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Rosmarinic acid attenuates 2,4-dinitrofluorobenzene-induced atopic dermatitis in NC/Nga mice

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ABSTRACT

Atopic dermatitis (AD) is one of the most common skin diseases, and its incidence is increasing in industrialized countries. Furthermore, the epicutaneous application of a hapten, such as 2,4-dinitrofluorobenzene (DNFB), evokes an AD-like lesion in NC/Nga mice under specific pathogen-free (SPF) conditions. Rosmarinic acid (RA) is a secondary metabolite that is frequently found in herbs, and has anti-inflammatory, anti-oxidant, and anti-microbial effects. In this study, we studied whether RA is an effective treatment against DNFB-induced AD-like skin lesions in NC/Nga mice. RA at 1 or 5 μM was found to suppress the productions of interferon (IFN)-γ and interleukin (IL)-4 significantly by activated $CD4^+$ T cells. Furthermore, an intraperitoneal injection of RA at 10 or 50 mg/kg significantly inhibited skin lesion development and ear thickness and total serum IgE level increases in DNFB-treated NC/Nga mice. In addition, intraperitoneal administered RA at 10 or 50 mg/kg significantly inhibited the infiltrations of $CD4^+$ T, $CD8^+$ T, and mast cells into DNFB-induced skin lesions in NC/Nga mice. This study suggests that RA suppresses the development of AD-like dermatitis in DNFB-treated NC/Nga mice by reducing IFN-γ and IL-4 production by activated T cells and total serum IgE levels.

1. Introduction

Atopic dermatitis (AD) is characterized by chronic and relapsing inflammatory dermatitis, immunological disturbance, and pruritic and eczematous skin lesions. Currently, AD is one of the most common skin diseases (10–20% of children worldwide are affected) and its incidence is increasing in industrialized countries [1]. AD has a complex pathogenic mechanism that includes abnormal genetic and immunologic pathways and exposure to environmental or chemical agents [2,3], and thus, its pathogenesis has not been clearly defined.

In AD skin, scratching induces the productions of cytokines, chemokines and upregulates the expressions of adhesion molecules. These processes are followed by the infiltration of skin lesions by lymphocytes, mast cells, eosinophils, and neutrophils. AD is a biphasic inflammatory skin disease that can be considered to have two distinct phases [4]. In the acute phase, AD skin lesions are infiltrated with $CD4^+$ T cells, which predominantly secrete the Th2 cytokines IL-4, IL-5, and IL-13, whereas in the chronic phase, Th1 cells secrete IFN-γ. The chronic phase of AD involves Th1-type inflammation and delayed-type hypersensitivity (DTH) due to an IFN-γ response that triggers tissue remodeling and dermal thickening via collagen accumulation [5,6].

NC/Nga mice have been most extensively studied as an animal model of AD. These mice spontaneously develop AD-like eczematous skin lesions when kept in air-uncontrolled conventional housing, but not when maintained under specific pathogen-free (SPF) conditions [7]. Given the similarity between the clinical symptoms displayed by NC/Nga mice and AD in humans, models based on these mice are considered to provide important information regarding the understanding of AD [7]. Furthermore, repeated treatment with DNFB (2,4-dinitrofluorobenzene) evokes AD-like skin lesions in NC/Nga mice under specific pathogen-free (SPF) conditions [8–10].

Rosmarinic acid (α-*o*-caffeoyl-3,4-dihydroxyphenyl-lactic acid; RA) is a naturally occurring hydroxylated phenolic compound found in Lamiaceae herbs, such as rosemary, sweet basil, and perilla [11–13]. RA has many biological activities, which include anti-inflammatory, anti-oxidant, anti-angiogenic, and anti-tumor activities [14–17]. In addition, RA has the ability to block complement fixation, attenuate T cell receptor mediated signaling (by inhibiting the activities of PLC-γ1 and ITK), and suppress IKK-β downstream signaling during the tumor necrosis factor (TNF)-α-induced upregulation of CCL11 (a potent

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chemoattractant and an activator of eosinophils, basophils, and T-helper 2 (Th2) cells) [18–20]. Moreover, it was reported in a clinical study that RA has an AD-ameliorating effect [21]. However, the nature of the mechanism responsible for this effect remains unclear. Thus, in the present study, we investigated whether RA suppresses the formation of DNFB-induced AD-like skin lesions in NC/Nga mice and sought to reveal the mechanism involved.

2. Materials and methods

2.1. Animals

Six-week-old male NC/Nga mice were purchased from Japan SLC (Shizuoka, Japan) and maintained under SPF conditions. Animals were housed in an air-conditioned animal room (25 ± 1 °C; relative humidity 40 ± 5%) and fed a laboratory diet and distilled water. The experimental protocol used in this study complied with the guidelines issued by the ethical committee for animal welfare at Kyung Hee University [KHUASP (SE)-09-005], and all procedures were conducted in accordance with the guidelines issued by the U.S. National Institute of Health.

