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The suppressive effect of puerarin on atopic dermatitis-like skin lesions through regulation of inflammatory mediators in vitro and in vivo

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ABSTRACT

Atopic dermatitis (AD) is one of the common inflammatory immune disorders. Puerarin is the main isoflavonoid obtained from the root of *Pueraria lobata* and has been known have ameliorative effects on diverse inflammatory diseases. However, the effects of puerarin on AD have not been uncovered. 2,4-dinitrochlorobenzene (DNCB) was used to induce atopic dermatitis(AD)-like skin lesions on BALB/c mice for 17 days. Further, the BALB/c mice were orally administered puerarin. Puerarin ameliorated DNCB-induced AD-like symptoms in the mice by regulating skin thickness, degranulation of mast cells, and serum immunoglobulin E (IgE). Human keratinocytes (HaCaT cells) were also used to clarify the effects of puerarin on the secretion of pro-inflammatory cytokines. Puerarin inhibited the secretion of inflammatory cytokines and chemokines. The aim of this study was to investigate the protective and alleviative effect of puerarin on AD in vitro and in vivo. The results in this study indicated that puerarin ameliorates AD-like skin lesion and skin inflammation via regulation of various atopic and inflammatory mediators. Therefore, puerarin might be useful in treating AD and other skin diseases.

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1. Introduction

Atopic dermatitis (AD) is a highly pruritic chronic inflammatory skin disease. The clinical symptoms of AD are known to relapse, and include, pruritic eczematous skin lesions, dry skin, skin hypersensitivity, and skin thickening [1,2]. Throughout the World, AD affects approximately 15–30% of children and about 2–10% of adults, and the prevalence rate of this disease is increasing annually [3]. AD is caused by a complex interaction between various factors, such as genetic factors, environmental factors, pharmacological disorders, skin barrier defects, and the immunological system [4]. Although the major cause of AD is still unknown, the onset of AD appears to be caused more accurately by allergic sensitization by environmental factors or allergens [1,5]. In early AD progression, extrinsic

or environmental triggers damage the skin barrier. This damage aggravates immunoglobulin E (IgE)-mediated sensitization, serious forms of skin inflammation, and immune responses [6]. Moreover, AD develops owing to the imbalance between T-helper (Th)-1 cells and Th2 cells in the immune system [7]. Patients with AD commonly have increased levels of serum IgE and Th2-mediated inflammatory cytokines, such as interleukin (IL)-4, IL-5, and IL-13. Th2 cytokines directly affect skin cells, such as keratinocytes. The affected keratinocytes produce pro-inflammatory cytokines and chemokines that induce the invasion of immune cells into inflammatory skin lesions [8]. Chronic AD skin lesions are characterized by increased epidermal thickness and, activation and infiltration of mast cells. Furthermore, the expressions of several inflammatory mediators such as protease-activated receptor 2 (PAR2), nuclear factor- κ B (NF- κ B), thymic stromal lymphopoietin (TSLP) and mitogen-activated protein kinases (MAPKs), are related to the severity of inflammation of AD [9].

Puerarin, one of the major isoflavonoid compounds, is isolated from the root of *Pueraria lobata* (known as Gegen in China), Gegen is widely used in traditional Chinese medicine. Until now,

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numerous medicinal properties of puerarin have been extensively identified [10]. It has been widely used in the treatment of cardiovascular and neuronal diseases. Moreover, puerarin has been known to have anti-oxidant, anti-inflammatory, anti-cancer, and anti-diabetic effects and reduce the risk of diabetic complications. Furthermore, it has been used in alcoholic liver injury, bone formation, Parkinson's disease, Alzheimer's disease, and allergic inflammation. It is also used frequently to treat fever, diarrhea, emesis, and toxicosis.

However, there is no research on the effects of puerarin on AD in vivo and in vitro. Thus, the aim of our present study was to investigate the anti-inflammatory effect and the mechanism of puerarin on DNCB-induced AD in vivo and TNF- α /INF- γ -induced skin inflammations in vitro.

2. Materials and methods

2.1. Cell culture and reagents

Human keratinocytes, HaCaT, were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The HaCaT cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Hyclone), and 1% of 100 units/ml of penicillin-streptomycin, and incubated at 37 °C in a humidified 5% CO₂ incubator.

Puerarin (purity, >98%) was purchased from Sigma-Aldrich and dissolved in sterile PBS solution.

2.2. MTT assay

The MTT assay was used to investigate the cell viability of puerarin on the HaCaT cells. Briefly, cells in 100 μ L culture medium were seeded at 1×10^4 cells/well in 96-well plates, and incubated for 24 h at 37 °C; puerarin was treated with several concentration (10 μ M–100 mM). MTT solution was added to the well plates, and the cells were further incubated over 4 h at 37 °C. The crystallized formazan was dissolved in DMSO and read at 540 nm using a microplate reader.

2.3. Real-time PCR (polymerase chain reaction)

The dorsal skin tissues were collected in each mouse at the end of the experiment. The HaCaT cells were harvested after reagent treatment. Total RNA was isolated from the skin tissues and cells using Trizol reagent. The cDNA was synthesized using the Super Script TM III kit, according to the manufacturer's protocol. The mRNA expression was quantitatively determined using Power SYBR Green PCR Master Mix (Applied Biosystems). The primer sequences used for real-time PCR are shown in Table 1.

2.4. Western blot analysis

The HaCaT cells were pre-treated with puerarin for 2 h and stimulated with TNF- α /INF- γ (each 10 ng/ml) for 30 min during incubation at 37 °C. The total proteins were extracted from the cells using a lysis buffer. The lysate was subjected to SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were probed with primary antibodies at 4 °C overnight. After antibody probing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody. The membranes were visualized using an enhanced chemiluminescence detection kit (Millipore, MA). Membranes were exposed to Fusion Fx gel documentation system (Vilber Lourmat, Marne-la-Vallee).

