

Original Article

Effects of Echinocystic Acid on Atopic Dermatitis and Allergic Inflammation of the Skin and Lungs

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ABSTRACT

Background: Echinocystic acid (ECA), a pentacyclic triterpene enriched in various herbs, promotes anti-inflammatory and antioxidant activity; however, its therapeutic effects on atopic dermatitis (AD) or atopic march and the underlying mechanisms of action have not yet been fully elucidated.

Purpose: This study aimed to elucidate the effects and molecular mechanisms of ECA on AD and allergic inflammation.

Methods: We evaluated the inhibitory effects of ECA using a house dust mite (HDM)-induced AD mouse model and human keratinocytes.

Results: The results revealed that ECA improved AD symptoms by decreasing epidermal/dermal thickness, immune cell infiltration, and restoring skin barrier function, as well as an imbalanced immune response. In addition, repeated epicutaneous HDM challenges aggravated allergic inflammation in mice lungs, which was caused by the infiltration of immune cells and collagen deposition, whereas ECA alleviated these symptoms. Moreover, ECA suppressed the expression of T helper cell-derived cytokines, phosphorylation of extracellular signal-regulated kinase, and signal transducer and activator of transcription 1 in the skin and lungs of mice with HDM-induced AD, as well as inhibited the translocation of nuclear factor- κ B in HaCaT keratinocytes.

Conclusion: This is the meaningful study to demonstrate that ECA improves allergic inflammation of the skin and lungs through recovery of the skin barrier, regulation of immune balance, and alleviation of lung inflammation, suggesting that ECA has therapeutic potential as an antiatopic and antiallergic agent that blocks the progression of AD to atopic march.

Introduction

Atopic dermatitis (AD) is the most common chronic skin inflammatory disease, affecting up to 20% of children and adolescents worldwide (Kowalska-Oledzka et al., 2019). The development of AD in infancy and subsequent comorbidities such as allergic rhinitis and allergic asthma in later childhood is called the atopic march (AM). The development of AD and AM is strongly influenced by a wide range of factors, mainly

highlighted by structural abnormalities in the stratum corneum, the outer layer of the epidermis, and immune dysregulation, resulting in not only a vulnerable skin barrier but also chronic immune activation that contributes to the development of asthma and allergies (Yang, Fu, et al., 2020).

The skin of patients with AD displays impaired barrier function, thus allowing for easy entry of allergens, pathogens, and other environmental insults, as well as increased transepidermal water loss (TEWL) which is

Abbreviations: AD, atopic dermatitis; AEDS, atopic eczema/dermatitis syndrome; AM, atopic march; DEX, dexamethasone; ECA, echinocystic acid; ERK, extracellular signal-regulated kinase; H&E, hematoxylin and eosin; HDM, house dust mite; IFN, interferon; IgE, immunoglobulin E; IKK, I κ B kinase; IL, interleukin; JAK, Janus kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF- κ B, nuclear factor- κ B; STAT, signal transducer and activator of transcription; TAK1, transforming growth factor β -activated kinase 1; TEWL, transepidermal water loss; TNF, tumor necrosis factor; Th, T helper; TSLP, thymic stromal lymphopoietin.

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observed in both lesional and non-lesional skin (Drislane et al., 2020; Zheng et al., 2011). This is not merely an epiphenomenon but rather the “driver” of the progression of AD and is now considered the primary mechanism of developing the disease (Elias et al., 2009). These skin irregularities are owing to dysfunctional filaggrin, a critical component of the cornified envelope, and disordered tight junctions (Yang, Seok, et al., 2020). In addition, the barrier of defective skin promotes allergen sensitization and leads to increased immunoglobulin E (IgE) levels, airway hyper-responsiveness, and airway inflammation (Kim et al., 2018). Activated macrophages and mast cells by allergens mediate inflammatory cells, such as T helper (Th)2 cells, eosinophils, and neutrophils, resulting in airway hyper-responsiveness, airway narrowing, lung inflammation, and allergic asthma symptoms (Sun et al., 2020). Th cell subsets that secrete specific cytokines play a key role in regulating the immune response and inflammatory processes in AD (Gavrilova, 2018). After exposure to allergens, keratinocytes in the skin of patients with AD produce distinct cytokine and chemokine profiles that promote Th2 cell-predominant inflammatory responses in acute lesions of AD, followed by chronic AD characterized by prominent Th1-induced inflammation (Akdis et al., 2020). Typically, patients with extrinsic AD have elevated Th2- and decreased Th1-expressing cells in their peripheral blood, as well as elevated interleukin (IL)-4, IL-5, and IL-13 expression (Humeniuk et al., 2017). The Th1:Th2 cell ratio is regulated by a dynamic and reversible balance; however, the balance of this ratio is skewed, and polarity shifts to the other side in response to foreign antigens (Wei et al., 2018).

Although topical steroids and immunosuppressant agents are widely used to treat AD, they are associated with long-term cutaneous adverse effects, including skin burning, irritation, atrophy, and telangiectasia (Hwang et al., 2019). Hence, the pharmacological activities of bioactive components from natural resources have received considerable attention for the development of better therapeutic options for sustainable treatment. Echinocystic acid (ECA) is a natural pentacyclic triterpene enriched in herbs such as *Eclipta prostrata* L. (Asteraceae), and reportedly has anticancer (Tong et al., 2004), antiatherosclerotic, antioxidant (Lai et al., 2014), and anti-inflammatory (Joh et al., 2014; Ma et al., 2016) effects. Furthermore, many reports have shown that plant-derived triterpenoids possess a wide spectrum of pharmacological properties associated with allergic disorders, such as anaphylaxis (Kang et al., 2021b), allergic rhinitis (Piao et al., 2020), and asthma (Kamaraj et al., 2021), supporting their evidence-based therapeutic effects in allergic diseases. However, the inhibitory effect of ECA on the progression of AD and the AM remains unclear, and the underlying mechanisms of action are not well understood. The aim of this study was to elucidate the effects of ECA as well as its molecular mechanisms in managing AD and the spontaneous AM using a house dust mite (HDM) (*Dermatophagoides farinae*) body-induced NC/Nga mouse model and human keratinocytes.

