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RESEARCH ARTICLE

Effect of Oral Administration of Nutmeg Extract on American House Dust Mite (*Dermatophagoides farinae*) Extract-induced Atopic Dermatitis-like Skin Lesions in NC/Nga Mice

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Abstract The effects of oral administration of nutmeg extract (NE) on atopic dermatitis (AD) were evaluated using NC/Nga mice treated with American house dust mite (*Dermatophagoides farinae*) extract. In this mouse model of AD, NE suppressed transepidermal water loss, erythema, and the production of serum immunoglobulin E, interleukin (IL)-4, and interferon-γ by auxillary lymph node cells. In addition, NE reduced epidermal thickening and inflammatory cell infiltration into the skin. In skin lesions, NE suppressed IL-13 and tumor necrosis factor (TNF)-α mRNA expression. In the spleen, NE upregulated T-box transcription factor expressed in T cells and downregulated GATA-binding protein 3, which are involved in T helper cell differentiation. Our findings suggest that NE ameliorates AD and may be a potential nutraceutical candidate for treating AD.

Keywords: nutmeg (*Myristica fragrans* Houtt), atopic dermatitis, *Dermatophagoides farinae* extract, NC/Nga mice

Introduction

Atopic dermatitis (AD) is a chronic, relapsing allergic skin disease caused by complicated interactions of genetic factors with environmental factors (e.g., food allergens, pollen, hair, mites, microbes, chemicals, sweat, dust, and clothes), immunological factors [immunoglobulin E (IgE) overproduction, local and systemic immunologic dysfunction leading to T helper type 1 cell (Th1)/T helper type 2 cell (Th2) imbalance], and skin barrier dysfunction caused by ceramide deficiency (1). Typical symptoms of AD are severe pruritus, inflammation, erythema, edema, eczema, papules, ichthyosis, and lichenification (2).

The pathogenesis of AD is not fully understood, but elevated serum IgE and Th2-predominant immune responses are associated with the systemic immunologic response, and biphasic Th cell responses are associated with skin lesions (3,4). Naive $CD4^+$ T cells stimulated with allergen express GATA-binding protein 3 (GATA-3) in the presence of interleukin (IL)-4 and develop into Th2 cells that secrete cytokines such as IL-4 and IL-13 (5). However, in the presence of IL-12, naive $CD4^+$ T cells stimulated with allergen express T-box expressed in T cells (T-bet) and preferentially differentiate into Th1 cells that secrete cytokines such as IL-2 and interferon (IFN)-γ (6). The impaired epidermal barrier associated with AD facilitates penetration of allergens (7). Skin barrier dysfunction therefore plays an important role in the aggravation of AD (8).

Nutmeg (*Myristica fragrans* Houtt.) is a plant cultivated in Indonesia, South Africa, India, and other tropical areas. Nutmeg has traditionally been used as a spice, but also possesses bioactive properties including carminative, astringent, hypolipidemic, antithrombotic, antiplatelet aggregation, antifungal, aphrodisiac, anxiogenic, antidiarrheal, and anti-inflammatory activities (9). In addition, studies have reported that macelignan, a major bioactive compound of nutmeg,

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exerts antioxidant, antibacterial, and anti-inflammatory actions (10,11). However, the effect of nutmeg on AD has not been evaluated. In the present study, the effect of oral administration of nutmeg on AD was evaluated using NC/Nga mice with American house dust mite (*Dermatophagoides farinae*) extract (DFE)-induced AD.

Materials and Methods

Plant material and preparation of nutmeg extract The dried seed kernels of nutmeg were collected in Jakarta, Indonesia. A voucher specimen has been deposited in the Department of Biotechnology, Yonsei University (Seoul, Korea). The ground nutmeg (100 g) was extracted with 95% ethanol (400 mL), and the extract (18.3 g) was concentrated with vacuum evaporator and freeze-dried. The extract was then separated by open column chromatography using a silica gel column (60, 70-230 mesh, Merck & Co., Whitehouse Station, NJ, USA) and eluted with *n*-hexane and ethyl acetate solution (5:1, v/v) to obtain 2 fractions (Fr. 1 and Fr. 2). Fraction 2, which was free of safrole and myristicin, was freeze-dried and used as nutmeg extract (NE) for this study.

