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Ophiopogonin D ameliorates DNCB-induced atopic dermatitis-like lesions in BALB/c mice and TNF- α -inflamed HaCaT cell

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ABSTRACT

Atopic dermatitis (AD) can occur in both children and adults, and the symptoms include itching and eczema, which in turn cause patients to suffer. Ophiopogonin D (OP-D) is a steroidal glycoside from *Radix Ophiopogon japonicus*, which is well known as an effective anti-inflammatory herbal medicine in many Asian countries. In this study, we aimed to investigate the anti-inflammatory effects of OP-D, using an AD mouse model and inflamed HaCaT cells. Through a histopathological analysis, we were able to confirm the suppressive effects of OP-D on skin thickening and the mast cell activation in AD-like mouse back skin tissues stimulated by DNCB. In addition, we detected significant decreases in cytokine expression levels through multiplex assessment assays of the OP-D-treated mice blood. We observed the anti-inflammatory effect of OP-D in the spleen, causing weight loss in the spleen and in the mRNA expression levels related to diverse cytokines. In human keratinocytes inflamed by TNF- α , OP-D inhibited p38 and ERK protein activation and showed a reduction of NF- κ B nuclear translocation. Furthermore, OP-D attenuated pro-inflammatory cytokine mRNA expressions in TNF- α -inflamed HaCaT cells. Accordingly, we came to the conclusion that OP-D is a potential natural drug which can be used in order to treat inflammatory skin diseases, such as AD.

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1. Introduction

Atopic dermatitis (AD) is recognized as a global health problem because it has been extensively increasing worldwide and currently has a global prevalence rate of 20%. It is an inflammatory skin disease that can occur in both children and adults accompanied by itching, eczema, hyperkeratosis and a thickened stratum spinosum, which is associated with epidermal immunity. _ENREF_1_ENREF_1 An imbalance of inflammatory factors or a damaged skin barrier, linked to the excessive production of IgE by external stimuli, can cause AD [1]. AD can be caused by regulatory T cell imbalances, and

in patients with dermatitis, the increased differentiation of helper T(Th)2 cytokines, such as interleukin (IL)-4 and IL-5, which both inhibit Th1 cell differentiation, and are defining characteristics[2]. Among the cells that make up skin, keratinocytes are stimulated by Th2 cytokines, allowing various immune factors to penetrate into damaged skin areas [2,3]. Tumor-necrosis factor-alpha (TNF- α) is an innate immune cytokine that mediates immune responses. It binds to receptors on the cell surfaces and activates the mitogen-activated protein kinases (MAPKs), specifically extracellular-signal-regulated kinase 1/2 (ERK1/2), Jun N-terminal kinase (JNK) and p38. It also activates the nuclear factor kappa B (NF- κ B) signal. Furthermore, the transcription factor NF- κ B induces the expression of genes, including diverse cytokines, and is a factor involved in inflammatory conditions [4,5].

Currently, most AD medications consist of corticosteroids and are in the forms of oral medications, ointments and UV phototherapy [6]. However, phototherapy has a disadvantage in that it has a lengthy process, and the corticosteroid medications can cause side effects, such as skin infections [7]. Therefore, there is a need to

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find safer and more effective anti-dermatitis materials and natural compounds derived from herbal medicines that can be potential sources of therapeutic improvements for AD [4].

Ophiopogon japonicus is a herbal medicine prescribed by traditional practitioners for inflammatory diseases, such as bronchitis and asthma [9]. Recent studies have shown that it also has anti-oxidant and anti-thrombin effects [10]. Ophiopogonin D (OP-D) is a steroidal glycoside from *Radix Ophiopogon japonicus*. In our previous study, Ophiopogonin D (OP-D) was detected by UPLC/ESI-QTOF-MS analysis in *radix Ophiopogon japonicus* extract [9]. In another recent study, it was found to have anti-inflammatory and platelet-coagulation inhibitory effects [11]. However, the full effects of OP-D on AD have not been uncovered. Therefore, we aimed to test whether Ophiopogonin D is effective in cases of inflammatory skin disease, especially AD, considering that it has a structure similar to those of corticosteroids, which act as anti-inflammatory agents in general AD therapies. Within this study, we confirmed its inhibitory effects on AD symptoms, using an AD-like mouse model with an *in vitro* study, and clarified what effects OP-D had on certain signaling pathways within the human keratinocyte cell line, HaCaT cell.

2. Materials and methods

2.1. Reagent

Ophiopogonin D (OP-D) was obtained from Chemfaces (Wuhan Chemfaces Biochemical Co., Ltd., China) (Supplementary Fig. S1B). Human Tumor necrosis factor alpha (TNF- α) was obtained from Peprotech (Neuilly/Seine, France). Antibodies to b-actin and specific rabbit antibodies were purchased from Sigma-aldrich (St. Louis, MO). MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-[2H]-tetrazolium bromide) was obtained from Invitrogen (Waltham, MA). Extracellular signal-regulated kinases 1 and 2 (ERK1/2), phospho-ERK1/2, p38, phospho-p38, p65, phospho-p65, I κ B were purchased from cell signaling technology (Danvers, MA). β -actin and Lamin B were purchased from Santa cruz (TX, USA).