2.2. RA treatment

Rosmarinic acid (97%) was purchased from Sigma-Aldrich (St. Louis), dissolved in EtOH, and further diluted with phosphate-buffered solution (PBS) at 5%. Mice were randomly divided into four groups, that is, an untreated control, a DNFB only, a DNFB plus RA (10 mg/kg), and a DNFB plus RA (50 mg/kg) group. RA was administered intraperitoneally at 10 or 50 mg/kg daily and DNFB was administered as described below.

2.3. DNFB-induced dermatitis

AD-like skin lesions were induced by the repeated application of 25 μl of 0.15% DNFB in acetone/olive oil (3:1) to the inner and outer surfaces of the ears and 100 μl of the same solution applied to shaved back skin once on days 1 and 4. On days 9, 13 and 17, sensitized mice were challenged by applying 0.2% DNFB to the back and ear skin surfaces. Control groups were treated with the same volumes of vehicle. Ear thicknesses were measured with a thickness gauge (Digimatic Indicator, Mitsutoyo, Tokyo) on days 1, 2, 4, 5, 6, 8, 9, 10, 12, 13, 14, 16, and 17. The severity of dermatitis was macroscopically assessed by the scoring atopic dermatitis (SCORAD) method. The degree of each symptom was scored as 0 (absence), 1 (mild), 2 (moderate) and 3 (severe). This scoring was based on the severity of erythema, edema, oozing, crust, excoriation and lichenification. The total score (minimum 0 and maximum 18) of the six symptoms of each mouse was taken as the score for that mouse. Assessment was performed by an investigator who was blind to the grouping of the animals.

2.4. MTT assay

Purified $CD4^+$ T cells seeded in 96-well flat-bottom culture plates were cultured for 50 h at 37 °C in a 5% CO_2 atmosphere and treated with various concentrations of RA. After 50 h, the culture supernatant was removed and the cells were washed with 100 μl of PBS at room temperature. Then 200 μl of medium and 20 μl of MTT solution (5 mg/ml) were added for 4 h at 37 °C (5% CO_2) and the resultant formazan crystals were then dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA). After 30 min at room temperature, the optical density of the solution was determined with enzyme-linked immunosorbent assay (ELISA) reader (EL 800) (Bio-Tek, Winooski, VT, USA) [22]. The absorbance of six wells for each treatment was averaged and expressed as a percentage of the mean of control untreated wells.

2.5. Measurement of cytokine production in vitro

T lymphocytes were isolated from the draining lymph nodes of mice, and $CD4^+$ T cells were purified using a Biomag separation column (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Isolated $CD4^+$ T cells (1.5×10^5) were cultured in 96-well flat-bottom culture plates (Corning, NY, USA) in RPMI-1640 medium with 10% fetal bovine serum, 50 μM β-mercaptoethanol and 2 mM glutamine, then stimulated with immobilized anti-CD3 antibody (BD Pharmingen, San Diego, CA, USA) (5 μg/ml) and the soluble form of purified anti-CD28 antibody (BD Pharmingen, San Diego, CA, USA) (2 μg/ml). Cultures were treated with different doses (1 or 5 μM) of RA for 50 h at 37 °C. The productions of IL-4 and IFN-γ after T cell activation were quantified by ELISA (Biolegend, CA, USA).

2.6. Serum IgE measurements

On day 17, 24 h after the final DNFB application, total serum samples were prepared. Total serum IgE concentration was quantified using BD OptEIA™ mouse ELISA kits (BD Pharmingen, San Diego, CA, USA).

2.7. Histological analysis

Right ears were removed 24 h after final DNFB application and fixed with 4% paraformaldehyde in PBS (pH 7.4). Ear sections were washed in sucrose solution with several changes (10% sucrose in PBS for 4 h, 15% sucrose in PBS for 4 h, and 20% sucrose in PBS overnight) and frozen in OCT compound. Cryostat sections (5 μm) were mounted on Superfrost Plus slides and dried overnight at room temperature, before being fixed in ice-cold acetone for 10 min. Slides were rehydrated in PBS and blocked with 5% normal goat serum in PBS for 1 h at room temperature. After washing with PBS, sections were incubated with primary antibody (rat anti-CD4, anti-CD8 antibody: BD Pharmingen, CA) overnight at room temperature. They were then washed with PBS and incubated with an FITC-labeled anti-rat antibody for 2 h at room temperature. Cell nuclei were counterstained with DAPI (Molecular Probes, Eugene, OR). Sections were mounted with glycerol mounting medium (Dako, CA, USA), and images were captured using a confocal microscopy system (Olympus, Tokyo). Skin sections were also stained with hematoxylin and eosin for inflammatory cells. Mast cells were stained using toluidine blue (TB). Acetone fixed frozen slides were rehydrated and immersed in a TB solution (0.1% TB (Sigma, MO, USA) in 70% EtOH) for 3 min, followed by rinsing three times in H_2O and dehydrated in graded series of EtOH (95–99% EtOH). At last, the sections were cleared in xylene and coverslipped in Vectashield anti-fading solution (Vector Laboratories, CA, USA). Numbers of $CD4^+$ T cells, $CD8^+$ T cells, and mast cells are expressed as average total counts in 5 fields of 100 μm^2 (×400).

