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Abstract. *Background/Aim: Pantoea agglomerans LPS (immunopotentiator from Pantoea agglomerans 1: IP-PA1) has been reported to have anti-inflammatory effects in in vitro and in vivo models. The aim of the present study was to investigate the effects of orally-administered IP-PA1 on atopic dermatitis (AD) symptoms induced by Dermatophagoides farinae body extract (DFE) in NC/Nga mice. Materials and Methods: Using the NC/Nga AD murine model, mice were orally administered 0.1% (High) or 0.01% (Low) water-containing IP-PA1. Skin lesion assessment and blood collection from the caudal vein was performed on days 0, 7, 21 and 31. On day 31, all mice were sacrificed and blood, skin, spleen, as well as intestine samples, were obtained. Results: Assessment score of the skin lesion and serum immunoglobulin E (IgE) level of both IP-PA1 groups were significantly lower than that of the DFE group on days 14 and 21. The serum periostin and thymus and activation-regulated chemokine (TARC) level of IP-PA1-Low group was significantly lower than that of the DFE group on day 31. On histological examination of the skin, hyperplasia of epidermal and dermal layers and infiltration of inflammatory cells were suppressed by IP-PA1 administration. Deposition of periostin was observed in the DFE group skin tissue. Moreover, the $CD4^+/CD8^+$ ratio of splenic T-cells increased by IP-PA1 administration. Conclusion: IP-PA1 administration may have an inhibitory effect on AD skin lesions.*

Industrial advances have resulted in the addition of pollutants, chemicals and artificial additives to our surroundings and food. Many people are exposed to these harmful substances, even in their home environment. House dust contains an allergen from *Dermatophagoides farinae*. Many studies have reported that the number of patients suffering from allergic diseases, such as atopic dermatitis (AD), is increasing worldwide (1, 2).

AD is widely recognized as a chronic and multi-factorial inflammatory skin disease caused by toxic substances accompanied by severe itching, erythema and skin hypersensitivity symptoms. AD is the result of a complex interrelation of immunological, psychological, environmental and genetic factors.

Regarding immunological actions in the body resulting in AD symptoms, AD skin lesions are characterized by the presence of inflammatory cell infiltrate in skin tissues, such as eosinophils, monocytes, macrophages, mast cells and T-cells. Animal studies have shown that AD is associated with increased immunoglobulin E (IgE) levels and many types of inflammatory cytokines in sera and skin lesions (3).

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 have already been used as markers of AD for clinical diagnosis. In skin tissues, periostin is critical for the amplification and persistence of allergic inflammation by automatically communicating between fibroblasts and keratinocytes. Blocking the interaction between periostin and its receptor, αv integrin, or knocking-out periostin gene (*POSTN*) expression results in the improvement of AD inflammation in NC/Nga mice. Thus, periostin is involved in the pathogenesis of AD and is critical for the amplification of chronic inflammation in the skin (4-6).

NC/Nga mice, originated from the Japanese mice Nishiki–Nezumi, were established as an inbred strain by Kondo *et al*. (7) at the Nagoya University in 1955. NC/Nga AD mice

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exhibit various histopathological and pathophysiological changes when compared to normal mice.

When NC/Nga mice were kept in conventional rooms, various dermatitis symptoms were observed on the skin. Clinical signs and symptoms observed in mice begin from the age of 8 weeks in all progeny, with itching, erythema and haemorrhage on their faces, ears, neck and dorsal skin. A human AD-like NC/Nga mouse model can be induced by continuous topical application of *Dermatophagoides farinae* body extract (DFE).

An increase in serum IgE and histamine levels is an important feature in the pathophysiology of NC/Nga AD mice. In addition, this model also displays Th2 immune responses with an overproduction of Th2 cytokines (interleukin (IL)-4, IL-5 and IL-13). Also, Th2-specific chemokines, such as TARC, are highly expressed in the AD-like mouse model (8, 9).

Pantoea agglomerans, a Gram-negative bacterium, has a symbiotic relationship with plants. For example, it is a major bacterium that is attached to all wheat produced in the United States, Canada, Australia and Japan. Furthermore, live bacterium of *P. agglomerans* is used in Europe as a bioformula to prevent fungal infections in fruits, such as apples and pears.

This bacterium contains lipopolysaccharide (LPS) that can activate a small number of phagocytes (100 pg/ml), such as macrophages, *in vivo*, in cellular membranes as observed in other Gram-negative bacteria.