2.5. Animals

Four-week-old male BALB/c mice weighing 20 ± 2 g were purchased from SAMTAKO Bio Korea. The mice were housed in stainless steel cages in a controlled environment with a temperature of 22 ± 2 °C and $55 \pm 5\%$ humidity under a 12 h light/12 h dark cycle. They were fed a standard laboratory diet and water ad libitum. The research was carried out in accordance with the ethical regulations with the approval of the Animal Experiment Ethics Committee of Chonbuk National University (Confirmation No. CBNU 2018-007).

2.6. DNCB-induced AD-like skin lesion

The animals were randomly divided into six groups of five mice each: Normal group: normal control group with a vehicle treatment; DNCB-induced group: negative control group, mice sensitized with DNCB and treated with water; P 10 group: mice sensitized with DNCB and treated with 10 mg/kg puerarin; P 30 group: mice sensitized with DNCB and treated with 30 mg/kg puerarin; P 50 group: mice sensitized with DNCB and treated with 50 mg/kg puerarin; and dexamethasone group: positive control group, mice sensitized with DNCB and treated with 1 mg/kg dexamethasone.

AD was induced in the mice by treatment with DNCB (Sigma-Aldrich). On day 0, the hair on the dorsal skin of the mice in all groups was shaved using an electric razor. During days 1–3, 200 μ L of 1% DNCB dissolved in acetone/olive oil (4:1,v/v) was applied once daily to the dorsal skin of the mice for 3 days to induce dermatitis in the DNCB-induced, puerarin groups, and dexamethasone groups. The normal group received a vehicle treatment (only acetone/olive oil). After this initial sensitization treatment, the mice were dorsally treated with 0.5% DNCB in acetone/olive oil at 2 days of intervals for 2 weeks. The puerarin groups (10 mg/kg, 30 mg/kg, and 50 mg/kg) and dexamethasone group (1 mg/kg) were orally administered with their respective drug concentrations daily for 2 weeks from day 4. The mice were sacrificed on day 17 of the experiment.

2.7. Measurement of dorsal skin thickness and clinical score of the AD-like skin lesions

The dorsal skin thickness of each mouse was measured thrice using a micrometer (Mitutoyo, Japan) on the last day of the experiment. The severity of AD was evaluated as the average of the individual skin symptom scores assessed as 0 (none), 1 (mild), 2 (median) and 3 (severe) for each mouse on the last day of the experiment.

2.8. Histological analysis

At the end of the experiments, the tissue specimens from the dorsal skin of each mouse were obtained, fixed in 10% formalin,

Table 1
Primers utilized for real-time PCR.

Gene	Forward	Reverse
hIL-5	GCTAGCTCTGGAGCTGCCT	CTTCAGTGCACAGTTGGTGA
hIL-6	CTCCACAAGCGCCTTCGGTC	TGTGTGGGGCGGCTACATCT
hTNF- α	TGGAGTGATCGGCCCCAG	ACAGGCTTGCTACTCGGGGT
mTNF- α	TAGCCAGGAGGGAGAACAGA	TTTCTGGAGGGAGATGTGG
m IL-1 β	CTCCATGAGCTTTGTACAAGG	TGCTGATGTACCAAGTTGGGG
mIL-6	GACAACCACGGCTTCCTCA	GGTACTCCAGAAGACCAAGGA
mIL-4	ATGGGTCTCAACCCCAAGCTA	TGCATGGCGTCCCTTCTCT
mIL-31	GCATAAAGCAGGCATCTGGG	ACTGTGTCTCGCTCAACAC
GAPDH	GTTAGGAAAGCCTGCCGGTG	GCATACCCCGAGGAGAAATC

embedded in paraffin wax and serially sectioned at 6 μ m. The sections were stained with haematoxylin and eosin (H&E) and toluidine blue (TB) for determining the number of mast cells. The TB-stained mast cells were counted by random selection of five tissue sections. Histological changes were observed using a microscope and photographed.

2.9. Immunohistochemistry (IHC)

The slide was deparaffinized by dipping twice in xylene, rehydrated in ethanol and rehydrated with water. Antigen retrieval was performed in sodium citrate buffer (10 mM citrate buffer with 0.05% Tween-20, pH = 6.0) for 10 min in the microwave. Endogenous peroxidase activity was blocked using 3% H₂O₂ (Dako). The slides were then pre-blocked with a protein blocking serum (universal Quick kit) for 10 min, followed by overnight incubation with a specific antibody at 4 °C. After incubation, the slides were washed in TBS, and the following procedure was performed using a LSAB2 kit (Dako), according to the manufacturer's instructions. The chromogenic substrate of all the slides was 3,3'-diaminobenzidine, which was then counterstained with hematoxylin (Sigma-Aldrich). Finally, the slides were mounted using a Faramount aqueous mounting medium and covered slip.

2.10. Enzyme-linked immunosorbent assay (ELISA)

We collected blood samples from the mice after sacrifice. Serum samples were obtained after centrifugation at 3000 rpm for 10 min. Using a mouse IgE ELISA kit (Biolegend, San Diego, CA), total serum

IgE levels were analyzed, according to the manufacturer's instructions.

2.11. Statistical analysis

All values were expressed as means $\pm$ S.D. Statistical significance was determined using the one-way analysis of variance (ANOVA). 'P'-values lower than 0.05 were considered statistically significant.