Animals Twenty specific pathogen-free 8-week-old female NC/Nga mice (Central Lab Inc., Seoul, Korea) were housed in a controlled environment (22±2°C, 55±15% relative humidity, 12 h light/dark cycle). After a 1-week acclimation, the mice were fed a standard diet (Cargill Agri Purina, Seongnam, Korea) and water. The animal experiments were approved by the Institutional Animal Care and Use Committee at Yonsei University in Seoul, Korea.

Induction of AD-like skin lesions AD-like skin lesions were induced in the mice using the procedure of Yamamoto *et al.* (12). After the hair was removed from the skin, barrier disruption was achieved by topical application of 4% sodium dodecyl sulfate. After 2 h, DFE-containing cream (100 mg; Biostir, Hiroshima, Japan) was topically applied to the skin. This procedure was repeated 3 times/week for 3 weeks. Induction of AD was then confirmed by macroscopic observation (e.g., erythematous eruptions).

After AD induction, the 20 mice were divided into 4 groups according to treatment: saline only (control group, *n*=5); saline with daily DFE treatment (DFE group, *n*=5); NE (50 mg/kg/day) with daily DFE treatment (NE 50 group, *n*=5); or NE (150 mg/kg/day) with daily DFE treatment (NE 150 group, *n*=5). The mice were injected by oral gavage for 2 weeks. After 2 weeks, transepidermal water loss (TEWL), skin hydration, and erythema were determined, and blood was drawn. After the experiments were completed, the 14-week old mice were sacrificed by diethyl ether inhalation. Hair was removed from the dorsal skin with depilatory cream (Reckitt Benckiser, Massy, France) and the spleen, axillary lymph nodes (ALNs), and dorsal cutaneous tissues were removed and stored at −80°C until analysis.

Measurement of total serum IgE Blood was collected with glass capillary tubes (Chase Scientific Glass, Rockwood, TN, USA) from the retro-orbital plexus. Serum was obtained by centrifugation at 800×*g* for 10 min and stored at −80°C until analysis. Total serum IgE levels were determined using a mouse IgE enzyme-linked immunosorbent assay (ELISA) kit (Immunology Consultants Lab, Newberg, OR, USA) according to the manufacturer's instructions.

Analysis of skin barrier function To evaluate skin barrier function at the end of the experiment, TEWL was determined using a Tewameter TM 300 (Courage & Khazaka, Cologne, Germany), skin hydration was determined using a Corneometer CM 825 (Courage & Khazaka), and erythema was determined using a Mexameter MX 18 (Courage & Khazaka) according to the manufacturers' instructions. These measurements were performed in a controlled environment (22±2°C and 55±15% relative humidity).

Histological observation of the skin Dorsal cutaneous tissue was fixed with 10% neutral formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin (H&E). To detect mast cells and eosinophils, sections were stained with toluidine blue and Congo red, respectively, and observed with an Eclipse TE2000U inverted microscope with twin CCD cameras (magnification ×200; Nikon, Tokyo, Japan).

Measurement of cytokine levels in axillary lymph nodes To study ALN cytokine production, the ALNs of mice from each group were removed. After isolating the cells, a cell suspension was prepared with 2% fetal bovine serum containing Dulbecco's modified Eagle's medium (Invitrogen Life Technology, Carlsbad, CA, USA). The lymph node cells (5×10^5 cells/mL/well) were plated in a 24-well flat-bottom microplate and restimulated with 1 μg/mL *D. farinae* body (Dfb) powder (Biostir, Hiroshima, Japan). After a 48 h incubation, culture supernatants were collected, and IL-4 and IFN-γ levels were determined using the mouse ELISA IL-4 kit (ID Labs, London, ON, Canada) and mouse ELISA IFN-γ kit (Assay Designs, Ann Arbor, MI, USA).

Measurement of mRNA levels of inflammatory cytokine using reverse transcription The mRNA levels of tumor necrosis factor (TNF)-α and IL-13 in skin lesions were

evaluated by reverse transcription-PCR (RT-PCR). Total RNA was isolated from dorsal cutaneous tissue with Trizol reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. The RNA (2 μg) was transcribed with oligo (dT) and reverse transcriptase premix (Elpis-Biotech, Daejeon, Korea). The resulting cDNA was amplified by PCR using a PCR premix (Elpis Biotech). Amplification conditions consisted of pre-denaturation at 95°C for 5 min; 35 cycles of denaturation at 95°C for 30 s, annealing at 58°C (IL-13) or 64°C (TNF-α) for 1 min, elongation at 72°C for 1 min; and a final elongation step at 72°C for 5 min. PCR reactions were carried out in a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). Amplification products were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining and UV illumination. Primer sequences were: IL-13, 5'-GCTCTGGGCTTCATGGCGCT-3' (forward) and 5'-GAAGGGGCCGTGGCGAAACA-3' (reverse); TNF-α, 5'-GCGGAGTCCGGGCAGGTCTA-3' (forward) and 5'-GGGGGCTGGCTCTGTGAGGA-3' (reverse); glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-CCCACTAACATCAAATGGGG-3' (forward) and 5'-ACACATTGGTAGGAACA-3' (reverse).

