Phytomedicine 67 (2020) 153137

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed

Original Article

I. inflexus (Thunb.) Kudo extract improves atopic dermatitis and depressive-like behavior in DfE-induced atopic dermatitis-like disease

Sun Haeng Park[a], Seol Jang[a], Jeong Eun An[a], Byung kil Choo[b], Ho Kyoung Kim[a,*]

[a] Herbal Medicine Research Division, Korea Institute of Oriental Medicine, 1672 Yuseong-daero, Yuseong-gu, Daejeon 305-811, South Korea
[b] Department of Crop Agriculture and Life Science, Chonbuk National University, 567 Baekje-daero, Deokjin-gu, Jeonju 561-756, South Korea

ARTICLE INFO

Keywords:
I. inflexus (Thunb.) Kudo extract
Atopic dermatitis
NC/Nga mice
Depression
Inflammation

ABSTRACT

Background: Atopic dermatitis (AD) is a chronic, relapsing inflammatory skin disease, which is caused by several genetic, immunological, and environmental factors. In addition to skin manifestations, AD is associated with an increased risk of depression and suicidal ideation. Furthermore, this association is underappreciated and therefore insufficiently studied.

Hypothesis/Purpose: We investigated the association between AD and depression and the effect of *I. inflexus* (Thunb.) Kudo extract (IIE) treatment in a *Dermatophagoides farinae* extract (DfE)-induced mouse model of AD.

Study Design: We evaluated the effects of IIE on depressive behavior in AD mice using four experimental groups: normal (untreated), AD mice (untreated Dfe-induced), IIE-treated (Dfe-induced AD mice), and positive control (tacrolimus-treated Dfe-induced AD mice).

Methods: An AD model was established by the application of 4% sodium dodecyl sulfate to the shaved dorsal neck skin and ears of NC/Nga mice 1 h before application of 100 mg DfE twice per week for 3 weeks. After the first week of DfE application, mice were treated with IIE every day for the remaining 2 weeks. We performed behavioral testing, histology, ELISA, and western blotting to assess depressive-like behavior and neuroinflammatory responses and to measure IgE, histamine, corticosterone, and serotonin levels.

Results: Compared with normal mice, AD mice showed more scratching behavior, increased ear swelling, and higher serum levels of IgE and histamine. AD mice also exhibited evidence of depressive-like behavior in the open-field and sucrose preference tests as well as altered serum corticosterone and brain serotonin concentrations. Histopathological analyses revealed increased infiltration of inflammatory cells and mast cells into the skin and ear tissue and elevated microglia activation and neuroinflammatory response in the brains of AD mice. Topical application of IIE reversed the effects of AD on scratching behavior, ear swelling, open-field locomotion, sucrose preference, and levels of IgE, histamine, corticosterone, serotonin, and inflammatory markers. Moreover, IIE treatment reduced inflammatory cytokine responses in keratinocyte cells.

Conclusion: IIE is a candidate anti-AD therapy due to its ability to exert neuroprotective and antidepressant effects.

Introduction

Atopic dermatitis (AD) is a common chronic inflammatory dermatitis characterized by an impaired epidermal barrier, dry skin, pruritus, eczematous lesions, abnormal immune responses, and IgE-mediated allergies to various exogenous antigens. Like many other dermatologic conditions, registry- and questionnaire-based studies indicate that AD is associated with poor quality of life, depression, and anxiety in children and adults with a direct correlation to disease severity (Slattery et al., 2011). Compared with non-AD individuals, AD patients are more likely to report clinician-diagnosed depression and anxiety and have a higher prevalence of suicidal ideation and depressive symptoms (Slattery et al., 2011); however, the mechanisms underlying this association remain unknown.

Mast cells are central players in skin stress responses and are involved in AD-related neurogenic inflammation. Increased mast cell numbers and nerve contacts are observed in lesional AD skin (Järvikallio et al., 2003). Recent studies indicate that neurohypophyseal hormone, a neuropeptide involved in behavioral regulation, is downregulated in lesional AD skin (Deing et al., 2013). Furthermore,

Abbreviations: AD, atopic dermatitis; DfE, *Dermatophagoides farinae* extract; IIE, *I. inflexus* (Thunb.) Kudo extract; KIOM, Korea Institute of Oriental Medicine

* Corresponding author.
E-mail address: hkkim@kiom.re.kr (H.K. Kim).

https://doi.org/10.1016/j.phymed.2019.153137
Received 10 June 2019; Received in revised form 21 October 2019; Accepted 17 November 2019

mast cells produce corticotropin-releasing hormone, which has also been identified in microglial cells where it regulates immune cells and contributes to the pathogenesis of neurodegenerative diseases, including depression. Thus, AD may contribute to depression by increasing neuroinflammation.

Isodon inflexus (Thunb.) Kudo (*Lamiaceae*) is widely utilized in Korea, China, and Japan for medicinal purposes. *I. inflexus* is used in Korean traditional folk medicine to treat gastrointestinal disorders, tumors, and various inflammatory diseases (Lee et al., 2008a, b). *I. inflexus* is a rich source of diterpenoids, such as ordinin, oridonin, ponicidin, maoecrystal P, and eriocalyxin B, and some of these components have led to development of therapeutic agents (Huang et al., 2006; Xie et al., 2012). In *in vitro* studies, *I. inflexus* component compounds show inhibitory activity against nitric oxide production in macrophages and NF-κB activation (Xu et al., 2016; Lee et al., 2008a, b). Rosmarinic acid (RosA, α-o-caffeoyl-3, 4-dihydroxyphenyl lactic acid) and chlorogenic acid (CGA) are important components of *Lamiaceae*. Several in vivo and *in vitro* studies of RosA and CGA have focused on delineating their effects on allergic and anti-inflammatory responses (Tsang et al., 2016; Zhao et al., 2008). Levels of IgE, histamine, interleukin (IL) − 1β, IL-6, and tumor necrosis factor (TNF)-α were reduced by RosA administration, which is a potent chemoattractant and an activator of eosinophils, basophils, and T-helper lymphocytes (Tsang et al., 2016; Fukui et al., 2009; Oh et al., 2011; Lee et al., 2006). In a clinical trial, RosA significantly reduced AD severity and erythema on the antecubital fossa in AD patients (Lee et al., 2008a, b). Interestingly, RosA was also found to be effective in preventing neuronal cell death in an *in vitro* model of neuronal death (Lee et al., 2008a, b).

Here, we investigated the association between AD and depressive-like behavior in a mouse AD model. Furthermore, we tested the ability of *I. inflexus* extract (IIE) to alleviate AD, decrease depressive-like behavior, and reduce neuroinflammatory responses.

Materials and methods

Animals

Eight-week-old male NC/Nga mice were purchased from Central Lab Animal Inc. (Seoul, Korea) and housed under environmentally controlled, pathogen-free conditions throughout the experiments. Mice were given sterilized standard laboratory diet (Purina 38057, Cargill Agri Purina Inc., Sungnam, Korea) and tap water *ad libitum* and provided with corncob natural bedding material (BioCOB 10384197, BioSystems Corporation Pte Ltd, Peninsula Plaza, Singapore). Mice were acclimatized for 7 d before the experiments. All procedures conformed to regulatory standards and were approved (No. 17–101) by the Institutional Animal Care and Use Committee at the Korea Institute of Oriental Medicine (KIOM).

Preparation of IIE

I. inflexus (Thunb.) Kudo was obtained from Dr. B. Y. Choo. (CheonBuk National University, Jeonju, Korea). A voucher specimen (no. KIOM 53) was deposited at the herbarium of the Mibyeong Research Center at the KIOM. Dried *I. inflexus* (Thunb.) Kudo plants (100 g) were extracted two times in 1 L of 70% ethanol for 120 min followed by filtration through filter paper (Whatman No. 1). After filtration, specimens were concentrated using a rotary evaporator (Eyela N-1000, Rikakikai Co., Tokyo, Japan) under reduced pressure. Extracts were lyophilized and stored at − 20 °C until use.