2.8. Statistical analysis

Results are expressed as means ± SEMs. The significances of changes were evaluated using the Student's *t*-test. Differences between experimental groups were evaluated using analysis of variance. p values of <0.05 were considered significant.

3. Results

3.1. RA suppressed IL-4 and IFN-γ levels in activated $CD4^+$ T cells

A recent study showed that RA has anti-inflammatory effects as well as anti-oxidant effects [14,15,17]. We investigated whether RA affected inflammatory cytokine production by $CD4^+$ T cells. To exclude the possibility of a toxic effect of RA on inflammatory cytokine production, we checked the viabilities of $CD4^+$ T cells treated with RA using MTT

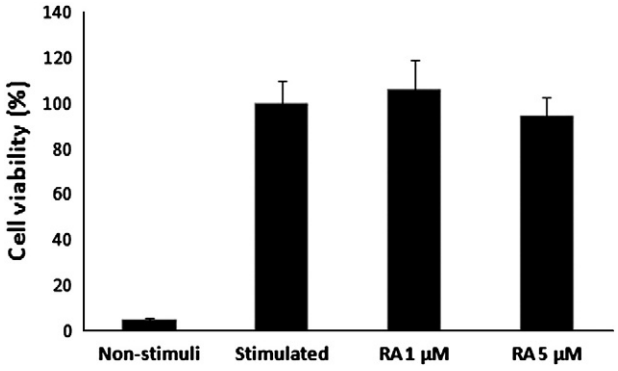


Fig. 1. Viability of CD4$^+$ T cells pre-treated with RA. For MTT assays, purified lymph node CD4$^+$ T cells (1.5×10^5) were pre-treated with RA for 20 min, stimulated with anti-CD3 (5 μg/ml) and anti-CD28 (2 μg/ml) and cultured in 96-well flat bottomed culture plates for 50 h at 37 °C in a 5% CO_2 atmosphere. Non-stimuli is no antibody stimulation. The results shown are means ± SEM ($n=6$). *$p<0.05$ versus untreated controls.

assays. The viabilities of purified lymph node CD4$^+$ T cells treated with different doses of RA (1 or 5 μM) for 50 h were not significantly different from untreated controls by the MTT assay (Fig. 1). Furthermore, 1 or 5 μM RA did not affect CD4$^+$ T cell proliferation, and treatment with 1 or 5 μM RA for 50 h resulted in 96–97% viability (Fig. 1).

AD exhibits Th1 or Th2 dominant inflammation dependent on stage [4]. Human Th1 and Th2 cells are characterized by distinct cytokine production profiles [23]. Furthermore, Th1 and Th2 cells have distinctive cytokine production profiles [10]. Th1 cells produce large amounts of IFN-γ and IL-2, whereas Th2 cells produce IL-4, IL-5 and IL-13 [9]. Therefore, we investigated whether RA regulated the Th1 and Th2 responses of CD4$^+$ T cells. Lymph node CD4$^+$ T cells were pre-treated with RA at the concentration used in the previous MTT assay (1 or 5 μM), and then stimulated with anti-CD3 and anti-CD28 antibody for 50 h. Supernatants were then collected and IL-4 and IFN-γ productions were measured by ELISA. It was found that the productions of both were significantly and dose-dependently reduced in RA-treated CD4$^+$ T cells versus non-treated controls (Fig. 2). These findings show that RA significantly inhibited the productions of IL-4 and IFN-γ in activated CD4$^+$ T cells.

3.2. DNFB-induced AD symptoms are ameliorated by RA

AD-like skin lesions can be induced in NC/Nga mice by the repeated application of a chemical hapten, such as DNCB (dinitrochlorobenzene) or DNFB [8]. Therefore, we investigated whether RA suppresses these DNFB-induced AD-like skin lesions. In the present study, mice treated with DNFB for 2 weeks exhibited pruritus and eruptions. However, the intraperitoneal administration of RA (10 or 50 mg/kg) to mice every day significantly relieved lesion severities (Fig. 3). Furthermore, DNFB-induced ear swelling was found to increase after five challenges over 2 weeks, and treatment with RA at 10 or 50 mg/kg i.p. every day significantly suppressed DNFB-induced ear swelling in a concentration-dependent manner (Fig. 4).