Materials and methods

HDM-induced AD model

Six-week-old male NC/Nga mice (20–25 g body weight; n = 30) were purchased from Daehan BioLink Inc. (Eumsung, Korea), a branch of Charles River Japan (Kanagawa, Japan), and housed under constant conditions at a humidity of 40–60%, temperature of 20–25 °C, and a 12 h light/dark cycle. The mice were randomly assigned to one of five groups (n = 6 per group): the control, HDM-induced AD, dexamethasone (DEX, 5 mg/kg) oral administration, and ECA (4 and 8 mg/kg)-treated groups. The ECA concentration was determined according to previous studies (Hyam et al., 2013; Li et al., 2016) and lower concentrations were used for safety. DEX, an immune suppressor, was used as a positive control. Animal experiments were performed as previously described (Kang et al., 2021a; Lee et al., 2015) with modifications. HDM (*Dermatophagoides farinae*) body ointment (100 mg) (Biostir-AD; Biostir, Hyogo, Japan) were topically applied to the shaved area (3 × 4 cm²) of the

dorsal surface to induce AD-like skin lesions. The HDM application was repeated twice weekly for 8 weeks. To disrupt the skin barrier, shaved dorsal skin was applied with 150 µl of 4% sodium dodecyl sulfate 3 h prior to HDM application. After AD-like symptoms were induced by the first challenge for 4 weeks, the mice were treated with ECA (oral administration) and DEX (dissolved in phosphate-buffered saline [PBS], oral administration) 4 h after HDM treatment, once a day. The control group received the same volume of PBS. At the end of the experiment, the mice were sacrificed. The experimental scheme for the *in vivo* study is summarized in Fig. 1B. After sacrificing the mice, skin tissues were subjected to histological and western blot analyses. All procedures involving animals were performed in accordance with university guidelines and were approved by the Institutional Animal Care and Use Committee of Korean Medicine, Sangji University (Wonju, Korea; approval no. 2019-17, 05-12-2019).

AD severity scoring

The AD severity index assesses the intensity of skin symptoms, scarring/dryness, erythema/hemorrhage, excoriation/erosion, and edema, which are scored as follows: 0, none; 1, mild (< 20%); 2, moderate (20–60%); or 3, severe (> 60%). The index was evaluated weekly throughout the experiment. The scores were determined in agreement among three observers and were calculated as the sum of the individual scores for each mouse and group and expressed as means with standard deviations.

HaCaT keratinocytes culture and treatment

The human keratinocyte cell line HaCaT was generously provided by Professor Jae-Young Um (Kyung Hee University, Republic of Korea) and was grown at 37 °C in Dulbecco's Modified Eagle Medium supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% fetal bovine serum in a humidified atmosphere of 5% CO₂. HaCaT keratinocytes were seeded (1 × 10⁵ cells per well), stabilized for 24 h, treated with ECA (5 and 10 µM) for 1 h, and then stimulated with 10 ng/ml tumor necrosis factor (TNF)-α/interferon gamma (IFN-γ) at 37 °C in humidified air with 5% CO₂. ECA was dissolved in dimethyl sulfoxide (DMSO) for the *in vitro* experiments. Cells treated with DMSO were used as controls.

Measurement of cell viability

HaCaT keratinocytes were seeded (5 × 10⁴ cells per well) in a 96-well culture plate and stabilized for 24 h. After incubation for 24 h with various concentrations of ECA, the cells were treated with 50 µl of MTT (5 µg/ml) for 4 h. The purple formazan precipitates were dissolved in 100 µl DMSO, and the optical density was measured at 540 nm using a microplate reader.

Statistical analysis

Data are expressed as the mean ± standard deviation of triplicate experiments. Statistically significant differences were compared using one-way analysis of variance with Dunnett's post-hoc test. *p* < 0.05 was considered statistically significant. Statistical analyses were performed using GraphPad Prism (version 5, GraphPad Software, San Diego, CA).

The detailed protocols are described in the Supplementary Materials online.

Results

Effect of ECA on severity of skin symptoms in HDM-induced AD mice

Repeated topical application of HDM resulted in AD-like skin lesions, including erythema, excoriation, edema, dryness, and erosion, with

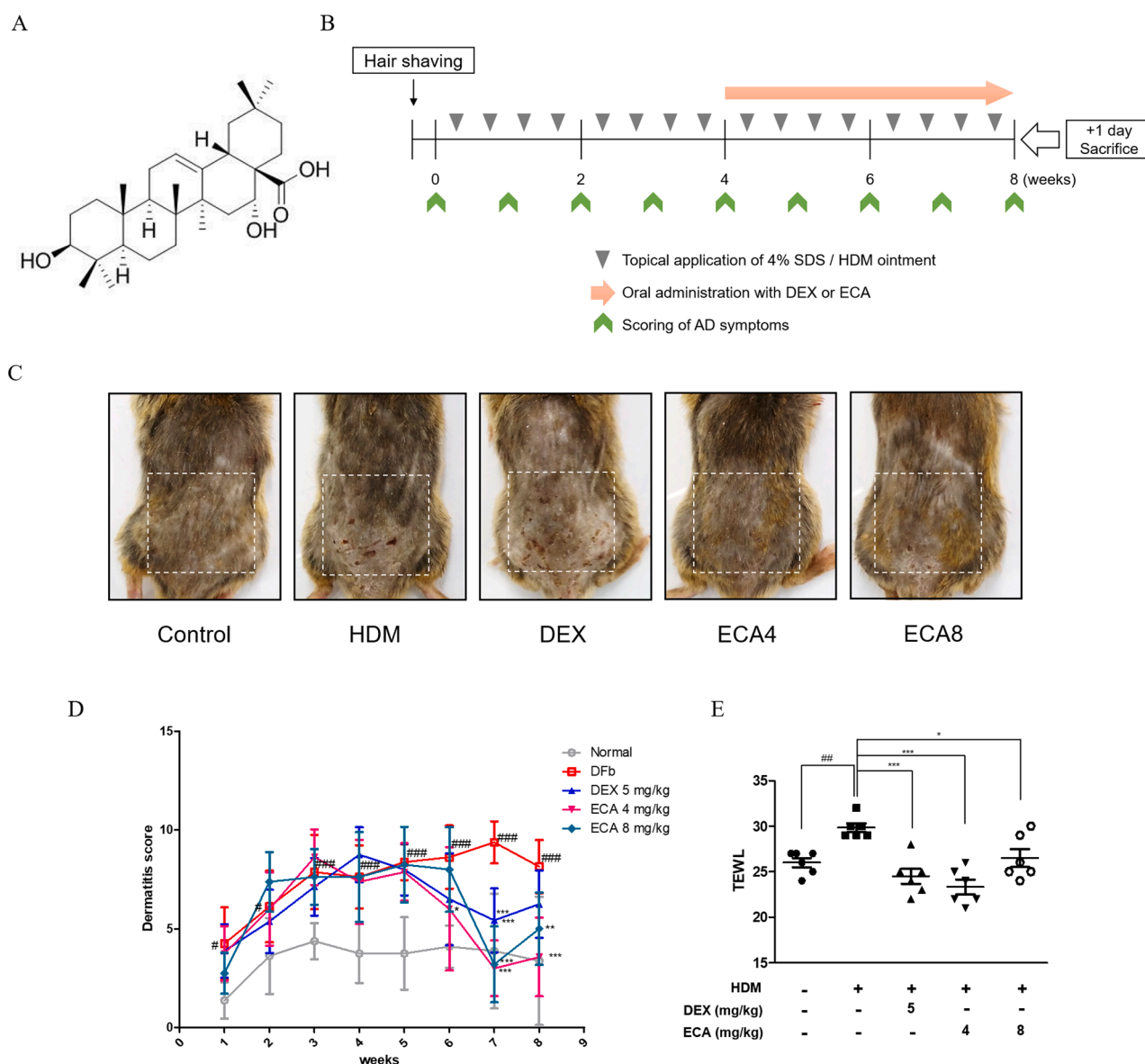


Fig. 1. Effects of ECA on the clinical features of HDM-induced AD skin in NC/Nga mice. (A) Chemical structure of ECA. (B) Schematic representation of the experiment. (C) Clinical features of AD-skin symptoms. (D) Dermatitis scores were recorded once a week for 8 weeks. (E) TEWL was measured at end of 8 weeks. The data shown represent mean $\pm$ S.D. (n = 6) of three 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. HDM-induced AD group.

