

Perilla leaf extract prevents atopic dermatitis induced by an extract of *Dermatophagoides farinae* in NC/Nga mice

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Abstract

Background: ~~Background:~~ Perilla (*Perilla frutescens* Britton) leaf comprises many types of active components, mainly flavonoids, and acts as an anti-inflammatory agent in *in vitro* and *in vivo* atopic dermatitis (AD) models.

Objective: We investigated the effects of orally administered perilla leaf extract (PLE) on the symptoms of AD induced by *Dermatophagoides farinae* extract (DFE) in NC/Nga AD model mice.

Methods: The mice were allowed free intake of 0.5% PLE. Skin lesions were assessed, and blood was sampled from the caudal vein on days 0, 7, 14, 21, and 31. On day 31, all mice were sacrificed to obtain blood, skin, spleen, and intestinal tissue samples.

Results: The assessment scores of the skin lesions and total serum IgE levels of PLE-treated mice (PLE group) were significantly lower than DFE-treated mice (DFE group) on days 7, 14, and 21. On day 31, the serum periostin and thymus and activation-regulated chemokine (TARC) levels in the PLE group were significantly lower than those in the DFE group. Histological analysis of the skin revealed that hyperplasia of the epidermal and dermal layers and infiltration of inflammatory cells (cell infiltration in corium tissues) were suppressed by PLE. Periostin deposition was observed in the skin tissue obtained from the DFE group. Moreover, the CD4⁺/CD8⁺ ratio of splenic T cells was suppressed in the PLE group but not in the DFE group.

Conclusion: The results indicated that PLE may inhibit the symptoms of AD.

Keywords: Perilla leaf extract, NC/Nga mice, atopic dermatitis, IgE antibody, periostin, thymus and activation-regulated chemokine

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Introduction

Industrial advances add pollutants, chemicals, and artificial additives to our environment and food sources. Many people are exposed to these harmful substances via dust, even in their homes; for example, house dust contains an allergen from the house dust mite *Dermatophagoides farinae*. Thus, the number of patients suffering from allergic diseases such as atopic dermatitis (AD) is increasing worldwide.^{1,2} AD is a chronic and multifactorial inflammatory skin disease caused by toxic substances. The symptoms of AD include severe itching, erythema, and skin hypersensitivity. AD is caused by a complex interrelation of immunological, psychological, environmental,

and genetic factors. The skin lesions of AD are characterized by the presence of infiltrating inflammatory cells such as eosinophils, monocytes, macrophages, mast cells, and T cells. Studies in animals show that AD is associated with increased IgE levels and many types of inflammatory cytokines in the serum and skin lesions.^{3,4}

Recent reports indicate that specific matrix proteins and chemokines, namely periostin, thymus and activation-regulated chemokine (TARC), and thymic stromal lymphopoietin (TSLP) are closely related to AD. These mediators serve as markers for the clinical diagnosis of AD.⁵ In skin tissues, periostin is critical for the amplification and persistence of allergic inflammation

by communicating between fibroblasts and keratinocytes. Inhibiting the interaction between periostin and its receptor integrin alpha v or knocking out periostin gene expression mitigates AD-associated inflammation in NC/Nga mice. These findings indicate that periostin contributes to the pathogenesis of AD and is critical for amplifying chronic inflammation of the skin.^{6,7}

NC/Nga AD model mice originated from Japanese fancy mice (Nishiki-Nezumi) and were established as an inbred strain by Kondo et al. at Nagoya University in 1955. NC/Nga AD model mice exhibit various histopathological and pathophysiological changes.⁸ When NC/Nga AD model mice are housed in conventional laboratory animal facilities, they uniformly exhibit various symptoms of dermatitis that begin at 8 weeks of age, including itching, erythema, and hemorrhage on their face, ears, neck, and dorsal skin. It has been reported that human AD-like traits in the NC/Nga AD model mice can be induced by continuous topical application of an extract prepared from the bodies of *D. farinae* (*D. farinae* extract; DFE).⁹ An increase in the total serum IgE and histamine levels is an important feature in the pathophysiology of NC/Nga AD model mice, and these mice display Th2 immune responses characterized by the overproduction of Th2 cytokines [interleukins (IL)-4, -5, and -13].¹⁰