2.2. Animals

We used six-week old female BALB/c mice (Daehan Bio Link Co., Ltd, Eumseong-gun, Chungcheonbuk-do, South Korea) for this study, and all animal studies were performed according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC) from Kyung Hee University (confirmation number: KHUASP(SE)-17-090). Animals were housed in a specific-pathogen free (SPF) animal room under the condition of a 12 h light-dark cycle at moderate temperature (21–23 °C) and humidity levels (55–60%). All mice were acclimated for a week before the experiment. During the acclimation period, all mice were fed with standard rodent chow [28.507% of protein, 13.496% of fat and 57.996% of carbohydrates (LabDiet, St. Louis, MO, USA)] and water ad libitum.

2.3. Atopic dermatitis induction and sample treatment

After being randomly divided into four groups (normal-treated with acetone/olive oil 4:1 solution, DNCB-challenged with only DNCB, OP-D low-1 μ M of OP-D and DNCB, OP-D High-10 μ M OP-D and DNCB), the mice backs were shaved and then topically treated with 200 μ L of 1% DNCB (dissolved in acetone: olive = 4:1) once a day for 4 days, excluding the normal group. The normal group received a vehicle treatment (only acetone/olive oil). After three days, 200 μ L of OP-D 1 μ M, 10 μ M and saline/olive oil solution were then topically treated to the mice back skin once a day for 12

days. Before the sample treatment, 100 μ L of 4% SDS was applied to enhance skin barrier perturbation. After allowing enough time for absorption, 200 μ L of 0.5% DNCB solution was applied to maintain conditions for 12 days. The mice were sacrificed on the 20th day of the experiment. Blood samples were collected by orbital puncture, and dorsal skins were collected to observe any histopathological changes [12,13]._ENREF_1.

2.4. Histological analysis

The mice skin samples were fixed in 10% formalin and immersed in paraffin. Paraffin samples were sectioned at a 6 μ m thickness, and stained with Hematoxylin & eosin (H&E). Histopathological features were then detected using light microscopy. After blue staining, using toluidine, we counted the number of mast cells [13,14]._ENREF_2.

2.5. Cytokine assay

Mice plasma was collected from each group blood. The collected blood was centrifuged at 1000 $\times$ g for 10 min in EDTA-coated tubes. The Human Cytokine 6-plexExpress (TNF- α , Eotaxin, IL-4, IL-5, IL-9 and IL-13; Bio-Rad, Hercules, CA) immune assay was performed with a 1 $\times$ 96-well multi plate according to the manufacturer's guidelines. The results were analyzed with Bio-plex (Bio-Rad, CA, United states).

2.6. Immunofluorescence assay

The cells were treated with or without OP-D in confocal dishes. After treatment, cells were fixed with 10% formalin for 10 min. Fixed cells were treated with 0.2% Triton X in PBS for 20 min, and blocked with 5% BSA solution in PBS for 1 h. Blocked cells were incubated overnight with anti-p65 antibody at 4 °C, and incubated with Alexa-Fluor-488 antibody for 1 h at room temperature. The nucleus was stained with a nuclear marker (4,6-diamidino-2-phenylindole) (DAPI; sigma-Aldrich, St. Louis, MO, USA) for 5 min at room temperature [17].

2.7. Immunoblotting assay

Cells were lysed with a cell lysis buffer (Cell Signaling Technology; Danvers, MS, USA). Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) was used to measure protein concentrations. Each sample was separated in a SDS-PAGE gel, and then electro-transferred to a nitrocellulose membrane. The membranes were blocked with 5% BSA in TBS-T. Primary antibodies, i.e. p-ERK1/2, ERK1/2, p-p38, p38, p-p65, p65, I κ B and β -actin (All were 1:3000), were incubated with the membrane at 4 °C overnight. Membranes were extensively washed and then incubated with the following secondary antibodies: goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP [18].

2.8. Statistical analysis

All data were presented as the mean $\pm$ SEM. The Graph Pad Prism 5 software package (Graph Pad Software, San Diego, CA, USA) was used to produce the statistical analysis of the results. An unpaired *t*-test was conducted to analyze Western blot bands and real-time PCR results. *P* value less than 0.05 was considered statistically significant, and all significant data were analyzed in triplicate.

3. Results

3.1. Effect of OP-D on TNF- α stimulated HaCaT cell pro-inflammatory cytokine expression

To investigate the cytotoxicity of OP-D on human keratinocyte (HaCaT) cells, a MTT assay was utilized. OP-D did not affect cell viability up to 1 μ M for 5 h (Fig. 1A). TNF- α was used to induce inflammation in HaCaT cells, which represents inflammatory skin disease in an *in vitro* model. We performed real-time PCR in order to confirm the suppressive effect of OP-D on TNF- α -induced cytokine expression levels in HaCaT cells. We found that TNF- α , IL-6, IL-1 β and IL-17 expression levels are significantly up-regulated by a TNF- α treatment on HaCaT cells. In particular, TNF- α , IL-6 and IL-1 β mRNA expressions significantly decreased by an OP-D treatment (Fig. 1B, C and D). We only observed a tendency to reduce within IL-17 levels (Fig. 1E).