higher skin severity scores observed in mice with HDM-induced AD than in control mice. Oral administration of ECA improved these skin symptoms and dermatitis scores compared with those of untreated mice, as depicted in Fig. 1D. Next, as an indicator of skin barrier function and hydration, TEWL in the mice dorsal skin was measured at the end of the experiment. The results showed that TEWL was significantly higher in the dorsal skin of mice with HDM-induced AD than in control mice, whereas treatment with ECA significantly decreased the TEWL value (Fig. 1E).

Effect of ECA on epidermal and dermal thickness and inflammatory cell infiltration in HDM-induced AD mice

Histological analysis of hematoxylin and eosin (H&E)-stained dorsal skin sections showed hyperkeratosis, epidermis thickening, and dense inflammatory cell infiltration into the skin of HDM-induced AD mice compared with those of control mice. However, oral administration of ECA significantly decreased epidermal thickness compared with that

observed in HDM-induced AD mice (Fig. 2A and D). The numbers of infiltrating mast cells and eosinophils were significantly increased in the dermis of HDM-induced AD mice compared with those observed in the skin of control mice (Fig. 2B and C). However, the infiltration of mast cells and eosinophils in the skin of mice in the ECA-treated groups was remarkably reduced (Fig. 2E and F).

Effect of ECA on skin barrier function and inflammatory cytokine production in HDM-induced AD mice

To investigate the role of ECA in barrier dysfunction, filaggrin expression in the dorsal skin of mice was determined. In comparison to control mice, the protein expression of filaggrin in the dorsal skin was decreased by HDM, indicating impaired skin barrier function. However, oral administration of ECA enhanced filaggrin expression compared with that observed in untreated mice with HDM-induced AD (Fig. 3A and B). These results suggest that ECA improves skin barrier function by restoring filaggrin expression and skin hydration. Crucial pro-

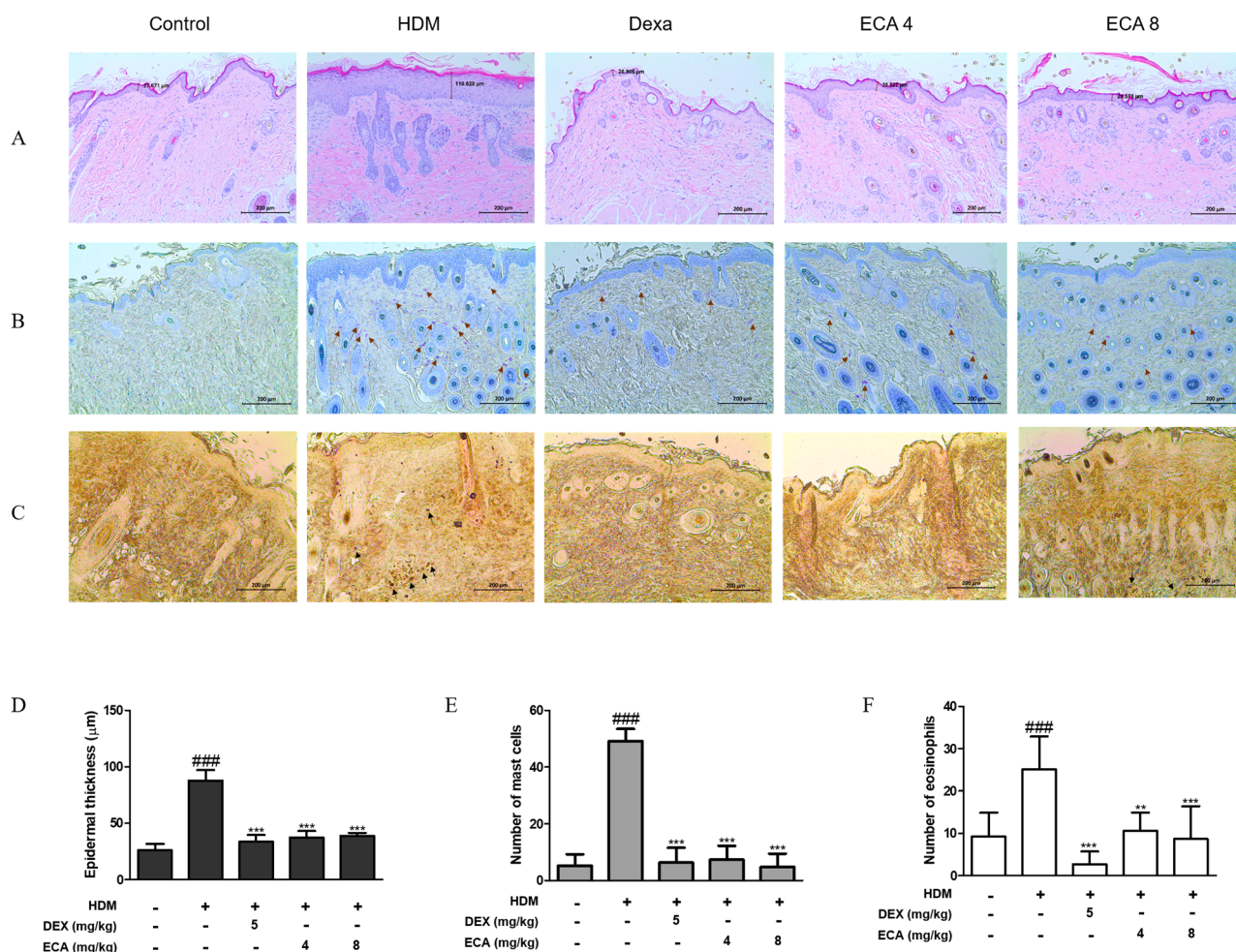


Fig. 2. Effects of ECA on the histological alterations in HDM-induced AD skin in NC/Nga mice. The sections were stained with (A) H&E and (B) toluidine blue. Brown arrows indicated stained mast cells. (C) The sections were stained with Congo Red. Black arrows indicated stained eosinophils (scale bar = 200 μm). (D) Epidermal thickness in H&E stained sections were measured under a microscope. (E) Mast cells and (F) eosinophils were counted with a microscope. The data shown represent mean ± S.D. (n = 6) of three independent experiments. ^{###} $p < 0.001$ vs. the control group; ^{**} $p < 0.01$, and ^{***} $p < 0.001$ vs. HDM-induced AD group.

inflammatory cytokines, such as thymic stromal lymphopoietin (TSLP) and TNF- α , which are involved in skin inflammation, were identified in the dorsal skin of HDM-induced AD mice. TSLP and TNF- α levels were significantly increased in mice from the aforementioned group compared with those from the control group. However, oral administration of ECA significantly inhibited these cytokine levels in HDM-induced AD mice (Fig. 3C and D), suggesting that ECA attenuates skin inflammatory responses elicited by HDM.