High-performance liquid chromatography (HPLC)

HPLC analysis was performed on a Waters e2695 liquid chromatography system (Waters Co., Milford, MA, USA), equipped with a Waters 2998 photodiode array detector. Data processing was performed using Empower software (Waters Co.). Column separation utilized a Phenomenex Luna C18 column (250 mm × 4.6 mm; 5-μm particle size; Phenomenex, Torrance, CA, USA). The mobile phases were 0.1% (v/v) trifluoroacetic aqueous solution (A) and acetonitrile (B). The gradient conditions involved holding the starting mobile phase at 90% A and 10% B and applying a gradient of 30% A and 70% B for 30 min. A wash with 100% B was applied for 8 min, followed by equilibration at 90% A and 10% B for 8 min. The flow rate was 1.0 ml/min, and the injection volume for all the samples was 20 μl. The absorbance was detected by an ultraviolet (UV) wavelength detector monitored at 330 nm. Sample peaks were compared to the retention time of the standard compound and the UV spectra in the chromatogram. All calibration curves were calculated by peak areas of standard solutions with concentrations ranging from 1 to 250 μg/ml and revealed good linearity (Table 1). HPLC-grade reagents, acetonitrile, and water were obtained from J. T. Baker (Phillipsburg, NJ, USA).

Table 1
Calibration curve parameters used to analyze IIE.

Compound	Linear range (μg/ml)	Regression equation[a]	R^{2b}
CGA	1–250	y = 28431 x - 71048	0.9999
RosA	1–250	y = 23451 x - 19812	1.0000

[a] In the regression equation y = ax + b, y: peak area, x: concentration (μg/ml).
[b] R^2: the square value of the correlation coefficient of the equation.

Induction of AD and IIE administration

AD was induced in NC/Nga mice. After shaving the dorsal neck and ears, we applied 100 mg of ointment containing crude *Dermatophagoides farinae* extract (DfE; Biostir Inc., Kobe, Japan) twice per week for 3 weeks. The skin dermal barrier was disrupted by applying 150 μl of 4% sodium dodecyl sulfate (SDS) to the shaved area 1 h before application of DfE. After the first week of DfE application, the dorsal skin and ears were topically treated with 1 mg or 3 mg of IIE dissolved in a 4:1 ratio (v/v) of acetone and olive oil or 100 μg tacrolimus containing 0.1% protopic ointment as a positive control (Oshio et al., 2009; Park et al., 2019). As a negative control, another group of mice (normal group) was maintained under the same environmental conditions without sensitization. Fig. 1A shows the experimental design.

Measurement of ear thickness and AD severity

Ear thickness was measured twice per week using a digital caliper (CAS©, Seoul, Korea; Oshio et al., 2009). Dermatitis severity was evaluated at the site of dorsal skin and ear lesions based on erythema/hemorrhage, edema, scarring/dryness, and excoriation/erosion, which were individually scored as 0 (no symptoms), 1 (mild), 2 (moderate), or 3 (severe). Total clinical dermatitis severity scores for each mouse were defined as the sum of the individual scores and ranged from 0 to 12 (Matsuoka et al., 2003).

Behavioral testing

Scratching

Scratching behavior was measured twice per week by counting the number of times mice scratched their dorsal skin or ears during a 20-min period (Yamaguchi et al., 2008).

Open-field test

Locomotor activity was assessed in an open-field test on the final day of the experiment. The open field consisted of an open-air black acrylic box (30 × 30 × 30 cm). At the beginning of the test, each

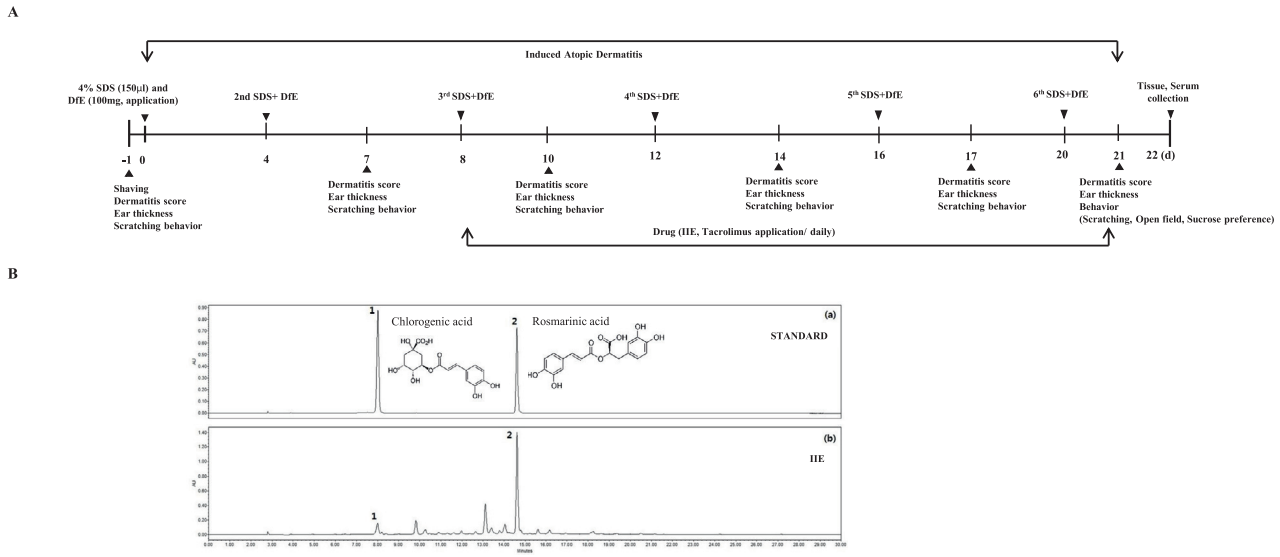

Fig. 1. (A) Experimental timeline and (B) analysis of *I. inflexus* constituents using HPLC. HPLC chromatogram of a standard mixture (a) and *I. inflexus* (b) at 330 nm. The peaks represent CGA (1) and RosA (2).

Table 2
Quantification of IIE components.

Compound	Retention time (min)	Area (mAU)	Amount (mg/g)	Average amount (mg/g)	R.S.D. (%)[a]
CGA	8.59	1311843	4.73	4.76	0.95
	8.56	1320870	4.76		
	8.51	1330190	4.79		
	8.52	1307127	4.71		
	8.50	1339364	4.82		
RosA	15.08	8972896	37.28	37.05	0.49
	15.07	8934928	37.12		
	15.03	8928130	37.09		
	15.04	8855719	36.79		
	15.00	8902154	36.98		

[a] Relative Standard Deviation.

Table 3
Body weight.

Group	Body weight Basal	Day 7	Sacrifice
Normal	26.00(25.97, 27.15)	26.75(26.4, 27.50)	28.30(27.37, 29.20)
AD	26.50(25.35, 27.57)	26.45(25.27, 27.97)	26.00(24.82, 27.07)
Tacrolimus	24.85(24.05, 28.15)	25.95(25.17, 27.70)	25.75(25.37, 28.45)
IIE 1%	23.85(23.30, 25.60)	25.15(24.52, 26.20)	25.70(25.00, 26.92)
IIE 3%	24.20(23.57, 26.00)	25.95(24.17, 26.82)	26.15(25.80, 26.32)

Data as median ± IQ (25%, 75%).
Mortality 0/8 (each group).

mouse was introduced into the left rear corner of the open field and allowed to explore freely. Total distance traveled and the number of center crosses were measured during a 30-min period (Kim et al., 2018). Mouse behavior was recorded using Ethovision XT9® video tracking software (Noldus, Wageningen, Netherlands).

Sucrose preference

Sucrose preference tests were performed on the last day of the experiment. Mice were deprived of water and food 20 h prior to the sucrose preference test. Mice were then given concurrent access to one bottle of 1% (w/v) sucrose solution and one bottle of tap water for 1 h. Consumption of the 1% sucrose solution and water was measured by weighing the bottles before and after the test (Tianzhu et al., 2014).