3. Results

3.1. Effects of puerarin on TNF- α /IFN- γ -induced cytokine secretion in the HaCaT cell line

Epidermal keratinocytes are known to play various roles in the immune diseases associated with allergy, AD and other skin diseases [11]. Therefore, we investigated the effect of puerarin on the cell viability in HaCaT cells using an MTT assay. Puerarin treatment ranging from 10 μ M to 10 mM did not show cytotoxicity in the HaCaT cells (Fig. 1A). To assess whether puerarin inhibits the expression of TNF- α /IFN- γ -induced pro-inflammatory cytokines, we performed real-time PCR. TNF- α /IFN- γ -stimulated the HaCaT cells significantly increased the mRNA levels of cytokines IL-5, TNF- α , and IL-6; the expression levels of cytokines IL-5, TNF- α , and IL-6 were suppressed by the puerarin treatment (10 μ M, 100 μ M, 1 mM and 10 mM) (Fig. 1B–D). To support the effect of puerarin on inflammatory mediator expression in HaCaT cells, we observed TNF- α /IFN- γ -induced chemokines secretion in HaCaT cells (Supplementary Fig. S1). Puerarin effectively inhibited TNF- α /IFN-

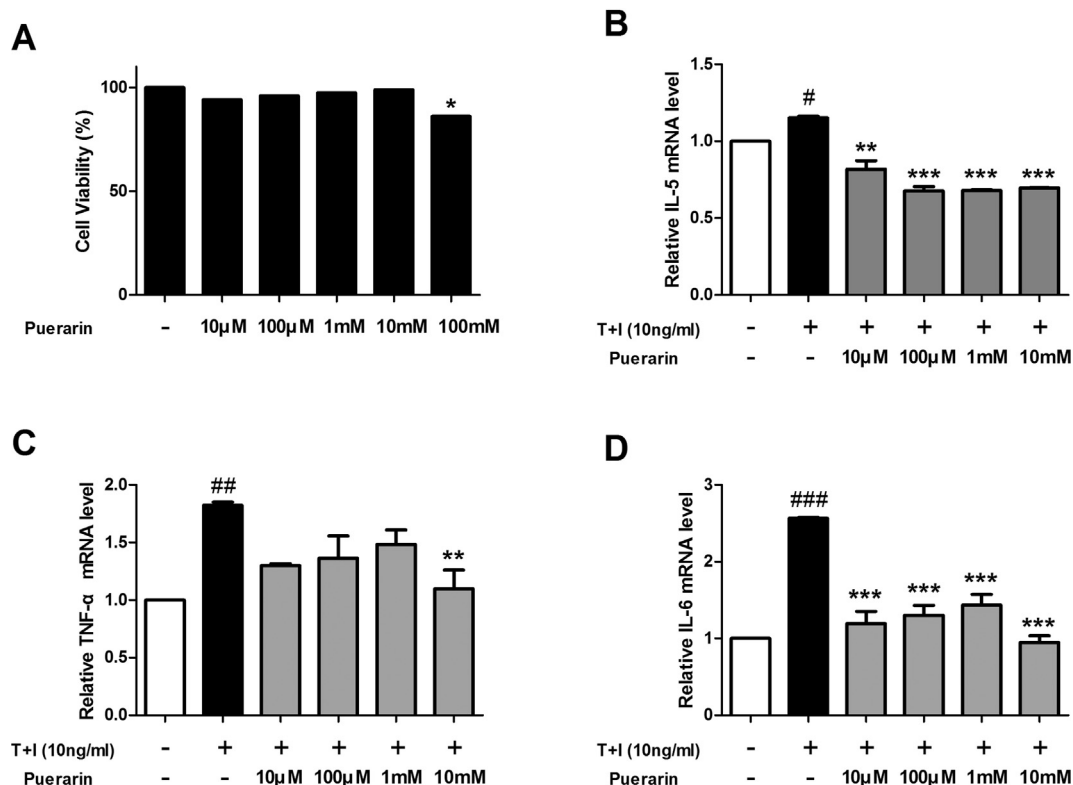


Fig. 1. Puerarin decreased pro-inflammatory cytokines in the HaCaT cells.

HaCaT cells were incubated for 24

h in the presence of several concentrations of puerarin (10 μ M–100 mM). Cell viability was measured using an MTT assay (A). The HaCaT cells were pretreated with the indicated concentrations of puerarin for 2 h and then stimulated with TNF- α /IFN- γ for 30 min. The mRNA expression levels of IL-5 (B), TNF- α (C) and IL-6 (D) were determined using real-time PCR. The data are shown as mean $\pm$ SD of the three experiments. * p < 0.05 or *** p < 0.001 versus the no-treatment condition, ** p < 0.01 or *** p < 0.001 versus the TNF- α /IFN- γ -treatment only condition.