The oral administration of LPS derived from the immunopotentiator *P. agglomerans* 1 (IP-PA1), which was derived from fermented wheat extracts, exerts effects against hyperlipidemia, ulcer, infectious disease, diabetes, allergy and tumors.

IP-PA1 has peaks around molecular weights of 5,000 and 45,000 and a lipid A structure, which is a characteristic cell-wall component of Gram-negative bacteria. Its oligosaccharide chain consists of a unit structure of rhamnose and glucose. This part serves as a ligand for Toll-like receptor (TLR) 4 on dendritic cells or macrophages, activates transcription factor nuclear factor-kappa B (NF-κB), facilitates the production of tumor necrosis factor-alpha (TNFα) and nitric oxide (NO) and activates natural immunity. Moreover, differences in the structure and activity between *P. agglomerans*-derived LPS and Escherichia coli-derived LPS have been reported (10, 11).

The aim of the present study was to investigate the effects of orally-administered IP-PA1 on AD symptoms induced by DFE in NC/Nga mice.

Materials and Methods

Materials. LPS derived from IP-PA1 (fermented wheat extract powder; Lot 310PS01, 6.8 mg IP-PA1/g) was manufactured by MACROPHI INC. (Takamatsu, Japan).

DFE ointment (Biostir-AD®) was purchased from Biostir Inc. (Kobe, Japan). IgE ELISA kit was purchased from Shibayagi Co. LTD. (Gunma, Japan). Periostin and TARC ELISA kits were purchased from R&D Systems Inc. (Minneapolis, MN, USA). CD8-PE monoclonal antibodies and CD4-Alexa monoclonal antibodies were purchased from Bio Legend Japan Co. LTD. (Tokyo, Japan). Ten percent neutral-buffered formalin and ammonium chloride haemolysing Tris buffer (ACTB; 0.17 M NH_4Cl, 10 mM Tris HCl and 0.25 mM ethylenediaminetetraacetic acid (EDTA)) were purchased from Wako LTD. (Osaka, Japan). Sodium dodecyl sulfate (SDS) and fetal bovine serum (FBS) were purchased from Sigma-Aldorich Japan Co., LTD. (Tokyo, Japan). Haematoxylin and eosin (HE) solution was purchased from Bio Optica Co. LTD. (Milano, Italy). Periostin was purchased from Novocastra Leica Biosystems Co., LTD. (Buffalo Grove, IL, USA). Diaminobenzidine (DAB) and EnVision™/Horeseradish peroxidase (HRP) were purchased Dako Japan Co., LTD. (Tokyo, Japan).

Animals. Ten-week-old NC/Nga male mice were purchased from Charles River Japan, INC. (Kanagawa, Japan). They were housed in individual cages under conventional conditions with 12-h light–dark cycle, 23±1˚C temperature and 55±15% humidity. At the end of experiments, animals were sacrificed with ether anesthesia.

The present investigation conforms to the Guiding Principles for the Care and Use of Experimental Animal of Hokkaido Pharmaceutical University (published 1998, revised in 2001 and 2007). The protocol approval number is 14013.

IP-PA1 and DFE treatment. After 1 week of acclimation, mice were divided into four groups (n=8 per group): (i) control (distilled water (DW)), (ii) DFE, (iii) DFE + IP-PA1-L (0.01%) group (0.68 μg/ml of IP-PA1in DW free intake) and (iv) DFE + IP-PA1-H (0.1%) group (6.8 μg/ml of IP-PA1 in DW free intake). AD-like skin lesions were induced in NC/Nga mice using DFE ointment (Biostir-AD) as described in the manufacturer's instructions.

The dorsal hair of mice was shaved using electric clipper (PHILIPS, Amsterdam, Holland). After complete dorsal hair removal, for the disruption of skin barrier, 150 μl of 4% SDS was topically applied to the dorsal skin of all mice. After 2 h, 100 mg of DFE ointment was applied to shaved dorsal surfaces, twice a week for 3 weeks to induce AD-like symptoms on the skin (Figure 1).

Assessment of dermatitis score. The skin lesion dermatitis score of AD-like mice was measured once per week according to a slight modification in criteria described previously as follows: scores of 0 (no symptoms), 1 (mild symptoms), 2 (moderate symptoms) and 3 (severe symptoms) for each of three indications and symptoms, including erythema/haemorrhage, edema, excoriation/erosion and scaling/dryness. The range of dermatitis score is from 0 to 12. Mice were also photographed once per week using a digital camera (Olympus XZ-10; Olympus Inc., Yokohama, Japan).