Measurement of IgE, histamine, corticosterone (CORT), serotonin, and chemokine/cytokine levels by enzyme-linked immunosorbent assay (ELISA)

Blood or tissue samples were collected on the final day of the experiment and maintained at −80 °C until use (Cho et al., 2018). Levels of IgE (Abcam, Cambridge, UK), histamine (Oxford Biomedical Research Inc., Oxford, MI, USA), CORT (LDN, Nordhorn, Germany), serotonin (LDN), macrophage-derived chemokine (MDC), thymus and activation-regulated chemokine (TARC), and thymic stromal lymphopoietin (TSLP; R&D Systems Inc., Minneapolis, MN, USA) were quantified using ELISA kits according to the specific manufacturer's instructions. Absorbance was measured using a Multiskan GO microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). All experiments were performed in triplicate with at least three biological replicates.

Histopathological analysis

The mice were euthanized after the 3-week experiment, and samples were then obtained for histopathological analysis. For analysis of epidermal thicknesses (μm) and numbers of infiltrating inflammatory cells (cells/mm^2 of dermis), paraffin-embedded dorsal skin and ear tissue were serially sectioned at 3–4 μm. Representative sections were stained with hematoxylin and eosin (H&E). Measurements were performed using a computer-assisted image analysis program (*i*-Solution FL ver. 9.1, IMT *i*-solution Inc., Burnaby, BC, Canada).

To quantify mast cell numbers, paraffin-embedded dorsal skin and ear tissue sections were stained with toluidine blue and observed under a light microscope (Model Eclipse 80*i*, Nikon, Tokyo, Japan). Measurement was performed using a computer-assisted image analysis program according to previously reported methods.

For immunofluorescence analysis, cryosectioned hippocampal tissue was incubated with cluster of differentiation 68 (CD68) antibody (Abcam, Cambridge, UK) followed by Alexa Fluor® 488 goat anti-mouse IgG secondary antibody (Thermo Fisher Scientific) or ionized calcium-binding adapter molecule 1 (Iba1) antibody (Novus Biologicals, Littleton, CO, USA) followed by Alexa Fluor® 546 donkey anti-goat IgG secondary antibody (Thermo Fisher Scientific). Sections were mounted and counterstained with Vectashield® mounting medium containing DAPI (Vector Laboratories, Burlingame, CA, USA). All incubation steps were performed in a dark humidified chamber.

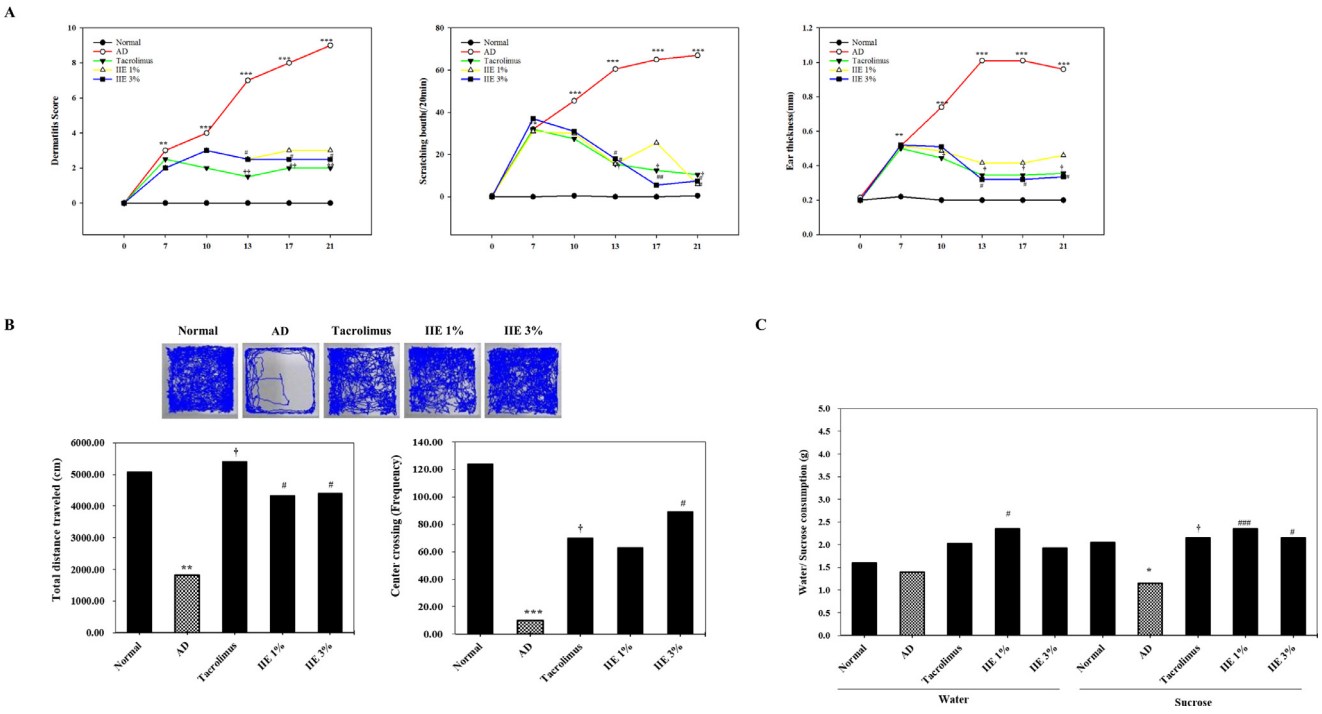

Fig. 2. Effect of IIE on the development of AD and depressive-like behavior in NC/Nga mice. (A) Clinical dermatitis severity score, ear thickness, and scratching behavior. (B) Behavior in the open-field and sucrose preference tests. Data as median ± IQ. $^{***}p < 0.001$ *vs*. normal; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ *vs*. AD.

Western blot analysis

Western blotting of tissue samples was performed according to a previously described method (Park et al., 2018). Briefly, brain tissue was homogenized in PRO-PREB protein extraction buffer (iNtRON Biotechnology, Seongnam, Korea) to obtain extracts of brain proteins for bicinchoninic acid assay (BCA) protein quantitation. Brain protein was then fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes using a Trans Blot® TURBO Transfer stack (Bio-Rad, Hercules, CA, USA). Transferred blots were incubated overnight at 4 °C with antibodies against TNF-α, interferon-gamma (IFN-γ), NF-κB, β-actin (Cell Signaling Technology, Beverly, MA, USA), CD68, and Iba1(Abcam) followed by horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 1 h. Transferred blots were developed using enhanced chemiluminescence (ECL) substrate solution (GE Healthcare Life Sciences, Buckinghamshire, UK) for 5 min. Specific bands were scanned and measured using an ImageQuant LAS 4000 apparatus (GE Healthcare Life Sciences).

Cell culture

HaCaT cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco BRL, Gaithersburg, MD, USA) containing 1% penicillin (Gibco BRL), streptomycin (100 μg g/ml, Gibco BRL), and 10% fetal bovine serum (Gibco BRL) at 37 °C in a humidified incubator with 5% CO_2 (Chot et al., 2017). Cells (3×10^5 cells) were seeded in wells of 24-well culture plates and incubated for 24 h. The following day, cells were stimulated with TNF-α (10 ng/ml, R&D Systems) and IFN-γ (10 ng/ml, R&D Systems). Cells were subsequently incubated with IIE, CGA, or RosA, dexamethasone for 24 h. The production of MDC, TARC, and TSLP in the supernatants was measured using ELISA kits (R&D Systems) according to the manufacturer's protocol.

Data analysis

Values are expressed as median ± interquartile range (IQR). Statistical comparisons were performed using Wilcoxon rank sum test and Kruskal-Wallis test using SigmaPlot software (version 13.0, Systat Software Inc., San Jose, CA, USA). Differences were considered statistically significant at $p < 0.05$.