3.3. RA reduced DNFB-induced serum IgE levels

AD is characterized by elevated total serum IgE levels, and IgE synthesis by B cells is probably modulated by the IL-4 secreted by Th2 cells [23]. In the present study, IL-4 production by activated CD4$^+$ T cells was reduced by RA (Fig. 2). Therefore, we investigated whether RA suppresses IgE in total serum. After the fifth DNFB treatment, serum samples were collected and total serum IgE levels were measured by ELISA. In mice topically treated with DNFB, total serum IgE levels were higher than in untreated controls (Fig. 5). However, treatment with α-RA at 10 or 50 mg/kg i.p. every day significantly reduced total serum IgE levels as compared with DNFB alone (Fig. 5).

3.4. RA reduced the infiltrations of inflammatory cells into DNFB-induced skin lesions

The dermal infiltration of inflammatory cells is an important feature of AD. Cell types contained in infiltrates include lymphocytes and mast cells. In the present study, histological analysis revealed that topical DNFB elicited the infiltrations of inflammatory cells into ear skin lesions, and that numbers of these cells were significantly

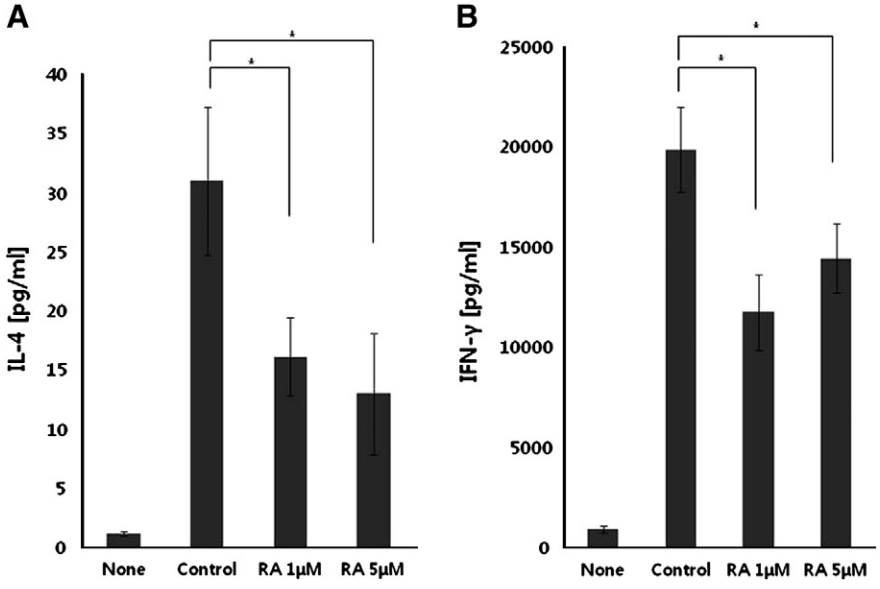
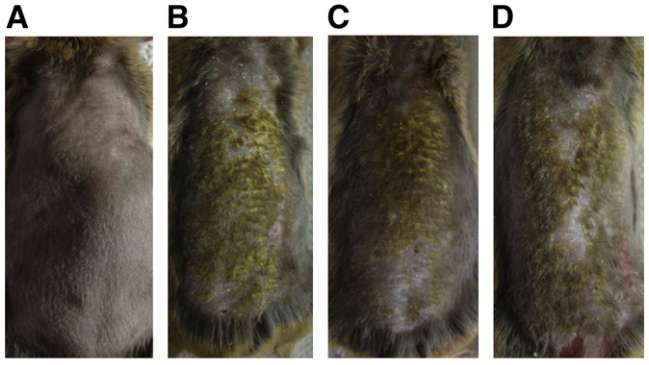


Fig. 2. Measurements of cytokine production by CD4$^+$ T cells. Cells were purified using a Biomag separation column. Isolated cells (1.5×10^5) were cultured in 96-well plates, pre-treated with different doses of RA (1 and 5 μM) for 20 min, then stimulated with immobilized anti-CD3 (5 μg/ml) and soluble anti-CD28 (2 μg/ml) antibody for 50 h at 37 °C in a 5% CO_2 atmosphere. Supernatants were then collected and interferon (IFN)-γ (A) and interleukin (IL)-4 (B) levels were quantified by ELISA. None (no antibody stimulation), Control (stimulated with anti-CD3 and anti-CD28), RA 1 and 5 μM (antibody stimulation with RA 1 or 5 μM, respectively). The absorbances of six wells per treatment were averaged. Bars represent means ± SEM. *$p<0.05$ (vs. the DNFB (+) group).