Perilla (*Perilla frutescens* Britton) is consumed as a vegetable or applied as a herbal medicine in Japan as well as many other Asian countries. It is believed that the original purpose for its use was not only its typical flavor but also its antidotal property of preventing food poisoning. With a focus on various inflammatory diseases caused by immune disorders, perilla leaf extract (PLE) is used to treat allergies, cold, bronchial asthma, seasonal allergic rhinoconjunctivitis, and chronic gastritis. One mechanism by which PLE exerts its potent anti-inflammatory effect is via the suppression of macrophages by tumor necrosis factor- α (TNF- α). The main active ingredients of PLE are polyphenols, including rosmarinic acid, luteolin, and apigenin.^{11,12}

The aim of the present study was to investigate the effects of orally administered PLE on the symptoms of AD induced by DFE in NC/Nga mice.

Methods

Materials

Parilla Leaf Extract (PLE powder, Lot 310PS01; Amino UP Chemical Co., LTD, Sapporo, Japan) was extracted from the leaves of perilla (*P. frutescens* Britton). DFE ointment (Biostir-AD) was purchased from Biostir Inc. (Kobe, Japan). IgE and an enzyme-linked immunosorbent assay (ELISA) kit were purchased from Shibayagi Co. Ltd. (Gunma, Japan). Periostin and a TARC ELISA Kit were purchased from R&D Systems Inc. (USA). Fluorochrome-conjugated monoclonal antibodies against CD8-phycoerythrin and CD4-Alexa Fluor were purchased from Bio Legend Co., Ltd. (USA). Neutral-buffered formalin (10%) and erythrocyte ammonium chloride hemolyzing Tris-buffer (ACTB) (0.17 M NH₄Cl, 10 mM Tris-HCl, and 0.25 mM EDTA and 0.17 M NH₄Cl, 10 mM Tris-HCl, and 0.25 mM EDTA, respectively) were purchased

from Wako LTD (Osaka, Japan). Sodium dodecyl sulfate (SDS) and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (USA). Hematoxylin and eosin (H&E) solution was purchased from Bio Optica Co. Ltd. (Italy). An antibody against periostin was purchased from Novocastra Inc. (Germany). Secondary antibodies (EnVision) and 3,3'-diaminobenzidine (DAB) were purchased from Dako Japan Co. Ltd. (Tokyo, Japan).

Mice

NC/Nga AD model mice (male, 10-weeks old) were purchased from Charles River Japan Inc. (Kanagawa, Japan). The mice were housed individually in cages under conventional conditions with a 12-h light-dark cycle, 23 \pm 1°C, and 55 \pm 15% relative humidity. At the end of experiment, the animals were sacrificed using ether.

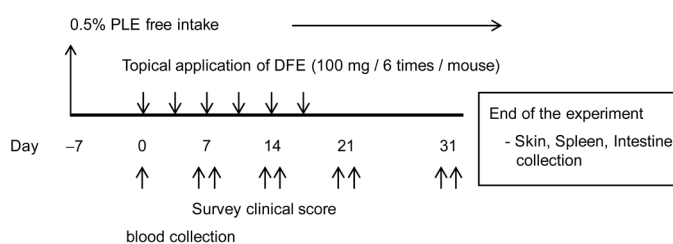
The present investigation (protocol approval number 14012) conformed to the Guiding Principles for the Care and Use of Experimental Animals of Hokkaido Pharmaceutical University (published 1998, revised 2001 and 2007).

PE and DFE treatment

After one week of acclimation, mice were divided into three groups (n = 8 per group) as follows: (1) Control group (Distilled Water, DW), (2) DFE group (DW), (3) DFE + PLE group (free access to 0.5% PLE in DW). AD-like skin lesions were induced in NC/Nga mice using DFE ointment (Biostir-AD) according to the manufacturer's instructions. Dorsal hair was completely removed using an electric clipper (Philips, Amsterdam, Holland), and the integrity of the skin barrier was disrupted using the topical application of 150 μ l of 4% SDS to the dorsal skin of all mice. To induce AD-like symptoms on the skin, 100 mg of Biostir-AD was applied 2 h after shaving the dorsal surfaces and repeated twice a week for three weeks (Figure 1).