Effect of ECA on STAT1 expression, ERK pathway, and AD-related cytokines in skin of HDM-induced AD mice

To investigate the possible protective mechanisms of ECA against AD-induced skin barrier disruption and immune dysregulation, we confirmed the involvement of signal transducer and activator of transcription (STAT), extracellular signal-regulated kinase (ERK), and mitogen-activated protein kinase (MAPK). HDM resulted in strong phosphorylation of both STAT1 and ERK protein expression levels relative to those observed in control mice. However, these expressions were suppressed in mice with HDM-induced AD following oral administration of ECA (Fig. 4A). These data indicate that HDM-induced AD skin barrier dysfunction and skin inflammation involve the activation of STAT1 and the ERK/MAPK pathway, which are inhibited by ECA. As shown in Fig. 4B–E, mRNA expressions of IL-4, IL-13, TSLP, and IL-1 β were significantly higher in the dorsal skin of HDM-induced AD mice

than those observed in control mice. Oral administration of ECA significantly suppressed the expression of these cytokines in the skin of HDM-induced AD mice. Overall, treatment with ECA-modulated cytokine expression in the skin of AD mice.

Effect of ECA on lung inflammation in HDM-induced AD mice

Following the assessment of H&E-stained lung sections, mixed peribronchial and perivascular inflammatory cellular infiltrates with basal membrane thickening were observed in ECA-treated mice with HDM-induced AD, reflecting compromised lung function (Fig. 5A). Moreover, amorphous eosinophilic amyloids were observed in these lung tissue samples, indicating pulmonary amyloidosis (Fig. 5B). Consistent with the altered peripheral changes in the lungs of these animals, marked collagen deposition was observed, indicating pulmonary inflammation, such as fibrosis (Fig. 5C). However, ECA treatment alleviated these alterations in the lung tissues of these mice (Fig. 5A–C) and improved the histological lung injury score and collagen content (%) in HDM-induced AD mice (Fig. 5D–E). These results showed that spontaneous lung inflammation occurred in mice with HDM-induced AD, and that these alterations were alleviated by treatment with ECA.

To confirm the role of ECA in STAT1 expression and the ERK pathway in alleviating inflammation in the lungs of HDM-induced AD mice, the protein levels of phosphorylated STAT1 and ERK were measured. HDM induced significant phosphorylation of STAT1 and ERK,

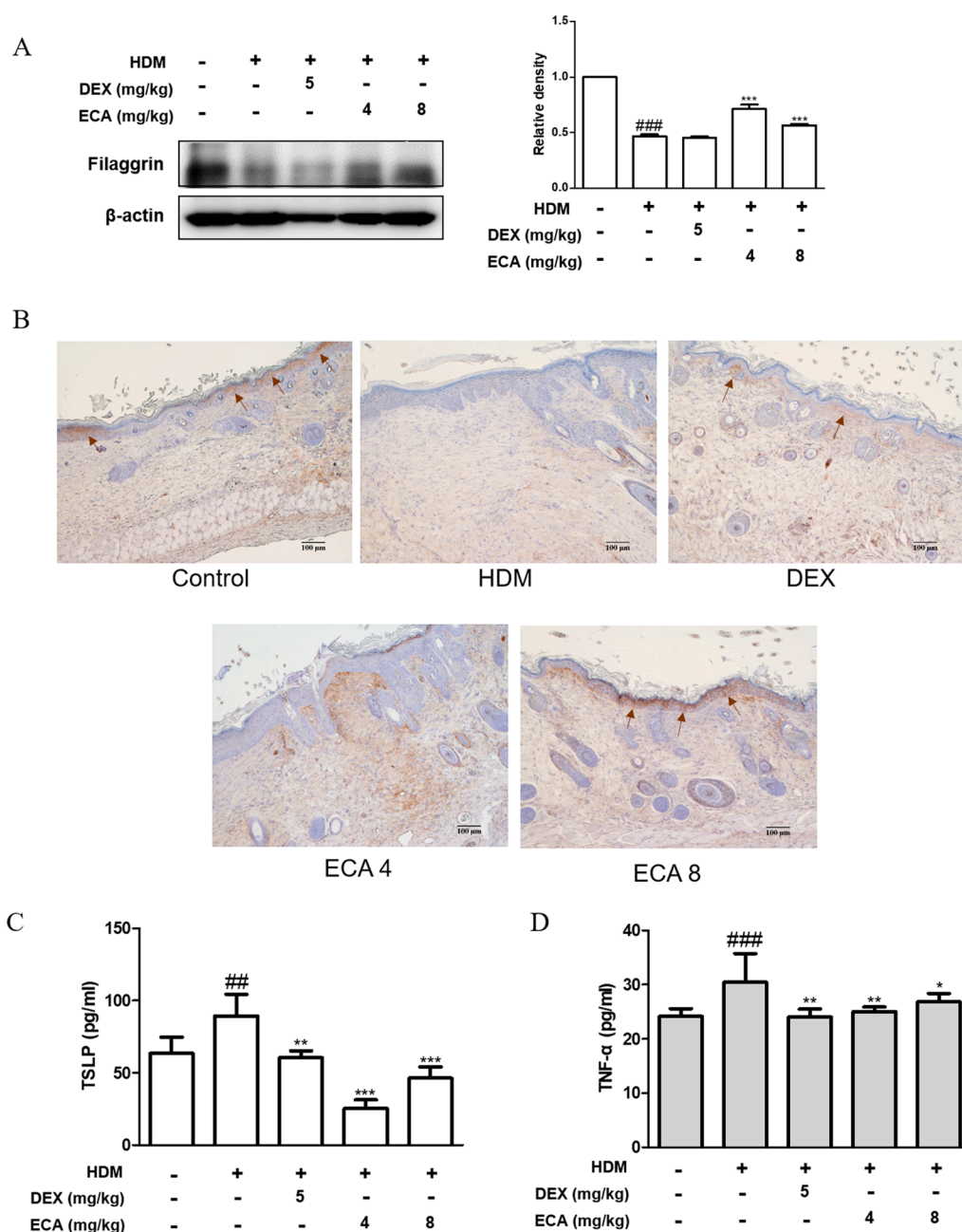


Fig. 3. Effects of ECA on the skin barrier function in HDM-induced AD skin in NC/Nga mice. (A) Total proteins were prepared and western blotted for filaggrin using specific antibodies. β -actin was used to normalize the protein expression. Densitometric analysis was performed using Bio-Rad Quantity One® Software. (B) Skin sections were stained with filaggrin using immunohistochemical staining. Brown arrows indicated stained. Bars indicate 100 μ m. Dorsal protein levels of (C) TSLP and (D) TNF- α were determined by using ELISA kits. The data shown represent mean $\pm$ S.D. ($n = 6$) of three independent experiments. ## $p < 0.01$, ### $p < 0.001$ vs. the control group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. HDM-induced AD group.

