

Rosa davurica Pall. improves DNCB-induced atopic dermatitis in mice and regulated TNF- α /IFN- γ -induced skin inflammatory responses in HaCaT cells

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ABSTRACT

Purpose: *Rosa davurica* Pall., is mainly distributed in Korea, Japan, northeastern China, southeastern Siberia, and eastern Asia. It has been extensively used to treat various kinds of diseases by reason of the significant antioxidant, antiviral and anti-inflammatory activities. However, the pharmacological mechanism of *Rosa davurica* Pall. in atopic dermatitis (AD) is still ill defined and poorly understood. This study was to examine the anti-inflammatory effects and its mechanism on AD of *Rosa davurica* Pall. leaves (RDL).

Methods: To evaluate the therapeutic potential of RDL against AD, we have investigated the effects of RDL on the inflammatory reactions and the productions of inflammatory chemokines and cytokines that were induced by tumor necrosis factor- α (TNF- α)/interferon- γ (IFN- γ) in HaCaT cells. Furthermore, we examined the effects of RDL on the signaling pathways of mitogen-activated protein kinase (MAPK) and nuclear factor-kappa B (NF- κ B). For the *in-vivo* studies, RDL extract was topically applied to the dinitrochlorobenzene (DNCB)-induced AD mice, then its therapeutic effect was evaluated physiologically and morphologically.

Results: After the stimulation of HaCaT cells with TNF- α /IFN- γ , RDL considerably reduced the release of inflammatory mediators such as nitric oxide (NO), PEG₂ and other cytokines. RDL also reduced the phosphorylations of MAPK and NF- κ B in TNF- α /IFN- γ -stimulated HaCaT cells. *In vivo* topical application of RDL to DNCB-induced AD mice significantly reduced the dorsal skin and ear thickness, clinical dermatitis severity, and mast cells. Treatment with RDL also markedly decreased the levels of serum IgE, IL-6 and the number of WBCs in the blood.

Conclusion: Our studies indicate that RDL inhibits the AD-like skin lesions by modulating skin inflammation. Consequently, these results suggest that RDL may be served as a possible alternative therapeutic treatment for skin disorder such as AD.

Introduction

Atopic dermatitis (AD) is a multifactorial chronic relapsing skin disorder (Leung et al., 2004). AD affects almost 15% to 20% of children and 1% to 3% of adults globally, which has continued to increase for the

last several decades. Symptoms of AD include itchy skin, red or brownish, pruritus, eczematous lesions, dry, cracked or inflamed skin as well as hypersensitive skin, especially at night. AD commonly starts in early childhood and the so-called 'atopic march' which is typically presented by a sequence of immunoglobulin E (IgE) responses and atopic

Abbreviations: AD, Atopic dermatitis; ALT, Alanine transaminase; AST, Aspartate transaminase; BSA, Bovine serum albumin; BUN, Blood urea nitrogen; COX, Cyclooxygenase; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DNCB, 2, 4-dinitrochlorobenzene; ELISA, Enzyme-linked immunosorbent assay; FBS, Fetal bovine serum; H&E, Hematoxylin and eosin; IFN- γ , interferon- γ ; IgE, Immunoglobulin E; IL-6, Interleukin 6; iNOS, inducible NO synthase; MAPK, activated mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; NF- κ B, Nuclear factor-kappa B; NO, Nitric oxide; PBS, Phosphate-buffered saline; RDL, *Rosa davurica* Pall. Leaves; TARC, Thymus and activation regulated chemokine; TB, Toluidine blue; TNF- α , tumor necrosis factor; WBC, White blood cell.

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clinical symptoms (Nutten, 2015). It is known that the cause of AD is a complex interrelationship of various causes such as environmental, genetic, psychological, abnormal skin barrier, and immunological factors. AD patients suffer discomfort in daily life from insufficient sleep due to severe itching, difficulties in social relationships, and economic problems due to treatment costs (Leung, 2000). Current therapies to reduce inflamed skin, rash and itching usually involve the administrations of topical application or oral medicines. (Simpson et al., 2017). Although these treatments are known to be effective and beneficial, their side effects have also been reported in long-term use (Akhavan and Rudikoff, 2008; Czarnowicki et al., 2017; Chia and Tey, 2015; D'Auria et al., 2016). Therefore, there is an increased need and demand for other therapeutic agents having optimal safety and efficacy.

Rosa davurica Pall., is mainly distributed in Korea, northeastern China, Japan, southeastern Siberia, and eastern Asia, and it has been used in traditional herbal medicine. Its therapeutic indication can include various diseases, such as dyspepsia, gastroenterologia, arteriosclerosis, pulmonary tuberculosis, frostbite and menoxenia (Kuang et al., 1989). *Rosa davurica* contains a large number of active components, including vitamins, flavonoids, tannins, and polyphenols, such as quercetin, hyperin, tiliroside and catechin (Kuang et al., 1989; Huo et al., 2017), and has significant anti-inflammatory and antioxidant activities (Cho et al., 2003; Jiao et al., 2004; Jung et al., 2011; Kim et al., 1999). We have previously reported that the extract of *Rosa davurica* Pall. Leaves (RDL) containing seven main ingredients, possess antimicrobial and anti-inflammatory effects (Hwang et al., 2020). Although the therapeutic effects of RDL on various kinds of diseases have been suggested, its anti-AD effect and the mode of action have not been clearly reported yet. Therefore, we have designed the experiments for anti-inflammatory effects and the anti-AD effects of RDL using TNF- α /IFN- γ -stimulated HaCaT keratinocytes and DNCB-induced AD mice models, respectively. Based on this study, RDL has improved the clinical symptoms of *P. acnes*-induced mice models as well as suppressed *P. acnes*-induced chemokine productions by the mechanism of MAPK and NF- κ B pathways in HaCaT cells.

Materials and methods

Materials and extract preparation

The specimen, *Rosa davurica* Pall. leaves (RDL) were provided by Jungsun, Korea. The leaves were briefly rinsed with distilled water and then dried. The dried leaves were grinded into fine powder and extracted with 70% methanol in shaking incubator for 24 h. The RDL extract was filtered and evaporated at low temperature. Finally, the specimen was freeze-dried, powdered, and stored until use at -70°C .