The skin lesion dermatitis score of the NC/Nga AD model mice was measured once a week according to a slight modification of the criteria described previously, using the following scores: 0, no detectable symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms for each of three indications and symptoms, including erythema/hemorrhage, edema, excoriation/erosion, and scaling/dryness. The range of dermatitis score was 0 to 12. The mice were photographed once a week using a digital camera (Olympus XZ-10, Olympus Inc., Yokohama, Japan).

Figure 1. Experimental design



Histological analysis

On day 31, all mice were sacrificed and skin, spleen, and ileum tissues were harvested, fixed in 10% neutral-buffered formalin, embedded in paraffin, and thin-sectioned (5 μ m). The sections were stained with HE. Periostin staining was performed by deparaffinizing the paraffin-embedded skin tissue sections using xylene. The sections were then treated with citric acid buffer/0.1% Tween for 20 min at 90°C, acidified, and then blocked with 3% bovine serum albumin. Periostin reagent was diluted 100-fold, reacted with the sections for 60 min at room temperature, reacted with Envision (anti-Rabbit), and the color was developed using 3,3'-diaminobenzidine. Skin lesions were examined using a light microscope (Olympus AX70, Olympus, Tokyo, Japan) equipped with 40 \times and 100 \times objective lenses.

Analyses of serum levels of IgE, periostin, and TARC

Blood samples were collected from the caudal vein on days 0, 7, 14, 21, and 31. The sera obtained from whole blood were separated by centrifugation at 12,000 rpm for 5 min and stored at -80°C. The total serum IgE levels on days 0, 7, 14, 21, and 31 and those of periostin and TARC on days 0 and 31 were detected using ELISA kits.

Preparation of splenic lymphocytes for flow cytometry

To analyze CD4⁺ and CD8⁺ expression by splenic lymphocytes, spleens were cut into pieces with scissors in cold phosphate-buffered saline (PBS, pH 7.2) and homogenized using glass slides. Homogenized spleen cells were passed through a 70- μ m nylon cell strainer (Becton, Dickinson and company, USA), and lymphocytes were centrifuged twice for 5 min at 300 \times g. Lymphocytes were washed with ACTB. FBS (10%)/PBS (pH 7.2) was added to the splenic lymphocytes (1 \times 10⁶ cells/ml) with 5 μ l of either CD4-Alexa Fluor 488 or CD8-phycoerythrin monoclonal antibodies and incubated at 4°C for 30 min. The lymphocytes were rinsed five times with 10% FBS/PBS and centrifuged at 300 \times g for 5 min. The stained lymphocytes were counted using Gallios flow cytometry software (Beckman Coulter, Inc., USA). Each analysis, including the control samples, was based on at least 1 \times 10⁴ events, excluding dead cells, and gated according to forward angle light scatter to eliminate residual erythrocytes.

Statistical Analysis

Results are expressed as the mean \pm standard error (SE). One-way analysis of variance followed by Tukey's honestly significant difference test was used to evaluate the significance of differences among multiple groups, and * P < 0.05, ** P < 0.01 were considered statistically significant differences.