whereas ECA suppressed their expression (Fig. 5F). These data show that ECA is capable of preventing the development of lung inflammation and progression to the AM through the inhibition of STAT1 or the ERK pathway in HDM-induced AD mice. The epithelial cell-derived cytokine, TSLP, is a potent inducer of type 2 inflammation at barrier sites, and Th2 cells are increased in the circulation with activated multiple cytokine axes (Czarnowicki et al., 2017). In particular, Th2 cytokines induce AD and the AM via a TSLP-dependent mechanism (Zhu et al., 2011). We confirmed that TNF- α , IL-6, IL-4, IL-5, and IL-13 were markedly upregulated in the lung tissues of HDM-induced AD mice compared with the control group. However, ECA treatment downregulated the expression of these genes (Supplementary Fig. 1). These results suggest that treatment with ECA attenuated lung inflammation and blocked AM progression through the modulation of AD-related inflammatory cytokine expression in the lung tissue and skin of HDM-induced AD mice.

Effect of ECA on production of inflammatory cytokines and expression of filaggrin in TNF- α /IFN- γ -stimulated HaCaT keratinocytes

An MTT assay was conducted to determine the cytotoxicity of various ECA concentrations on human keratinocytes (HaCaT). After 24 h of treatment with ECA, cell viability steadily increased at 25 μ M and then declined at 50 and 100 μ M (Fig. 6A). Therefore, ECA concentrations of 5 and 10 μ M were selected for subsequent assays. The production levels of pro-inflammatory cytokines (TNF- α , IL-6, and IL-1 β) were significantly increased following stimulation with TNF- α /IFN- γ in HaCaT keratinocytes compared with the normal group. However, ECA treatment significantly reduced TNF- α , IL-6, and IL-1 β production compared to that in TNF- α /IFN- γ -stimulated HaCaT keratinocytes (Fig. 6B-D). Moreover, our results showed that the expression of filaggrin was diminished by stimulation with TNF- α /IFN- γ , whereas it was significantly increased upon treatment with ECA in HaCaT keratinocytes (Fig. 6E).

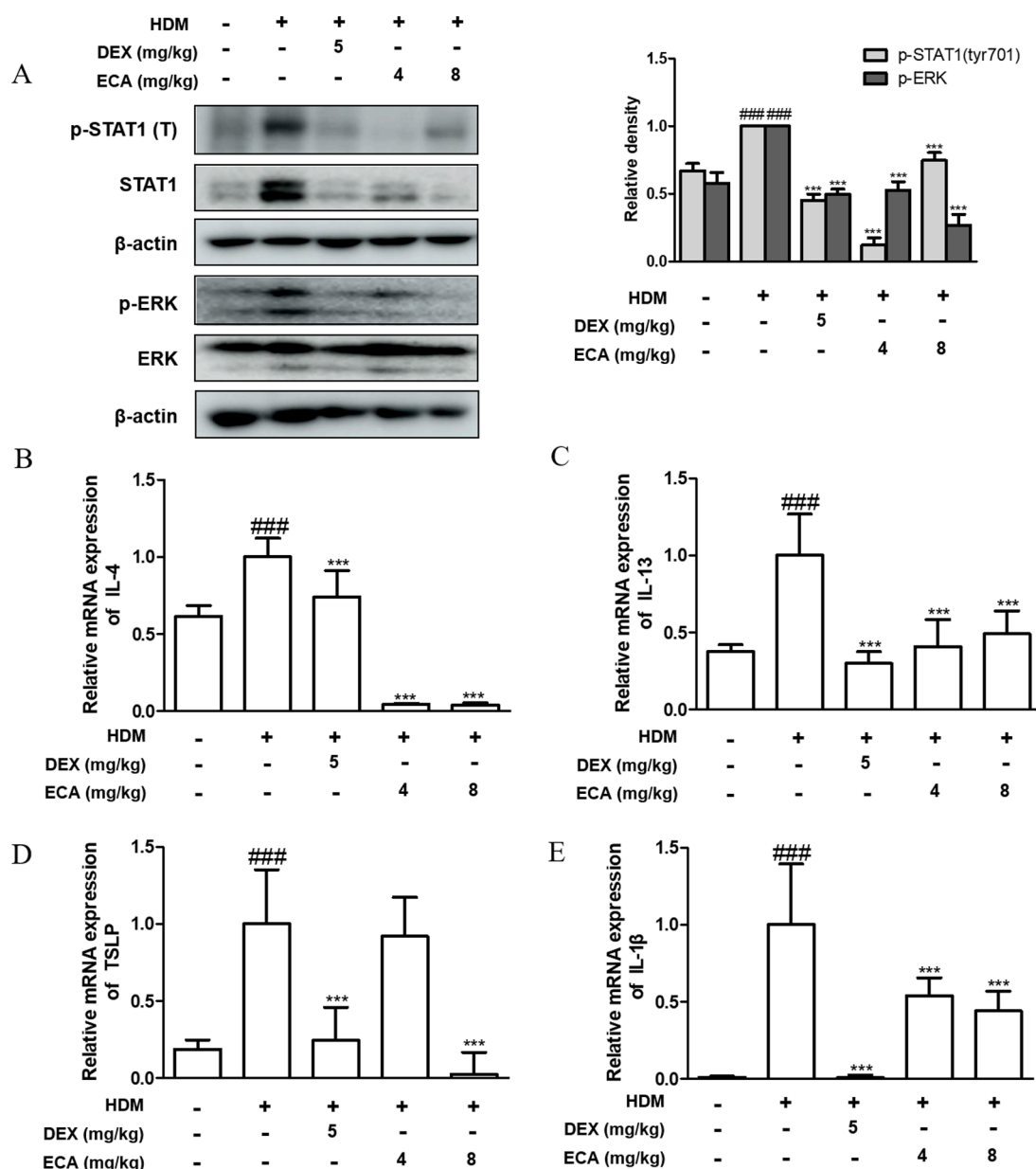


Fig. 4. Effects of ECA on STAT1 and ERK activation and AD-related cytokines in HDM-induced AD skin in NC/Nga mice. (A) Total proteins were prepared and western blotted for p-STAT1 (tyr701), STAT1, p-ERK, and ERK using specific antibodies. β -actin was used to normalize the protein expression. Densitometric analysis was performed using Bio-Rad Quantity One® Software. Total RNA prepared from the dorsal tissue, and the levels of (B) IL-4, (C) IL-13, (D) TSLP, (E) IL-1 β were determined by qRT-PCR. The data shown represent mean $\pm$ S.D. (n = 6) of three independent experiments. ### $p < 0.001$ vs. the control group; *** $p < 0.001$ vs. HDM-induced AD group.