Cell culture and MTT assay

The human keratinocytes, HaCaT cells were carefully cultured in DMEM, comprising 10% (v/v) FBS, and 100 $\mu\text{g}/\text{ml}$ antibiotics at 37°C . The cell viability was assessed using MTT (Sigma-Aldrich, St. Louis, MO, USA) assay. HaCaT cells (1×10^5 cells/well) were seeded into 24-well plates and maintained at 37°C in 5% CO_2 . After the incubation for 24 h, cell culture medium was replaced with serum-free culture medium containing various concentrations of RDL for 24 h. Then, MTT dye was added and the culture dishes were further incubated at 37°C for an additional 3 h. The supernatants were then carefully removed and insoluble formazan crystal was dissolved in DMSO (Sigma-Aldrich, St. Louis, MO, USA). The absorbances were determined at 540 nm using a spectrophotometer (PowerWaveTMXS, BioTek Instruments, Inc., Winooski, USA). DMEM, FBS and antibiotics were purchased from Gibco-BRL (Grand Island, NY, USA).

Cytokine measurement using ELISA assay

The supernatants were collected and TARC and IL-6 secretions were measured using their respective ELISA kits (eBioscience, San Diego, CA, USA). For this, the cells (1×10^5 cells/well) were seeded in each well of 6-well culture plates. After the adaptation process, cells treated with RDL for 1 h at 37°C . Then, cells were stimulated with TNF- α or IFN- γ (10 ng/ml) for 24 h. Cytokine levels were assessed using the ELISA kits according to the manufacturer's instructions.

Western blot analysis

The cell pellets were incubated with RIPA buffer (TransLab, Daejeon, Korea) containing protease inhibitor cocktail. The protein concentration of the samples were determined using the Bradford assay. The proteins were separated using 12% SDS-PAGE gel and then transferred to polyvinylidene fluoride (PVDF) membrane (Bio-Rad, C.A. USA). The membrane was blocked in 5% BSA. The blocked membrane was probed with specific primary antibodies for overnight at 4°C and horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Chemiluminescence was detected ChemiDoc XRS (Bio-Rad, California, USA). The protein bands were analyzed using a NIH Image software (Image J).

Detection of Nitric Oxide

Nitric oxide was assessed by measuring nitrite in the culture medium using the Griess reagent (Molecular Probes #G-7921, Inc, Eugene, OR) following the manufacturer's protocol. The supernatant was mixed with Griess reagent for 10 min, then nitric oxide was measured by the absorbance at 540 nm.

PGE₂ assay

PGE₂ levels in culture medium were evaluated by ELISA kits (R&D Systems) according to the specific manufacturer's protocol.

Animals

Five-week-old BALB/c mice were obtained from Samtako BioKorea (Osan, Korea). The mice were acclimatized to laboratory condition for 1 week before the experiment. The BALB/c mice were randomly assigned into five groups: (1) Negative control (NC), (2) DNCB + vehicle, (3) DNCB + 1 mg/kg RDL, (4) DNCB + 10 mg/kg RDL, and (5) DNCB + 1.5 mg/kg dexamethasone (Sigma-Aldrich Inc.). All animal groups were kept on temperature ($23.5 \pm 0.5^{\circ}\text{C}$), humidity (35 – 60%), water/food with 12 h of light/dark cycle. The animal procedures were approved by the Institutional Animal Care and Use Committee of Gyeongsang National University, and certification number: GNU-180730-M0034.

AD models and drug treatment

DNCB-induced AD mice model has been employed for the present study using the method as described elsewhere (Yang et al., 2019) with a little modification. Briefly, 200 μl of 0.5% DNCB dissolved in acetone: olive oil (3:1) vehicle was topically applied on a daily basis for sensitization to the ear and the dorsal skin of mice for experimental days 1–3. On days 8–35, the mice were then challenged with either 20 μl or 200 μl of 1% DNCB on the induced skin sites for three times a week. For RDL group, the same skin sites were additionally administered with the topical treatment of RDL for everyday during the experiment. For dexamethasone (positive control) group, the mice were intraperitoneally injected with 1.5 mg/kg of dexamethasone for three times a week.

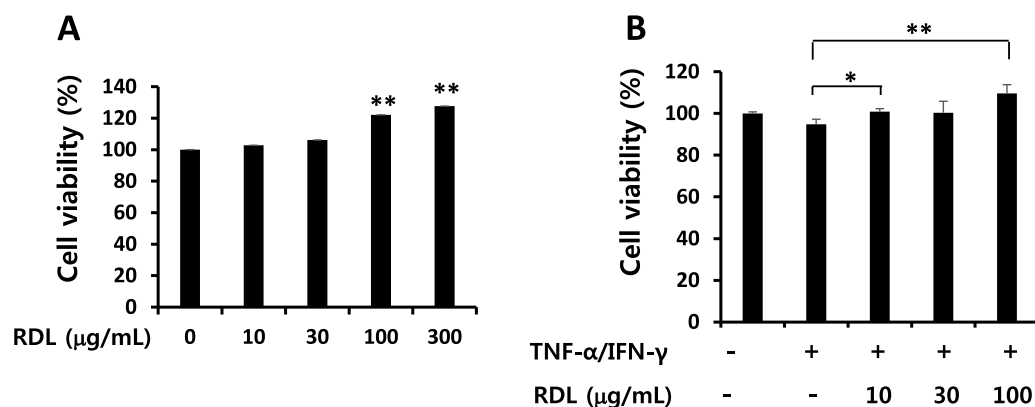


Fig. 1. Effects of RDL on the viability of HaCaT cells. (A) HaCaT cells were treated with the various concentrations of RDL for 24 h. (B) Effect of RDL (0–100 µg/ml) on HaCaT cell viability with or without TNF-α/IFN-γ (10 ng/ml each) in HaCaT cells. Cell viability was determined by the MTT assay. The cells were pretreated with RDL for 1 h and then stimulated with or without TNF-α/IFN-γ for 24 h. Values represent the mean ± SD. * $p < 0.05$ and ** $p < 0.01$ compared with the vehicle control group or TNF-α/IFN-γ-treated group.