Results

IIE quality evaluation

The main components of IIE, as identified by HPLC, were CGA and RosA with retention times of approximately 8.5 and 15.0 min, respectively (Fig. 1B). Calibration curves of the two compounds showed high linearity, with $R^2 \geq 0.999$ for the five different concentration ranges tested (Table 1). The IIE contained 4.76 ± 0.05 mg/g CGA and 37.05 ± 0.18 mg/g RosA (Table 2). The extract yield was 10%.

Effect of IIE on behavior in NC/Nga mice

We assessed dermatitis severity, scratching behavior, and ear swelling to determine whether DfE application caused cutaneous damage in our mouse model of AD. We observed no toxicity signs, such as loss of body weight or mortality, in any of the groups (Table 3). The AD mice showed significantly less locomotor activity and sucrose intake, which are markers of depression, than the normal mice. However, IIE treatment significantly reduced skin damage and depression-like behavior. These results suggest that skin damage and depressive-like behavior were induced in our AD model and were also alleviated by IIE treatment.

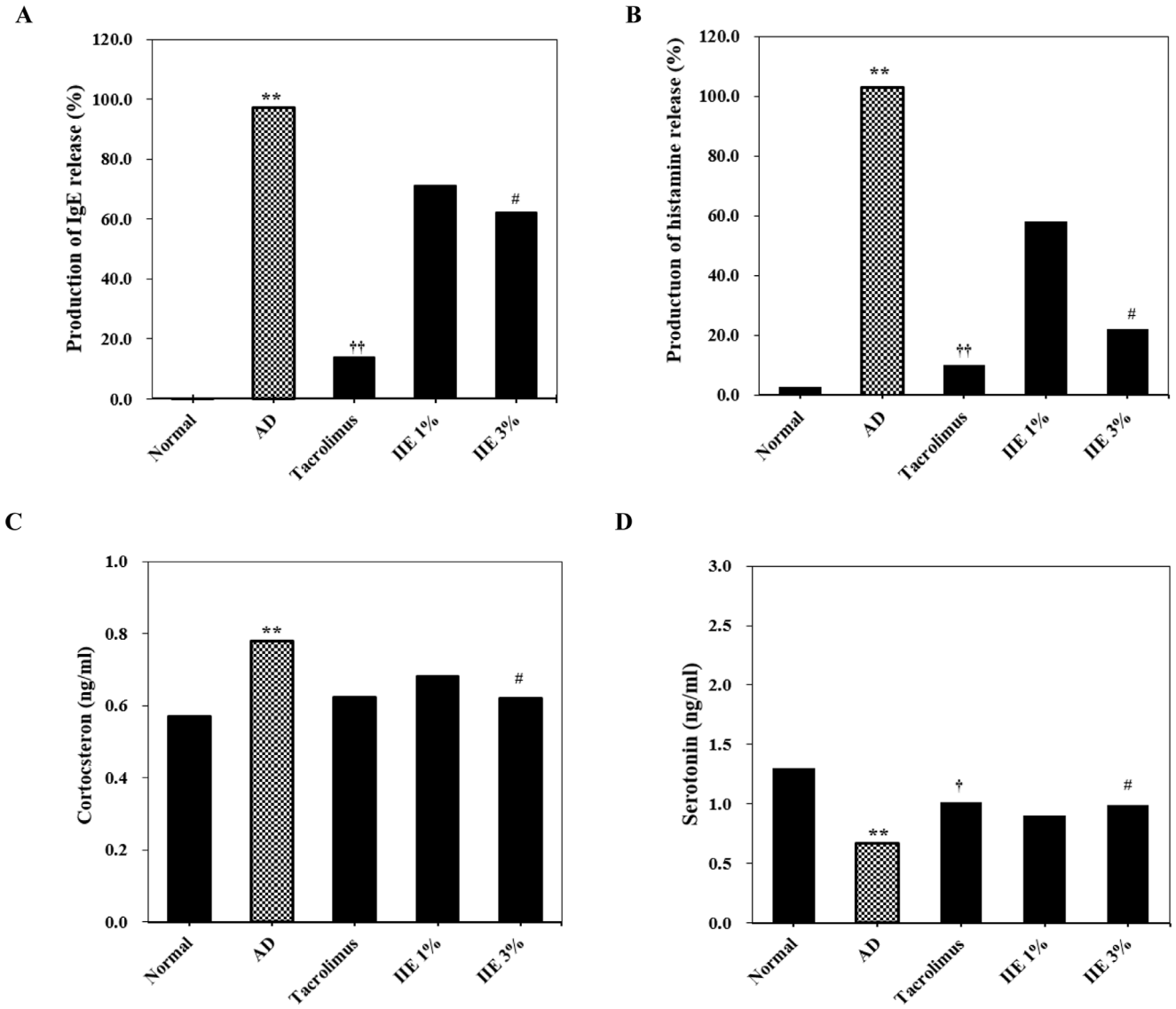

Fig. 3. Effect of IIE on levels of IgE, histamine, and depression-related hormones in NC/Nga mice. Serum concentrations of (A) IgE, (B) histamine, and (C) CORT and brain concentrations of (D) serotonin. Data as median ± IQ. $^{**}p < 0.01$ *vs.* normal; $^{\dagger\dagger}p < 0.01$ and $^{\#}p < 0.05$, *vs.* AD.

Effect of IIE on levels of IgE, histamine, CORT, and serotonin in NC/Nga mice

AD mice showed increased serum concentrations of IgE, histamine, and CORT and reduced levels of serotonin in the brain compared with normal mice; however, IIE treatment significantly reversed these changes.

Effect of IIE on inflammation and mast cell accumulation in NC/Nga mice

In AD mice, H&E staining revealed an increase in inflammatory cell infiltration into the epidermis and dermis (Figs. 2–4). Compared to normal mice, AD mice also showed increased dorsal skin and ear epidermal thickness, which are histological signs of chronic inflammation. However, IIE treatment reduced epidermal thickness and the number of inflammatory cells. Furthermore, AD mice showed increased infiltration of mast cells in the dorsal skin and ear tissue, but this was also reduced by IIE treatment.

Effect of IIE on microglial neuroinflammation in NC/Nga mice

In addition, we found double immunostaining of the macrophage marker CD68 and microglia marker Iba1 in AD mice, revealing activation of macrophages and microglia cells in the CA1, CA2, and CA3 regions of the hippocampus in AD mice compared to normal mice (Fig. 5A–C). This activation was reduced by IIE treatment of AD mice (Fig. 5).

We also examined neuroinflammatory responses using western blotting and found that AD mice exhibited elevated expression of TNF-α, IFN-γ, CD68, Iba1 and NF-kB in brain, which was reversed by IIE treatment (Fig. 6A and B).

Effect of IIE components on production of chemokines/cytokines by keratinocytes

We used ELISAs to investigate whether treatment with IIE components CGA and RosA inhibits TNF-α/IFN-γ-stimulated production of MDC/CCL22, TARC/CCL17, RANTES/CCL5, and TSLP in HaCaT cells. The levels of all of these chemokines were significantly downregulated in TNF-α and IFN-γ-stimulated cells treated with CGA and RosA