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Group	NO. of Mice	After 5th treatment SCORAD
Negative	5	0 ± 0.0
DNFB	5	15.6 ± 0.34
DNFB + Rosmarinic acid 10 mg/kg	5	10.6 ± 0.51 *
DNFB + Rosmarinic acid 50 mg/kg	5	15.0 ± 0.52 *

*P < 0.05 versus positive

Fig. 3. Comparisons of AD-like skin lesions in NC/Nga mice after the 5th DNFB challenge. AD was induced by the topical application of 0.15% DNFB (acetone 3/olive oil 1) on dorsal skin and right ears on experimental days 1 and 5. On days 9, 13, and 17, sensitized mice were challenged with 0.2% DNFB. Five mice were allocated to each of the following four groups: (A) acetone 3/olive oil 1; (B) acetone 3/olive oil 1 containing 0.15–2% DNFB; (C) DNFB plus RA (10 mg/kg); and (D) DNFB plus RA (50 mg/kg). (E) The severity of atopic dermatitis was macroscopically assessed by SCORAD. The degree of each symptom was scored as 0 (absent), 1 (mild), 2 (moderate) and 3 (severe). The total score from the six symptoms of each mouse was taken as the score for that mouse. Data are presented as mean ± SEM of five determinations. *$p<0.05$ versus positive.

increased after fifth DNFB challenge. However, the number of infiltrated cells was remarkably lowered by RA treatment at 10 or 50 mg/kg every day (Fig. 6). In addition, $CD4^+$ T and $CD8^+$ T cell numbers in ear skin lesions were significantly reduced by RA (Fig. 7). AD patients with IgE-mediated allergy exhibit enhanced cytokine

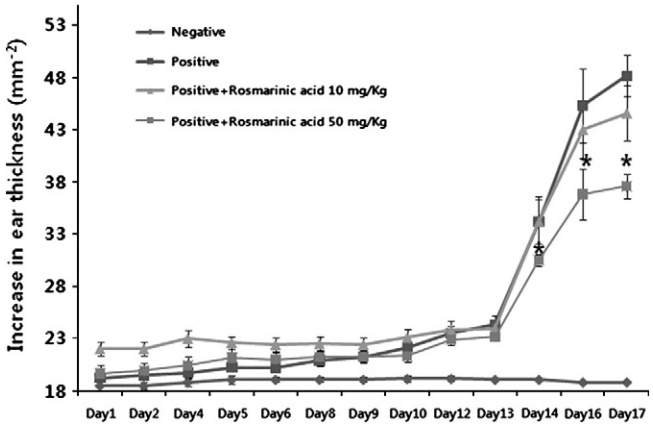

Fig. 4. AD was induced by the repeated application of DNFB on both outer and inner right ear surfaces. Ear swelling was measured daily using a thickness gauge. RA was administered intraperitoneally at concentrations of 10 or 50 mg/kg. Five mice were allocated to each of the four groups: Negative (acetone 3/olive oil 1); Positive (acetone 3/olive oil 1 containing 0.15–2% DNFB); DNFB plus RA (10 mg/kg); and DNFB plus RA (50 mg/kg). *$p<0.05$ (vs. the DNFB (+) group).

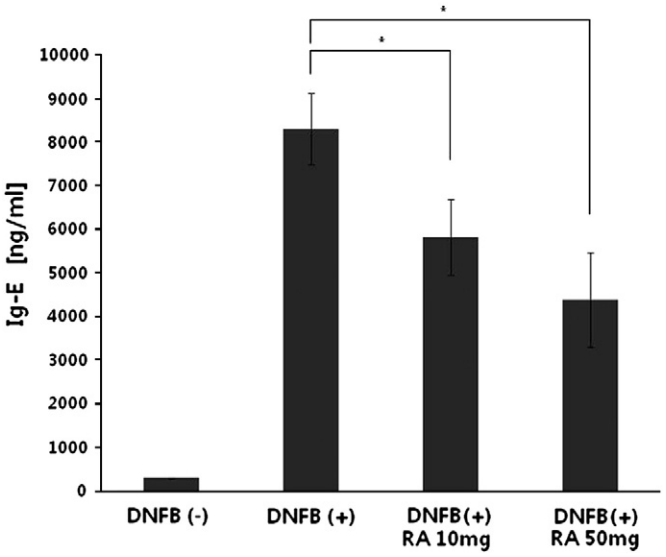

Fig. 5. Effects of the intraperitoneal administration of RA on IgE production in the DNFB-induced NC/Nga mouse AD model. Total serum samples were prepared from blood collected after last DNFB application and serum IgE concentrations were determined by ELISA. Five mice were allocated to each of four groups: Negative (acetone 3/olive oil 1); Positive (acetone 3/olive oil 1 containing 0.15–2% DNFB); DNFB plus RA (10 mg/kg); and DNFB plus RA (50 mg/kg). The absorbances of serum samples from five mice per group were averaged. Bars represent means ± SEM. *$p<0.05$ (vs. the DNFB (+) group).