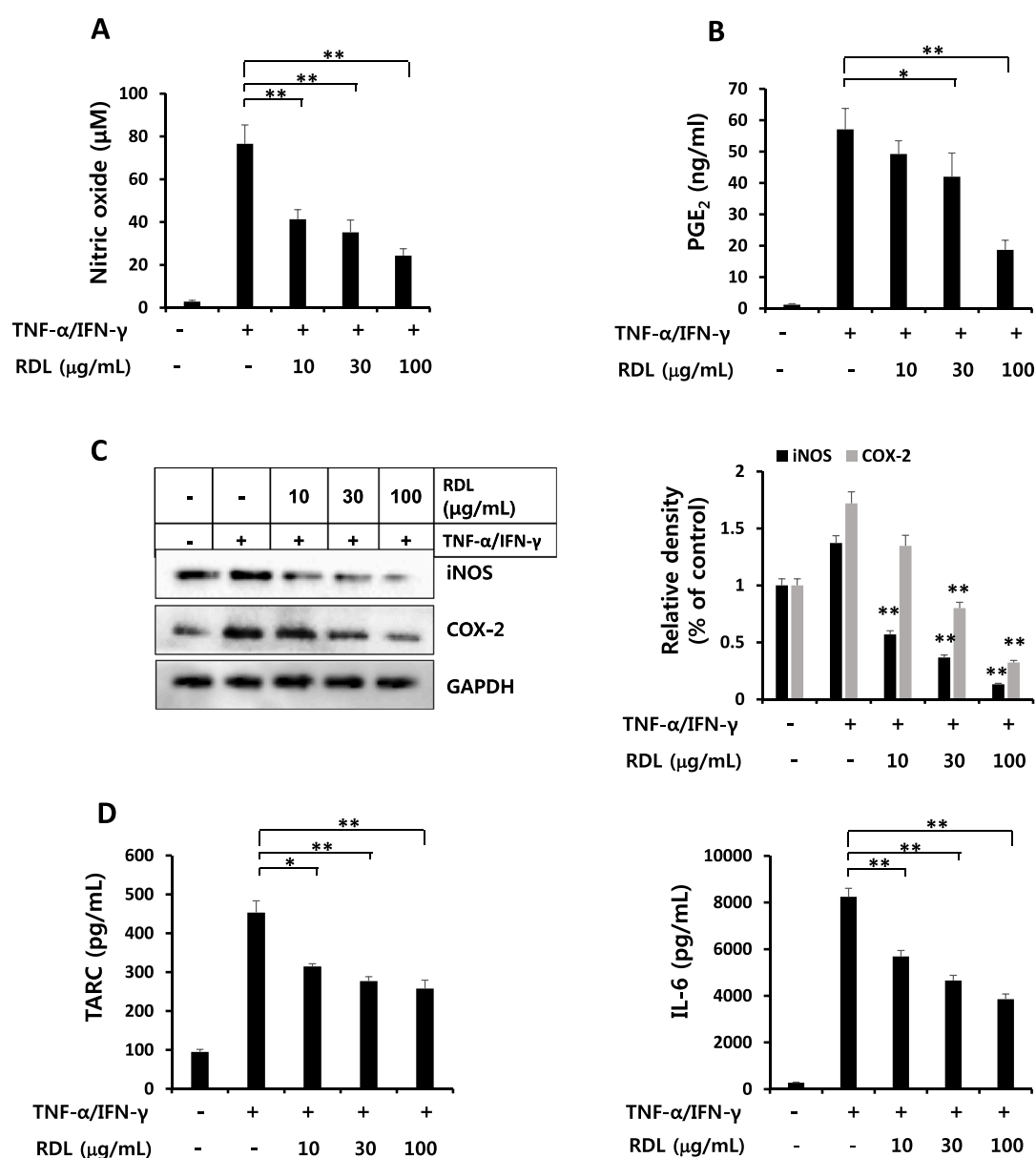


Fig. 2. Effects of RDL on the production of inflammatory mediators in TNF-α/IFN-γ-stimulated HaCaT cells. Effects of RDL on the (A) NO, (B) PGE₂ (C) iNOS and COX-2, (D) TARC and IL-6 were determined by the culture supernatants. For the RDL treatment, the cells preincubated with the RDL (10, 30, and 100 µg/ml) at 1 h before the treatment of TNF-α/IFN-γ (10 ng/ml each) for 24 h. Values represent the mean ± SD. * $p < 0.05$ and ** $p < 0.01$ compared with TNF-α/IFN-γ treated group.

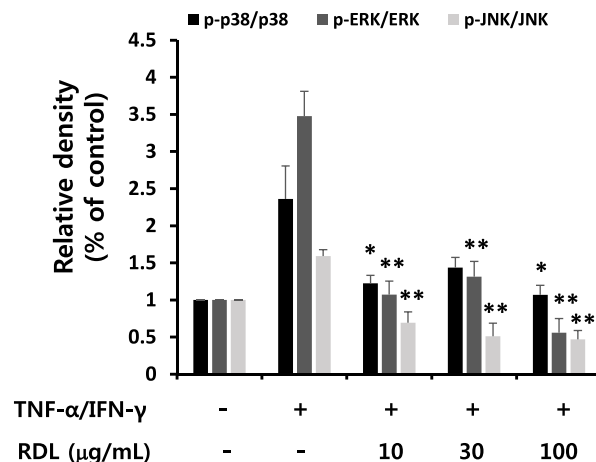
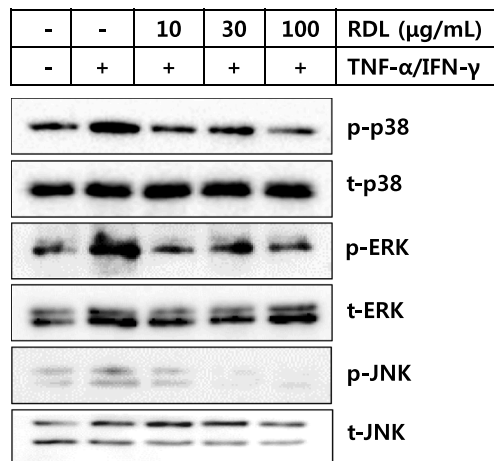
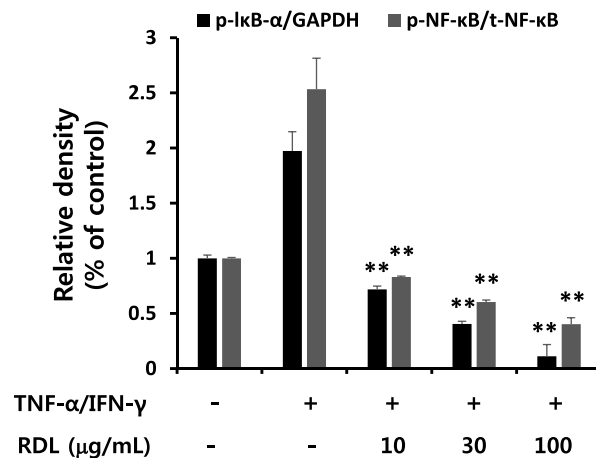
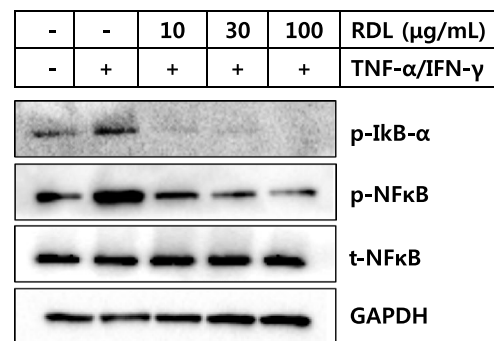
A**B**

