

α -Lipoic acid suppresses the development of DNFB-induced atopic dermatitis-like symptoms in NC/Nga mice

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Abstract: Atopic dermatitis (AD) is a common skin disease that has complex pathogenic mechanisms. Under specific pathogen-free conditions, repeated epicutaneous treatment of 2,4-dinitrofluorobenzene (DNFB) evokes AD-like clinical symptoms in NC/Nga mice. α -Lipoic acid (α -LA; 1, 2-dithiolane-3-pentanoic acid) is a dietary component that is synthesized in bacteria, yeast, plants, and mammals. α -LA and its reduced form, dihydrolipoic acid, are powerful antioxidants that have many physiological functions, including free radical scavenging of reactive oxygen species, generation of cellular antioxidants, chelation of metal ions, and inflammatory suppression. In this study, we investigated whether α -LA suppresses AD-like skin lesions induced by repeated DNFB application in NC/Nga mice. α -LA significantly suppressed production of interferon (IFN)- γ

and interleukin (IL)-4 by activated CD4⁺ T cells. We found that the oral administration of α -LA reduced AD-like clinical symptoms and inhibited increases of epidermal thickness in DNFB-induced AD-like skin lesions of NC/Nga mice. Furthermore, total serum IgE levels were dramatically reduced by topical α -LA treatment. Our findings suggest that oral administration of α -LA suppresses the development of AD in DNFB-treated NC/Nga mice and reduces IFN- γ and IL-4 production from activated CD4⁺ T cells as well as total serum IgE levels.

Key words: 2,4-dinitrofluorobenzene – atopic dermatitis – CD4⁺ T cells – NC/Nga – α -Lipoic acid

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Introduction

Atopic dermatitis (AD) is a chronically relapsing inflammatory skin disease with a clinical presentation of skin erythematous, edema, lichenification by cutaneous hypersensitivity and intense pruritus (1). AD affects 10–20% of children and 1–3% of adults worldwide (2,3). AD has complex pathogenic mechanisms that include abnormal genetical and immunological pathways and exposure to environmental or chemical agents (4,5). Therefore, its pathogenesis has not been clearly defined. AD is a biphasic inflammatory skin disease that is largely separated into two distinct phases (6). Acute AD skin lesions reveal Th2-dominant inflammation by infiltrated CD4⁺ T cells and eosinophils, which produce Th2 cytokines such as IL-4, IL-5, and IL-13. In contrast, the chronic phase involves Th1-type inflammation and delayed-type hypersensitivity by an interferon (IFN)- γ response that triggers tissue remodelling and dermal thickening via increased collagen accumulation (7,8). The NC/Nga mice display clinical symptoms similar to those of AD in human. Therefore, they have been used as an animal model of AD (9). Under specific pathogen-free (SPF) conditions, they maintain a healthy condition. But in non-sterile conditions, AD-like skin lesions spontaneously progress beginning at the age of 6–7 weeks (10,11). Similar to non-sterile conditions, administration of a chemical hapten such as 2,4-dinitrofluorobenzene (DNFB) and dinitrochlorobenzene (DNCB) produces AD-like skin lesions in NC/Nga mice in SPF conditions (12). DNFB is a chemical hapten that activates T cells (13). It binds Lys and Cys residues in epidermal proteins. α -Lipoic acid (α -LA; 1, 2-dithiolane-3-pentanoic acid) and its reduced form, dihydrolipoic acid (DHLA), have been called ‘uni-

versal antioxidants’ that function in both hydrophobic and hydrophilic phases (14). α -LA recycles other antioxidants such as vitamin C, vitamin E, and glutathione (GSH) (15). It also scavenges free radicals and modulates the activity of NF- κ B. Because of its therapeutic effects, α -LA has been used to treat diverse disease conditions such as ischemia–reperfusion injury, heavy-metal detoxification, diabetes, diabetic neuropathy, and asthma (16,17). Furthermore, a recent study reveals that an α -LA-based PPAR γ agonist has anti-inflammatory effects in skin diseases by inhibiting keratinocyte proliferation (18). Thus, we investigated whether α -LA suppresses DNFB-induced AD in the NC/Nga AD model.