expression in skin lesions and the surface expression of FcεRI on mast cells. Furthermore, elevated IgE levels might participate in cutaneous inflammation caused by the activation of mast cells via Fc receptors in AD patients.

Thus, we examined the effect of RA on skin lesion infiltration by mast cells using toluidine blue. Numbers of infiltrating mast cells in lesions were found to obviously decrease after RA treatment at 10 or 50 mg/kg (Fig. 8).

4. Discussion

AD is the most commonly encountered skin disease and is characterized by erythema, edema, excoriation, and scaling [24]. Furthermore, the incidence of AD continues to increase in industrialized countries [7]. Although topical steroid therapy is essential for the treatment of AD, it cannot be administered long-term due to its side effects, and thus, new types of treatment are required.

RA has been reported to have anti-oxidant and anti-inflammatory effects, and is used in traditional medicine to treat inflammatory disorders and arthritis [25,26]. Recently, it was reported that RA has as AD-mitigating effect *in vivo* [21], but the mechanism involved was not elucidated.

Therefore, in this study, we examined the mechanisms by which RA improves AD using a DNFB-induced AD model in NC/Nga mice. Our findings indicate that RA reduces the severities of AD-like skin lesions by suppressing total serum IgE levels and the productions of IFN-γ and IL-4 by activated $CD4^+$ T cells, and by reducing lesion infiltration by $CD4^+$ T, $CD8^+$ T and mast cells.

T cells play critical roles in the pathogenesis of AD. More specifically, activated $CD4^+$ T cells induce the differentiation of Th1 cells to IFN-γ and Th2 cells that secrete IL-4, IL-5, and IL-13 [27]. It is well known that IFN-γ is elevated during the chronic phase in AD, and that IL-4 secretion is increased during the acute phase [28]. Recently, it was reported that RA inhibits T cell activation via the inactivation of $p56^{lck}$. In the present study, treatment with 1 or 5 μM RA did not affect $CD4^+$ T cell viability or proliferation (Fig. 1; the concentrations used in experiments to measure cytokine production). Furthermore, we found that RA dose-

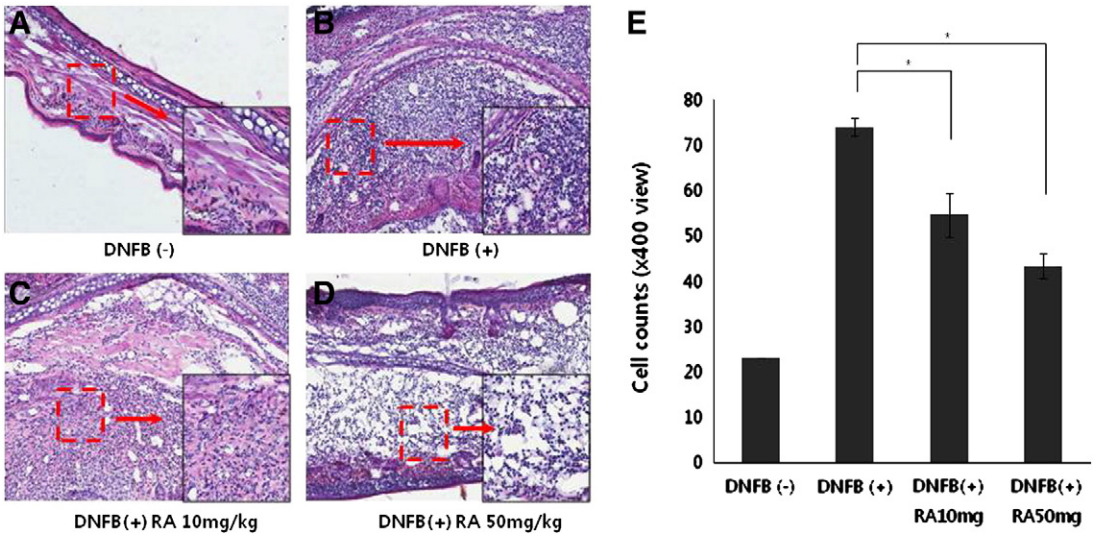

Fig. 6. Effect of RA on numbers of inflammatory cells in DNFB-treated AD-like skin lesions. On day 17 of the experiment, the numbers of inflammatory cells in ear skin lesions were counted as described in Materials and methods. (A) DNFB (−), (B) DNFB (+), (C) DNFB (+) + RA 10 mg/kg and (D) DNFB (+) 50 mg/kg. (E) The data are the representative results of three independent experiments. Numbers of inflammatory cells are presented as means ± SEM (5 fields/group). *$p<0.05$ (vs. the DNFB (+) group).

dependently suppressed the productions of IFN-γ and IL-4 by activated lymph node $CD4^+$ T cells (Fig. 2).