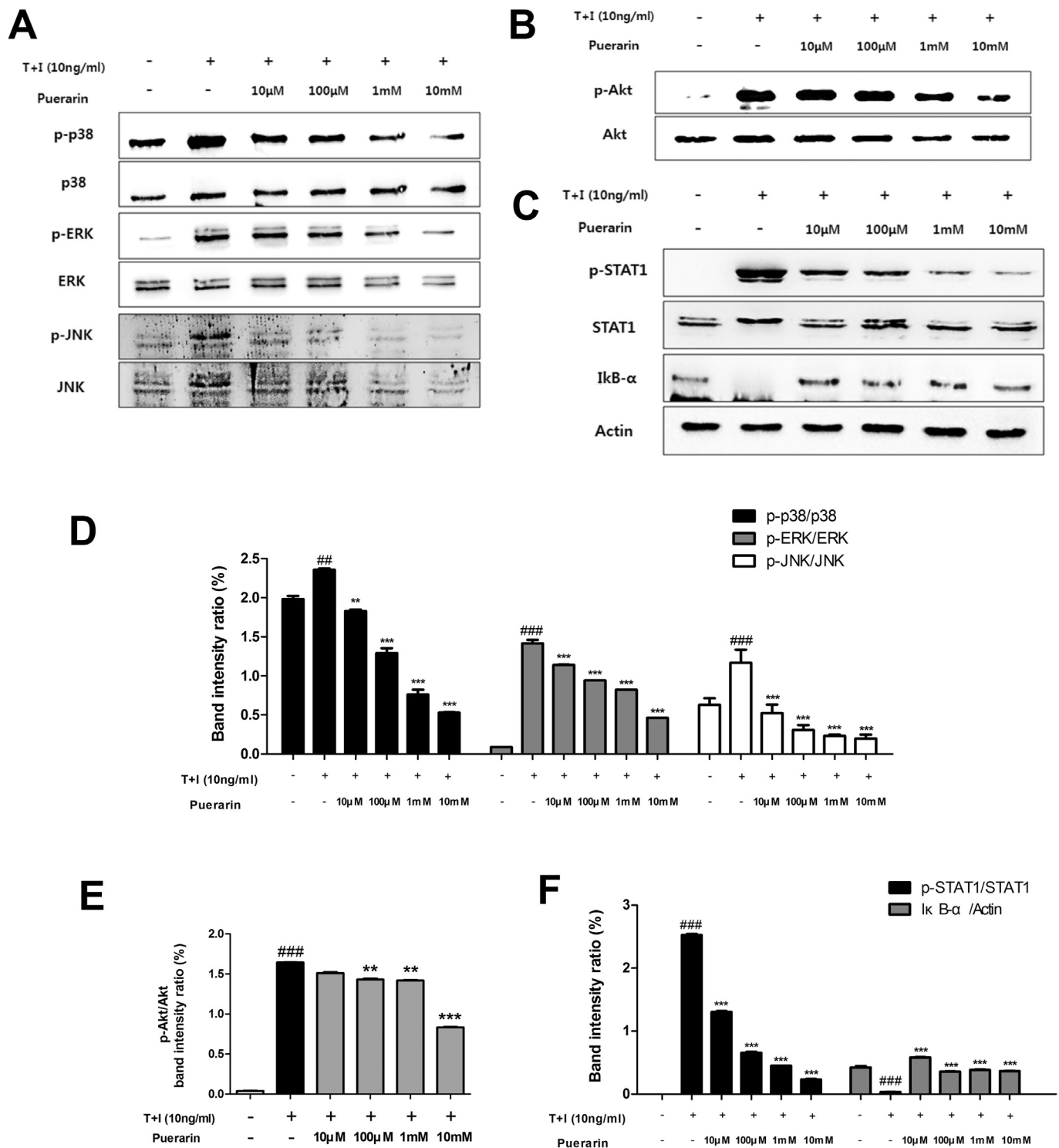


Fig. 2. Puerarin suppressed inflammation through MAPKs, Akt, STAT-1, and IκB-α pathway.

HaCaT cells were incubated with various concentrations of puerarin for 2 h and stimulated with TNF-α/IFN-γ for 30 min. The expressions of the phosphorylated and total forms of p38, ERK, and JNK were evaluated using Western blot analysis (A). The bar graph for the density of the band is as follows: p-p38/p38, p-ERK/ERK, and p-JNK/JNK (D). The expressions of the phosphorylated and total forms of Akt were analyzed using Western blot analysis (B). The bar graph for the density of the band is as follows: p-Akt/Akt (E). The expressions of the phosphorylated and total forms of STAT1 and degradation of IκB-α were measured using Western blot analysis (C). The bar graph for the density of the band is as follows: p-STAT1/STAT1 and IκB-α/Actin (F). The data are presented as mean ± SD of the three experiments. ###*p* < 0.001 versus no-treatment condition, ***p* < 0.01 or ****p* < 0.001 versus only TNF-α/IFN-γ-treatment condition.