Fig. 3. Effects of RDL on the phosphorylations of MAPK and NF-κB pathways in TNF-α/IFN-γ-stimulated HaCaT cells. The cells were preincubated with various concentrations of RDL (10–100 μg/ml) for 1 h and then treated with TNF-α/IFN-γ (each 10 ng/ml) for 30 min. Cell lysates were prepared for the analyses by Western blot. (A) Effects of RDL on the phosphorylations of P38, ERK, JNK, and (B) NF-κB pathways. Relative abundances of each protein were calculated for the indicated protein ratios. Values represent the mean ± SD. * $p < 0.05$ and ** $p < 0.01$ compared to the TNF-α/IFN-γ-treated control group.

Clinical skin score

Clinical skin score was evaluated once a week, according to the scoring system (Leung et al., 1990). Briefly, the total score of dermatitis clinical severity was the sum of individual scores: 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of five symptoms, which were erythema/hemorrhage, edema/hematoma, excoriation/erosion, itching/dryness, and lichenification.

Histopathological analysis

Before sacrifice, the tissues (dorsal skin and ear lesions) and the organs (lymph nodes and spleen) of each mouse were measured for the thickness using a micrometer. The weights of mice and their organs were determined using an electronic balance. Then the obtained tissues were fixed in 10% formalin solution. After fixing, the paraffin-embedded tissues were serially sectioned into 5 μm thickness. Representative sections were stained with hematoxylin and eosin (H&E) and evaluated for any histological changes. To quantify mast cell numbers, paraffin-embedded tissue sections were stained with toluidine blue (TB) and

visualized under the microscope. To measure the mast cells per 0.025 mm² sample was counted at 400 × magnification. The images were analyzed using ImageJ software.

Blood analysis

Collected blood samples were placed in an EDTA containing tube (BD Science, NJ, USA). Any hematological changes of the samples were evaluated using a HEMAVET 950 analyzer (Drew Scientific, Inc., Oxford, USA) according to the manufacturer's instructions.

Measurement of IgE, IL-6 levels and blood biochemical analysis

Whole blood samples were centrifuged and the serums were collected. The levels of IgE and IL-6 in mouse serum were assessed using ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's recommendation. In addition, the functional indices of liver (ALT and AST) and kidney (creatinine and BUN) were determined using commercial kits with IDEXX VetTest Analyzer (IDEXX laboratories, Inc., Maine, USA).