IL-4 (a Th2-type cytokine) induces class switching from IgM secreting B cells to IgE secreting B cells. Furthermore, elevated serum IgE levels have several effects on skin inflammation [29–31]. In particular, IgE expression is known to cause both acute and chronic phase skin symptoms [29–31], and repeated painting with DNFB increases serum IgE levels in NC/Nga mice [4,8,9,32]. We also found that total serum IgE levels were significantly increased by repeated DNFB treatment in NC/Nga mice, as was IL-4 production by activated

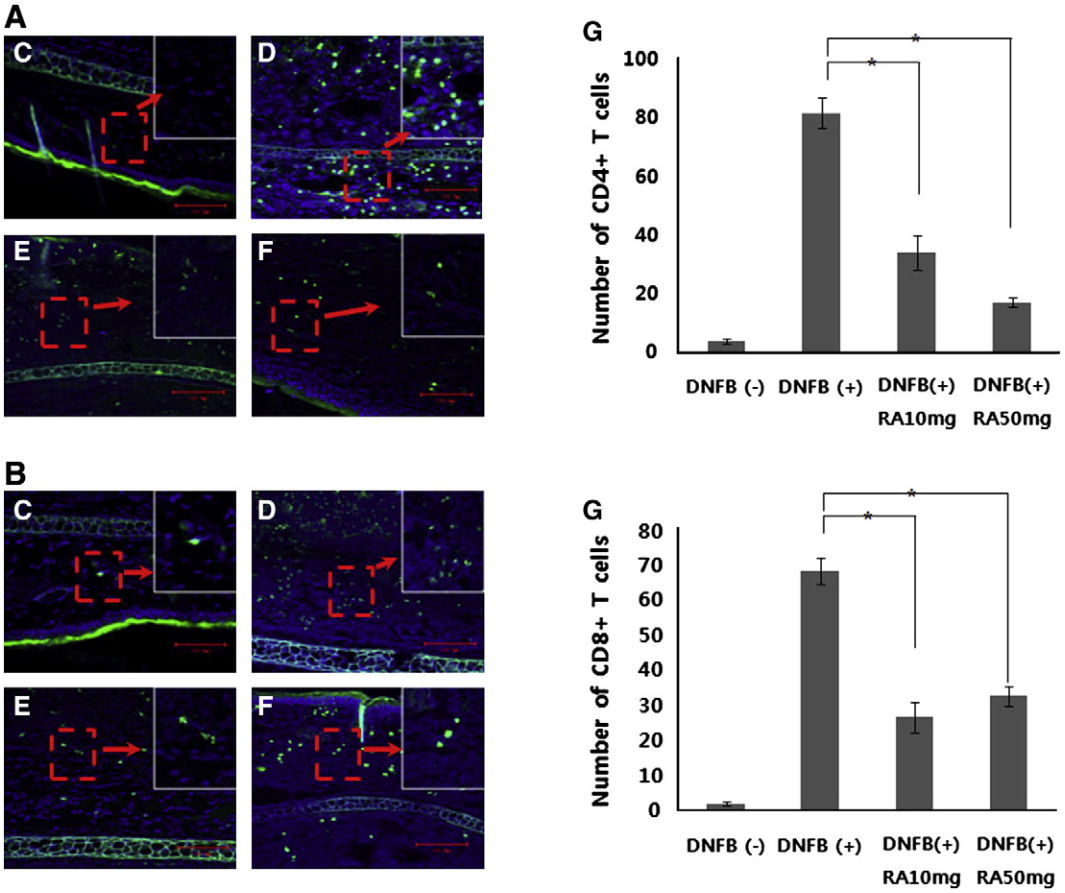

Fig. 7. Effect of RA on numbers of infiltrating $CD4^+$ T (A) and $CD8^+$ T cells (B) in skin lesions. On day 17 of the experiment, the numbers of $CD4^+$ T and $CD8^+$ T cells in skin lesions were counted, as described in Materials and methods. (C) DNFB (−), (D) DNFB (+), (E) DNFB (+) + RA 10 mg/kg and (F) DNFB (+) 50 mg/kg. (G) The data are the representative results of three independent experiments. Numbers of inflammatory cells are presented as means ± SEM (5 fields/group). *$p<0.05$ (vs. the DNFB (+) group).