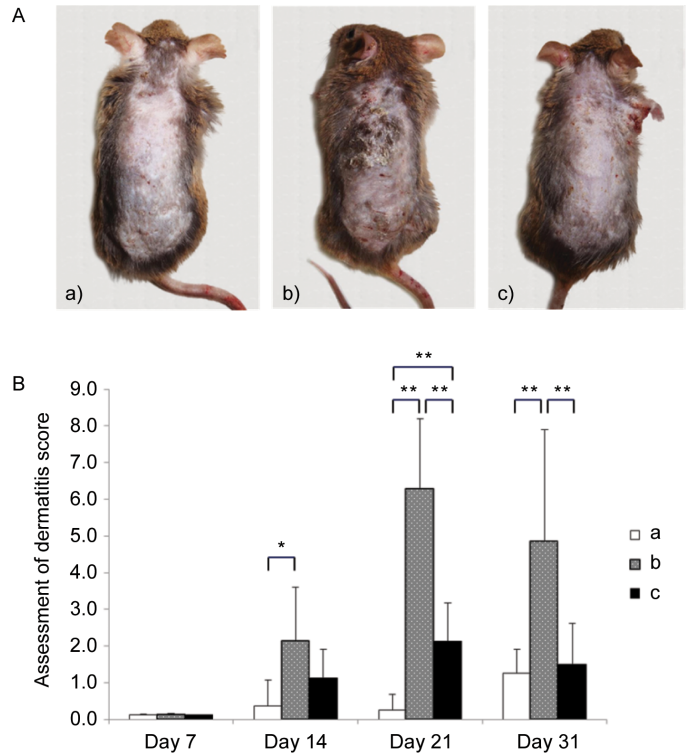
Results

Assessment of skin lesions and dermatitis score

To investigate the effects of PE on DFE-induced AD-like symptoms in NC/Nga mice, the skin lesions were evaluated according to their dermatitis scores. On day 31, the dorsal skin of the DFE group exhibited erythema, erosion, and dryness compared with that of the control group (Figure 2A).

Figure 2. Effects of perilla leaf extract (PLE) on dorsal skin lesions
A: Representative changes of *Dermatophagoides farinae* extract (DFE)-induced dorsal skin lesions. Images were acquired 31 days after sensitization of each group of mice. a) Control group, b) DFE group, c) DFE + PLE group, N = 8 for all groups

B: Atopic dermatitis scores were evaluated on days 7, 14, 21, and 31. Data are presented as the mean \pm standard error. * P < 0.05, ** P < 0.01. a) Control group, b) DFE group, c) DFE + PLE group, N = 8 (duplicate) for all groups



In comparison, the severity of AD-like symptoms was reduced, although the dermatitis score increased gradually from days 14 to 31. The dermatitis score of the DFE group was significantly higher than that of the control or PE group on days 21 and 31 (Figure 2B).

Histological analysis

Skin, spleen, and intestinal sections collected on day 31 were subjected to H&E staining to detect cells infiltrating the tissues. Some mice exhibited splenic germinal-center hypertrophy, extramedullary hematopoiesis, and hemosiderin, although there was no difference in these findings among the groups (data not shown). Germinal-center hypertrophy was observed in tissue samples from the ileum of the DFE group but not in those of the control or PLE group (data not shown). Epithelial and dermal thickening and inflammatory-cell infiltration were detected in the DFE group, although they were not as evident in the control or PLE group (Figure 3A). The number of cells that infiltrated the corium tissues (CIT) in a 90-mm² area was significantly lower in the PLE group in comparison with the DFE group (Figure 3B). We detected an accumulation of periostin, mainly between the epithelium and dermis, in the DFE group (Figure 4).

Table 1. Effect of PLE on the CD4⁺/CD8⁺ ratio of splenic T lymphocytes

	CD4 ⁺ (%)	CD8 ⁺ (%)	CD4 ⁺ /CD8 ⁺ ratio (%)
Control	8.80 ± 1.03	6.89 ± 0.65	1.28 ± 0.16
DFE	8.21 ± 1.14	4.45 ± 1.00	1.82 ± 0.32 *
DFE + PLE	8.49 ± 1.71	5.29 ± 0.59	1.50 ± 0.07

DFE, *Dermatophagoides farinae* extract; PLE, perilla leaf extract
CD4⁺ and CD8⁺ cells were extracted from the spleen and were determined using flow cytometry. Data are presented as the mean ± standard error (SE). N = 3 (duplicate) for all groups, *P < 0.05 vs Control

Figure 3. Effects of perilla leaf extract (PLE) on inflammatory cells in dorsal skin tissues

A: Histological features of the dorsal skin lesions (Hematoxylin and eosin staining). a) Control group, b) *Dermatophagoides farinae* extract (DFE) group, c) DFE + PLE group. Scale bars = 100 μm
B: Number of cell infiltrations in corium tissues in a 90-mm² area of each skin lesion (**P < 0.01).