Measurement of Th1/Th2-related transcription factors in the spleen To determine T-bet and GATA-3 protein levels, the spleen was homogenized with RIPA lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.25% deoxycholate, 1 mM EDTA, and 1 mM PMSF). After incubation on ice for 10 min, the homogenized suspension was centrifuged, and the supernatant was used to determine protein concentrations. To determine T-bet and GATA-3 levels, total protein (30 μg) was resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes (Whatman GmbH, Dassel, Germany). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h and then incubated with the primary rabbit polyclonal anti-GATA-3 and anti-T-bet/Tbx21 antibodies (Abcam, Cambridge, UK) for 16 h at 4°C. After 3 washes in Tris-buffered saline containing 0.1% Tween 20, the membranes were incubated with secondary antibodies (Bethyl Laboratories, Montgomery, TX, USA) for 2 h. Proteins were detected by enhanced chemiluminescence (Amersham Biosciences, Little Chalfont, UK) and visualized with a LuminoImager (LAS-3000; Bio Imaging Analysis System; Fuji Film, Tokyo, Japan).

Statistical analysis Results are expressed as mean± standard deviation (SD). Data analysis was performed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA). Group results were compared by the Scheffé test; $^{\#}p<0.01$, $^{*}p<0.05$, and $^{**}p<0.01$ were considered significant.

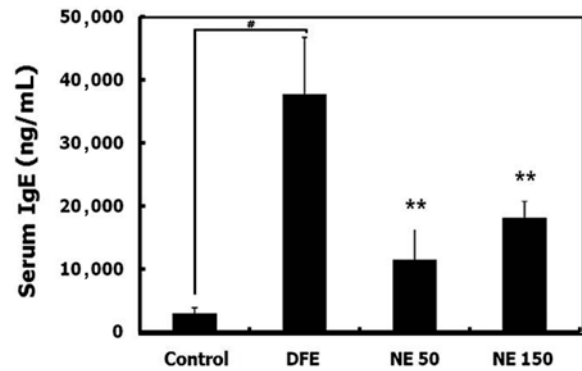

Fig. 1. Effect of NE on total serum IgE in NC/Nga mice. Mice received saline without DFE treatment (control group), with daily DFE treatment (DFE group), 50 mg/kg/day NE (NE 50 group), or 150 mg/kg/day NE (NE 150 group) with daily DFE treatment. Results are expressed as mean±SD (n=5). $^{\#}p<0.01$ compared with control group; $^{*}p<0.05$ and $^{**}p<0.01$ compared with DFE group

Results and Discussion

NE reduced total serum IgE levels in DFE-treated NC/Nga mice When activated, B cells in lymphoid organs (e.g., spleen, lymph nodes) secrete IgE and differentiate into plasma cells in the presence of Th2 cytokines such as IL-4 and IL-13. Patients with AD exhibit elevated serum IgE levels, and several studies have demonstrated that serum IgE levels are elevated in NC/Nga mice with AD-like skin lesions (3,4). To investigate the effect of NE on IgE production in DFE-treated NC/Nga mice, total serum IgE concentrations were determined by ELISA. DFE treatment resulted in a marked elevation of total serum IgE. However, mice that were also treated with NE (50 or 150 mg/kg/day) showed significantly lower ($p<0.01$) IgE levels (Fig. 1). These results indicate that oral administration of NE suppresses DFE-induced IgE production in NC/Nga mice.

NE ameliorated skin barrier disruption Skin barrier disruption in patients with AD facilitates allergen penetration (3,4), leading to aggravation of AD. To evaluate the effect of NE on skin barrier function, TEWL and erythema were evaluated. Although DFE markedly increased TEWL and erythema, mice that received NE by oral administration showed significantly reduced TEWL and erythema in skin lesions. These results suggest that NE ameliorates DFE-induced skin barrier dysfunction in this mouse model of AD.