Histological analysis using HE stain. On day 31, all mice were sacrificed and the skin, spleen and ileum samples were obtained. These tissues were fixed in 10% neutral-buffered formalin and samples were embedded in paraffin and thin sections (5 μm) were cut. Sections were stained with HE solution. Skin lesions were histologically examined using a light microscope (Olympus AX70, Olympus, Tokyo, Japan) with 40× and 100× objective lenses.

Serum IgE, periostin and TARC measurement. Blood samples were collected five times during this experiment (days 0, 7, 14, 21 and

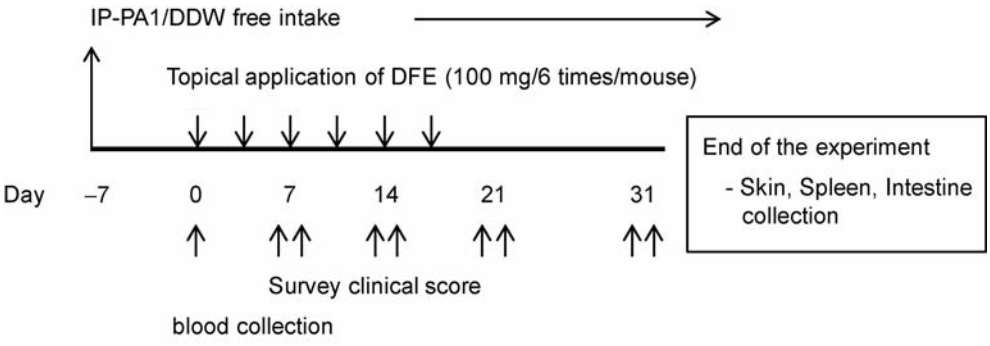

Figure 1. *Experimental design*.

31) from the caudal vein of mice. Serum was obtained from whole blood, separated by centrifugation at 12,000 rpm/min for 5 min and stored at –80˚C. Serum levels of IgE (days 0, 7, 14, 21 and 31), periostin (days 0 and 31) and TARC (days 0 and 31) were detected using ELISA kits.

Preparation of spleen lymphocytes for flow cytometry. To analyze $CD4^+$ and $CD8^+$ spleen lymphocytes, spleens were cut into pieces with scissors in cold phosphate-buffered saline (PBS, pH 7.2) and de-centralized using a glass slide. De-centralized spleen cells were passed through a 70-μm nylon cell strainer (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and its lymphocytes were centrifuged twice at 300 × *g* for 5 min. Lymphocytes were washed with erythrocyte ACTB. Ten percent FBS/ PBS were added to spleen lymphocytes suspended at 1×10^6 cells/ml with 5 μl of either CD4-Alexa Fluor 488 or CD8-PE monoclonal antibodies and incubated at 4˚C for 30 min.

Lymphocytes were rinsed five times with 10% FBS/ PBS and centrifuged at 300 × *g* for 5 min. Stained lymphocytes were counted by Gallios™ flow cytometry (Beckman Coulter, Inc., Brea, CA, USA). Each analysis, including control samples, was based on at least 1×10^4 events exclusive of dead cells, whereas gating on the basis of forward angle light scatter eliminated residual erythrocytes.

Statistical analysis. Results are expressed as means ± (standard error of the mean (S.E). One-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference (HSD) test was used for comparing differences among multiple groups. Differences were considered significant at $^{**}p<0.01$ and $^{*}p<0.05$.

Results

Assessment of skin lesions and dermatitis score. To investigate the effects of IP-PA1 on DFE-induced AD-like symptoms in NC/Nga mice, skin lesions were evaluated by dermatitis scores.

On day 31, the dorsal skin of mice in the DFE group showed erythema, erosion and dryness; these symptoms were not observed in the control group (Figure 2A). In contrast, the severity of AD-like symptoms was suppressed in the IP-PA1 groups but the dermatitis score gradually increased from day 14 to 31. The dermatitis score was significantly higher in the DFE group than in the control or IP-PA1 groups on day 21 and 31 (Figure 2B).