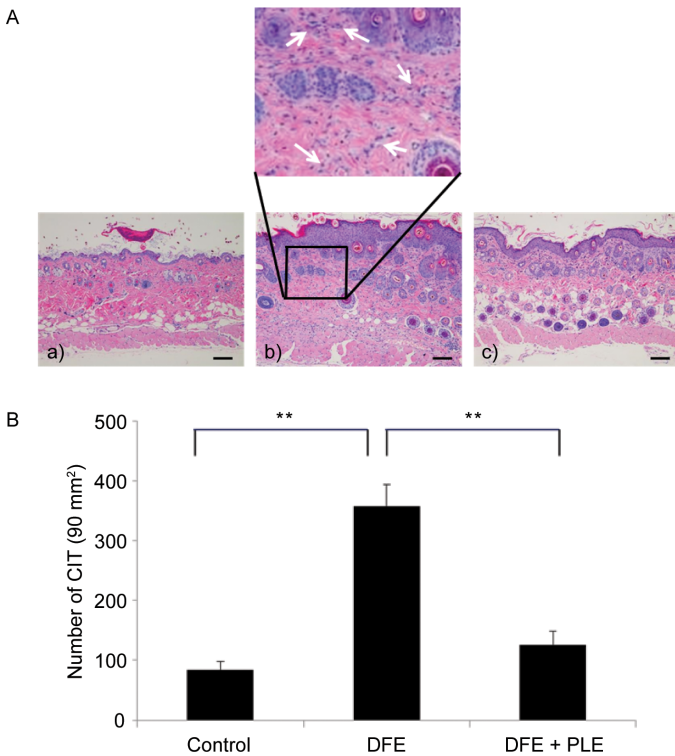


Figure 4. Immunohistochemical analysis of periostin levels of dorsal skin lesions a) Control group, b) *Dermatophagoides farinae* extract (DFE) group, c) DFE + perilla leaf extract (PLE) group. Scale bars = 100 μm

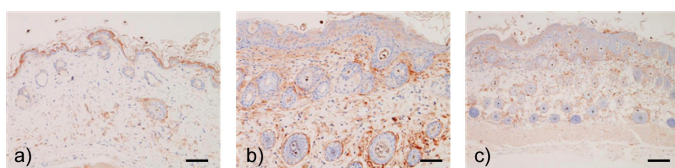
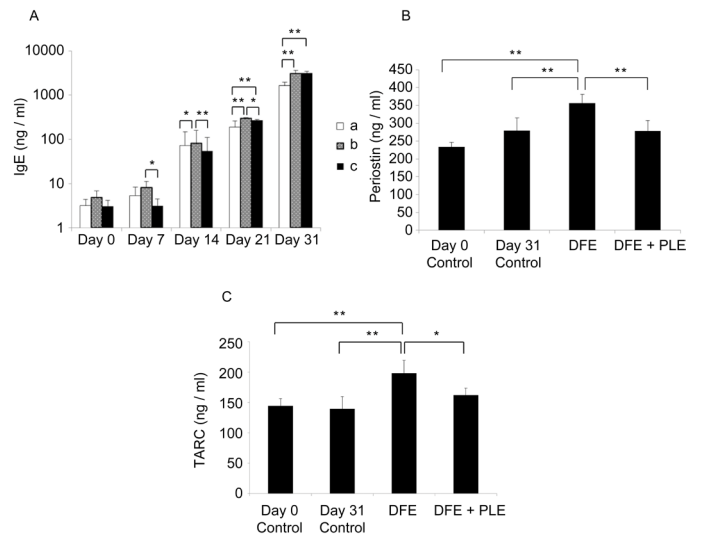


Figure 5. Effects of perilla leaf extract (PLE) on the serum levels of markers of inflammation

A: The total serum IgE level was measured using an enzyme-linked immunosorbent assay (ELISA) on days 0, 7, 14, 21, and 31. Data are presented as the mean ± standard error (SE), *P < 0.05, **P < 0.01 a) Control group, b) *Dermatophagoides farinae* extract (DFE) group, c) DFE + PLE group, N = 8 for all groups