3.2. Effect of OP-D on the MAPKs and NF- κ B signaling pathway in TNF- α stimulated HaCaT cells

We assessed what affects OP-D might have on the MAPKs pathway and NF- κ B signals, the two main mediators of inflammatory reactions. OP-D effectively inhibited phosphorylation of ERK and p38 protein expression levels (Fig. 2A). There were significant decreases in p-ERK and p-p38 in comparison to the total ERK and total p38 levels ratios (Fig. 2B). When HaCaT cells are stimulated by TNF- α , NF- κ B became separated from I κ B and moved into the nucleus which resulted in transcription facilitation of the genes encoding pro-inflammatory factors, such as diverse cytokines. In addition, I κ B is phosphorylated and decomposed by proteasome in the cytosol. In whole-cell fractions, OP-D has a suppressive effect on I κ B degradation (Fig. 2A). The relative levels of proteins were calculated as the I κ B/ β -actin ratio (Fig. 2C). In the nuclear fractions,

OP-D effectively inhibited NF- κ B translocation in TNF- α -treated HaCaT cells (Fig. 2A). Nuclear NF- κ B protein intensity were normalized with lamin B, and OP-D showed significant decrease in NF- κ B protein levels in the nucleus (Fig. 2C). Additionally, by means of an immunofluorescence assay, we observed the noticeable mitigation effect of OP-D on NF- κ B translocation into the nucleus, which was induced by TNF- α in HaCaT cells (Fig. 2D). This indicates that OP-D reduced the pro-inflammatory cytokines expressions through a mitigation effect on MAPKs and NF- κ B signaling pathways, which were activated by TNF- α in HaCaT cells.

3.3. Effect of OP-D on DNCB-induced AD-like mouse skin and mast cell activation

The experimental schedule to induce AD with the mouse model has been described in the materials & methods section (Supplementary Fig. S1). Topically-applied OP-D onto the DNCB-induced AD model noticeably suppressed clinical symptoms, compared to the DNCB-challenged group only (Fig. 3A). The effect of OP-D on the histopathological features was characterized by H&E staining (Fig. 3B). In the H&E staining results, while the normal group showed narrow thickened ranges in the epidermis and dermis, the DNCB-challenged group demonstrated greater thickening of skin in comparison to the normal group. The epidermal thickness of the DNCB-challenged group ($130.6 \pm 7.7 \mu$ m) was increased by nearly three times in comparison to that of the normal group ($42.2 \pm 7.4 \mu$ m). In the OP-D-treated group, skin thickening was significantly mitigated with the OP-D 1 μ M group ($101.1 \pm 1.9 \mu$ m) and the OP-D 10 μ M group ($96.4 \pm 4.2 \mu$ m) (Fig. 3C). The effect of OP-D on mast cell activation upon the DNCB treatment was determined by counting the cells under toluidine blue staining (Fig. 3C). The DNCB-sensitized group (102.7 ± 3.07) showed approximately 4.5 times more stained mast cells than the normal group (24 ± 2.7). The OP-D (OP-D 1 μ M: 59.7 ± 5.5 , OP-D 10 μ M:

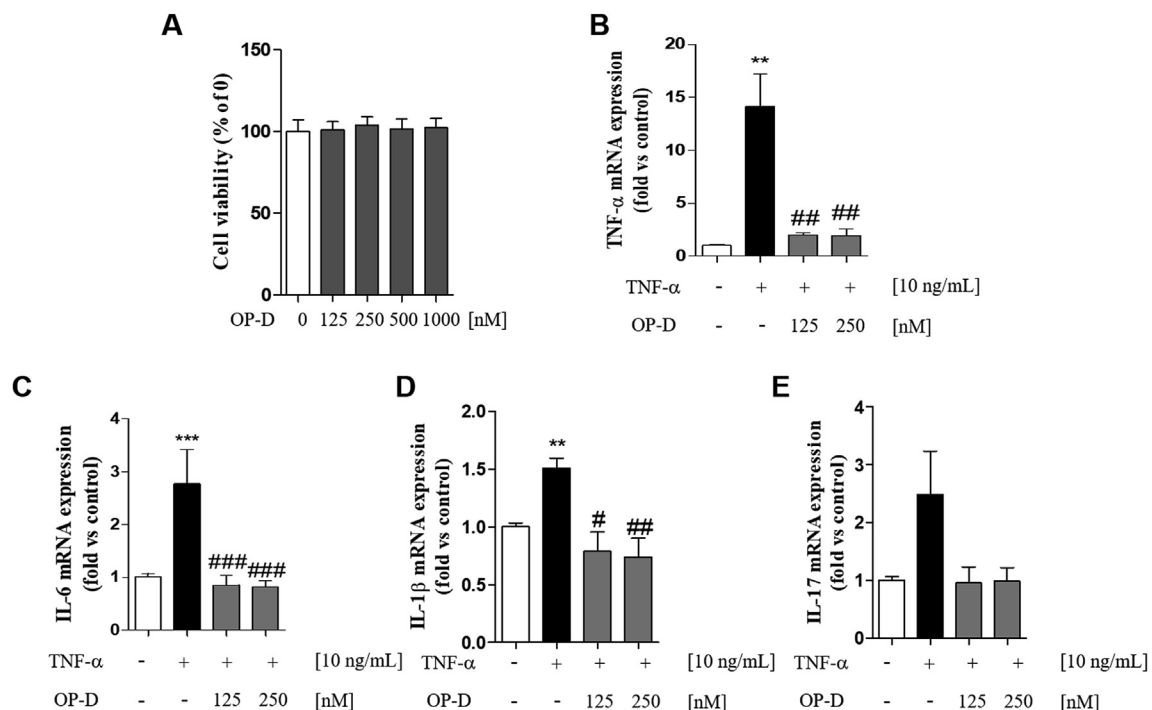


Fig. 1. Effect of OP-D on cytokine mRNA expression levels in HaCaT cells

(A) The cell viability of HaCaT cells with OP-D over the 5 h period was determined by MTT assay. (B)–

