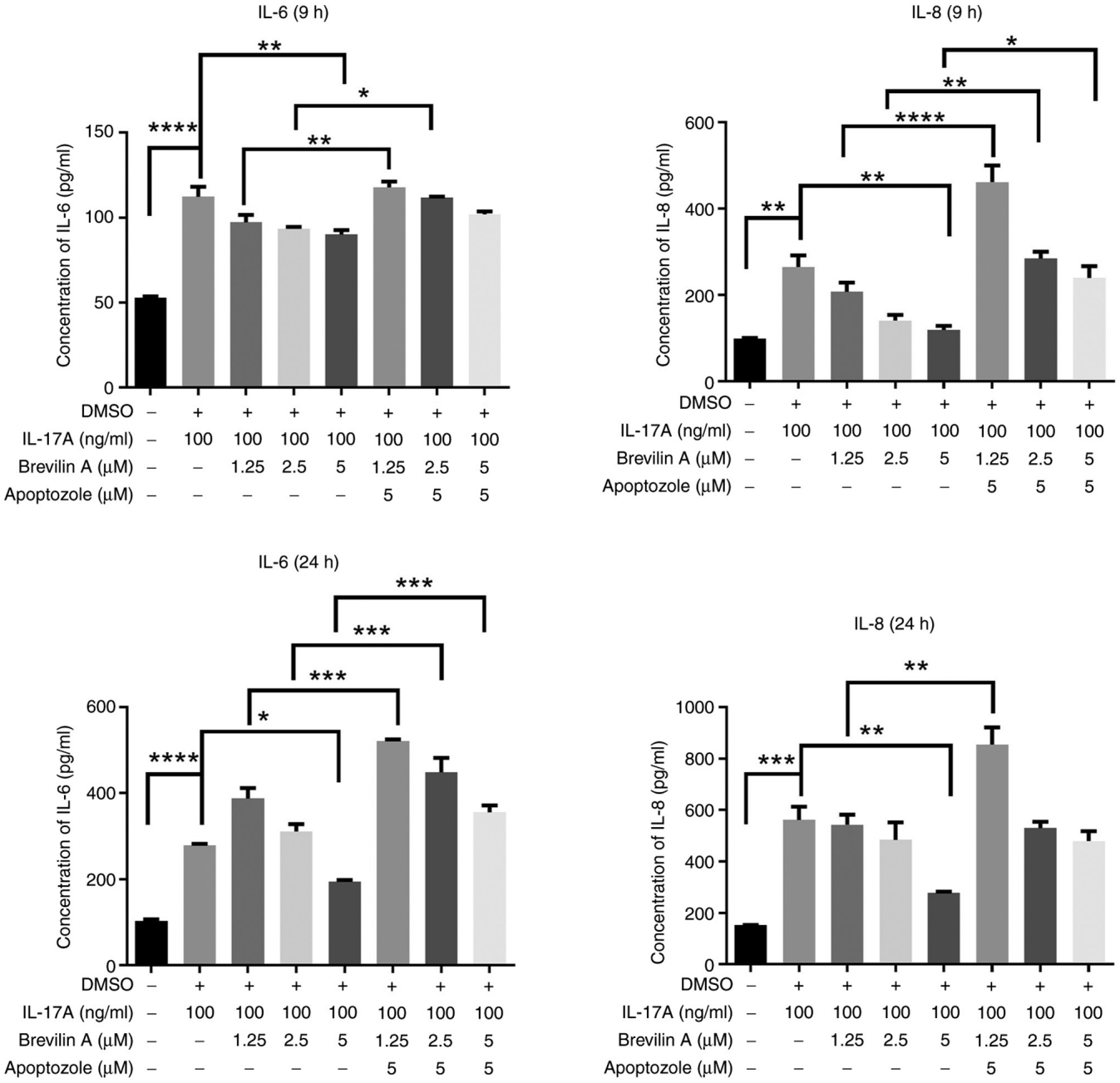

Figure 6. Effects of brevilin A treatment on anti-inflammatory cytokines were reversed by apoptozole treatment. HaCaT cells were seeded at a density of $2x10^5$ cells/well in a 24-well plate, and were co-treated with IL-17A (100 ng/ml), brevilin A (1.25, 2.5 and 5 μM) and apoptozole (5 μM). Samples were collected after 9 and 24 h of treatment. Levels of IL-6 and IL-8 were measured by ELISA. Data are presented as the mean ± SEM (n=3). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

CYY and SJY conceptualized the study. CLL, KYY, WNH, and ECL designed the methodology. KCW, ECL, HSH, TYC and CLL conducted the formal analysis. KCW, KYY, TYC, HSH, WNH and SJY carried out the investigation. CLL and SJY prepared the original draft of the manuscript, and CYY reviewed and edited it. SJY and CYY were responsible for project administration and secured funding for the study. CLL, SJY and TYC confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The animal experimental protocol was approved by the Institutional Animal Care and Use Committee of Kaohsiung Veterans General Hospital (2021-A039).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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