B: The level of serum periostin was measured using ELISA on day 31. Data are presented as the mean ± SE, **P < 0.01. N = 8 for all groups C: The level of serum thymus and activation-regulated chemokine was measured using ELISA on day 31. Data are presented as the mean ± SE, *P < 0.05, **P < 0.01. N = 8 (duplicate) for all groups



Serum levels of IgE, periostin, and TARC

The total serum IgE level of the DFE group significantly increased in comparison with the control group from days 14 to 31 and significantly reduced in comparison with the DFE group on days 7, 14, and 21 (Figure 5A). The serum levels of periostin (Figure 5B) and TARC (Figure 5C) significantly reduced in the PLE group in comparison with the DFE group on day 31 (P < 0.01 and P < 0.05, respectively).

Ratios of CD4⁺ to CD8⁺ T cells in splenic lymphocytes

The CD4⁺/CD8⁺ ratio of T lymphocytes of the DFE group significantly increased in comparison with the control group on day 31 (P < 0.05). The CD4⁺/CD8⁺ ratio of T lymphocytes in the DFE + PLE group was lower in comparison with the DFE group on day 31 (Table 1).

Discussion

The present study, which employed NC/Nga AD model mice, provides important information that enhances the understanding of the cause of basic and clinical symptoms of AD induced by DFE. The continuous topical application of DFE led to high levels of IgE in the blood and skin tissues of NC/Nga AD model mice. IgE is secreted from B cells in response to IL-4 and stimulation by exogenous antigens. IgE-mediated activation of mast cells leads to the release of numerous chemical mediators that induce clinical symptoms of inflammation, which are reflected in the NC/Nga AD model mice.¹³ Here, we show that when low skin lesion scores are compared with the total serum IgE levels, the association of

serum periostin and TARC levels with AD becomes apparent (Figure 5B,C). The extracellular matrix protein periostin was discovered by Kudo et al. in 1999, and Izuhara et al. subsequently demonstrated that periostin plays a critical role in the chronic inflammation of AD. Th2 cytokines IL-4 and IL-13 stimulate fibroblasts to produce periostin, which is deposited in the skin tissues of patients with AD.¹⁴ NC/Nga mice are well-known spontaneous allergy models when they are housed in conventional rooms. Furthermore, AD symptoms can be induced quickly by the continuous topical application of DFE as prophlogistic material. Hence, AD symptoms and markers were increasingly observed in the mice belonging to the control group.

TARC is a chemokine designated CCL17, and its receptor is designated CCR4. Dendritic cells, lymphocytes, vascular endothelial cells, and fibroblasts produce TARC, and TARC production in the skin, which increases in the presence of TSLP produced by keratinocytes, induces the migration of Th2 cytokines. The levels of ceramides in the stratum corneum in AD are insufficient to maintain the skin's barrier function, allowing easier entry of bacteria and chemicals. For example, inflammation progresses when allergens invade the skin and are recognized as foreign bodies by immunocompetent cells. Moreover, Th2 cells are activated and produce IL-4 and IL-13. Th2 cells act on fibroblasts, which leads to the production of periostin.

Periostin binds to integrin alpha v, which increases the production of the inflammatory mediator TSLP and induces TARC production by various cells such as dendritic cells. This series of events once again activates Th2 cells and cytokine production, establishing a vicious cycle of inflammation that maintains AD without a new invasion of allergens.^{14,15} TARC levels detected in the blood are significantly higher in patients with AD in comparison with healthy individuals and correlate with the severity of AD. The levels of TARC in the blood differ from those of IgE and rapidly and sensitively reflect the progression status of AD. Therefore, periostin and TARC are gaining the attention of researchers and clinicians in Japan as new diagnostic markers for allergic diseases.^{5,16}

The histopathological analysis of skin conducted here shows that the appearance of lesions related to inflammation (epithelial and dermal hypertrophy) and CIT was inhibited by PLE (Figure 3A,B). These findings are consistent with those of Tai et al., who reported epithelial and dermal hypertrophy and infiltration of inflammatory cells such as macrophages and T cells in lesions of NC/Nga AD model mice.^{7,17} Moreover, they found that PLE inhibits the deposition of periostin in skin tissues. These findings, taken together with those of the present study, suggest that PLE inhibits the exacerbation of skin lesions and the migration of inflammatory cells through the involvement of mechanisms such as the production of periostin by fibroblasts or TARC by Langerhans cells in the skin. Furthermore, Figure 5A shows that during the initial stages in NC/Nga AD model mice, PLE significantly inhibited IgE production, although the level of inhibition was not statistically significant on day 31. The present findings that PLE significantly decreased skin scores and reduced the levels of periostin and TARC on day 31 suggest

an association between PLE and AD pathology as well as IgE levels.