(D) Cells were treated with OP-D for 4 h before being treated with TNF- α for 1 h. TNF- α , IL-6, IL-1 β and IL-17 mRNA expression levels were detected by RT-qPCR. The data are shown as the mean $\pm$ SEM. # p < 0.05, ## p < 0.01, ### p < 0.001 in comparison to the TNF- α -treated group only ** p < 0.01, *** p < 0.001 compared with the control (non-treated) group.

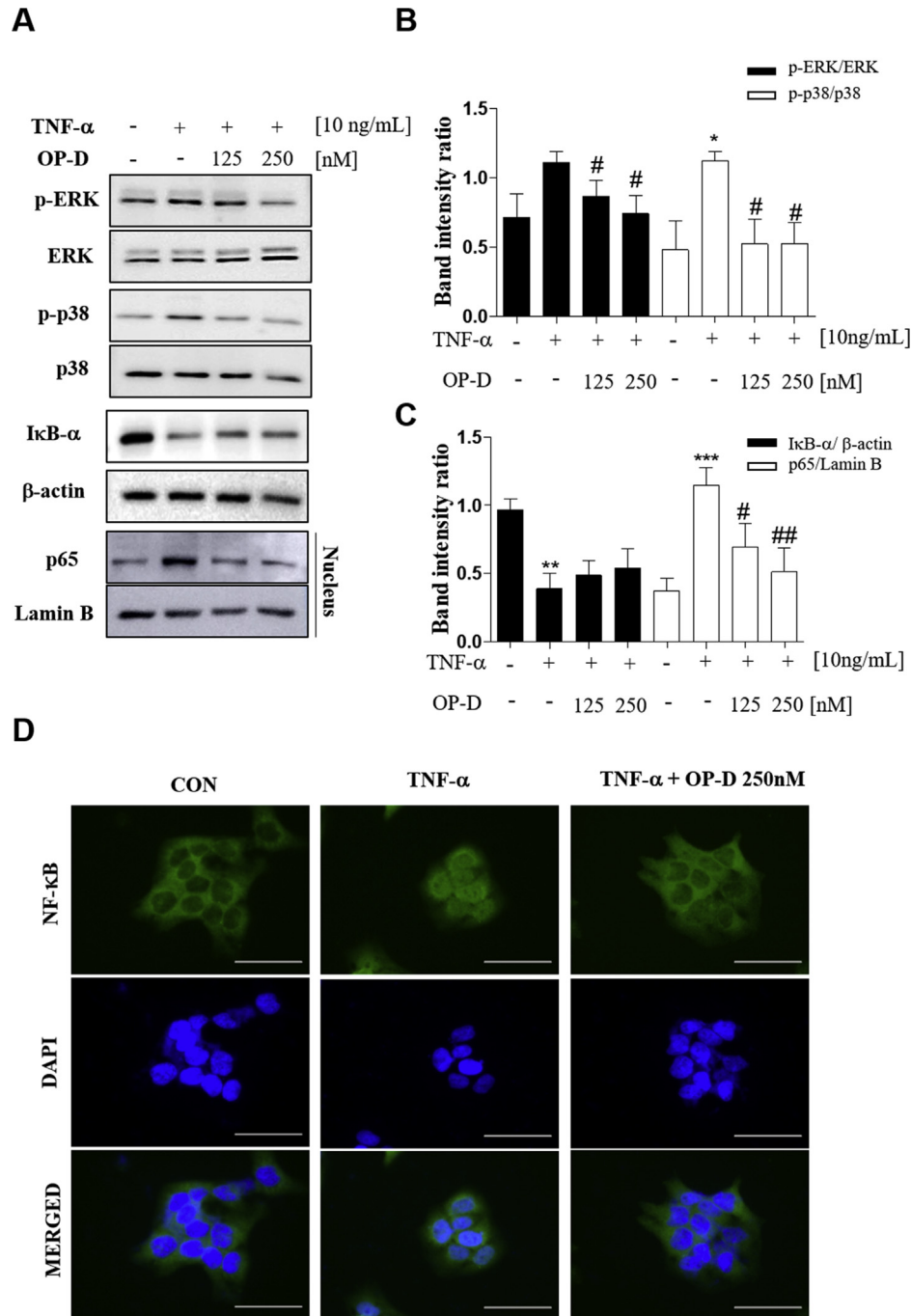


Fig. 2. Effect of OP-D on MAPKs pathway and NF- κ B translocation in HaCaT cells

(A) Cells were treated with OP-D for 4 h before treated with TNF- α for 15 min. The expression levels of phosphorylated ERK, phosphorylated p38, I κ B- α and nuclear NF- κ B were detected by Western blot. (B and C) The ratio of p-ERK/ERK, p-p38/p38, I κ B- α / β -actin and nuclear p65/lamin B was determined by image J. (D) Nuclear p65 in TNF- α -stimulated HaCaT cells were detected by immunofluorescence analysis using microscopy (the magnification is 400 x, scale bar: 50 μ m). Cells were treated with OP-D for 4 h before being treated with TNF- α for 1 h. The data are shown as the mean $\pm$ SEM. * p < 0.05, ** p < 0.01 in comparison to the TNF- α -treated group only. * p < 0.05, ** p < 0.01, *** p < 0.001 compared with the control (non-treated) group.