Histological analysis. Histopathological analysis of the spleen revealed that germinal centre hypertrophy, extramedullary hematopoiesis and hemosiderin were observed in some mice but no differences were observed between the groups. For the ileum tissue, although germinal center hypertrophy was observed in the DFE group, it was not observed in the control or IP-PA1 groups (data not shown). With regard to the skin tissue, epithelial and dermal thickening and inflammatory cell infiltration was noted in the DFE group; however, these pathological findings were not observed in the control and IP-PA1 groups (Figure 3A). The number of cell infiltration in corium tissues (CIT), which are inflammatory cells, were counted in an area of 300 μm × 300 μm (900 μm^2) from the skin tissues photos. CIT was significantly decreased in IP-PA1 groups than that in the DFE group (Figure 3B).

Serum IgE, periostin and TARC levels. The serum IgE level of the DFE group was significantly increased than that in the control group from day 14 to 31. On the other hand, serum IgE levels were significantly decreased in IP-PA1 groups than those in the DFE group on days 7, 14 and 21 (Figure 4A).

The serum periostin and TARC levels of the DFE group were significantly increased compared to those in the control group on day 31 ($p<0.01$). In addition, serum periostin levels were significantly decreased in IP-PA1 groups ($p<0.01$) than those in the DFE group on day 31. TARC levels tended to decrease on day 31 after IP-PA1 administration (Figure 4B).

$CD4^+/CD8^+$ ratio of spleen lymphocytes. The $CD4^+/CD8^+$ ratio of T lymphocytes of the DFE group was significantly increased than that in the control group on day 31 ($p<0.05$). The $CD4^+/CD8^+$ ratio of T lymphocytes in IP-PA1 groups show a significant ($p<0.01$ and $p<0.05$) increase on day 31 as well (Table I).