NE suppressed dermal infiltration of inflammatory cells and epidermal thickening in skin lesions In AD, skin lesions show increased infiltration of eosinophils, mast cells, and macrophages, which are associated with inflammation

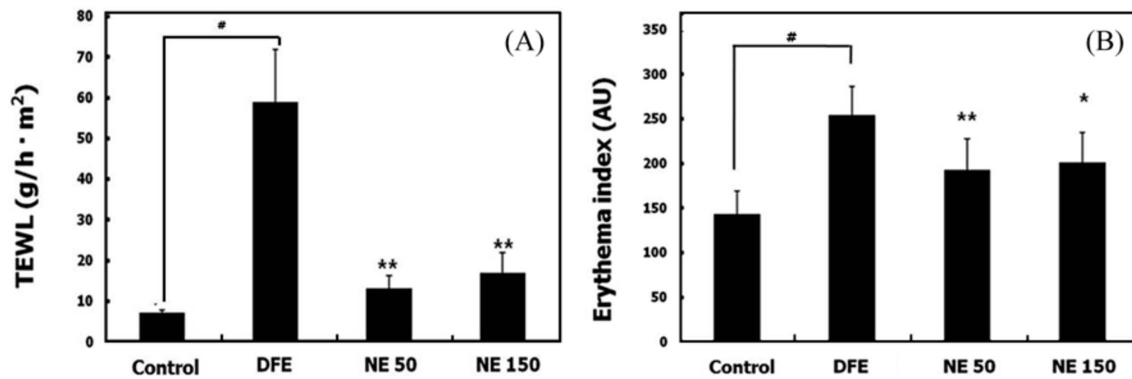

Fig. 2. Effect of NE on the skin barrier dysfunction in AD, as assessed by transepidermal water loss (TEWL, A) and erythema (B) measurements. Results are expressed as mean±SD (n=5). $^{\#}p<0.01$ compared with control group; $^{*}p<0.05$ and $^{**}p<0.01$ compared with DFE group