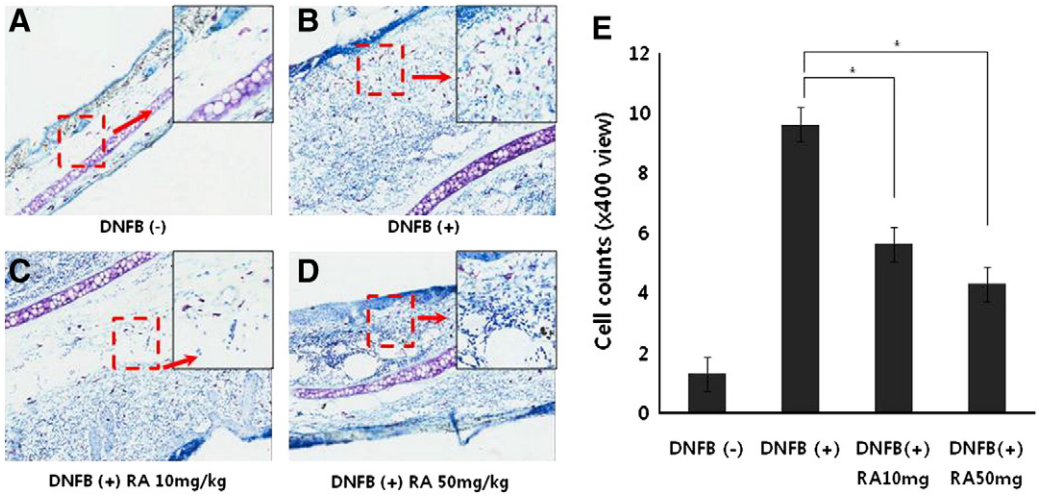

Fig. 8. Effect of RA on numbers of infiltrating mast cells in skin lesions. On day 17 of the experiment, numbers of mast cells in skin lesions were counted as described in Materials and methods. (A) DNFB (−), (B) DNFB (+), (C) DNFB (+) + RA 10 mg/kg and (D) DNFB (+) 50 mg/kg. (E) The data are the representative results of three independent experiments. Numbers of inflammatory cells are presented as means ± SEM (5 fields/group). $^{*}p<0.05$ (vs. the DNFB (+) group).

$CD4^+$ T cells obtained from these mice (Fig. 5). In contrast, RA treatment significantly reduced levels of total serum IgE in DNFB-treated mice and IL-4 production by activated $CD4^+$ T cells (Fig. 5).

AD patients tend to scratch, which causes mechanical injury and induces the productions of inflammatory cytokines and chemokines. Levels of chemokines and adhesion molecules, like ICAM-1 and VCAM-1 on endothelial cells, are increased and recruited by a variety of leukocytes, such as lymphocytes, mast cells, eosinophils, neutrophils, to skin lesion [33–35]. Furthermore, the interaction between CCL17 and CCR4 is regulated by the infiltration of Th2 cells into AD lesions by chemotaxis [36] and in human AD, histological findings have shown that the majority of skin-infiltrating T cells in active AD lesion are $CD4^+$ T cells [37]. We also found that DNFB treatment increased the number of $CD4^+$ T cells infiltrating skin lesions. In contrast, RA treatments reduced infiltrating $CD4^+$ T cell numbers (Fig. 7). Recently, it was shown that allergen-primed $CD8^+$ T cells are required for the development of AD skin lesions in mice [38]. In the present study, RA significantly reduced skin lesion infiltration by $CD8^+$ T cells, but in mouse and rat models, allergic inflammation was found to be dependent on $CD4^+$ T cells, and $CD8^+$ T cells were found to have a negative regulatory effect [39,40]. We do not know whether infiltrating $CD8^+$ T cells provoke skin lesions, and further studies are required to clarify this point. In particular, mast cells are known to be pivotal effector cells in allergic disorders and during IgE-associated immune responses [41]. Upon activation, mast cells undergo degranulation and secrete arachidonic acid metabolites and various cytokines and chemokines, which can all have immunoregulatory effects [42]. The infiltration of the upper dermis by mast cells is a critical feature of AD skin lesions [43], and in the present study, RA significantly reduced the number of mast cells infiltrating DNFB-induced AD skin lesions (Fig. 8).

Summarizing, RA reduced the development of the AD-like skin lesions on NC/Nga mice induced by repeated DNFB treatment by suppressing total serum IgE levels and the productions of IFN-γ and IL-4 by activated $CD4^+$ T cells, and by reducing lesion infiltration by $CD4^+$ T, $CD8^+$ T and mast cells.

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