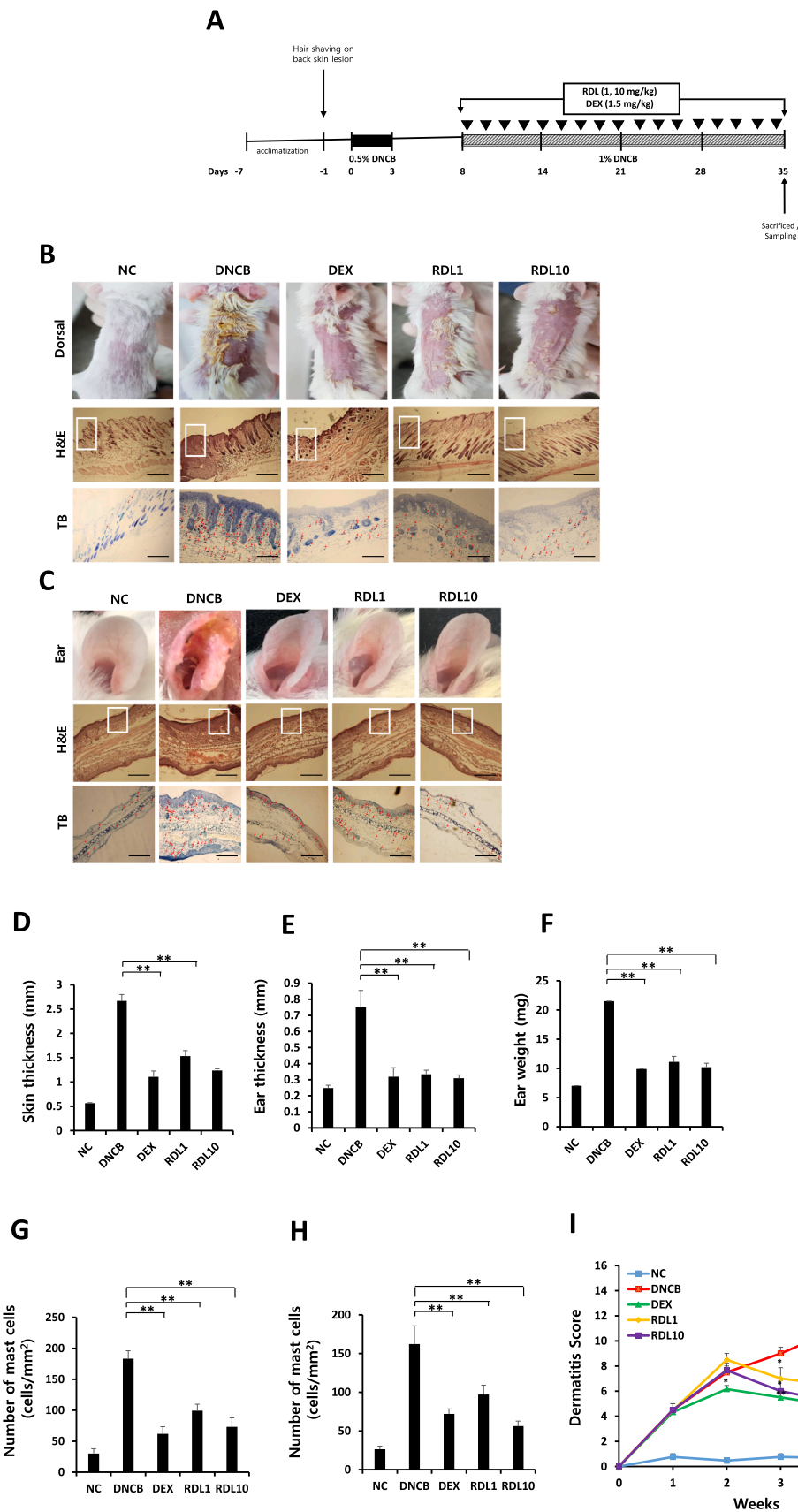


Fig. 4. Effects of RDL on pathological changes of DNCB-induced AD mice skin and ear lesions. (A) General schematic diagram for the experimental protocol. (B, C) Effects of RDL on clinical features of AD-like dorsal skin and ear lesions. Histopathological features of skin and ear lesions. The skin lesions were determined by H&E and TB stain (magnification, 200 × (scale bars, 100 μm)). The arrows indicate the TB-stained mast cells. (D) The dermatitis score was defined as the sum of scores. The numbers of mast cells of dorsal skin (E) and ear (F) were measured and graphed. (G-I) Thickness and weight were measured with a micrometer. Values represent the mean ± SD. * $p < 0.05$, ** $p < 0.01$ compared with the DNCB group.

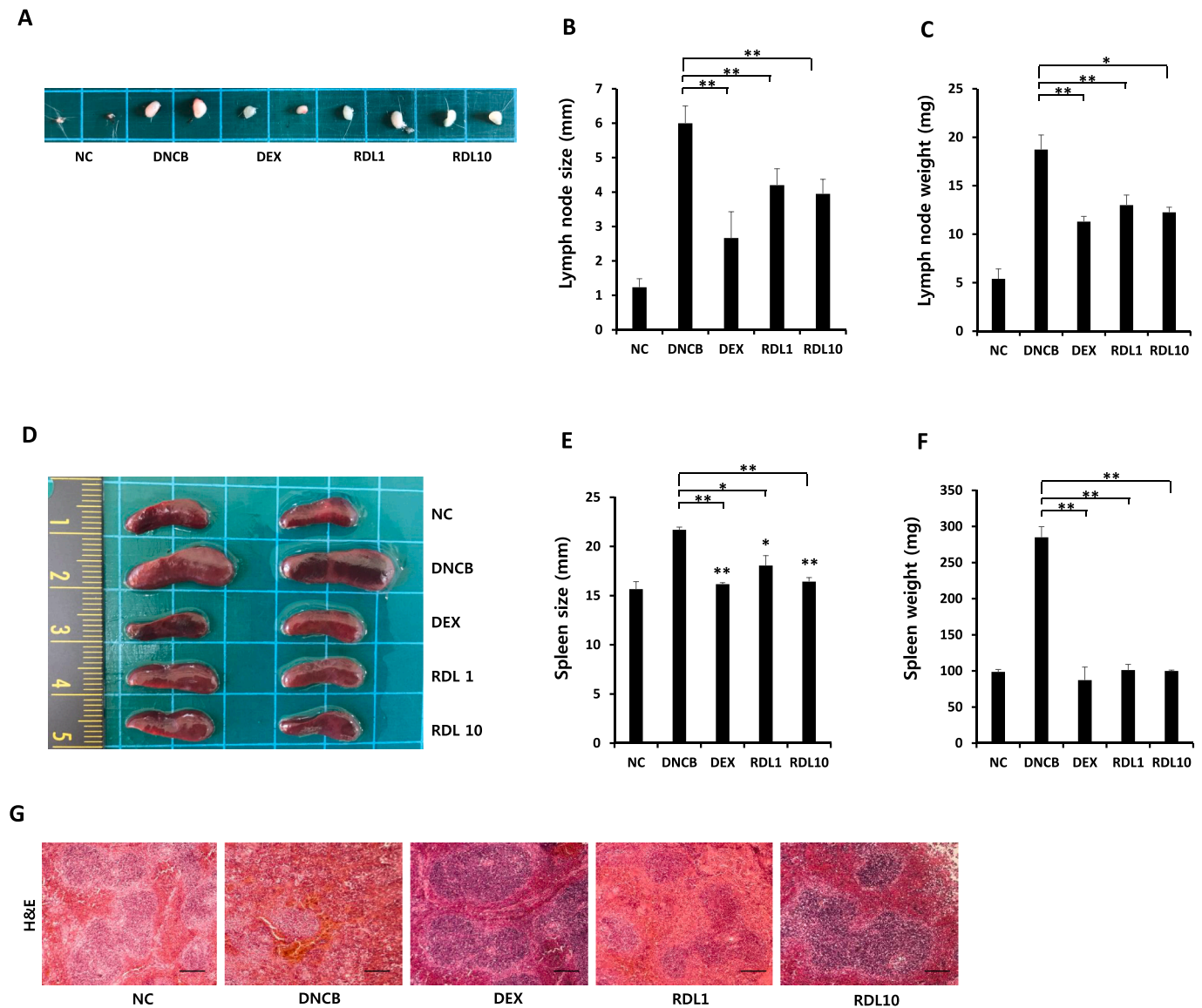


Fig. 5. Effects of RDL on DNCB-induced AD mice organ symptoms. Representative photographic images and the physical characteristics of lymph node (A–C) and spleen (D–F) were compared between control and treatments. (G) H&E staining of the spleen of mice showing the organization of red pulp (pink) and white pulp (purple). Values represent the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ compared with the DNCB group.

Statistical analysis

The results are expressed as the mean $\pm$ standard deviation (SD). For individual comparison, Student's unpaired t -test was used to assess the significance of differences between two mean values. * $p < 0.05$ and ** $p < 0.01$ were taken as statistically significant.

Results

Effects of RDL on HaCaT cell viability

To determine the concentration of RDL without toxicity, HaCaT cell viability was measured using MTT assay at various concentrations of RDL. From this, RDL did not exhibit any cytotoxicity on HaCaT cells up to 300 $\mu\text{g/ml}$ of RDL concentration (Fig. 1A). We have also examined the effect of RDL on HaCaT cells viability in the presence or the absence of TNF- α /IFN- γ . RDL showed no significant cytotoxic effect at the concentrations up to 100 $\mu\text{g/ml}$ upon TNF- α /IFN- γ stimulated HaCaT cells (Fig. 1B). Hence, we decided to use 10–100 $\mu\text{g/ml}$ of RDL for further experiments in this study.