Effect of ECA on activation of AD-related signaling pathways in TNF- α /IFN- γ -stimulated HaCaT keratinocytes

To elucidate the underlying mechanisms of ECA, the protein levels and phosphorylation of transforming growth factor β -activated kinase 1 (TAK1), a key signaling component of the MAPK and nuclear factor- κ B (NF- κ B) signaling pathways, and mitogen-activated protein kinase kinase (MEK)/ERK kinase were determined using western blot analysis. These results demonstrated that TAK1, MEK1/2, and ERK were phosphorylated by TNF- α /IFN- γ stimulation. However, phosphorylation was significantly inhibited following ECA treatment (Fig. 7A). NF- κ B, p65, and STAT1 are transcription factors fundamentally involved in AD-related signaling in TNF- α /IFN- γ -stimulated HaCaT cells. Therefore, we investigated whether ECA affected these transcription factors in HaCaT keratinocytes. As shown in Fig. 7B, TNF- α /IFN- γ induced the

phosphorylation of I κ B kinase (IKK) α/β and NF- κ B translocation, whereas treatment with ECA significantly suppressed the phosphorylation of IKK α/β and p65 translocation to the nucleus. Moreover, TNF- α /IFN- γ -induced phosphorylated STAT1 expression decreased in the ECA-treated groups (Fig. 7C).

Discussion

Recently, the comprehensive term “atopic eczema/dermatitis syndrome” (AEDS) was recommended by the European Academy of Allergy and Clinical Immunology, EAACI, nomenclature task force to cover the different subtypes of AD (Cuppari et al., 2016; Scheynius et al., 2002). AEDS highlights the fact that AD is not a single disease but rather an aggregation of several diseases, reflecting the heterogeneity of particular clinical symptoms. Patients with allergic AEDS have associated

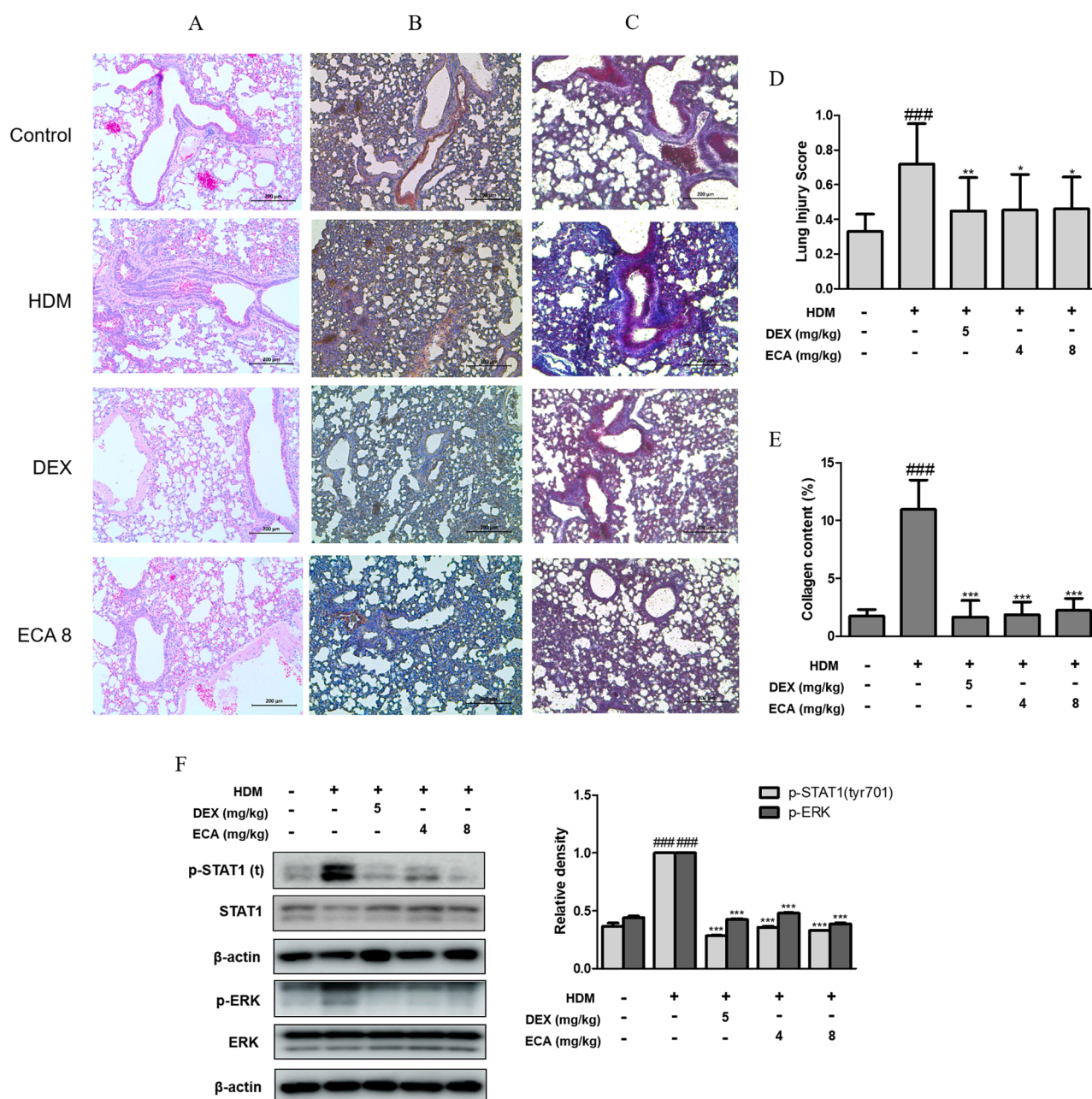


Fig. 5. Effects of ECA on histological alterations and STAT1 and ERK in HDM-induced AD lung in NC/Nga mice. Representative lung tissue images of histological examination (scale bar = 200 μ m). (A) The sections were stained with H&E, (B) Congo Red to detect amyloid plaques, and (C) Masson's trichrome staining to detect collagen deposition. (D) Histological injury scores of the lungs in different groups were quantified. (E) Quantification of collagen content (%) after Masson's trichrome staining. (F) Total proteins were prepared and western blotted using specific antibodies. β -actin was used to normalize the protein expression. Densitometric analysis was performed using Bio-Rad Quantity One® Software. The data shown represent mean $\pm$ S.D. ($n = 6$) of three independent experiments. ^{###} $p < 0.001$ vs. the control group; ^{*} $p < 0.05$, ^{**} $p < 0.01$, and ^{***} $p < 0.001$ vs. HDM-induced AD group.

respiratory diseases, such as allergic rhinitis or bronchial asthma, and show high total serum IgE levels (Wuthrich et al., 2003). This definition seems more appropriate for explaining the AM, which is characterized by the sequential development of symptoms, such as asthma, allergic rhinitis, or both, after AD onset. In this study, the effects of ECA and its mechanisms of action were investigated from the perspective of AD-induced skin barrier dysfunction, immune dysregulation, and secondary lung inflammation as symptoms of AM progression.