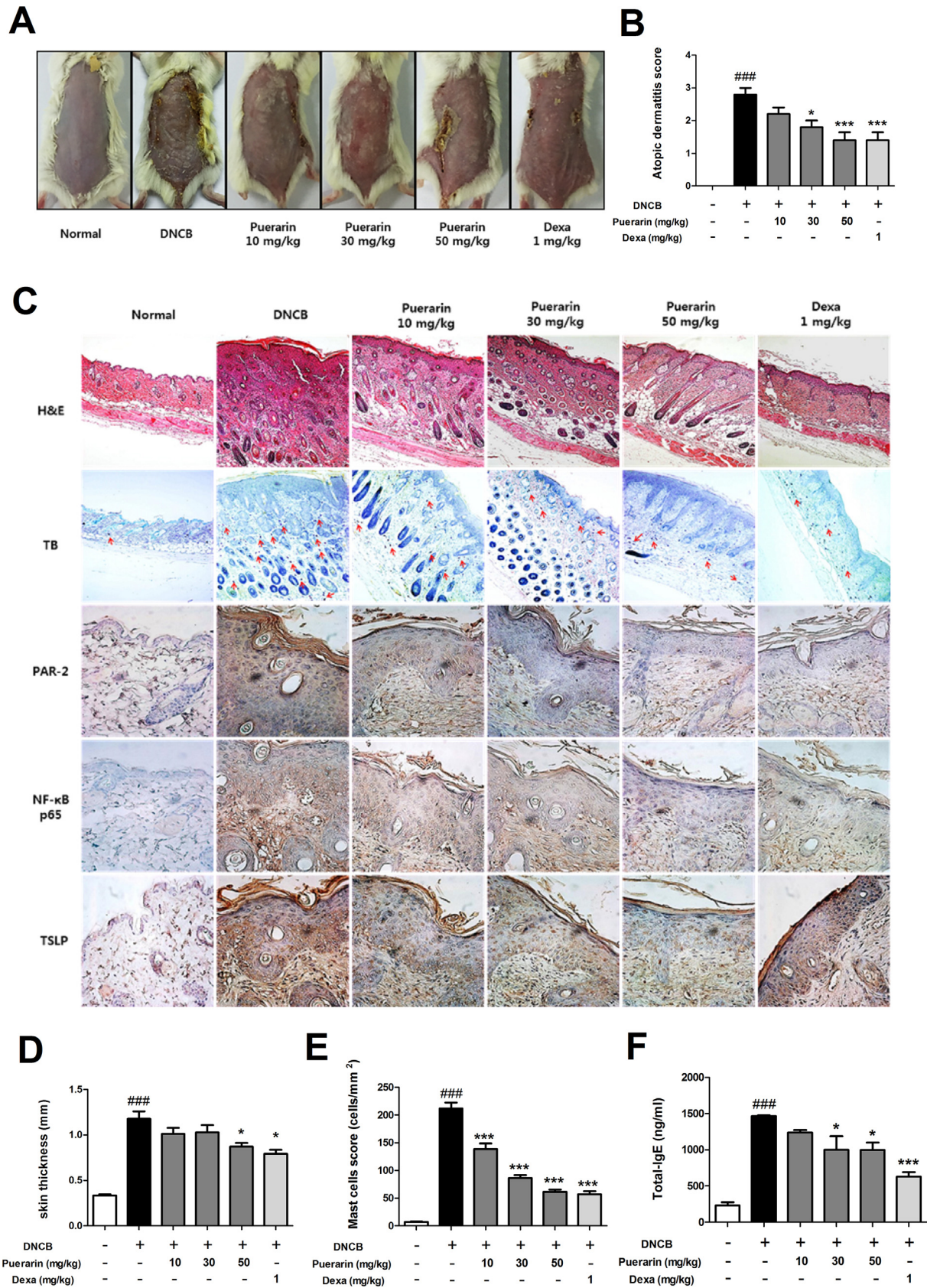


Fig. 3. Puerarin attenuated DNCB-induced clinical symptoms.

The photographs were obtained before the mice were sacrificed (A). The clinical severity of inflammation of each mouse dorsal skin lesion was evaluated (B). Mouse dorsal skins were removed after the experiment. The dorsal skin lesions were stained with H&E and TB. PAR2, NF-κB and TSLP expression levels in dorsal skin tissue were detected by immunohistochemical staining using anti-PAR2, anti-NF-κB and anti-TSLP antibody (C). The figures are representative of each group of mice (n = 5). The images were obtained at

γ -induced the secretion of chemokines (CCL2, CCL5, CCL17, CXCL8, CXCL10, and CXCL11) in HaCaT cells.

3.2. Effects of puerarin on TNF- α /IFN- γ -induced phosphorylation of MAPKs, Akt, STAT-1, and I κ B- α signal pathway in HaCaT cell line

We investigated whether puerarin affects the expression of the pathway of MAPKs, which are important mediators of AD, because phosphorylation of MAPKs (p38, ERK, and JNK) induces cytokine production [12]. The expression of active Akt is involved in promoting growth arrest and differentiation in keratinocytes. We analyzed the phosphorylation of MAPKs and Akt using Western blot analysis. We found that the TNF- α /IFN- γ -induced phosphorylation of MAPKs (Fig. 2A,D) and Akt (Fig. 2B,E) expressions were significantly suppressed by the puerarin treatment in a dose dependent manner, without affecting the total protein levels.

To assess whether puerarin affects the NF- κ B and STAT-1 signaling pathways, we determined the degree of I κ B- α degradation and STAT-1 phosphorylation using Western blot analysis. It is reported that NF- κ B and STAT-1 are related to the transcriptional regulation of TNF- α /IFN- γ -induced pro-inflammatory cytokines and chemokines in the HaCaT cells. Puerarin attenuated I κ B- α degradation, and suppressed phosphorylation of STAT-1 in a dose dependent manner, without affecting the total protein levels (Fig. 2C,F). These results suggested that puerarin inhibited the expression of cytokines and chemokines by blocking the activation of MAPKs, Akt, STAT1 and NF- κ B.

3.3. Effects of puerarin on DNCB-induced clinical signs and tissue injury in vivo

To examine the effect of puerarin on AD, the AD-like skin lesions of the mice were observed 18 days after the start of the experiment. The puerarin groups had noticeably improved AD-like skin symptoms compared with the DNCB-induced group (Fig. 3A). Severity score of skin lesions was assessed by reference to known standards. As shown in Fig. 3B, the severity score significantly decreased in the puerarin groups in a dose-dependent manner.

In H&E-stained skin tissues, the skin thickness in the DNCB-induced group was remarkably thicker than those in the normal group because DNCB induces skin hyperkeratosis and hypertrophy in mice. The skin thickness was attenuated by the oral puerarin administration. The effects of the oral administration of 50 mg/kg puerarin and 1 mg/kg dexamethasone significantly reduced the skin thickness (Fig. 3D). In the TB-stained skin tissues, the number of mast cells (red arrows) increased in the DNCB-induced group compared with that in the normal group. The oral administration of puerarin considerably inhibited the number of infiltrated mast cells into the dermis in a dose-dependent manner. In addition, oral dexamethasone administration markedly diminished the DNCB-induced infiltration of mast cells (Fig. 3E).

TSLP, an epithelial cell derived cytokine, plays an important role in the pathogenesis of AD by initiating Th2 cytokine production by activated mast cells. In previous studies, the expression of TSLP has been shown to be induced by the activation of PAR2 and NF- κ B [13]. We investigated the expressions of PAR2 and NF- κ B in dorsal skin tissues by IHC staining. As shown in Fig. 3C, the PAR2, NF- κ B, and TSLP expressions markedly increased in the DNCB-induced group compared with those in the normal group. The puerarin groups had significantly diminished levels of PAR2, NF- κ B, and TSLP expression

as compared with the DNCB-induced group in a dose-dependent manner.

IgE play a crucial role in AD progression, and IgE antibody production is known to be related to the Th2 immune reaction [14]. The serum total IgE level increased in the DNCB-induced group as compared with that in the normal group, the serum total IgE was significantly suppressed by the oral administration of puerarin in a dose-dependent manner (Fig. 3F).