Effects of RDL on the production of inflammatory mediators in TNF- α /IFN- γ -stimulated HaCaT cells

Previous report suggests that immune reaction is one of the key players in inflammatory skin disorders, such as AD and other skin diseases (Lee et al., 2018). It has a crucial role in initiating the inflammatory skin responses by releasing pro-inflammatory mediators, thereby contributing to many skin problems (Bae et al., 2015; Akram et al., 2016). To examine the therapeutic potential of RDL against AD, we evaluated the protective effect of RDL on the inflammatory responses in HaCaT human keratinocyte. The cells were pretreated with various concentrations of RDL (10, 30 and 100 $\mu\text{g/ml}$) for 1 h, then treated with TNF- α /IFN- γ for 24 h. The RDL pretreatment inhibited the releases of NO and PGE₂, as well as suppressed the expressions of iNOS and COX-2 in HaCaT cells, which were initiated by TNF- α /IFN- γ (Fig. 2A–C). Then, TNF- α /IFN- γ induced HaCaT cell supernatants were determined by ELISA for pro-inflammatory cytokine productions (Fig. 2D). Pro-inflammatory cytokines, including TARC and IL-6, which have been found to be secreted or produced in numerous inflammatory skin diseases, such as AD disorder (Tapia et al., 2007). This result (Fig. 2C)

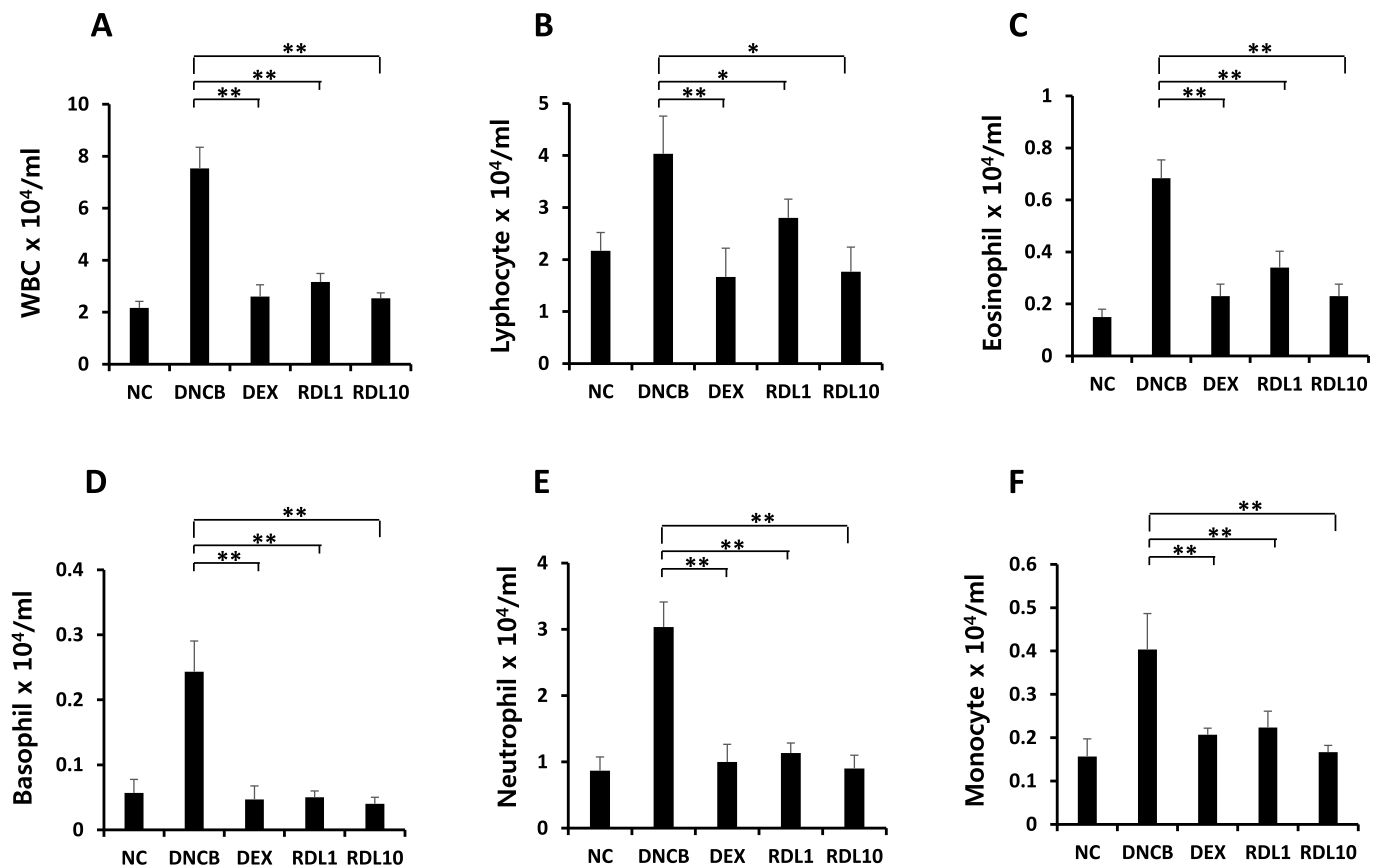


Fig. 6. RDL reduced leukocyte numbers in the blood. (A) WBC, (B) lymphocytes, (C) eosinophils, (D) basophils, (E) neutrophils, and (F) monocytes were analyzed using a HEMAVET 950. Values represent the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ compared with DNCB group.

shows that TARC and IL-6 were excessively produced upon by HaCaT cells stimulated with TNF- α /IFN- γ . Whereas, treatment with RDL significantly suppressed the production of TARC and IL-6. From this experiment, TNF- α /IFN- γ stimulated the productions of TARC and IL-6 from HaCaT cells (Fig. 2C). Whereas, they could be significantly modulated by the treatment of RDL.

Effects of RDL on MAPK and NF- κ B activation in TNF- α /IFN- γ -stimulated HaCaT cells

The production of inflammatory mediators by activated MAPK and NF- κ B pathways is also considered to be related to AD. Hence, we examined the effect of RDL on MAPK pathways (Fig. 3A). HaCaT cells were preincubated with RDL (10, 30 and 100 $\mu\text{g}/\text{ml}$) for 1 h, then treated with TNF- α /IFN- γ (each 10 ng/ml) for 30 min. Western blot analysis showed that levels of phosphorylated MAPK proteins (p-p38, p-JNK, and p-ERK) were increased in TNF- α /IFN- γ -stimulated HaCaT cells. However, the pretreatment of RDL significantly decreased the phosphorylation of MAPK. In the cytosol, the phosphorylation of NF- κ B was also enhanced in HaCaT cells stimulated with TNF- α /IFN- γ . Whereas, RDL pretreatment suppressed the activation of NF- κ B through reducing p-I κ B and p-NF- κ B, while having no effect on t-NF- κ B (Fig. 3B). These results indicate that RDL may prohibit inflammation reactions by blocking MAPK and suppressing NF- κ B activations.