Reduced expression of filaggrin, loricrin, and involucrin, the cornified-envelope proteins, has been demonstrated in lesioned skin of patients with AD, which contributes to skin barrier defects in this disease. In addition, the expression of these proteins has been shown to be

downregulated by Th2-type cytokines, including IL-4 and IL-13 (Combarros et al., 2020). Our results provide evidence that ECA restored HDM-induced impaired skin barrier function and TEWL by restoring filaggrin upregulation of (Figs. 1 and 3). The significance of filaggrin deficiency has been acknowledged in the progression to the AM, showing that filaggrin mutations predispose individuals to allergic rhinitis, asthma, and allergic sensitization (Cepelak et al., 2019). In line with this, our results support that filaggrin plays an important role in skin barrier function and the pathogenesis of AD, and is an indicator of the AM.

In patients with AD, TSLP is highly expressed in epithelial cells, especially keratinocytes, at the skin barrier surface as well as the lungs,

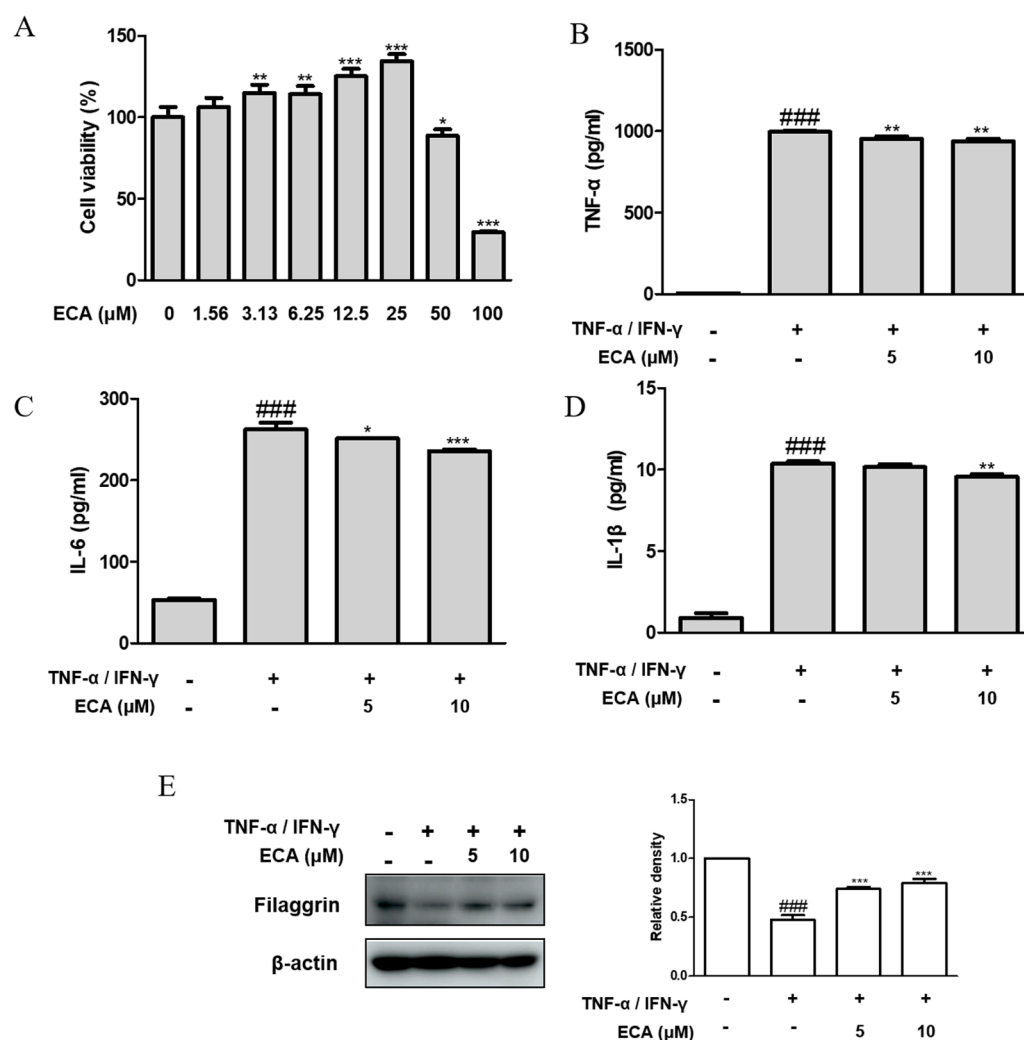


Fig. 6. Effects of ECA on AD-related cytokines and filaggrin in TNF- α /IFN- γ -stimulated HaCaT keratinocytes. (A) Cell viability in HaCaT keratinocytes was evaluated by MTT assay. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. untreated cells. (B-D) Cytokines productions were measured using ELISA kit. (E) Total proteins were prepared and western blotted for filaggrin. β -actin was used to normalize the protein expression. Densitometric analysis was performed using Bio-Rad Quantity One® Software. The data shown represent mean $\pm$ S.D. of three independent experiments. ### $p < 0.001$ vs. the control group; * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. TNF- α /IFN- γ -stimulated group.

eyes, and gastrointestinal tract. T-cell subsets from patients with Th2-dominant diseases express TSLP receptors, and TSLP directly enhances the expansion of activated Th2 cells and IL-4/IL-13 production (Sugaya, 2020). Interestingly, epidermal-derived TSLP is sufficient to trigger the AM, thereby sensitizing the lung airways to inhaled allergens in the absence of epicutaneous sensitization (Kim et al., 2018). Our findings showed that HDM increased TSLP and AD-related cytokines, notably Th2-type cytokines, in mice, suggesting an imbalanced immune response predominantly caused by TSLP-Th2 cells. However, ECA inhibited the HDM-induced imbalance of Th1/Th2 cell responses and reduced Th2 cell-type inflammation. These data suggest that in mice with AD, Th1- and Th2-type cytokines are associated with strong systemic allergic inflammation involving the skin and lungs, which is in agreement with a previous study that reported the presence of Th1/Th2 cell imbalance in patients with asthma (Zhu et al., 2016).