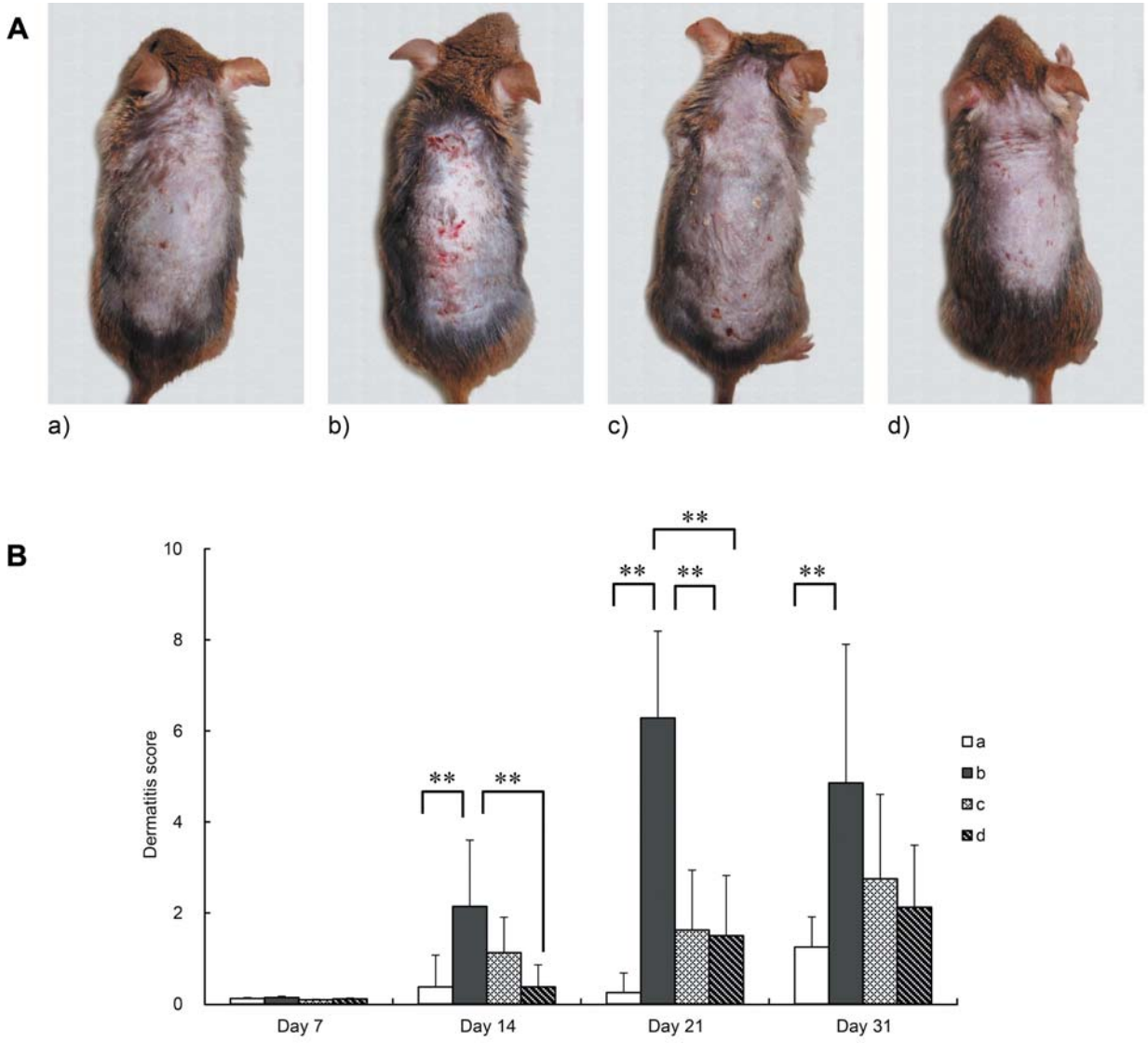

Figure 2. *Effect of IP-PA1 on the development of AD-like dorsal skin lesions in DFE-treated NC/Nga mice. A) Representative feature changes in DFE-induced skin lesions on the back. The photos were taken 31 days after sensitization of each group of mice. B) Changes in survey scores on the back were evaluated on days 7, 14, 21, and 31. Data are presented as mean±SE (**p<0.01 and *p<0.05). a, control group; b, DFE group; c, DFE+IP-PA1-L group; d, DFE+IP-PA1-H group. N=8 for all groups.*

Discussion

The AD-like mouse model based on NC/Nga mice provides important information regarding the understanding and investigation of basic and clinical AD symptoms induced by DFE. The continuous topical application of DFE leads to a high production of IgE in blood and skin tissues of mice. IgE is secreted from B cells by IL-4 and external antigen stimulation. The IgE-mediated mast cell activation leads to the release of many kinds of chemical mediators; thus, this AD-like mouse model is closely related to clinical inflammatory symptoms (12).

In our study, when low skin lesion scores and stable serum IgE values are considered from the viewpoint of the underlying mechanism, the relationship between serum periostin and TARC levels shown in Figure 2 and Figure 4 may be cited. Periostin, an extracellular matrix protein, was discovered by Kudo *et al*. in 1999 (7). Masuoka *et al*. (5) have clearly 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 deposits in the skin tissues of patients with AD (13, 14). TARC is a type of chemokine, also called CCL17. The receptor of TARC, CCR4 is produced by dendritic cells,