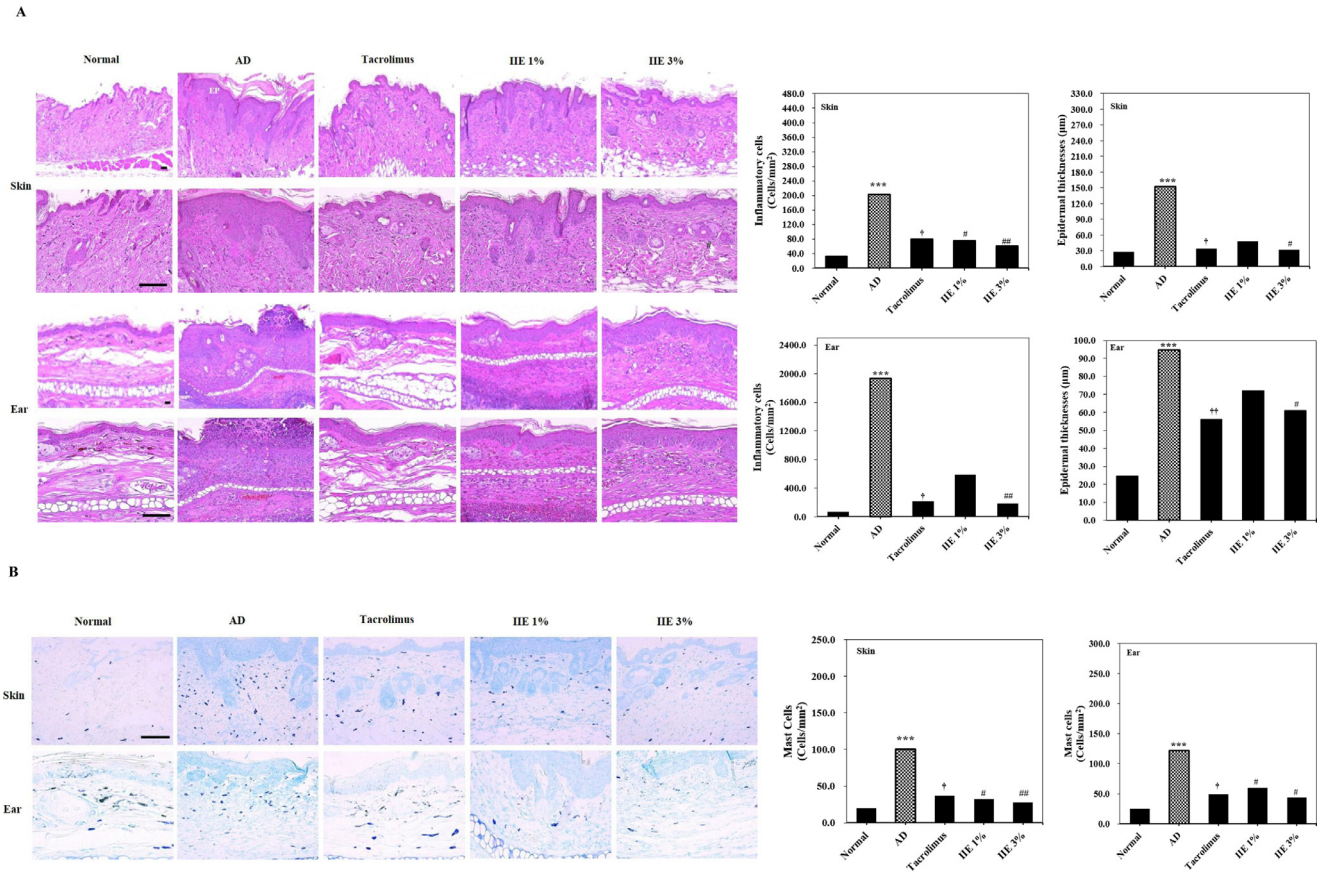

Fig. 4. Effect of IIE on inflammatory and mast cell infiltration in NC/Nga mice. Sections were stained with (A) H&E and (B) toluidine blue. Data as median ± IQ. $^{***}p < 0.001$ vs. normal; $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs.* AD. Scale bars = 80 μm.

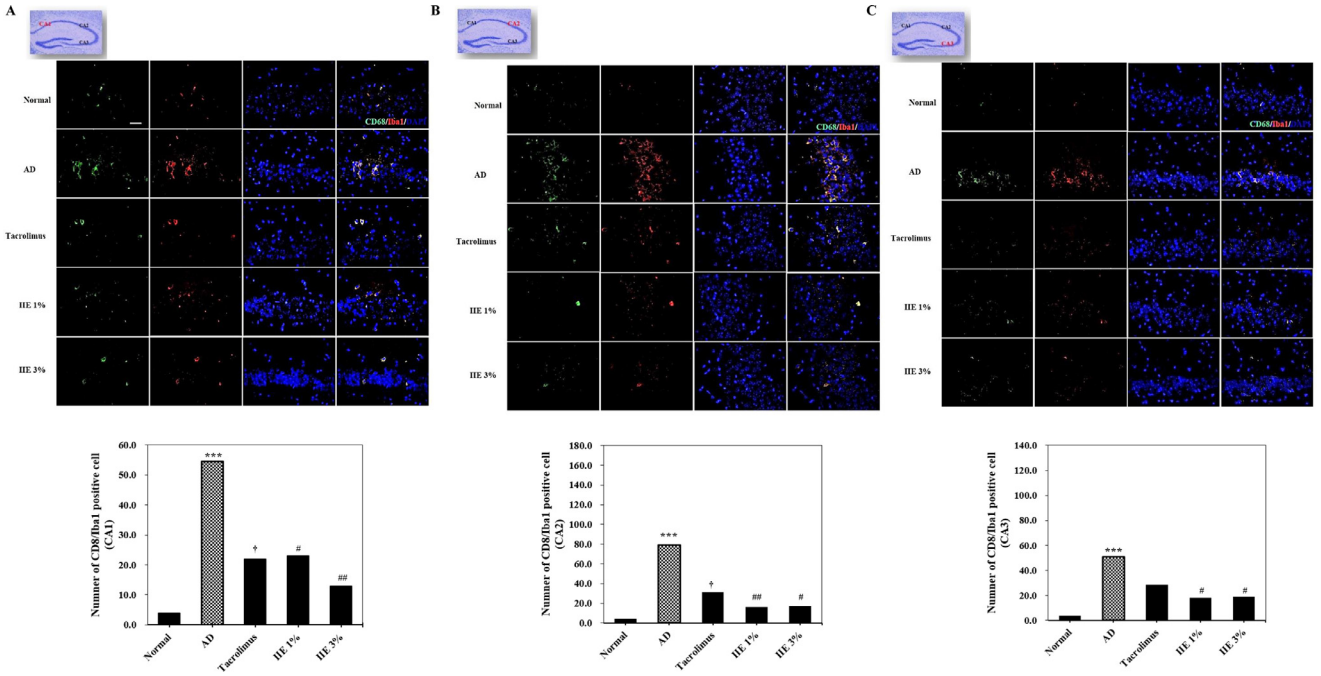

Fig. 5. Effect of IIE on microglia and macrophage expression in NC/Nga mice. Immunofluorescence double labeling of CD68 (green) and Iba1 (red) in the (A) CA1, (B) CA2, and (C) CA3 regions of the hippocampus. Data as median ± IQ. $^{***}p < 0.001$ *vs.* normal; $^{\dagger}p < 0.05$, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs.* AD. Scale bar = 30 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