60 $\pm$ 7.8) trials had a significant suppressive effect on the number of mast cells in the dermis in comparison to that in the DNCB group (Fig. 3D).

3.4. Effect of OP-D on the DNCB-induced cytokine levels in blood and spleen inflammation

As shown in Fig. 4, the eotaxin, IL-4, IL-13, TNF- α , IL-5 and IL-9

expression levels in mouse plasma were detected by the multiplex assessment method. DNCB treatment showed increase in the cytokines levels in comparison to the normal group, and OP-D was found to have a significant inhibitory effect on increased TNF- α , IL-4, and IL-5 expression levels in the DNCB-sensitized group. IL-13 and eotaxin production levels had a tendency to decrease with OP-D, and there was no suppressive effect on IL-9 expressions. To test the overall anti-inflammatory effects of OP-D, we observed

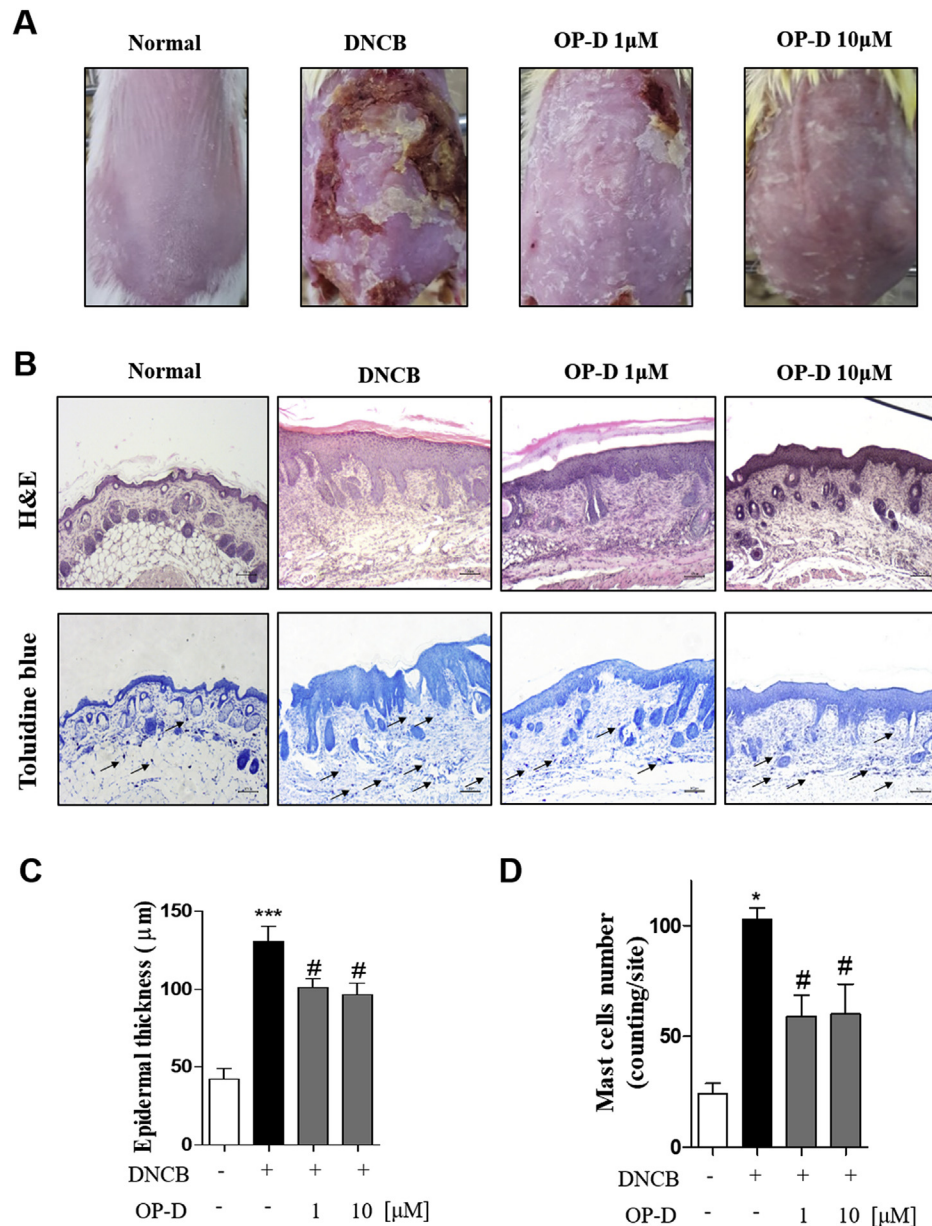


Fig. 3. The effect of OP-D on DNCB-induced AD mouse skin lesions

(A) Photographs for the clinical features of mouse dorsal skin lesion. (B) The representative images of dorsal skin were stained with hematoxylin and eosin ($n = 3$) (the magnification is 100 x, scale bar: 100 μ m). (C) Measurement of epidermis thickness. (D) The representative images of mast cells in dorsal skin were stained with toluidine blue (the magnification is 100 x, scale bar: 100 μ m). (E) The number of mast cells were counted ($n = 3$). Normal (treated with vehicle), DNCB (vehicle and DNCB-sensitized group), OP-D 1 μ M (treated with DNCB and Ophiopogonin 1 μ M group), OP-D 10 μ M (treated with DNCB and Ophiopogonin 10 μ M group). The data are shown as the mean $\pm$ SEM. # $p < 0.05$ in comparison to the DNCB group and, *** $p < 0.001$, * $p < 0.05$ compared with the normal group.