Materials and methods

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 $\pm$ 1°C; relative humidity 40 $\pm$ 5%) and fed a laboratory diet and distilled water. The experiments 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 US National Institute of Health guidelines.

α -LA treatment

The mice were randomly divided into four groups: control, DNFB, DNFB with α -LA (10 mg/kg), and DNFB with α -LA (50 mg/kg). Each group included five mice. α -LA (Sigma, St Louis, MO, USA) was dissolved in distilled water and adjusted to pH 7.0 by 1 M NaOH. The α -LA-treated mice received oral α -LA at 10 or 50 mg/kg every day.

Allergen sensitization and challenge

AD-like skin lesions were induced by the repeated application of 25 μ l of 0.15% DNFB in acetone/olive oil (3:1) to the inner and outer surfaces of the ears, and 100 μ l of the same solution applied to shaved back skin once on day 1 and 4. On days 7, 10, and 13, 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) every day. The severity of dermatitis was macroscopically assessed by the scoring AD (SCORAD) method (19). 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 as described previously (20). 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.

MTT assay

Purified CD4⁺ T cells seeded in 96-well flat-bottom culture plates (Corning 3595 96-well cell culture plate, Corning, NY, USA) were cultured for 50 h at 37°C in a 5% CO₂ atmosphere and treated with various concentrations of α -LA. After 50 h, the culture supernatant was removed and the cells were washed with 100 μ l of Phosphate buffered saline (PBS) at room temperature. Then 200 μ l of medium and 20 μ l of MTT solution (5 mg/ml) were added for 4 h at 37°C (5% CO₂), and the resultant formazan crystals were then dissolved in dimethyl sulfoxide (DMSO) (Sigma). 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) (21). The absorbance of six wells for each treatment was averaged and expressed as a percentage of the mean of control untreated wells.

Measurement of cytokine production *in vitro*

T lymphocytes were isolated from the 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 3595 96-well cell culture plate) in RPMI-1640 medium with 10% fetal bovine serum, 50 μ M β -mercaptoethanol and 2 mM glutamine then stimulated with immobilized anti-CD3 antibody (BD Pharmingen, San Diego, CA, USA) (5 μ g/ml) and the soluble form of purified anti-CD28 antibody (BD Pharmingen) (2 μ g/ml). Cultures were treated with different doses (1–100 μ M/ml) of α -LA for 50 h at 37°C. The productions of IL-4 and IFN- γ after T cell activation were quantified by ELISA (Biolegend, San Diego, CA, USA).

Serum IgE measurements

On day 13, 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). In summary, immunoplate (Corning 3590 96-well EIA/RIA plate) microwells were coated with 100 μ l per well of anti-mouse IgE monoclonal antibody diluted 250-fold with coating buffer. The plates were sealed and incubated overnight at 4°C. After the wells were aspirated and washed three times with 245 μ l of PBS containing 0.05% Tween 20 (washing buffer), the plates were blocked with 200 μ l of PBS containing 1% bovine serum albumin and

incubated for 1 h at room temperature. After 1 h, the wells were washed three times with washing buffer, and 100 μ l of each standard or serum samples diluted 100- or 200-fold with PBS with 1% BSA were introduced into the appropriate wells. After incubation for 3 h at room temperature, the seals were removed and the wells were washed five times with washing buffer. Then 100 μ l of prepared streptavidin-horseradish peroxidase-conjugated detection antibody were added to each well and incubated for 2 h at room temperature. After seven washes with washing buffer, 100 μ l of substrate solution (0.1 M sodium citrate, tetramethyl-benzidine dihydrochloride, and H₂O₂) were added. Plates were incubated for 40 min at room temperature in the dark. The enzyme reaction was terminated by adding 50 μ l of stop solution (1 M H₂SO₄) to each well, and the absorbance was immediately read at 450 nm on an ELISA reader (EL 800) (Bio-Tek).

Histological analysis

Right ears were excised from the mice 24 h after final DNFB application. The ears were fixed in 10% buffered formalin, embedded in paraffin and sectioned at 3 μ m, then stained with hematoxylin and eosin (H&E staining).