PLE suppresses systemic inflammatory reactions that occur in people with type I and type IV allergies induced by chemical substances. Moreover, intraperitoneal injection or the oral administration of PLE in NC/Nga AD model mice suppresses IgE production and induces Th1-type cytokines,^{11,18} suggesting that certain active compounds in PLE modulate the immune response. The main constituents of perilla include flavonoids, terpenoids, phenolics, cyanogenic glycosides, anthocyanins, saponins, polysaccharides, and amino acids.^{19,20} Among these compounds, plant flavonoids exert anti-inflammatory and anti-allergic activities. For example, the PLE component luteolin inhibits the secretion of inflammatory cytokines such as IL-1 β and TNF- α from mouse peritoneal macrophages. Similarly, the PLE component rosmarinic acid maintains inflammation in an allergy model by decreasing excessive levels of IgE, COX-2, and histamine.²¹⁻²³

In the present study, we focused on the expression of CD4⁺/CD8⁺ and found that PLE suppressed the increase in the CD4⁺/CD8⁺ ratio of T cells in DFE-stimulated mice to a level that was not statistically significant. These findings indicate that PLE may control the immune reactions of NC/Nga AD model mice at the cellular level because CD4⁺ T cells play an important role in the immune balance of the host defense response, and CD4⁺/CD8⁺ T cells migrate preferentially into the skin of people with AD.^{24,25} The PLE group tends to suppress the CD4⁺/CD8⁺ ratio more compared with the control group. It is assumed that this suppression is associated with the Th1 and Th2 balance. Bak et al. reported that the CD4⁺/CD8⁺ ratio and serum inflammatory cytokines (TNF α , IL-1 β , IL-6) elevated with AD progression in an AD model, wherein AD was induced using dinitrochlorobenzene (DNCB). CD4⁺ (T-helper) cells play an important role in the immune system, and the IL-12 cytokine induces the differentiation of these cells into two subfamilies (Th1 and Th2) of immune cells. Particularly, the Th2-type cytokines (IL-4, IL-5, IL-10, and IL-13) regulate IgE levels.²⁶ We did not measure these cytokines in this experiment, but it is known that PLE suppresses TNF α , IL-1 β , and IL-6 and is assumed to suppress the Th1-type inflammation. Moreover, findings of increased CD4⁺/CD8⁺ ratios in skin cells of animals with experimental AD are consistent with the findings of the present study. However, further research is required to determine the nature of the relationship between treatment with PLE and the suppression of the increase of splenic CD4⁺/CD8⁺ ratios and the alleviation of the symptoms of AD.

In the NC/Nga AD model mice employed here, a decrease in the blood periostin levels as well as changes in skin lesions and a decrease in the total serum IgE levels were detected. These results indicate that PLE prevents the pathology of AD. In particular, the characteristics of changes in the skin lesions of patients with AD resemble those observed in our NC/Nga AD model mice (pruritus, erythema, and hemorrhage). Because these skin lesions cause people with AD to suffer various types of stress, it is extremely important to inhibit the exacerbation of such superficial skin lesions. Therefore, in clinical practice, we expect that PLE may prevent the onset and progression of AD.

Conclusions

Oral administration of PLE inhibited the AD phenotype induced by DFE in the NC/Nga AD model mice. Because of the reduction of the total serum IgE levels, periostin, and TARC as well as the inhibition of changes in skin lesions, PLE may serve as an effective agent for preventing AD and inhibiting its progression. The reduction of the increase in the CD4⁺/CD8⁺ ratio indicates that PLE may affect certain cells of the immune system.

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