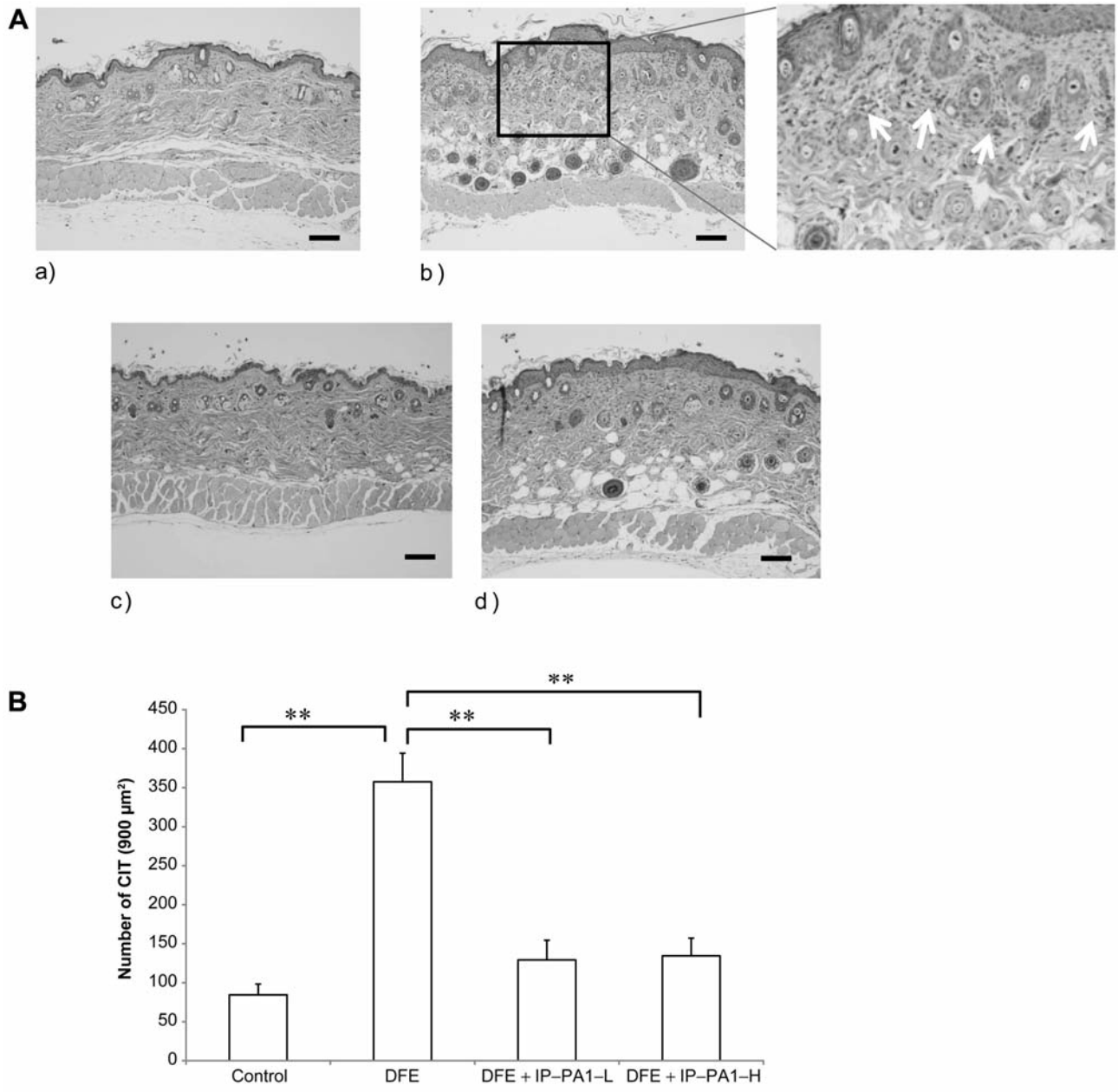

Figure 3. *Effect of IP-PA1 on histological features of DFE-induced AD-like model in NC/Nga mice. A) Back skin lesions were fixed with formaldehyde and determined after staining with hematoxylin and eosin (HE). a, control group; b, DFE group; c, DFE+IP-PA1-L group; d, DFE+IP-PA1-H group (Scale bars: 100 μm). B) Number of CITs were counted in an area of 300 μm × 300 μm (900 μm²) of each skin lesion (**p<0.01).*

lymphocytes, vascular endothelial cells and fibroblasts. Recently, it has been shown that TARC production in the skin increases with stimulation by thymic stromal lymphopoietin (TSLP) produced from keratinocytes, causing migration of Th2 cytokines. AD leads to insufficient ceramide levels in the skin's stratum corneum lowering the barrier function and allowing for easier entry of bacteria and chemical substances. The allergens that invade the skin are recognized as foreign bodies by immunocompetent cells and inflammatory reaction progresses. This leads to activation of Th2 cells causing the production of IL-4 and IL-13 (15).

Th2 cells act on fibroblasts; therefore, substances, such as periostin, are induced. Periostin binds to its receptor, α_v integrin, thus promoting the production of inflammatory