Statistical analysis

Data are means $\pm$ SEM. The significance of changes was evaluated by the Student's *t*-test. Differences between the two experimental groups were compared by analysis of variance. Statistical significance was defined as a *P* value of less than 0.05.

Results

Effects of α -LA on the proliferation of CD4⁺ T cells

A recent study shows that α -LA has not only antioxidant effects but also anti-inflammatory effects (22). We investigated whether α -LA treatment on CD4⁺ T cells would affect inflammatory cytokine production. We performed the MTT assay to assess viability of CD4⁺ T cells treated with α -LA. The viabilities of purified lymph node CD4⁺ T cells treated with different doses of α -LA (10–100 μ M) for 50 h were not significantly different in the MTT assay (Fig. 1); 10 μ M α -LA did not affect CD4⁺ T cell proliferation compared with that of non-treated CD4⁺ T cells. Also, treatment with 50 μ M and 100 μ M α -LA resulted in 96–97% viability in this study (Fig. 1).

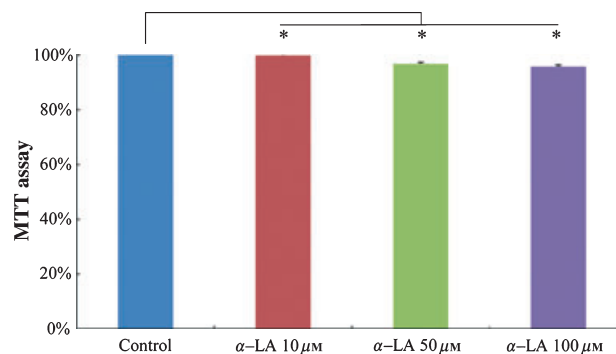


Figure 1. Viability of CD4⁺ T cells after pretreatment with α -lipoic acid (α -LA). For MTT assay, purified lymph node CD4⁺ T cells (1.5×10^5) were pretreated with α -LA for 20 min then stimulated with anti-CD3 (5 μ g/ml) and anti-CD28 (2 μ g/ml) and cultured in 96-well flat-bottom culture plates for 50 h at 37°C in a 5% CO₂ atmosphere. Relative viability of CD4⁺ T cells with different doses (1–100 μ M) of α -LA decreased in a dose-dependent manner: 100% with 10 μ M α -LA, 96.8% with 50 μ M α -LA and 95.7% with 100 μ M α -LA. Data are shown as means $\pm$ SEM (*n* = 6). **P* < 0.05 versus control.

α -LA suppressed both IL-4 and IFN- γ production in CD4⁺ T cells *in vitro*

AD exhibits Th1- or Th2-dominant inflammation dependent on phase. Human Th1 and Th2 cells are characterized by distinct cytokine production profiles (23). Th1 cells produce large amounts of IFN- γ and IL-2, whereas IL-4, IL-5, and IL-13 are produced by Th2 cells (24). Therefore, we investigated whether α -LA modulates the Th1 and Th2 responses of CD4⁺ T cells. Lymph node CD4⁺ T cells were pretreated with α -LA at the concentration used in the previous MTT assay, then stimulated with anti-CD3 and anti-CD28 antibody for 50 h. The supernatants were collected, and then IL-4 and IFN- γ production in CD4⁺ T cells were measured by ELISA. The production of IL-4 and IFN- γ was significantly and dose-dependently decreased in α -LA-treated CD4⁺ T cells compared with that in non-treated CD4⁺ T cells (Fig. 2). These data showed that α -LA treatment significantly inhibited IL-4 and IFN- γ production by activated CD4⁺ T cells.