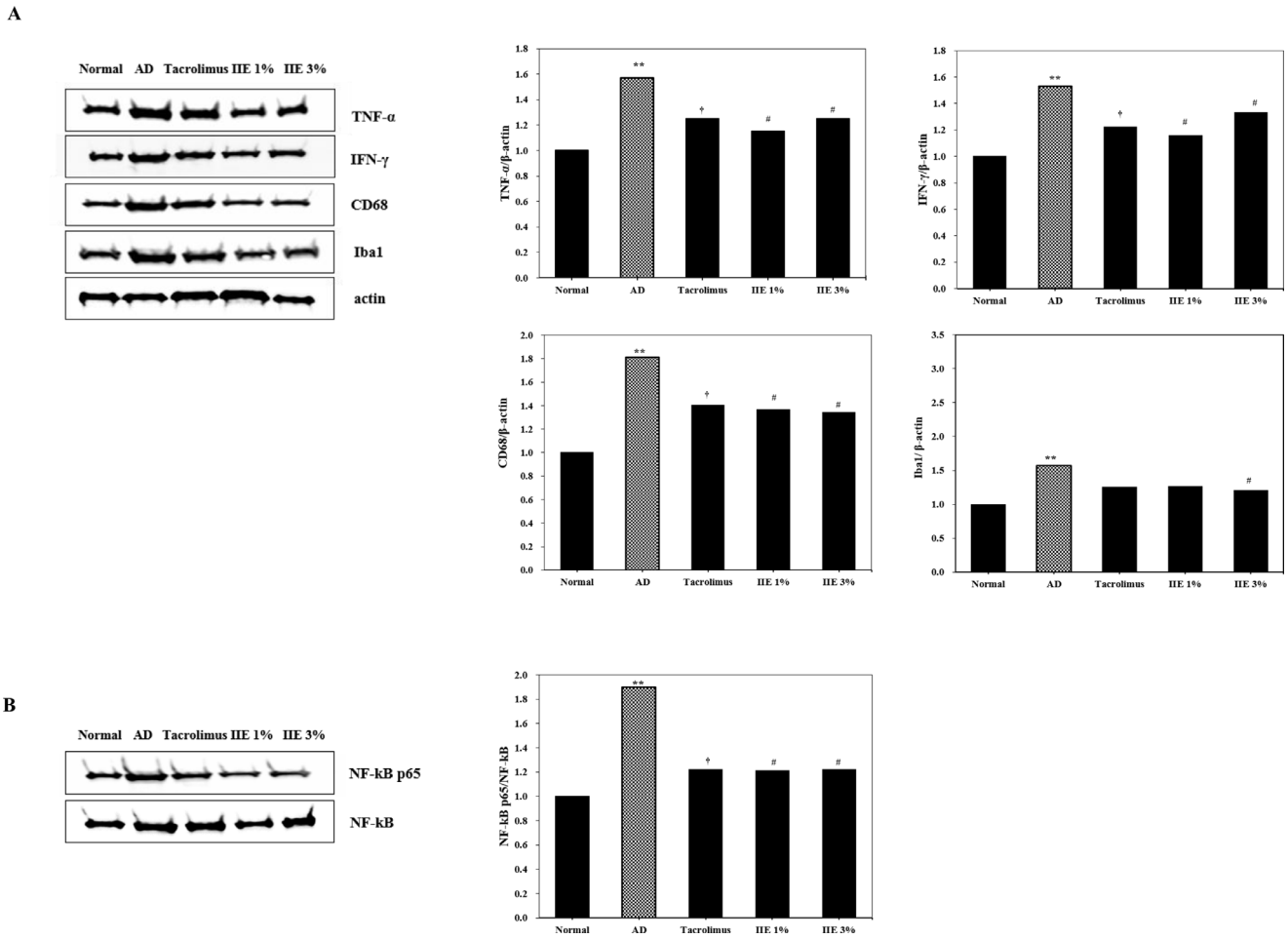

Fig. 6. Effect of IIE on neuroinflammatory response in NC/Nga mice. (A) Western blot of brain protein levels of TNF-α, IFN-γ, NF-κB, CD68, and Iba1. (B) Quantification of levels of TNF-α, IFN-γ, NF-κB, CD68, and Iba1. Values represent mean ± SEM. $^{**}p < 0.01$ *vs.* normal; $^{\dagger}p < 0.05$ and $^{\#}p < 0.05$ *vs.* AD.

compared to cells treated with only TNF-α and IFN-γ (Fig. 7).

Discussion

AD is a common chronic disease associated with an underappreciated increased risk of depression and suicide ideation. Our results suggest that AD induces neuroinflammation (i.e., increased macrophage and microglial activation and expression of proinflammatory cytokines) and increases depressive-like behavior in an AD mouse model. Interestingly, topical treatment of IIE appeared to alleviate AD symptoms and depressive-like behavior by regulating immune responses and neuroinflammation. These results provide new insight into the development of depression in AD patients.

Clinical studies indicate that AD patients experience more anxiety, depression, thought alterations, aggressive behavior, and suicide ideation compared with individuals without AD. In addition, consistent with observations that AD is associated with an increased likelihood of depression (Slattery et al., 2011), we found that AD mice not only exhibited higher clinical dermatitis severity scores, more scratching behavior, and increased ear swelling but also depressive-like behavior compared to normal mice. Importantly, IIE treatment alleviated not only the AD symptoms but the depressive-like behaviors as well.

Due to their roles in allergic inflammation, IgE and IgE-mediated mast cell activation contribute to AD. AD is an inflammatory disorder in which cytokines released from mast cells cause allergic inflammation, which is called the late-phase reaction (Sidler et al., 2017; Inada et al., 2004). High levels of IgE and histamine, which are indicative of allergic sensitization, are associated with skin barrier abnormalities and AD severity (Liu et al., 2011). Interestingly, previous studies report a significant correlation between serum IgE and salivary cortisol levels in an AD model (Toda et al., 2007). In the present study, AD mice showed high serum concentrations of IgE, histamine, and CORT; low brain concentrations of serotonin; and high numbers of infiltrating inflammatory cells and mast cells at the lesion sites. IIE treatment successfully reversed these changes.

In addition, previous studies demonstrated that corticotrophin-releasing hormone (CRH) augments skin-mast cell interactions and that CRH release is induced by activation of mast cells that are located perivascularly and close to nerves. Furthermore, mast cells are activated by nerve stimulation and proinflammatory mediators, including histamine, cytokines, prostanoids, and proteases (Alysandratos et al., 2012). In agreement with these findings, we found significantly higher expression of IgE and histamine, which are diagnostic markers of AD (Hattori et al., 2010), as well as CORT, which alters functional responses to serotonin in a stress-induced depression model (Karten et al., 1999). Previous studies have demonstrated a similar distribution under high-stress conditions. One such study showed CRH was produced by stressed epidermal, follicular, and mast cells under physiological stress (Slominski et al., 2013; Park et al., 2017). In addition, atopy stress enhances the disruption of symptoms and neuronal damage via a glucocorticoid-related mechanism, based on findings that were confirmed in a CORT-induced AD mouse model (Park et al., 2017). IIE treatment may therefore reduce the severity of AD-related depression symptoms by decreasing levels of stress- and depression-related hormones.

Many inflammatory mediators released by allergic-type immune cells, such as mast cells, act on pruriceptor neurons to sensitize itch. For