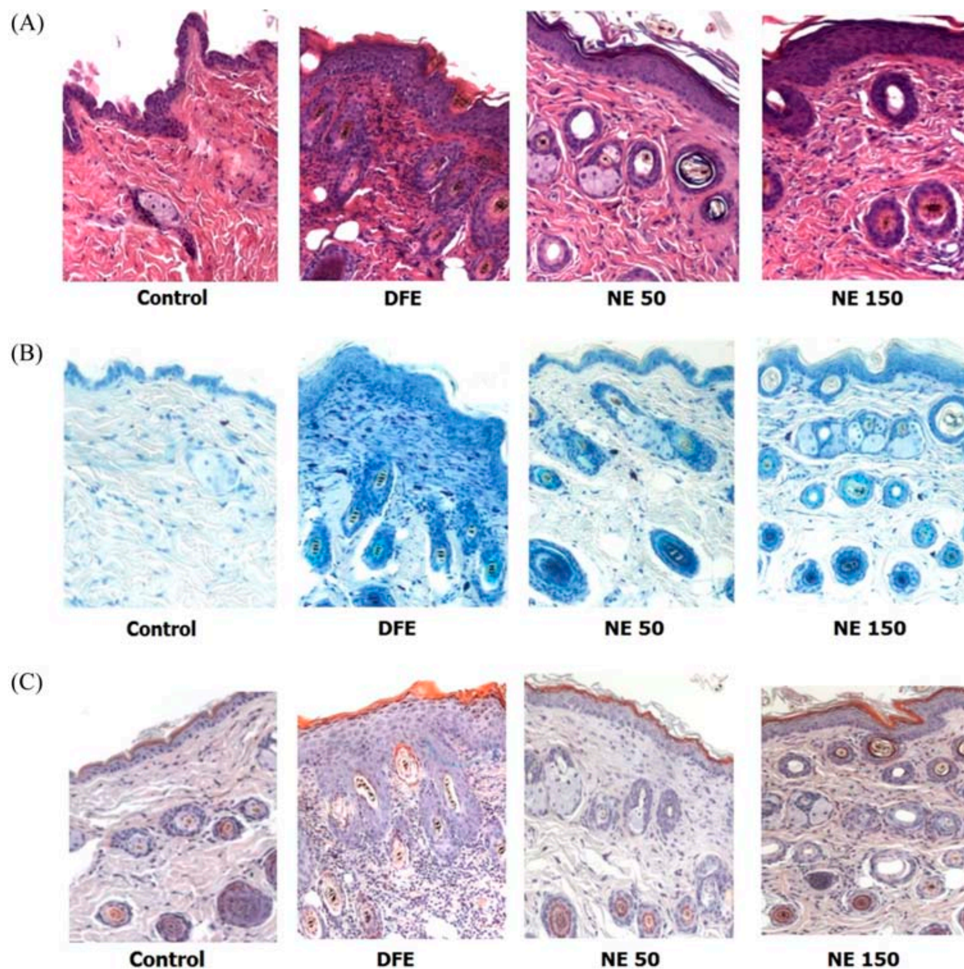

Fig. 3. Effect of NE on the dermal infiltration of inflammatory cells and epidermal thickening in skin lesions. Histological analysis was carried out by staining dorsal cutaneous skin sections with H&E (A), toluidine blue (B), and Congo red (C) (magnification, ×200).

and epidermal thickening. Histological analysis of the skin (H&E, toluidine blue, and Congo red staining) was performed to evaluate the effect of NE on the AD-like skin lesions. DFE strongly increased epidermal thickening and inflammatory cell infiltration, particularly mast cells and eosinophils, but these reactions were reduced in mice treated with NE (Fig. 3). These results indicate that NE can effectively suppress the development of AD in NC/Nga mice.

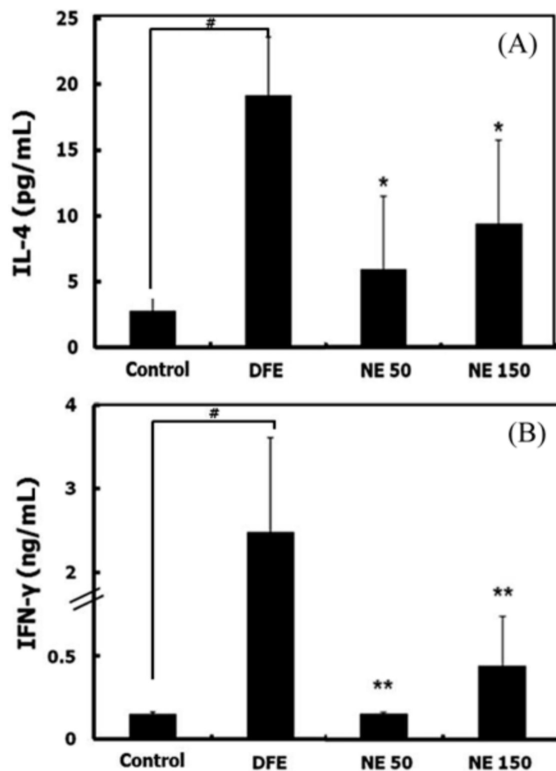

Fig. 4. Effect of NE on the production of inflammatory cytokines, IL-4 (A) and IFN-γ (B), in ALNs. Results are expressed as mean±SD. (n=5). #p<0.01 compared with control group; *p<0.05 and **p<0.01 compared with DFE group

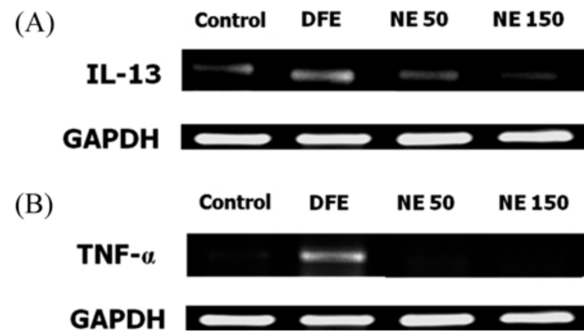

Fig. 5. Effect of NE on the expression of inflammatory cytokines, IL-13 (A) and TNF-α (B), in skin lesions.

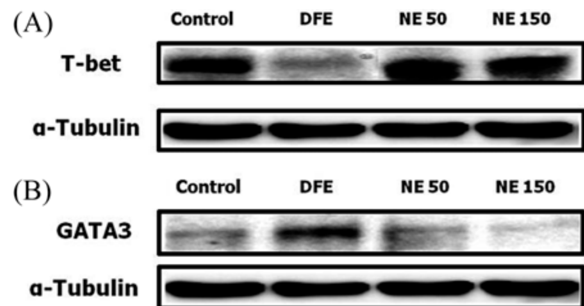

Fig. 6. Effect of NE on the expression of T-bet (A) and GATA-3 (B) in the spleen. α-Tubulin was a loading control.