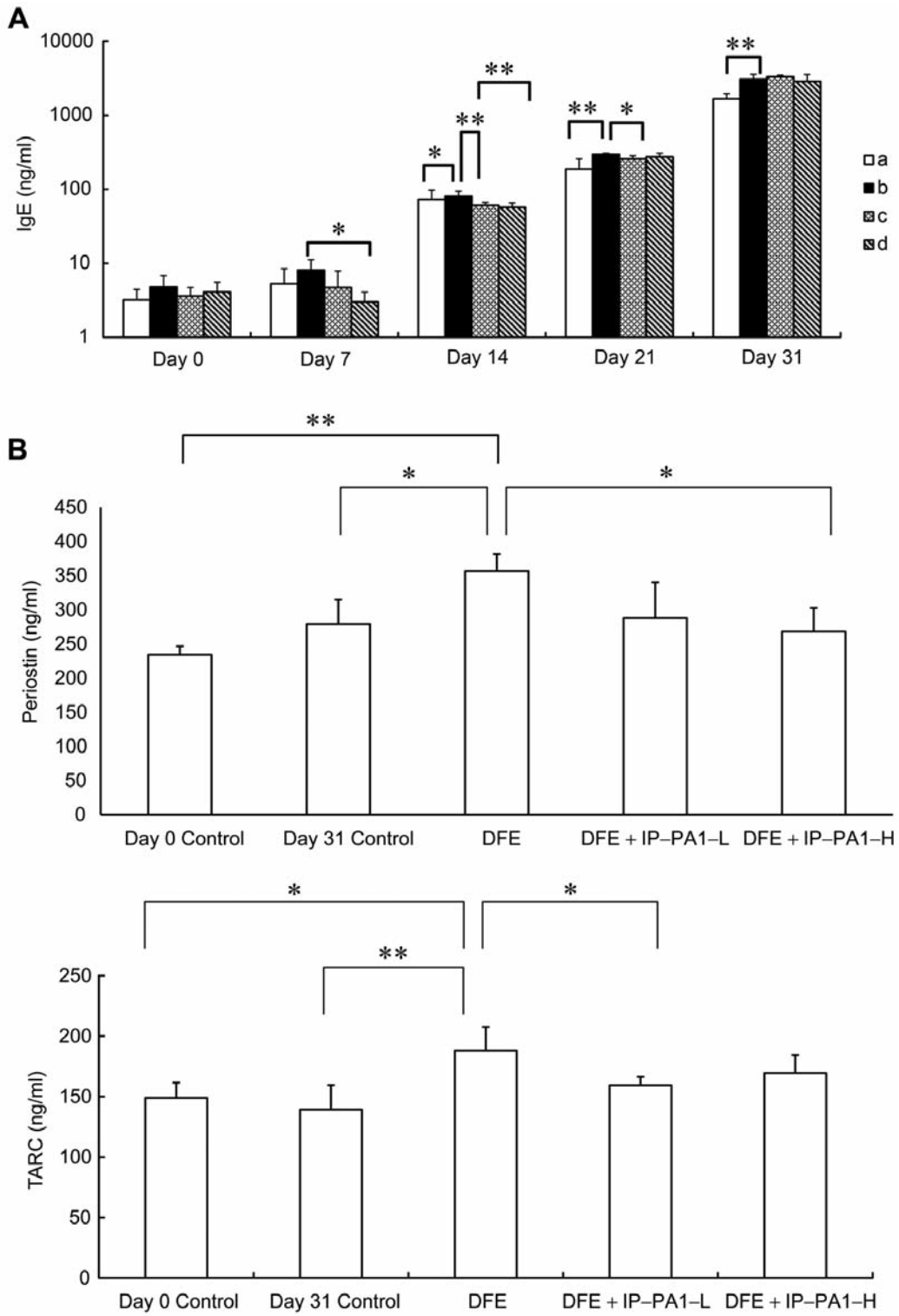

Figure 4. *Effect of IP-PA1 on serum IgE, periostin and TARC levels of DFE-induced AD-like model in NC/Nga mice. A) Effect of IP-PA1 on DFE-induced increase in the serum IgE level in NC/Nga mice. The level of serum IgE was measured with ELISA on days 0, 7, 14, 21 and 31. Data are presented as mean±SE (**p<0.01 and *p<0.05). a, control group; b, DFE group; c, DFE+IP-PA1-L group; d, DFE+IP-PA1-H group. N=8 for all groups. B) Effect of IP-PA1 on DFE-induced increase serum periostin and TARC level in NC/Nga mice. The level of serum periostin and TARC were measured with ELISA on day 0 and 31. Data are presented as mean±SE (**p<0.01 and *p<0.05). N=8 for all groups.*

Table I. *Effect of IP-PA1 on CD4+/CD8+ ratio of T lymphocytes.*

	(CD4+/CD8+) ratio (%)	
Control	1.28±0.15	*
DFE	1.82±0.31	**
DFE + IP-PA1-L	2.41±0.36	*
DFE + IP-PA1-H	2.40±0.59	

CD4+ and CD8+ cells were extracted from spleen and were determined by FACS analysis. Data are presented as mean±SE. N=3 for all groups (**p<0.01 and *p<0.05).

mediator TSLP and inducing TARC from various cells, such as dendritic cells. This series of events, once again, activates Th2 cells and cytokines establishing a vicious cycle of inflammation and causing AD to continue without new invasion of allergens (16).