DNFB-induced AD symptoms reduced by α -LA

AD-like skin lesions can be induced in NC/Nga mice by repeated application of a chemical hapten such as DNCB or DNFB (8). Therefore, we investigated whether α -LA suppresses these DNFB-

induced AD-like skin lesions. In this study, the group of mice treated with DNFB for 2 weeks exhibited AD-like skin lesions involving pruritus and eruptions. However, oral administration of α -LA (10 or 50 mg/kg) to mice significantly relieved lesion severities compared with the DNFB-treated group (Fig. 3). Furthermore, SCORAD scores assessing skin erythema, edema, oozing, crust, excoriation, and lichenification were dramatically reduced in α -LA-treated groups compared with that in the DNFB-treated group (Fig. 3). DNFB-induced ear swelling consistently increased with five challenges during 2 weeks. Oral administration of α -LA at 10 or 50 mg/kg significantly suppressed DNFB-induced ear swelling in a concentration-dependent manner (Fig. 4). In this study, histological analysis revealed that topical DNFB elicited infiltration of inflammatory cells in ear skin lesions, which was significantly increased by the fifth challenge of the DNFB-treated group. However, the number of infiltrated cells was remarkably decreased after oral administration of α -LA at 10 or 50 mg/kg (Fig. 5).

α -LA reduced DNFB-induced serum IgE production

AD is characterized by elevated total serum IgE levels, and IgE synthesis in B cells is probably modulated by IL-4 secreted from Th2 cells (25). Furthermore, in this study, IL-4 production by activated CD4⁺ T cells was reduced by α -LA treatment (Fig. 2),

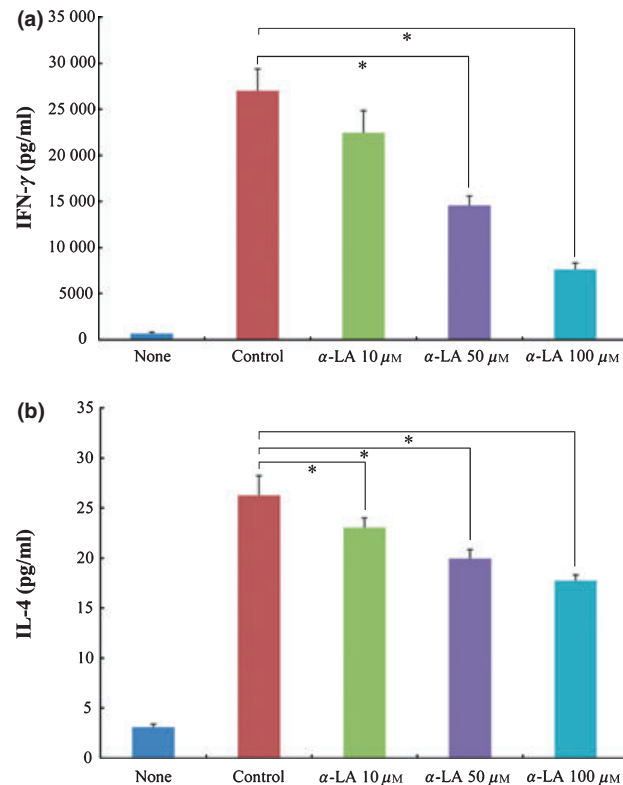


Figure 2. Measurements of cytokine production. CD4⁺ T cells were purified by using a Biomag separation column. Isolated CD4⁺ T cells (1.5×10^5) were cultured in 96-well plate and pretreated with different doses of α -lipoic acid (α -LA) (1–100 μ M) for 20 min then stimulated with immobilized anti-CD3 (5 μ g/ml) and soluble anti-CD28 (2 μ g/ml) antibody for 50 h at 37°C in a 5% CO₂ atmosphere. After 50 h, supernatants were collected and quantified by ELISA of interferon (IFN)- γ (a) and interleukin (IL)-4 (b). None (no stimulation), Control (stimulated with anti-CD3 and anti-CD28), α -LA 10, 50, and 100 μ M (antibody stimulation with α -LA 10, 50, and 100 μ M). The absorbance of six wells for each treatment was averaged, and each bar represents the mean $\pm$ SEM. * P < 0.05 versus control.