Effects of rdl on ad skin lesions of BALB/c mice induced by DNCB

We investigated the therapeutic effects of RDL on DNCB-induced AD model of dorsal skin and ears of BALB/c mice (Fig. 4A). Typical symptoms of AD, such as remarkably increased skin thickness and erythema,

were observed from the skin and ears of AD mice induced by DNCB (Fig. 4). Whereas, RDL pretreatment group showed effectively and significantly reduced skin and ear thickness throughout the study period. In addition, erythematic intensity was also alleviated by RDL (Fig. 4D-F). Histopathological evaluation demonstrated that RDL was highly effective at improving the symptoms DNCB-induced AD mice. Skin thickening of both epidermal and dermal tissues was noticeably increased in AD mice compared with those of non-treated NC group, and these changes were significantly reduced by RDL treatment. Furthermore, RDL-treated group showed the decrease of mast cell numbers in DNCB-induced AD mice (Fig. 4G and H). Clinical skin score of DNCB-induced AD mice were increased remarkably until 5 weeks after induction. However, the dermatitis severity was noticeably improved by RDL treatment (Fig. 4I). Collectively, these results support that RDL can be a good candidate for AD treatment.

Effects of RDL on DNCB-induced AD mice organ symptoms

To determine whether topical treatment with RDL affects systemic immune responses in DNCB-induced AD mice, we next examined spleen and local lymph node to quantify atopic symptoms in the mice models (Arts et al., 1996). AD often develops systemic immune responses by affecting the immune organs (Lee et al., 2016). We evaluated the morphological changes of organs in DNCB-induced AD mice. The lymph node and spleen of AD mice were heavier and larger than those of healthy NC group. Whereas, RDL treatment resulted in dose-dependent decreases of lymph node and spleen by weight and size (Fig. 5A-F). For further characterization of this pharmacological effect of RDL on the spleen in AD mice, we observed histopathological changes in the splenic tissues (Fig. 5G). AD mice has an irregular spleen organization described

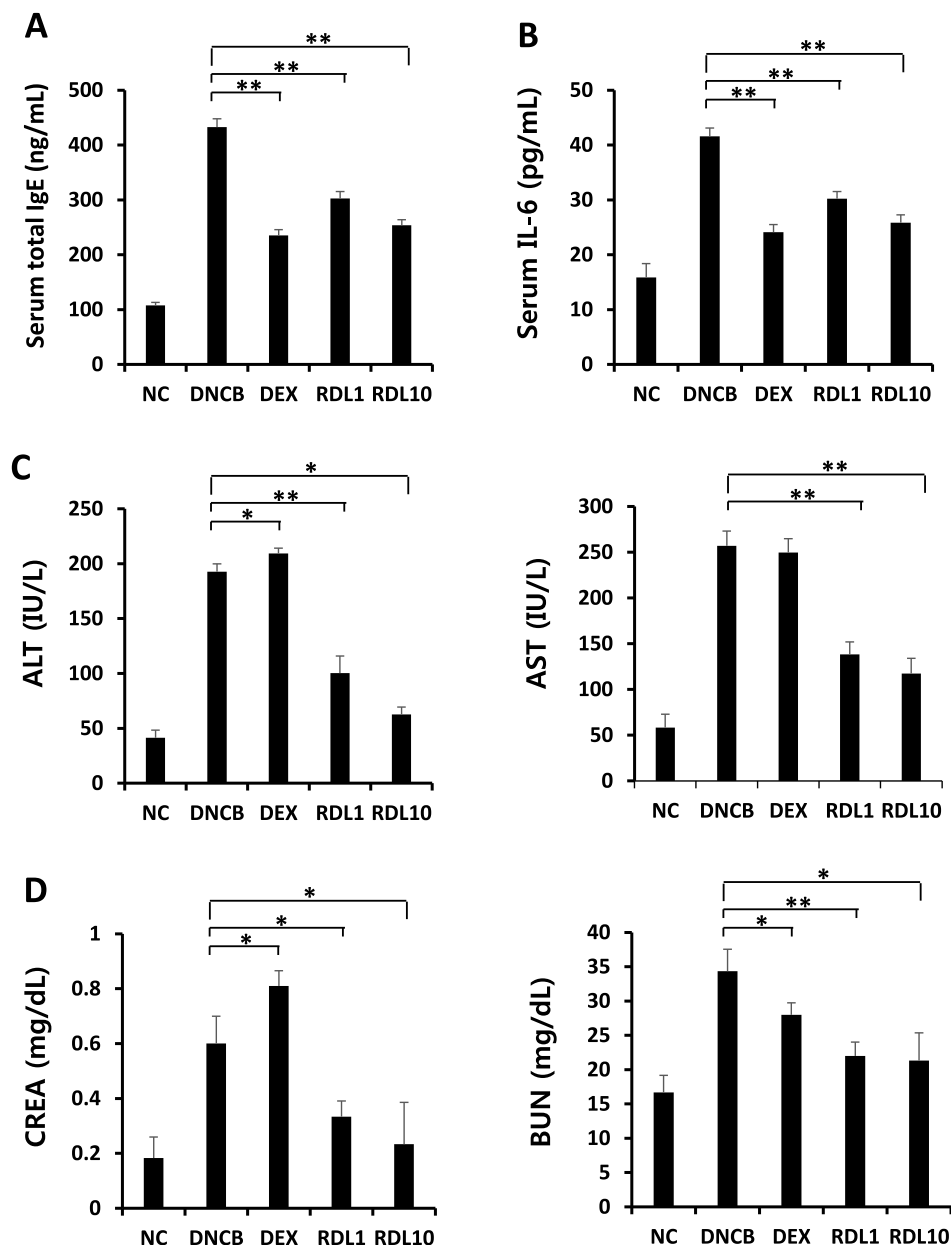


Fig. 7. Effects of RDL on serum IgE and IL-6 levels in DNCB-induced atopic dermatitis (AD) mice. (A) Total serum IgE levels. (B) Serum IL-6 levels. Effects of RDL on (C) serum ALT, AST, and (D) serum creatinine and BUN. The samples were analyzed by ELISA. The data were presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$ compared with the DNCB group.

by atrophy and decreased white pulp (purple areas) and an increase in red pulp of the spleen in comparison with NC group. Moreover, the spleen of RDL treated AD mice shows a white and red pulp distribution and tissues that were similar to NC group mice. In this study, RDL treatment prevented the abnormal structural changes of spleen in DNCB-induced AD mice. Collectively, RDL has an alleviation effect on the alterations of immune system in DNCB-induced AD mice.