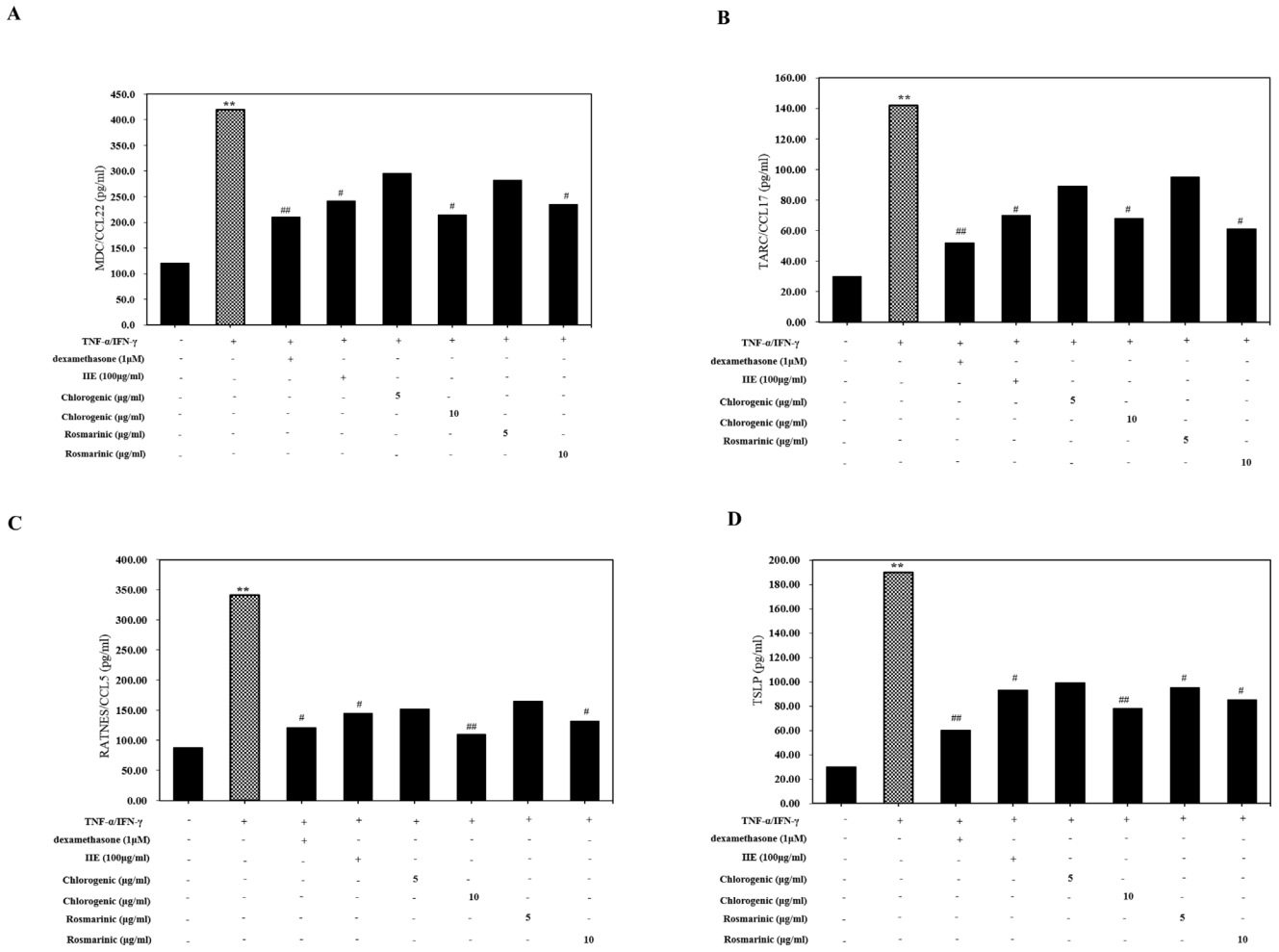

Fig. 7. Effect of IIE components on inflammatory chemokine responses in HaCaT keratinocytes. The production of (A) MDC/CCL22, (B) TARC/CCL17, (C) RANTES/CCL5, and (D) TSLP by HaCaT keratinocytes following 24 h of treatment with IIE, CGA, and RosA, dexamethasone was examined using ELISAs. Data as median ± IQ. $^{**}p < 0.01$ *vs*. control cells; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs*. TNF-α/IFN-γ-treated cells.

example, pruriceptor neurons possess receptors for and respond to histamine, serotonin, and cytokines (Voisin et al., 2017). Itch in AD can contribute to psychological stress. Furthermore, AD is associated with higher rates of psychiatric symptoms such as depression, anxiety, and suicidal ideation, as well as increased brain CRH and serotonin levels, which may exacerbate the symptoms of AD (Brunner et al., 2017; Vasiadi et al., 2012). Consistently, we found that AD mice exhibited altered levels of brain serotonin and serum corticosterone, suggesting that AD can give rise to depression symptom through abnormalities in serotonin and corticosterone production. AD-related psychological stress increases neuroendocrine dysfunction, exacerbates neuroinflammation, and potentially accelerates neurodegenerative diseases (Park et al., 2017). The brains of depressed patients show activation of microglia, which play important roles in regulating neuronal cell death, neurogenesis, and synaptic interactions, in addition to the generation of cytokines as part of the immune response, which then aggravate the condition. In the AD mouse brain, we observed the immune reactivity of CD68/Iba1-positive resident microglia and the expression of the neuroinflammatory markers TNF-α, IFN-γ, NF-κB, CD68, and Iba1. Importantly, IIE treatment reversed these AD-associated changes. Recent studies have uncovered potentially important roles of the nervous system and neuroimmune interactions in the development of allergic reactions (Voisin et al., 2017), and anti-depressants may affect the brain by protecting against neuroinflammation that accompanies depression (Kohler et al., 2016). Thus, our results indicate that AD may induce depression via altered CRH and serotonin concentrations, activation of microglia and macrophages, and neuroinflammation and that IIE treatment may alleviate these effects.

A previous report indicated that *Lamiaceae* contains CGA and RosA as the main constituents. Here, using HPLC analysis, we found that two main components of the IIE that reversed the AD characteristics in our model were also CGA and RosA. Furthermore, treatment with IIE reduced levels of TARC, MDC, RANTES, and TSLP in TNF-α/INF-γ-induced HaCaT cells. CGA has been shown to inhibit Th2 cytokine production, leading to reduced pulmonary eosinophilia and IgE serum levels (Kim et al., 2010). In addition, RosA suppresses Ikk-β downstream signaling following TNF-α-induced upregulation of CCL11, which is a potent chemoattractant and an activator of eosinophils, basophils, and T-helper 2 lymphocytes. T cell kinase able to regulate cell receptor signaling (Lee et al., 2006). Although several studies have reported an effect of CGA and RosA on the inflammatory response, no anti-inflammatory effects for these compounds within IIE have yet been demonstrated. Nonetheless, we speculate that the anti-inflammatory effects of CGA and RosA underlie the inhibitory effects of IIE on AD-like skin lesions.

Conclusion

AD may lead to depressive-like behavior though dysfunction of CRH and serotonin regulation in the brain. In a mouse AD model, topical IIE treatment not only improved skin symptoms and inflammation but also protected against neuroinflammation and depressive-like behavior in

AD. We suggest further studies to extend these findings and examine the potential for IIE as a treatment for AD and related depression in patients.

Conflict of interest

None to report.

Acknowledgments

This research was supported by the Bio & Medical Technology Development Program of the National Research Foundation funded by the Korean government (MSIP NRF-2015M3A9E3052336).

References

Alysandratos, K.D., Asadi, S., Angelidou, A., Zhang, B., Sismanopoulos, N., Yang, H., Critchfield, A., Theoharides, T.C, 2012. Neurotensin and CRH interactions augment human mast cell activation. PLoS ONE 7 (11), e48934.

Brunner, P.M., Silverberg, J.I., Guttman-Yassky, E., Paller, A.S., Kabashima, K., Amagai, M., Luger, T.A., Deleuran, M., Werfel, T., Eyerich, K., Stingl, G, 2017. Increasing comorbidities suggest that atopic dermatitis is a systemic disorder. J. Invest. Dermatol. 137 (1), 18–25.

Cho, K., Parveen, A., Kang, M.C., Subedi, L., Lee, J.H., Park, S.Y., Jin, M.R., Yoon, H., Son, Y.K., Kim, S.Y, 2018. Pyrus ussuriensis Maxim. leaves extract ameliorates DNCB-induced atopic dermatitis-like symptoms in NC/Nga mice. Phytomedicine 48, 76–83.

Deing, V., Roggenkamp, D., Kühnl, J., Gruschka, A., Stäb, F., Wenck, H., Bürkle, A., Neufang, G, 2013. Oxytocin modulates proliferation and stress responses of human skin cells: implications for atopic dermatitis. Exp. Dermatol. 22 (6), 399–405.

Fukui, N., Honda, K., Ito, E., Ishikawa, K, 2009. Peroxisome proliferator-activated receptor γ negatively regulates allergic rhinitis in mice. Allergol. Int. 58 (2), 47–53.

Hattori, K., Nishikawa, M., Watcharanurak, K., Ikoma, A., Kabashima, K., Toyota, H., Takahashi, Y., Takahashi, R., Watanabe, Y., Takakura, Y, 2010. Sustained exogenous expression of therapeutic levels of IFN-gamma ameliorates atopic dermatitis in NC/Nga mice via Th1 polarization. J. Immunol. 184 (5), 2729–2735.

Huang, S.X., Zhou, Y., Pu, J.X., Li, R.T., Li, X., Xiao, W.L., Lou, L.G., Han, Q.B., Ding, L.S., Peng, S.L., Sun, H.D., 2006. Cytotoxic ent-kauranoid derivatives from Isodon rubescens. Tetrahedron 62 (20), 4941–4947.

Inada, N., Shoji, J., Tabuchi, K., Saito, K., Sawa, M, 2004. Histological study on mast cells in conjunctiva of NC/Nga mice. Jpn. J. Ophthalmol., vol.48 (3), 189–194.