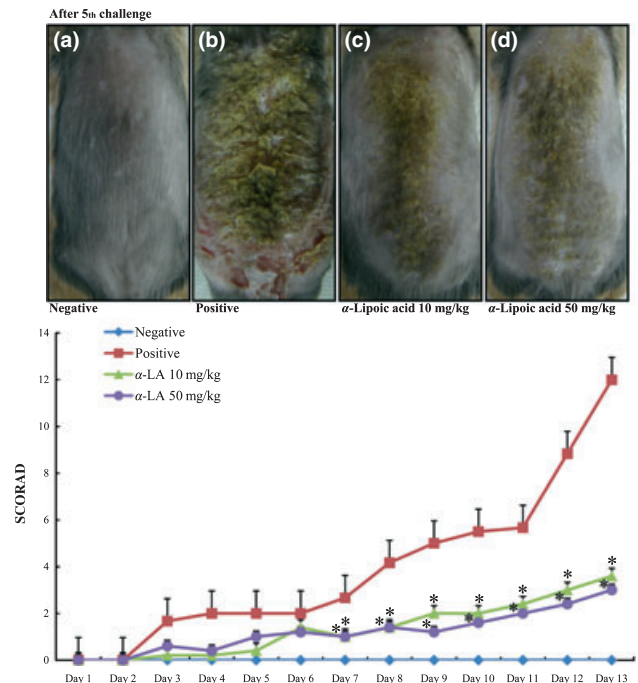


Figure 3. Comparisons of atopic dermatitis (AD)-like skin lesions of NC/Nga mice after the 5th challenge. AD was induced by topical application of 0.15% 2,4-dinitrofluorobenzene (DNFB) (acetone 3: olive oil 1) on dorsal skin and right ear on days 1 and 4. On days 7, 10, and 13, sensitized mice were challenged with 0.2% DNFB. Five mice were allocated to each of four groups: (a) acetone 3: olive oil 1; (b) acetone 3: olive oil 1 with 0.15–2% DNFB; (c) DNFB with α -lipoic acid (α -LA), 10 mg/kg; and (d) DNFB with α -LA, 50 mg/kg. The severity of AD was macroscopically assessed by scoring AD. 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 $\pm$ SEM of five determinations. * P $\leq$ 0.05 versus positive.

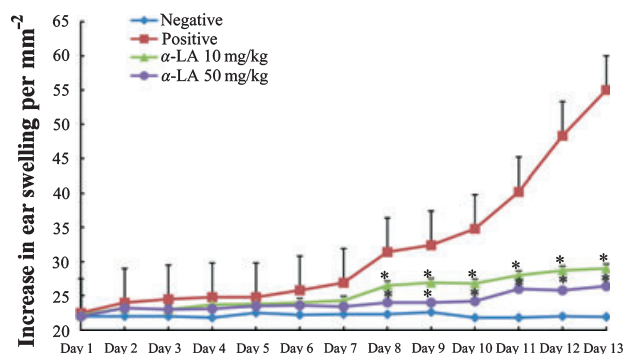


Figure 4. Atopic dermatitis was induced by repeated application of 2,4-dinitrofluorobenzene (DNFB) on both outer and inner surfaces of the right ear. Ear swelling was measured with a thickness gauge every day. α -lipoic acid (α -LA) was orally administered at concentrations of 10 or 50 mg/kg. Five mice were placed into each of four groups: Negative (acetone 3: olive oil 1); Positive (acetone 3: olive oil 1 with 0.15–2% DNFB); DNFB with α -LA, 10 mg/kg; and DNFB with α -LA, 50 mg/kg. Data are presented as mean $\pm$ SEM of five determinations. * $P \leq 0.05$ versus positive.