mouse spleen and analyzed the cytokine levels with real-time PCR. The DNCB-sensitized group showed an abnormally expanded spleen. OP-D remarkably reduced the spleen weight to body weight ratio compared to the DNCB-sensitized group only (Supplementary Fig. S2A). Moreover, OP-D showed a significant decrease in TNF- α , IL-1 β and IFN- γ mRNA expression levels (Supplementary Fig. S2B, C and D). These results suggested that OP-D reduced mast cell activation, skin thickening in AD-like mouse skin and cytokine expression levels in the blood increased by DNCB.

4. Discussion

Atopic dermatitis (AD) is a complex disease that includes

abnormal inflammatory pathways and immunological hyper-responsiveness [20,21]. It is accompanied by the collapse of the skin barrier, leading to dry itchy skin, which is exacerbated by scratching and the inflow of allergens through the damaged skin. However, the actual cause of AD is not known yet. To investigate the pathology and mechanism of contact allergic dermatitis, DNCB-induced mouse models are predominantly used. DNCB is known to cause skin inflammation or ear swelling and polarizes Th cells to stimulate Th2 cell differentiation which promotes inflammatory cell infiltration, accompanied by skin thickening [19].

Epidermal keratinocytes are enhancer cells involved in innate immunity, and function by producing antimicrobial peptides, in order to show inflammatory responses, as well as by facilitating the

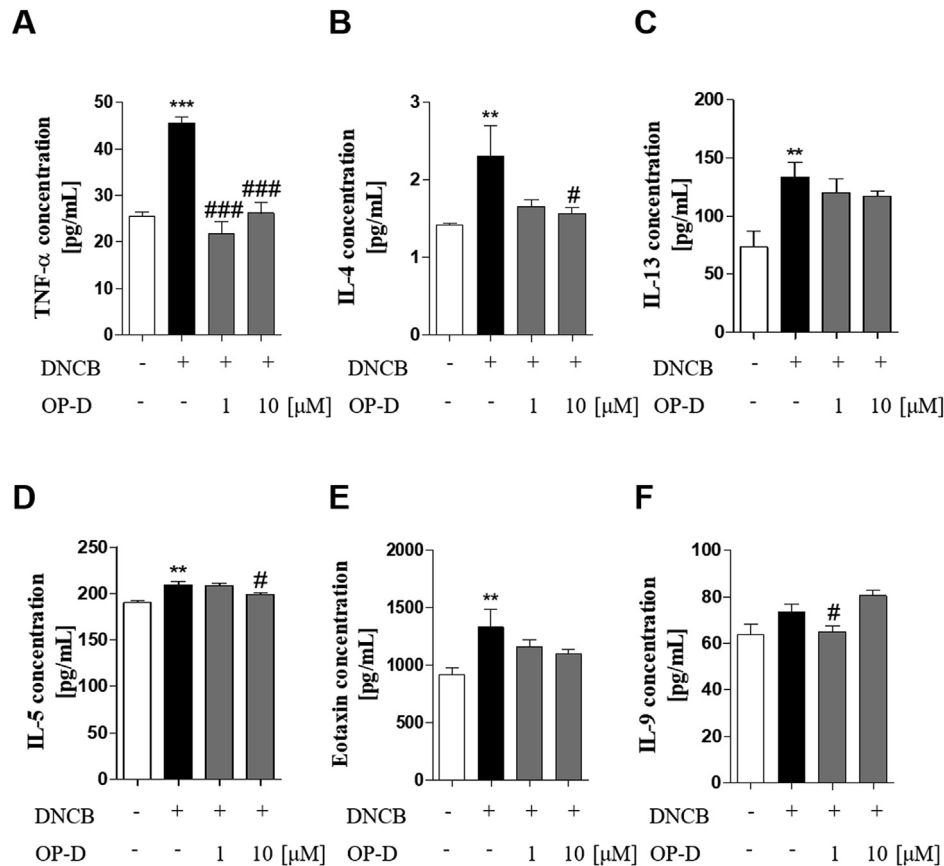


Fig. 4. Inhibitory effect of OP-D on secreted pro-inflammation cytokines in the DNCB-induced mice plasma (A)–(F) The levels of Eotaxin, IL-4, IL-13, IL-9, IL-5 and TNF- α were detected by multiplex ($n = 5-6$). The data are shown as the mean $\pm$ SEM. ### $p < 0.001$, # $p < 0.05$ in comparison to the DNCB group. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared with the normal group.

expression of TLRs. Under AD-like conditions, their ability to produce antimicrobial factors for innate immunity was deficient [22]. When exposed to pro-inflammatory cytokines, keratinocytes secrete specific chemokines and cytokines in order to stimulate T cell differentiation.