Among MAPKs, it was reported that ERK phosphorylation was induced by the HDM allergen (Utsunomiya et al., 2018), and blocking the ERK/NF- κ B signaling pathways alleviated airway inflammation in ovalbumin-lipopolysaccharide (LPS)-challenged BALB/c mice (Zhou et al., 2018). STAT is also regarded as a key transcription factor in cytokine-mediated inflammation (Howell et al., 2019), and the activation of STATs (Amano et al., 2015) and ERK (Tan et al., 2017) can downregulate filaggrin expression. As AD and the AM share similar pathological characteristics, we speculated that ECA might target the MAPK/STAT pathway as a possible mechanism of action. Our results showed that HDM induced the phosphorylation of ERK and STAT1 in the

skin and lung tissues of mice. However, these phosphorylation levels were reduced after treatment with ECA (Figs. 4 and 5), indicating the pivotal role of ERK and STAT1 in Th2 cell development and ultimately in the pathogenesis of the AM. STAT1 is reportedly involved in the pathogenesis of antigen-induced allergic rhinitis in BALB/c mice (Hattori et al., 2007) and animal asthma models (Chiba et al., 2011). In addition, *in vitro* studies have postulated a potential mechanism for the therapeutic effects of ECA, confirming its inhibitory effect on the TAK1-mediated MEK/ERK and IKK/NF- κ B pathways in TNF- α /IFN- γ -stimulated HaCaT cells. Furthermore, the inhibitory effect of ECA on the activation of STAT1 in TNF- α /IFN- γ -stimulated HaCaT cells correlated with the *in vivo* results (Fig. 7).

The AM mouse model was developed using an established AD mouse model, in which mice treated with mite antigens (*D. farinae* or *D. pteronyssinus*) were further treated daily with the same antigen intranasally for 3 days (Lee et al., 2015; Lee et al., 2020). However, our study had some limitations. The first is the phenotypic ambiguity of AM progression in this study. The effect of HDM-induced AD on the lungs might have been weaker than that in the aforementioned model; thus, a long-term experiment must be designed to evaluate the AM considering the progression and development of this disease. Second, there are no phenotype-specific biomarkers for AM. Recently, fatty acid-binding protein 5 was identified as a potential predictive biomarker for identifying individuals at high risk of developing the AM (Lee et al., 2020). Targeting specific markers that connect AD to the AM would be interesting in future studies. Notwithstanding these limitations, this study is

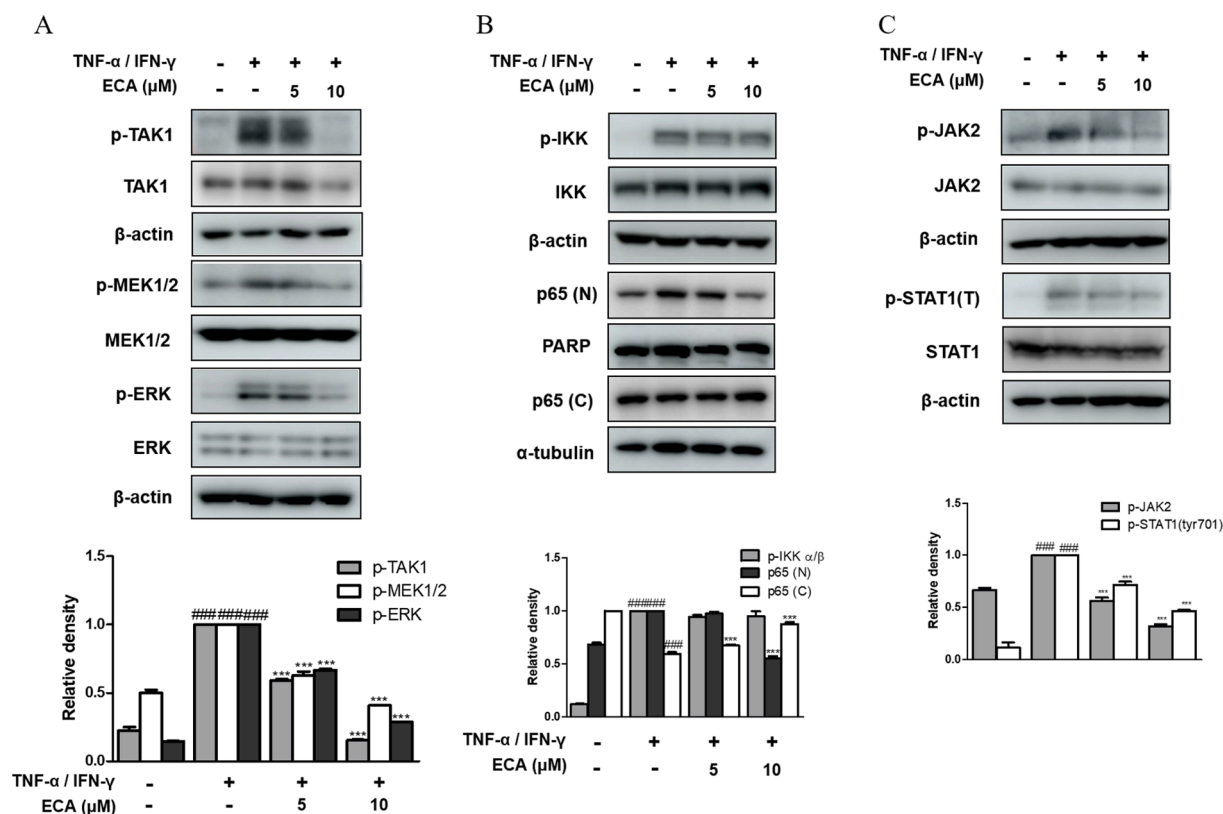


Fig. 7. Effects of ECA on TAK1/MEK/ERK and p65 NF-κB activation and JAK2/STAT1 in TNF-α/IFN-γ-stimulated HaCaT keratinocytes. Total proteins were prepared and western blotted for (A) p-TAK1, TAK1, p-MEK1/2, MEK, p-ERK and ERK and (B) p-IKK α/β and IKK α/β. Nuclear (N) and cytosol (C) extracts were isolated, and levels of p65 in fractions were determined by Western blotting. (C) Total proteins were prepared and Western blotted for p-JAK2, JAK2, p-STAT1, and STAT1. β-actin, PARP, and α-tubulin were used to normalized the protein expressions. Densitometric analysis was performed using Bio-Rad Quantity One® Software. The data shown represent mean ± S.D. of three independent experiments. ### $p < 0.001$ vs. the control group; *** $p < 0.001$ vs. TNF-α/IFN-γ-stimulated group.

meaningful in that it confirmed the development of allergic diseases, and shows that AD-induced skin affects allergic respiratory symptom progression. Conversely, ECA reportedly has a protective effect against LPS-induced lung inflammation by inhibiting LPS binding to Toll-like receptor 4 on alveolar macrophages (Joh et al., 2012), and 12-O-tetra decanoylphorbol-13-acetate-induced ear inflammation in mice (Joh et al., 2014) showing that ECA exerts its anti-inflammatory activity on lung or skin inflammation caused by other conditions. Notably, the therapeutic efficacy of ECA in the present study indicates its potential application in specific lung and skin diseases. This is the meaningful study on ECA in allergic disorders, and our findings expand the potential pharmacological and therapeutic effects of ECA on AD and the AM and will contribute to the establishment of an AM model. More experiments are required to better understand the effects of ECA on the AM.

CRediT authorship contribution statement

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

Competing Interests

The authors declare no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2022.154211.

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