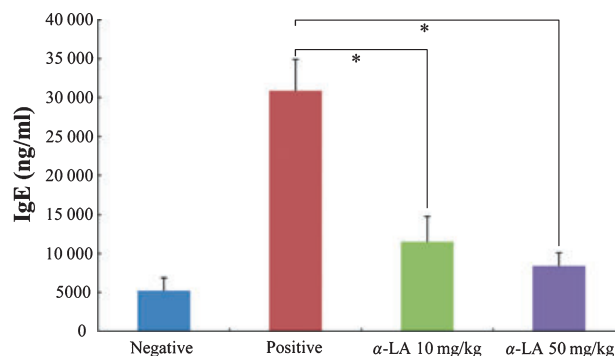


Figure 6. Effect of oral administration of α -lipoic acid (α -LA) on IgE production in 2,4-dinitrofluorobenzene (DNFB)-induced NC/Nga mouse atopic dermatitis model. Total serum samples were prepared from blood collected after last DNFB application, and serum IgE concentration was determined by ELISA. Five mice were allocated to each of four groups: Negative (acetone 3: olive oil 1); Positive (acetone 3: olive oil 1 with 0.15–2% DNFB); DNFB with α -LA, 10 mg/kg; and DNFB with α -LA, 50 mg/kg. The absorbance of the serum of five mice for each group was averaged, and each bar represents the mean $\pm$ SEM. * $P \leq 0.05$ versus positive.

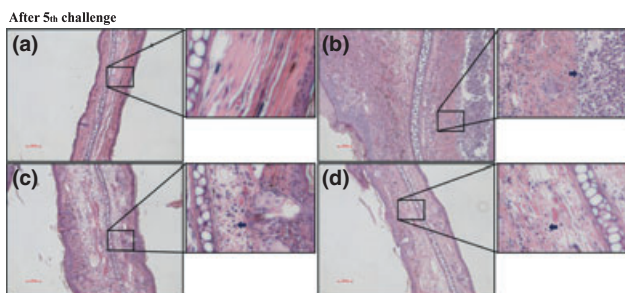


Figure 5. Histological analysis of 2,4-dinitrofluorobenzene (DNFB)-induced atopic dermatitis-like ear skin lesions of NC/Nga mice. (a) treated with acetone:olive oil (3:1); (b) treated with DNFB; (c) treated with DNFB with α -lipoic acid (α -LA), 10 mg/kg; and (d) treated with DNFB with α -LA, 50 mg/kg. Ear sections were stained with haematoxylin and eosin. Scale bar represents 100 μ m. Arrows indicate inflammatory cells.

and we investigated whether oral administration of α -LA suppresses IgE hyperproduction on the total serum. After the fifth DNFB treatment on day 13, serum samples were collected and total IgE levels were measured by ELISA. In the topical DNFB-treated group, total serum IgE levels increased about sixfold compared with those in the control group (Fig. 6). However, oral administration of α -LA at 10 or 50 mg/kg significantly reduced the total serum IgE levels when compared with DNFB-treated group (Fig. 6).

Discussion

AD is a complex multifactorial disorder influenced by genetically based immune abnormalities or various environmental factors (6). Also, most AD patients have an atopic background with familial histories of allergies and bronchial asthma (26). Therefore, AD treatment has not been clearly defined. Topical corticosteroid treatments are the first-line therapy for AD. However, repeated application of corticosteroids may be associated with skin atrophy (27). Therefore, there is a need for new kinds of treatment for AD. α -LA is a naturally occurring short chain fatty acid with sulphhydryl groups that is known as an ideal antioxidant that functions in both membrane and aqueous phases. α -LA and its reduced form DHLA have substantial antioxidant properties that involve free radical quenching, metal