3.4. Effects of puerarin on DNCB-induced mRNA level of inflammatory cytokine in skin tissue

To investigate the effects of puerarin on the inflammatory cytokine productions, dorsal skin lesions were collected. The levels of inflammatory cytokine were analyzed using real-time PCR. In skin lesions, the levels of cytokine such as TNF- α , IL-1 β , IL-4, IL-6, and IL-31 were increased in DNCB group compared with those in the normal group (Fig. 4A–E). The levels of the pro-inflammatory cytokines were significantly decreased in the puerarin groups as compared with those in the DNCB-induced group. Moreover, we measured mRNA expression of inflammatory chemokine such as CXCL9, CXCL10, and CXCL11 in skin tissue (Supplementary Fig. S2). Inflammatory chemokines were reduced by oral administration of puerarin. These results indicate that puerarin is superior to dexamethasone, which is a positive control, to inhibit the cytokine productions in dorsal skin lesion.

4. Discussion

Atopic dermatitis (AD), a chronic inflammatory skin disease, recurs in certain areas and is accompanied by severe itching and overall dry skin. The major causes of AD include genetic factors, environmental factors, and immunological and skin barrier disorders, and the severity of the disease is determined by the interactions between them. It is known that many cells (T lymphocytes, Langerhans cells, eosinophils, and keratinocytes) and factors (cytokines, IgE, and chemokines) are involved in the progression of AD [15]. Increased Th2-mediated cytokines, IgE, and mast cells are typical examples; however, the exact cause of the disease has yet to be elucidated.

Puerarin, Gegen's major isoflavonoid compound, is widely used as an alternative medicine in China. It has been proven to have broad pharmacological effects in many diseases. However, further studies are needed on the effects of puerarin on other, unknown diseases. To date, the molecular mechanisms and targets of specific pharmacological properties of puerarin in AD have not yet been known [10]. On that basis, we examined the effect of puerarin on AD and confirmed the potential of using puerarin for the treatment of AD.

Keratinocytes are cells essential for the formation of the stratum corneum and act as a primary derivative and target of the immune response that occurs in the skin. The keratinocytes of patients with AD produce many cytokines and chemokines, which promote inflammatory skin disease progression. When an itchy skin is scratched, a variety of pro-inflammatory cytokines and CC-chemokines are released from the keratinocytes, further exacerbating skin inflammation [16].

The secretion of pro-inflammatory cytokines is known to be caused by the activation of the MAPKs (p38, ERK and JNK) signaling pathway. Indeed, stimulation of TNF- α /IFN- γ in keratinocytes

400 $\times$ magnification. Dorsal skin thickness was measured using a micrometer (D). The number of mast cells was counted at five random sites per animal (E). Serum IgE was analyzed using ELISA (F). The data shown are the average of five samples per group and are presented as mean $\pm$ SD. ### p < 0.001 versus normal group, * p < 0.05, *** p < 0.001 versus DNCB-induced group.

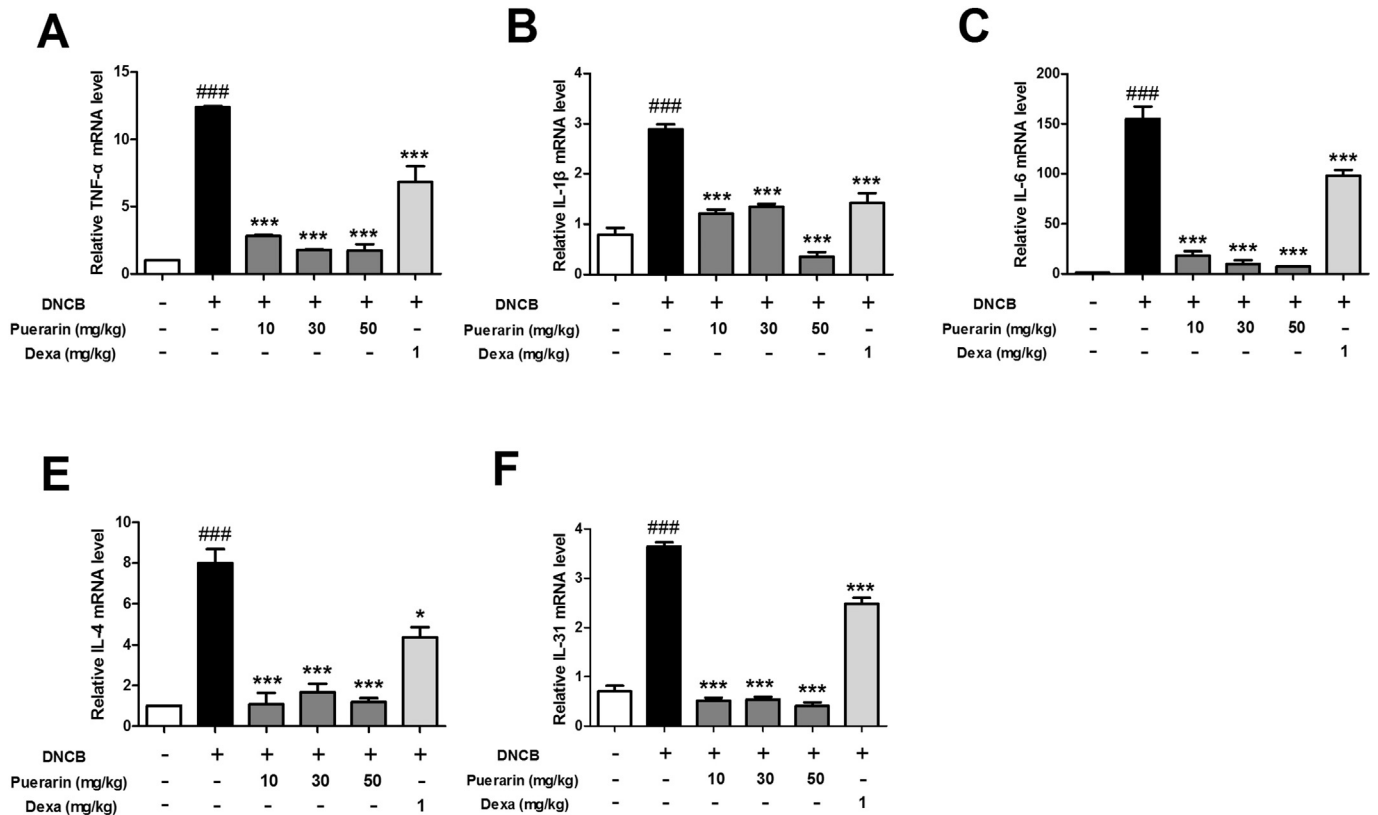


Fig. 4. Puerarin decreased pro-inflammatory cytokines in the DNCB-induced mice skin tissue.