Effects of RDL on the hematological changes in DNCB-induced AD mice

The numbers of most of white blood cells (WBCs) have been increased in AD disorder (Lubach et al., 1989; Hanifin et al., 2005). We performed a complete blood count, including the total number of WBCs and the numbers of each WBC subtypes, including monocytes, basophils, neutrophils, eosinophils, and lymphocytes of the mice (Fig. 6A-F). Upon the application of DNCB, the numbers of WBCs were increased up to

3-fold compared with those of NC group mice. However, the amplified WBCs were significantly reduced with the treatment of RDL, indicating that RDL suppress the inflammatory responses of AD mice.

Effects of RDL on the production of inflammatory mediators in DNCB-induced AD mice

AD is a skin disorder related with the markedly increased levels of IgE and inflammatory cytokines (Purushothaman et al., 2018). To understand the anti-inflammatory mechanisms of RDL, we monitored the inflammatory mediators using ELISA kits. The total IgE level was greatly upregulated in DNCB-induced AD mice nearly up to 4 fold comparing with that of non-treated control mice. However, the augmented IgE level was significantly suppressed by RDL treatment (Fig. 7A). Furthermore, the level of IL-6 was also markedly down regulated by the treatment in DNCB-induced AD-like skin inflammation (Fig. 7B). Thus, these results

demonstrate that RDL suppressed skin inflammation by suppressing the levels of serum IgE and pro-inflammatory cytokine IL-6 in DNCB-induced AD mice. We also examined the effects of RDL on liver and kidney functions, and found that RDL treatment significantly abrogated the drastic elevations of serum markers, such as ALT, AST, creatinine and BUN in DNCB-induced AD mice (Fig. 7C and D). In brief, the administration of RDL can dose-dependently attenuate the liver and kidney damages observed in DNCB-induced AD mice.

Discussion

AD is one of the most common chronic inflammatory skin diseases by complex interactions of abnormal immune network and IgE-mediated sensitization allergies (Kim et al., 2018). Many studies have suggested that the abnormal immunological reaction and various allergens are through pierce the skin, making it more easier to AD skin disease (Boguniewicz and Leung, 2011). AD pathology is often described by its characteristic symptoms, including barrier dysfunction and intercellular edema resulting in the elevated IgE levels and increased inflammatory cells (Cole et al., 2014). IgE is reported to play a key role in the pathogenesis of AD disease, including the functions of macrophages and eosinophils, dermal infiltration of (T helper) Th cells, imbalances of Th1 and Th2 cells (Jegal et al., 2017). Excessive production of cytokines by activated Th2 cells is also known to contribute to AD developments. Hence, the suppressions of these cytokines in the skin cells might be an attractive target for the treatment of AD by reducing the serum IgE levels of patients (Fang et al., 2015; Deo et al., 2010).

Human keratinocyte, HaCaT cell is one of the cell lines, which are commonly used for the study of AD symptoms due to the inflammatory responses stimulated with TNF- α /IFN- γ (Huang et al., 2016). The stimulation triggers the activation of various cell signaling mechanisms, which increase the expressions of pro-inflammatory mediators (Huang et al., 2016; Yang et al., 2018; Kim et al., 2015). Pro-inflammatory mediators along with the activation of monocyte and the expression of pro-inflammatory enzymes, play important roles in the process of inflammation. (Jeong et al., 2010; Yang et al., 2015).

Although in spite of the many reports on the pharmacological and biological activities of RDL, its beneficial effects against AD-related mechanisms have not been explored yet. For the present study, we stimulated HaCaT cells with TNF- α /IFN- γ and investigated the anti-inflammatory effect of RDL. Our results show that RDL can significantly reduce the releases of NO and PGE₂ in TNF- α /IFN- γ -induced HaCaT cells by inhibiting iNOS and COX-2 activities, respectively (Fig. 2A–C). RDL also suppressed the productions of TARC and IL-6 (Fig. 2D) of the HaCaT cells. Several reports have shown that MAPK activation can stimulate a number of inflammatory mediators, including iNOS and COX-2 (Senthil Kumar et al., 2010). Inducible nitric oxide synthase (iNOS) is induced by cytokines in inflammatory cells and related to the host cellular immune responses (Shah et al., 2014). COX-2 catalyzes the conversion of arachidonic acid to prostaglandin, and the levels of COX-2 activity can contribute to inflammatory pain (Giuliano and Warner, 2002). NF- κ B is a key transcription factor in the immune reactions involved in various skin diseases and regulates the transcription in the immune and inflammatory responses of various proinflammatory molecules in AD. In addition, NF- κ B is associated with I κ B, when I κ B- α is degraded, NF- κ B is activated along with inflammatory responses (Yang et al., 2018). Therefore, our present results demonstrate that RDL inhibits the release of chemokines and cytokines through the blockade of MAPK and NF- κ B pathways.

Recently, a number of mice model for AD have been developed, including DNCB-induced AD using BALB/c mice, in which many AD-like changes could be observed such as a thick epidermis, keratosis and infiltration of inflammatory cells. In another model, NC/Nga mice have specific pathogen-free conditions that develop AD-like skin lesion symptoms (Kang et al., 2011; Ki et al., 2016). In our study, the topical application of RDL could improve the atopic damages, in terms of skin

and ear thickness, pro-inflammatory chemokines and cytokines, mast cells, and serum IgE levels. Moreover, RDL has a beneficial effect on the spleen and lymph nodes, which may be involved in immune responses (Fig. 4–7). Our findings suggest that RDL has strong anti-atopic activities, as shown in DNCB-induced AD mice model as well as HaCaT human keratinocyte model.

Based on the results, RDL may be served as a possible therapeutic candidate for skin disorder such as AD.

Author contributions

E. K. investigation, supervision and project administration. D. H. H. mainly original draft preparation, and visualization. P. -O. K. performed the histological analysis. C. K. participated in focus group discussion.

Declaration of Competing Interest

None to report.

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