chelation and interaction with other antioxidants such as vitamin C, vitamin E, and GSH (15). Furthermore, neither animal nor human studies to date have shown harmful side effects of α -LA administration (14). Thus, α -LA has been used for therapeutic treatment of diabetes, ischaemia, and neurodegenerative diseases (16). A recent study shows that α -LA suppressed airway inflammation in a mouse model of asthma by inhibiting IgE synthesis and production of cytokines such as IL-4, IL-5, and TNF- α (17). Furthermore, a LA-based thiazolidinedione (TZD) ligands for PPAR inhibit proliferation of human keratinocytes and IL-2 production (18). This result suggests that α -LA may be useful in the treatment of diseases such as psoriasis and AD. Therefore, we investigated the effect of α -LA on the severity of AD-like skin lesions in DNFB-treated NC/Nga mice. In this study, treatment with 10, 50, and 100 μ M α -LA for 50 h did not affect CD4⁺ T cell viability (Fig. 1), and these test concentrations of α -LA were used in experiments to measure cytokine production. Activated CD4⁺ T cells led to the differentiation of Th1 cells that produce IFN- γ and Th2 cells that secrete IL-4, IL-5, and IL-13 (28). It is well known that IFN- γ is elevated in the chronic phase of AD lesions, whereas IL-4 secretion is increased in the acute phase (29). Furthermore, in a recent study, α -LA inhibited IFN- γ synthesis and cellular cytotoxicity in NK-cells (30). We also found that α -LA suppressed IFN- γ and IL-4 production by the activated lymph node CD4⁺ T cells in the dose-dependent manner (Fig. 2). Two hallmarks of AD are evaluated total serum IgE levels and dry itchy skin (31). The acute phase of AD involves Th2 dominant inflammation characterized by IL-4 that affects class switching that induces further rearrangement of Variable Diversity Joining to C γ 4 or C ϵ in the B cell so that the cell switches to IgG4 or IgE synthesis, respectively. Furthermore, IgE mediates mast cell production of various kinds of chemical mediators that trigger infiltration of eosinophils and lymphocytes into the inflammatory skin lesion (32). IgE hyperproduction is known to cause both acute and chronic AD-like skin symptoms, and it has been reported that repeated application of DNFB induces total serum IgE production in NC/Nga mice (33). In concordance with these studies, we found that total serum IgE levels were increased about sixfold by repeated application of DNFB in NC/Nga mice (Fig. 6). However, α -LA-treated mice displayed

three- to fourfold reduced levels of total serum IgE compared with DNFB-treated mice (Fig. 6). CD4⁺ T helper cells control the local inflammatory response via the production of various cytokines, such as IL-4 and IL-13, which induce various local responses. Induction of adhesion molecules, like ICAM-1 and VCAM-1 on endothelial cells and Fc receptors on eosinophils or chemokines, results in increased vascular permeability that provokes leucocyte rolling or infiltration of inflammatory cells (34). Interaction between CCL17 and CCR4 is regulated by the infiltration of Th2 cells in AD lesions by chemotaxis (35). Furthermore, dendritic cells (DC) are essential for the pathophysiology of AD. The expression of CCR7 allows epidermal CD1a-positive Langerhans cells or dermal DC to migrate towards draining lymph nodes (DLN) where they induce T cell activation by antigen presentation or bind IgE via upregulated Fc ϵ R1 in AD-like skin lesions (27). In this study, the number of infiltrated inflammatory cells in the right ear, which had been treated with DNFB, was significantly increased compared with that in non-treated NC/Nga mice (Fig. 5), while that in α -LA-treated groups was remarkably decreased (Fig. 5). Also, SCORAD was significantly decreased in α -LA-treated groups compared with DNFB-treated group, similar to previous histology data (Fig. 3). In this study, *in vitro* α -LA treatment suppressed IL-4 and IFN- γ secretion by activated lymph node CD4⁺ T cells (Fig. 2). Furthermore, IgE was reduced from hyperproduction levels to normal levels via the reduc-

tion of inflammatory cell infiltration and the inhibition of IL-4 production (Figs 5 and 6). Many diseases such as diabetes, cancer, atherosclerosis, and AD are affected by oxidative stress that results from increased levels of reactive oxygen species (ROS). ROS also promote the activities of pro-inflammatory redox-sensitive nuclear factors such as NF- κ B, which provokes the allergic inflammation (36,37). α -LA directly scavenges free radicals of ROS, promotes GSH synthesis and reduces the binding activity of NF- κ B (17). Furthermore, a recent study shows that α -LA treatment attenuates NF- κ B activation by enhancing the PI3K/Akt pathway (38). Further research is necessary to determine the molecular mechanisms of α -LA that modulate suppression of IgE overproduction, scavenging of ROS, cytokine production by CD4⁺ T cells, and chemoattraction in the NC/Nga AD model. Furthermore, studies in human AD are required to determine the contribution of α -LA. In conclusion, α -LA reduced the development of AD-like skin lesions in repeated DNFB treatment of NC/Nga mice through suppression of total serum IgE levels and IFN- γ and IL-4 production from activated CD4⁺ T cells.

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