TARC can be detected in blood and significantly higher TARC levels are identified in patients with AD than in healthy individuals; these levels also correlate with the severity of AD. Blood TARC level differs from blood IgE level in that it quickly and sensitively reflects the progression status of AD. Therefore, periostin and TARC have been recently gaining attention as new diagnostic markers for allergic diseases within Japan (17).

As shown in Figure 3, histopathological analysis of the skin shows that skin lesions related to inflammation (epithelium and dermis hypertrophy) and CIT were inhibited by IP-PA1. Kim *et al*. (18) also reported epithelium and dermis hypertrophy and infiltration of inflammatory cells, such as macrophages and T-cells, in the NC/Nga mouse skin lesion tissue.

Figure 4A shows that at the initial stage of this model, IP-PA1 significantly inhibited IgE production; however, inhibition stopped being statistically significant on day 31. The fact that IP-PA1 also significantly decreased skin scores and periostin and TARC levels on day 31 shows that the relationship between IP-PA1 and AD pathology, IgE values and other markers is interesting.

In the past, it has been reported that IP-PA1 suppressed IgE-related allergic reaction. For example, the administration of monoclonal anti-dinitrophenol (DNP)-IgE antibody to a mouse, followed by application of dinitrofluorobenzene to the auricle of the mouse, induced edema and inflammation on the auricle. However, when IP-PA1 was intradermally injected prior to experiment, edema on the auricle was suppressed. The regulatory mechanism of immune balance is considered to be present, which acts on T or B cells *via* macrophages, *i.e*. the production of Th1 cytokines (IL-12 and IFN-γ) shifts the immune system from Th2 to Th1 in case of inflammation, as observed in the present study.

LPS induces inflammation as an endotoxin; however, oral administration of LPS was recently reported to regulate intestinal homeostasis and prevent diseases, such as typhoid fever infection. Therefore, the role of LPS as a factor, and not just as an endotoxin, to regulate biological function *via* oral administration, has been attracting attention.

According to previous researches, NC/Nga mice have been found to be more sensitive to LPS as mice have less NKT cells compared to C57BL/6 mice and the production capacity of IFN-γ, a Th1 cytokine, was also low. However, in our experiment, continuous oral administration of LPS to NC/Nga mice repressed IgE-induced type I allergy. Although we were not able to elucidate its underlying mechanism, it is likely that IP-PA1 controls the biological immune system *via* certain mechanisms and prevents the occurrence of AD in our study model (10, 11, 19).

In the present study, no difference was observed between groups with either high or low concentrations of IP-PA1. Therefore, the anti-inflammatory effect or immunoregulation exerted by IP-PA1 was confirmed to be not necessarily dependent on the concentration.

With regard to IP-PA1 immune response, in the present study we only investigated the $CD4^+/CD8^+$ ratio of the spleen. Our results showed an increase in the $CD4^+/CD8^+$ ratio through DFE stimulation. This suggested that IP-PA1 may be controlling the immune reaction occurring *in vivo* in NC/Nga mice at the cellular level. This is because $CD4^+$ T-cells play an important role in the immune balance of the host defence reaction and $CD4^+$ and $CD8^+$ have been confirmed to be present in AD skin-penetrating cells, in particular (20, 21).

However, further detailed research is required to determine the nature of the relationship between the increase in the $CD4^+/CD8^+$ ratio in the spleen after IP-PA1 administration and alleviation of AD symptoms.

In our study, in addition to skin lesion changes and decrease in serum IgE levels, suppression of blood periostin levels was also exhibited. These results clarified that IP-PA1 works preventively against AD pathology. In particular, changes in skin lesions of patients with AD include the same itchiness, reddening and hemorrhage observed in our AD model. Because these skin lesions cause patients with AD to suffer various types of stress, it is extremely significant to inhibit the exacerbation of superficial skin lesions. Therefore, in clinical practice, we expect that IP-PA1 may be used to prevent AD onset and progression.

In conclusion, oral administration of IP-PA1 (both low and high dose) exhibited inhibitory effects on AD symptoms, induced by DFE in NC/Nga mice. The suppression of serum IgE, periostin and TARC levels was also confirmed in addition to inhibition of skin lesion changes, which suggest that IP-PA1 could be effective for the prevention of AD and

in inhibiting its exacerbation. As the $CD4^+/CD8^+$ ratio was increased, we believe that the underlying mechanism of IP-PA1 effect may be related to immune cells in the body.

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