NE attenuated inflammatory cytokine levels in ALNs Axillary lymph nodes are important secondary lymphoid organs involved in the local immune response in AD (13). The most important cytokine produced by Th2 cells in mediating IgE production is IL-4, which is increased systemically in AD as well as locally in acute skin lesions (4,14). IFN-γ is the most important cytokine produced by Th1 cells, and its expression is increased in chronic skin lesions in AD (4). Yamamoto *et al.* (12) reported that human AD-like dermatitis was observed in NC/Nga mice that underwent repeated applications of DFE ointment, and serum IgE and cytokine levels (IL-5, IL-13, and IFN-γ) were increased in ALN cells restimulated with DFE. To determine the effect of NE on the activation of Th2 and Th1 cells in the local immune response, ALN were restimulated with Dfb, and IL-4 and IFN-γ secretion were determined by ELISA. When ALN cells from DFE-treated mice were incubated with Dfb, IL-4 and IFN-γ levels were significantly increased. These findings suggest that Th2 and Th1 cells were activated and involved in the development and chronification of AD in this model. In contrast, ALN cells from mice treated with NE showed significantly reduced levels of IL-4 (p<0.05) and IFN-γ (p<0.01) (Fig. 4). These results suggest that oral administration of NE attenuates DFE-induced activation of Th2 and Th1 cells in ALNs in our model.

NE attenuated inflammatory cytokine mRNA levels in skin lesions The cytokine IL-13, which is released from Th2 cells and mast cells, activates B cells and stimulates IgE production (14). TNF-α is a cytokine produced by cells such as macrophages and keratinocytes (4). IL-13 and TNF-α recruit inflammatory cells to the skin, thus playing an important role in the inflammatory response of AD skin lesions (15). To confirm the effect of NE on this inflammatory response, IL-13 and TNF-α expression in skin lesions was determined by RT-PCR. Repeated applications of DFE increased IL-13 and TNF-α mRNA levels, but NE suppressed this response (Fig. 5). These results suggest that oral administration of NE suppresses inflammation in skin lesions of DFE-treated NC/Nga mice.

NE inhibited DFE-induced upregulation of GATA-3 and/or downregulation of T-bet in the spleen T-bet and GATA-3 are transcription factors that are specifically expressed in Th1 cells and Th2 cells, respectively. They play critical roles in the differentiation of naive T cells (16,17). In the presence of IL-4, naive $CD4^+$ T cells stimulated with allergen express GATA-3 and develop into Th2 cells that secrete cytokines such as IL-4 and IL-13. In

the presence of IL-12, naive $CD4^+$ T cells stimulated with allergen express T-bet and preferentially differentiate into Th1 cells that secrete cytokines such as IL-2 and IFN-γ (16,17). Therefore, the T-bet/GATA-3 ratio in the spleen reflects whether Th1 or Th2 predominates in the systemic immune response and is an indicator of Th1/Th2 imbalance. To investigate the effect of NE on the systemic immune response, GATA-3 and T-bet levels in the spleen were determined by Western blot analysis. DFE treatment decreased T-bet expression and increased GATA-3 expression in the spleen. However, compared with mice treated with DFE only, mice treated with NE showed increased T-bet expression and decreased GATA-3 expression (Fig. 6). These findings suggest that oral administration of NE inhibits the DFE-induced upregulation of GATA-3 and/or downregulation of T-bet in the spleen in our model of AD. However, it is not clear whether NE acts as a negative regulator of GATA-3 or a positive regulator of T-bet because of their mutual inhibition (17).

AD is a chronic, pruritic, relapsing, and inflammatory allergic skin disease that is strongly associated with IgE elevation and a Th2 cell-predominant immune response at the local and systemic levels. Thus, normalizing the Th2-biased systemic immune response may be important to prevent and treat AD. Taken together, our findings suggested that oral administration of NE to NC/Nga mice with DFE-induced AD-like skin lesions had a beneficial effect by preventing a Th2-predominant immune response. Although additional studies are necessary to further characterize the mechanisms underlying the ability of NE to modulate the Th2-biased immune response, our findings suggest that NE may serve as a potential candidate for the prevention and treatment of AD.

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