The cytokine mRNA expression levels of TNF- α (A), IL-1 β (B), IL-6 (C), IL-4 (D), and IL-31 (E) were determined using real-time PCR. The data are shown as the mean $\pm$ SD of the three experiments. ### p < 0.001 versus normal group, * p < 0.05, *** p < 0.001 versus DNCB-induced group.

activates the intracellular MAPK signaling pathway [43]. In the present study, we found that puerarin suppressed the mRNA levels of the cytokines and chemokines in TNF- α /IFN- γ -stimulated HaCaT cells. In addition, we have shown that puerarin diminished the activation of the MAPKs (p38, ERK and JNK) and Akt signaling pathways in the TNF- α /IFN- γ -stimulated HaCaT cells, decreased STAT-1, and increased the degradation of I κ B- α . Puerarin seems to decrease the expression of cytokines and chemokines by inhibiting STAT-1 and NK- κ B pathways as well as activation of MAPKs and Akt signaling pathways (Figs. 1 and 2). Based on these results, puerarin has a protective effect on skin inflammation by regulating pro-inflammatory cytokines and phosphorylation of MAPKs, STAT-1, and NK- κ B.

In AD, skin barrier function abnormalities, such as increase of skin hyperkeratosis, and increased skin surface pH, are observed [17]. Thus, we measured the skin condition in DNCB-induced dermatitis using the AD score (Fig. 3A and B).

When the skin barrier is damaged, it causes dermatitis due to secretion and generation of pro-inflammatory cytokines such as TNF- α and IL- β , at the damaged site [18]. Damage of the skin barrier causes a Th2 inflammatory response, increases the infiltration of mast cells, and increases the expression of many cytokines and chemokines.

When the pH of the skin surface is constantly increased by dermatitis, the activity of proteases, such as serine proteases, increases over the entire surface of the epidermis, resulting in an abnormal cohesion of the stratum corneum. Activation of various proteases causes inflammatory reaction and itching through PAR2 [19]. PAR2 is present in keratinocytes, mast cells, and vascular endothelial cells and, causes abnormalities in the skin barrier when

activated. In a recent study, it has been shown that the PAR2-NF- κ B signaling pathway is involved in the process of TSLP secretion. TSLP is an epithelial-derived cytokine that is required to cause or sustain Th2-type allergic inflammation. Thus, TSLP plays a crucial role in the pathogenesis of AD [20].

T lymphocytes are mainly infiltrated into atopic skin lesions. They can be divided into Th1 and Th2 cells depending on the function. Th2 cells are predominant in acute AD; Th2 cytokines, such as IL-4, IL-5, IL-6, IL-10, and IL-31, not only cause an allergic reaction but also decrease the innate immune response [21]. An increased IL-4 level in the acute phase of AD stimulates B lymphocytes to synthesize IgE. IgE activates mast cells and basophils [22].

In early lesions, Th2-immunoreactivity is predominant; however in chronic lesions, it is converted to a Th1-like response [23]. Previous studies have shown that CCL chemokines recruit eosinophils, basophils, mononuclear cells, and Th2 cells and thus have an important effect on allergic inflammation. Moreover, CXCL chemokines predominantly recruit Th1 lymphocytes and neutrophils. CXCL9, CXCL10, and CXCL11 attract type 1 T cells, and the production of these chemokines by keratinocytes in AD is increased [24] (Supplementary Fig. S1,S2).

In this experiment, oral administration of puerarin alleviated the AD-like symptoms by improving the skin severity score, skin thickness, and mast cell infiltration as well as decreasing the elevated serum IgE level with DNCB. The expression of PAR2, NF- κ B and TSLP also decreased with the oral puerarin administration (Fig. 3). In addition, the levels of cytokines and chemokines were remarkably suppressed by the oral administration of puerarin in DNCB-induced AD mice (Fig. 4).

In conclusion, puerarin may be an effective candidate for the treatment of AD. Further, puerarin ameliorated skin inflammation and AD-like skin lesions in vitro and in vivo through regulation of inflammatory and allergic mediators, such as pro-inflammatory cytokines, MAPKs, and IgE. However, the specific molecular mechanisms of puerarin and precise targets are not yet known, and further studies are required.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.bbrc.2018.03.018>.

Transparency document

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SUPPLEMENTARY MATERIALS AND METHODS

Real-time PCR (polymerase chain reaction)