TNF- α , a well-known potent inflammatory cytokine, is an important factor in controlling immune responses and cell differentiation, and it is involved in inflammation signal transduction by inducing the migration of NF- κ B into the nuclei of keratinocytes [8,22].

IL-17 is an important therapeutic target for AD treatment. Lack of IL-17A suppressed dermatitis through inhibition of IL-4 productions. Additionally, as it initiates the production of TNF- α , it eventually intensifies the impact of AD [23]. IL-6 and IL-1 β has been reported to increase with IL-17 stimulation in keratinocytes, and they are also associated with AD pathogenesis. IL-1 β is considered to be a potential early trigger for inducing AD-like epidermal inflammation in normal human epidermal keratinocytes [24].

The MAPKs and NF- κ B pathways are major intracellular signaling pathways that respond to inflammatory stimuli. The cascade transferred from the cytokine receptor leads to the phosphorylation of MAPKs, which are classified into three groups: ERK1/2, JNK, and p38. In particular, p38 is stimulated by using cytokines such as TNF- α and IL-17, and it is involved in the activity of NF- κ B with JNK. NF- κ B consists of p50 and p65, and it is present as a trimer with I κ B in inactivated state.

NF- κ B is activated and separated from I κ B, and moves into the nucleus in a phosphorylated form to act as a transcription factor, inducing the expressions of pro-inflammatory cytokines. The

isolated I κ B is also phosphorylated, ubiquitinated and degraded by cytosol proteasome. Consequently, nuclear NF- κ B promotes inflammation by transcription with various cytokines [25,26].

This study demonstrates the anti-inflammatory effect of OP-D on human keratinocytes, by inhibiting MAPKs and NF- κ B in the signaling pathways and the excessive cytokine expressions induced by TNF- α . One hypothesis is based on AD pathology, which leads to an imbalance in specific helper T cells, acute itching, the production of IL-4, IL-13 and other interleukins that increase the IgE levels. IgE can aggravate AD by stimulating mast cells in the skin. Mast cells are found around skin vessels, lymphatic vessels, glandular structures, and hair follicles, and are stimulated by the high-affinity IgE receptor on the cell surface releasing excessive histamine in the granule producing cytokines. In addition, histamine derived from mast cells produces pro-inflammatory factors of keratinocytes, and mast cells can be a marker showing the severity of AD [27].

In this study, we performed a local treatment of OP-D on DNCB-induced mice, and we were able to confirm that the topical administration of OP-D dose reduced the infiltration of mast cells and epidermal thickening.

Abnormally increased Th2 cytokines have been found in both acute and chronic AD patients. Some research has indicated that eczema-like features exist in a transgenic mouse model, engineered to express these cytokines excessively. In particular, IL-5 contributes to epidermal thickness [28].

IL-4 and IL-13, serving as key components in the pathogenesis of AD, induce keratinocyte differentiation, and are associated with excessive skin keratinization [7]. Eotaxin acts as a powerful chemo-attractant, leading to the activation of immune cells such as

eosinophils and basophils and invoking Th2 cytokines [29]. It has also been reported that the expression of eotaxin increases in the plasma of chronic AD patients [29,30]. _ENREF_19OP-D has a significant inhibitory effect on increased IL-4, TNF- α and IL-5 levels in DNCB-treated mice. The spleen, the largest lymphatic organ in the body, plays a significant role in the immune response and is also involved in T cell and B cell activation in an inflammatory stimulation. This showed a decrease in the expression levels of cytokines after analysis [31]. These results imply that OP-D inhibits the increase of certain Th 2 cytokines in the DNCB-treated mice body.

In conclusion, we believe that OP-D can be a potential natural compound for AD treatment. OP-D may also regulate inflammation signals in diverse inflammatory skin diseases and can be developed as an anti-allergic substance through the regulation of mast cell activation.

However, we would urge further studies in order to examine the effect of OP-D, especially related to the anti-allergic effects, such as the confirmation of IgE and histamine secretions and more efficient mechanism studies.

4.1. Authors's contributions

Eun-Jin An designed this study and wrote the manuscript. Yumi Kim contributed to the execution of the *in vitro* research. Seung-Hyun Lee and Seung-Han Choi contributed to execution of the *in vivo* research. Won Seok Chung and Hyeung-Jin Jang contributed the statistical analysis and wrote the manuscript. All authors read and approved the final manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2019.10.190>.

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Culture of keratinocyte

Human keratinocyte (HaCaT) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% and 1x antibiotic-antimycotic solution, and were incubated at 37 °C and 5% CO₂.

PCR primer for mRNA expression analysis and Real-Time qPCR

Real-time quantitative PCR was performed using the Universal SYBR Green Master Mix (Applied Bio systems, USA). Amplification of the cDNA was performed as follows: 95 °C for 15 min followed by 40 cycles at 95 °C for 30 s, at 59 °C for 30 s, and at 72 °C for 30 s. The real-time PCR analysis was performed on an Applied Bio-systems Step One system (Applied Bio-systems, USA). In this study, quantification based on the relative expression of a target gene versus GAPDH gene was utilized to determine the level of mRNA expression.