Järvikallio, A., Harvima, I.T., Naukkarinen, A, 2003. Mast cells, nerves and neuropeptides in atopic dermatitis and nummular eczema. Arch. Dermatol. Res. 295 (1), 2–7.

Karten, Y.J., Nair, S.M., van Essen, L., Sibug, R., Joëls, M, 1999. Long-term exposure to high corticosterone levels attenuates serotonin responses in rat hippocampal CA1 neurons. Proc. Natl. Acad. Sci. U. S. A. 96 (23), 13456–13461.

Kim, H.R., Lee, D.M., Lee, S.H., Seong, A.R., Gin, D.W., Hwang, J.A., Park, J.H, 2010. Chlorogenic acid suppresses pulmonary eosinophilia, IgE production, and Th2-type cytokine production in an ovalbumin-induced allergic asthma: activation of STAT-6 and JNK is inhibited by chlorogenic acid. Int. Immunopharmacol. 10 (10), 1242–1248.

Kim, Y.R., Park, B.K., Kim, Y.H., Shim, I., Kang, I.C., Lee, M.Y, 2018. Antidepressant effect of Fraxinus rhynchophylla Hance extract in a mouse model of chronic stress-induced depression. Biomed. Res. Int. 2018, 8249563.

Kohler, O., Krogh, J., Mors, O., Benros, M.E, 2016. Inflammation in depression and the potential for anti-inflammatory treatment. Curr. Neuropharmacol. 14 (7), 732–742.

Lee, C., Hong, S.S., Han, X.H., Jin, Q., Li, D., Kim, T.O., Kim, H.K., Lee, J., Kwon, S.H., Kim, Y.B., Lee, M.K., Hwang, B.Y, 2008a. A new abietane diterpenoid from Isodon inflexus. Arch. Pharm. Res. 31 (11), 1381–1384.

Lee, J., Jung, E., Kim, Y., Lee, J., Park, J., Hong, S., Hyun, C.G., Park, D., Kim, Y.S, 2006. Rosmarinic acid as a downstream inhibitor of IKK-beta in TNF-alpha-induced upregulation of CCL11 and CCR3. Br. J. Pharmacol. 148 (3), 366–375.

Lee, J., Jung, E., Koh, J., Kim, Y.S., Park, D, 2008b. Effect of rosmarinic acid on atopic dermatitis. J. Dermatol. 35 (12), 768–771.

Liu, F.T., Goodarzi, H., Chen, H.Y, 2011. IgE, mast cells, and eosinophils in atopic dermatitis. Clin. Rev. Allergy Immunol. 412 (3), 298–310.

Matsuoka H., Maki N., Yoshida S., Arai M., Wang J., Oikawa Y., Ikeda T., Hirota N., Nakagawa H., Ishii A. 2003. A mouse model of the atopic eczema/dermatitis syndrome by repeated application of a crude extract of house-dust mite Dermatophagoides farinae. vol. 58, no.2, 139–145.

Oh, H.-A., Park, C.-S., Ahn, H.-J., Park, Y.S., Kim, H.-M, 2011. Effect of Perilla frutescens var. acuta Kudo and rosmarinic acid on allergic inflammatory reactions. Exp. Biol. Med. 236 (1), 99–106.

Oshio, T., Sasaki, Y., Funakoshi-Tago, M., Aizu-Yokota, E., Sonoda, Y., Matsuoka, H., Kasahara, T, 2009. Dermatophagoides farinae extract induces severe atopic dermatitis in NC/Nga mice, which is effectively suppressed by the administration of tacrolimus ointment. Int. Immunopharmacol. 9 (4), 403–411.

Park, G., Lee, S.H., Oh, D.S., Kim, Y.U, 2017. Melatonin inhibits neuronal dysfunction-associated with neuroinflammation by atopic psychological stress in NC/Nga atopic-like mouse models. J. Pineal Res. 63 (2). https://doi.org/10.1111/jpi.12420.

Park, S.H., Jang, S., Son, E., Lee, S.W., Park, S.D., Sung, Y.Y., Kim, H.K, 2018. Polygonum aviculare L. extract reduces fatigue by inhibiting neuroinflammation in restraint-stressed. Phytomedicine 42, 180–189.

Park, S.H., An, J.E., Jang, S., Kim, J.Y., Lee, J.W., Kim, H.K, 2019. Gardenia jasminoides extract without crocin improved atopic dermatitis-like skin lesions via suppression of Th2-related cytokines in Dfe-induced NC/Nga mice. J. Ethnopharmacol. 241, 112015.

Sidler, D., Wu, P., Herro, R., Claus, M., Wolf, D., Kawakami, Y., Kawakami, T., Burkly, L., Croft, M, 2017. TWEAK mediates inflammation in experimental atopic dermatitis and psoriasis. Nat. Commun. 8 (15395). https://doi.org/10.1038/ncomms15395.

Slattery, M.J., Essex, M.J., Paletz, E.M., Vanness, E.R., Infante, M., Rogers, G.M., Gern, J.E, 2011. Depression, anxiety, and dermatologic quality of life in adolescents with atopic dermatitis. J. Allergy Clin. Immunol. 128 (3), 668–671.

Slominski, A.T., Zmijewski, M.A., Zbytek, B., Tobin, D.J., Theoharides, T.C., Rivier, J, 2013. Key role of CRF in the skin stress response system. Endocr. Rev. 34 (6), 827–884.

Tianzhu, Z., Shihai, Y., Juan, D, 2014. Antidepressant-like effects of cordycepin in a mice model of chronic unpredictable mild stress. Evid. Based Complement Alternat. Med. 2014, 438506.

Toda, M., Sakaguchi, Y., Morimoto, K, 2007. Correlation between serum IgE and salivary cortisol levels in subjects with type I allergic disorders. Int. J. Immunopathol. Pharmacol. 20 (1), 203–205.

Tsang, M.S., Jiao, D., Chan, B.C., Hon, K.L., Leung, P.C., Lau, C.B., Wong, E.C., Cheng, L., Chan, C.K., Lam, C.W., Wong, C.K, 2016. Anti-inflammatory activities of pentaherbs formula, berberine, gallic acid and chlorogenic acid in atopic dermatitis-like skin inflammation. Molecules 21 (4), 519.

Vasiadi, M., Therianou, A., Sideri, K., Smyrnioti, M., Sismanopoulos, N., Delivanis, D.A., Asadi, S., Katsarou-Katsari, A., Petrakopoulou, T., Theoharides, A., Antoniou, C., Papadavid, E., Stavrianeas, N., Kalogeromitros, D., Theoharides, T.C, 2012. Increased serum CRH levels with decreased skin CRHR-1 gene expression in psoriasis and atopic dermatitis. J. Allergy Clin. Immunol. 129 (5), 1410–1413.

Voisin, T., Bouvier, A., Chiu, I.M, 2017. Neuro-immune interactions in allergic diseases: novel targets for therapeutics. Int. Immunol. 29 (6), 247–261.

Xu, G.H., Cai, X.F., Jin, X., Lee, J.J, 2016. A new kaurane diterpenoid from Isodon inflexus. Nat. Prod.

Xie, W.D., Li, X., Zhao, J.H., Liu, Y.H., Row, K.H, 2012. Abietane diterpenoids from Isodon inflexus. Phytochemistry 81, 153–158.

Yamaguchi, J., Nakamura, F., Aihara, M., Yamashita, N., Usui, H., Hida, T., Takei, K., Nagashima, Y., Ikezawa, Z., Goshima, Y, 2008. Semaphorin3A alleviates skin lesions and scratching behavior in NC/Nga mice, an atopic dermatitis model. J. Invest. Dermatol. 128 (12), 2842–2849.

Zhao, Z., Shin, H.S., Satsu, H., Totsuka, M., Shimizu, M., 2008. 5-caffeoylquinic acid and caffeic acid down-regulate the oxidative stress- and TNF-alpha-induced secretion of interleukin-8 from Caco-2 cells. J. Agric. Food Chem. 56 (10), 3863–3868.