The dorsal skin tissues were collected in each mouse at the end of the experiment. The HaCaT cells were harvested after reagent treatment. Total RNA was isolated from the skin tissues and cells using Trizol reagent. The cDNA was synthesized using the Super Script™ III kit, according to the manufacturer's protocol. The mRNA expression was quantitatively determined using Power SYBR Green PCR Master Mix (Applied Biosystems). The following PCR primers were used: hCCL2, 5'- CAA ACT GAA GCT CGC ACT C -3' and 5'- CAT TTC CAC AAT AAT ATT TTA G -3'; hCCL5, 5'- CGC TGT CAT CCT CAT TGC TA -3' and 5'- GCA CTT GCC ACT GGT GTA GA -3'; hCCL17, 5'- CCA TTC CCC TTA GAA AGC TG -3' and 5'- CTC TCA AGG CTT TGC AGG TA -3'; hCXCL8, 5'- ACC GGA GCA CTC CAT AAG GCA -3' and 5'- AGG CTG CCA AGA GAG CCA CG -3'; hCXCL10, 5'- TTG CTG CCT TAT CTT TCT GAC TC -3' and 5'- ATG GCC TTC GAT TCT GGA TT -3'; hCXCL11, 5'- GGC TGT GAT ATT GTG TGC TAC -3' and 5'- GGA TTT AGG CAT CGT TGT CC -3'; mCXCL9 5'- GCA GTG TGG AGT TCG AGG AA -3' and 5'- TCT AGG CAG GTT TGA TCT CC -3'; mCXCL10, 5'- CTG AGT GGG ACT CAA GGG AT -3' and 5'- TCG TGG CAA TGA TCT CAA CAC -3'; mCXCL11, 5'- GGC AGA GAT CGA GAA AGC TT -3' and 5'- ATT GCC TGC ATT ATG AGG CG -3';

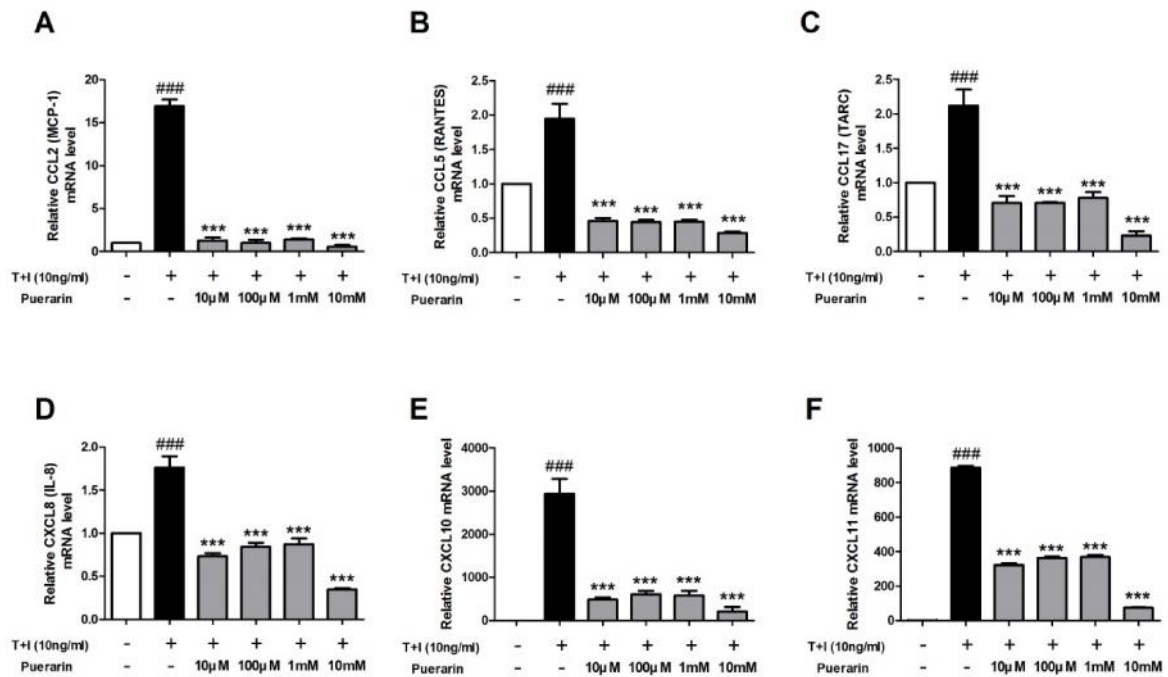


Fig. S1. Effects of puerarin on TNF- α /IFN- γ -induced chemokines in HaCaT cell line.

HaCaT cells were pretreated with the indicated concentrations of puerarin for 2 h and then stimulated with TNF- α /IFN- γ (each 10 ng/ml) for 30min. The mRNA expression levels of CCL2 (A), CCL5 (B), CCL17 (C), CXCL8 (D), CXCL10 (E) and CXCL11 (F) were analyzed using real-time PCR. The data are shown as the mean $\pm$ SD of the three experiments.

$p < 0.001$ versus no-treatment condition, *** $p < 0.001$ versus only TNF- α /IFN- γ treatment condition.

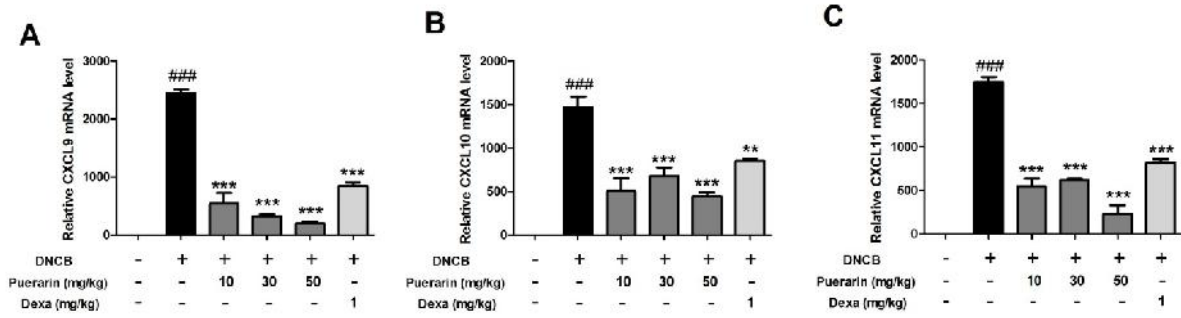


Fig. S2. Effects of puerarin on DNCB-induced mRNA level of chemokines in skin tissue.

The cytokine mRNA expression levels of CXCL9 (A), CXCL10 (B), and CXCL11 (C) were determined using real-time PCR. The data are shown as the mean $\pm$ SD of the three experiments. ### $p < 0.001$ versus normal group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus DNCB-induced group.