The following PCR primers were used:

Human IL-6, forward: 5-AGGGCTCTTCGGCAAATGTA-3,

reverse: 5-GAAGGAATGCCCATTAACAACAA-3.

Human IL-17, forward: 5-GGAACGTGGACTACCACATCAA-3,

reverse: 5-GCGCAGGACCAGGATCTCT-3.

Human TNF- α , forward: 5-GCAGGTCTACTTTGGGATCATTGT-3,

reverse: 5-GCGTTTGGGAAGGTTGGA.

Human IL-1 β forward: 5'-TCAGCCAATCTTCATTGCTCAA-3'

Reverse: 5'-TGGCGAGCTCAGGTACTTCTG-3'

Mouse TNF- α , forward: 5'-TCCAGGCGGTGCCTATGT-3'

Reverse: 5'-GCCCCTGCCACAAGCA-3'

Mouse IL-1 β , forward: 5'-CCATGGCACATTCTGTTCAA-3'

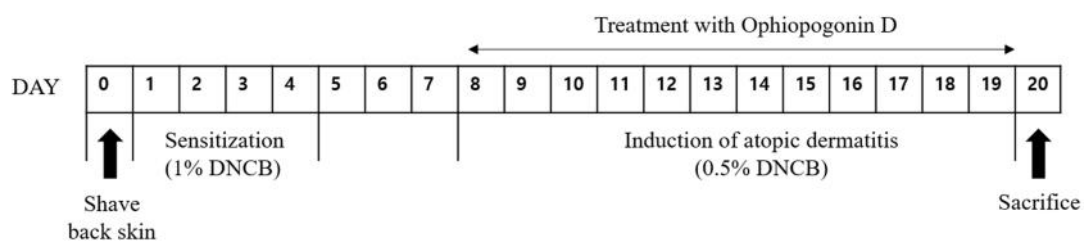
Reverse: 5'-GCCCATCAGAGGCAAGGA-3'

Mouse IFN- γ , forward: 5'-GAACGGACACACTGCATC-3'

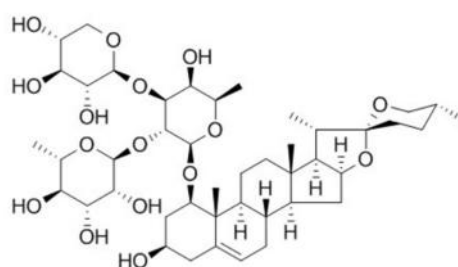
Reverse: 5'-GAGCTCATTGAATGCTTGG-3'

Supplementary Figure 1.

A



B



Ophiopogonin D

Fig. S1. Experimental design and chemical structure of Ophiopogonin D

- (A) Experiment design from shaving and inducing atopic dermatitis on mice back skin to sacrifice.
 (B) The chemical structure of Ophiopogonin D.

Supplementary Figure 2.

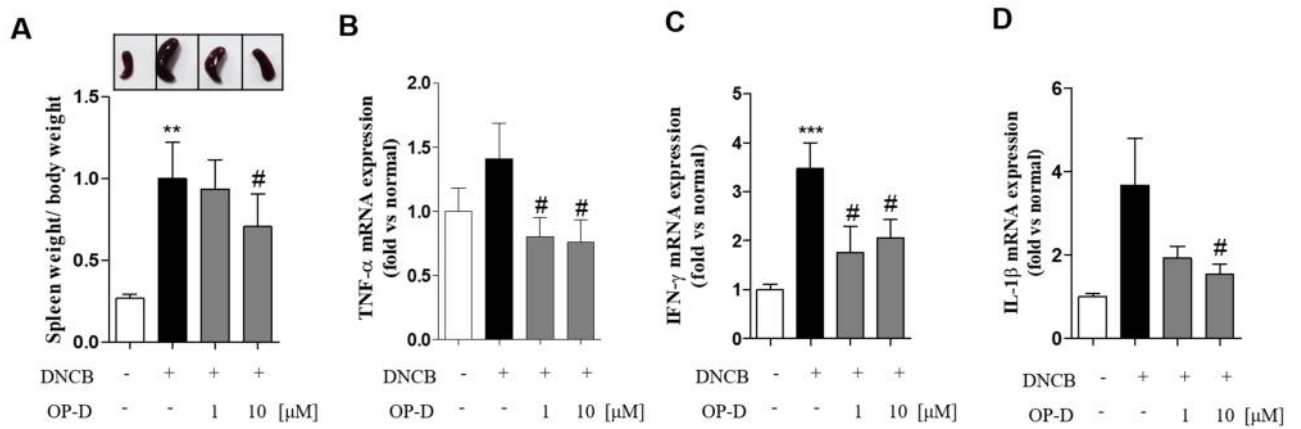


Fig. S2. Effect of OP-D on DNCB-induced mice spleen weight and cytokine expression.

(A) The spleen mass/ body weight was measured. (B)-(D) Relative mRNA expression in DNCB-induced mice spleen was measured by Real-Time PCR (n=3). The data are shown as the mean $\pm$ SEM. #p<0.05 in comparison to the DNCB group. *** p<0.001, ** p<0.01 compared with the normal group.

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