AGE-RELATED RESPONSE OF IL-4/LUC/CNS-1 TRANSGENIC MICE TO PHTHALIC ANHYDRIDE EXPOSURE

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Abstract: Age-related changes are associated with susceptibility to infection, malignancy, autoimmunity, response to vaccination and wound healing. To investigate the relationship of several pathological phenotypes of allergic inflammation to age, alterations in the IL-4 derived luciferase signal and general phenotype biomarkers were measured in young (2-month-old) and old (12-month-old) IL-4/Luc/CNS-1 transgenic (Tg) mice with phthalic anhydride (PA)-induced allergic inflammation for 2 weeks. There was no difference in the ear phenotypes and thickness between young and old mice, although these levels were higher in the PA-treated group than the acetone-olive oil (AOO)-treated group. The luciferase signal was detected in the mesenteric lymph node (ML), thymus and pancreas of both young and old PA-treated mice, but showed a greater increase in old Tg mice (except in the thymus). A greater increase in the epidermal thickness and dermal thickness was measured in old PA-treated mice than young PA-treated mice, while total mast cell number remained constant in both groups. Furthermore, the concentration of IgE was greater in young PA-treated mice than in old PA-treated mice, as was the expression of VEGF and IL-6. Taken together, the results of this study showed that an animal's age is an important factor that must be considered when PA-induced allergic inflammation in IL-4/Luc/CNS-1 Tg mice are investigated to screen for allergens and therapeutic compounds.

Key words: aging; skin inflammation; IL-4/Luc/CNS-1 transgenic mice; phthalic anhydride; IgE

INTRODUCTION

Several important changes in the innate and adaptive response associated with increasing age are defined as immunosenescence (Busse and Mathur, 2010; Milgrom and Huang, 2014). The clinical consequences of immunosenescence include enhanced susceptibility to various infections, increased rate of malignancies and autoimmune disease, decreased response to vaccination and delayed wound healing (Busse and Mathur, 2010; Franceschi et al., 1995). Immunosenescence also influences the development of three allergic disorders including asthma, allergic rhinitis and atopic dermatitis, as well as common complications such as comorbidities, polypharmacy and adverse effects of drugs (Milgrom and Huang, 2014).

Generally, in the elder group, the adaptive immunity generated by newly encountered antigens was found to be lower, and the thymus was gradually replaced by fatty acids in a group of elderly patients until the age of 60 (Hannoun et al., 2004; Milgrom and Huang, 2014). Moreover, a high level of HLA-DR and CD69 expression in T cells and an enhanced level of inflammatory cytokines in the plasma such as IL-6, IL-1β and TNF-α have been detected in elderly individuals (Franceschi et al., 2007). Depending on age, the profile changes from Th1 (IL-2, IL-12, IFN-γ, TNF-α) to Th2 cytokines (IL-4, IL-6, IL-8, TGF-β) leads to increased Th2 immune responses and allergic sensitization (Milgrom and Huang, 2014; Franceschi et al., 2007). Furthermore, the functional ability of neutrophils to kill phagocytosed organisms and remove reactive oxygen species decreased in elderly

individuals, although this cell number in bronchoalveolar lavage fluid increased (Milgrom and Huang, 2014). However, the number of mast cells in tissue was shown to decrease in an elderly group when compared with a young group (Gunin et al., 2011). Moreover, age influenced the anti- and proinflammatory cytokine expression pattern and final outcome, including reaction intensity and scale of fibrosis, in a hypersensitivity pulmonitis (HP) model of 3- and 18-month-old mice (Lemieszek et al., 2013). Moreover, greater elevation of pulmonary inflammation and IFN-γ and IL-5 expression occurred in older mice, while airway hyperresponsiveness (AHR) and IL-4 and IL-13 expression was higher in young mice (Busse et al., 2007). However, no other studies have investigated whether the allergic responses to PA sensitization are affected by aging in IL-4/Luc/CNS-1 Tg mice.

Therefore, we attempted to evaluate alterations in allergic phenotypes, including the IL-4-derived luciferase signal, in response to skin inflammation induced by repeated dermal exposure to PA using young and old IL-4/Luc/CNS-1 Tg mice. The results of the present study suggest that several pathological phenotypes of allergic inflammation and IL-4 responses to PA application can be affected by aging in IL-4/Luc/CNS-1 Tg mice.

MATERIALS AND METHODS

Experimental animals

The animal protocols used in this study were reviewed and approved for ethical and scientific care procedures by the Pusan National University-Institutional Animal Care and Use Committee (PNU-IACUC; Approval Number PNU-2013-0410). The young (2-month-old) and old (12-month-old) IL-4/Luc/CNS-1 Tg mice used in this study were kindly provided by the National Institute of Food and Drug Safety Evaluation of the Korea Food and Drug Administration (FDA) (Osong, Korea). All mice were provided with *ad libitum* access to a standard irradiated chow diet (Samtako, Korea) and water throughout the 2-week feeding study. This diet was composed of moisture (12.5%), crude pro-

tein (25.43%), crude fat (6.06%), crude fiber (3.9%), crude ash (5.31%), calcium (1.14%) and phosphorus (0.99%), as well as corn (546 g/kg), vegetable protein (316 g/kg), fish meal (34 g/kg), beet pulp (30 g/kg), animal fat and oil (36 g/kg), lysine (2 g/kg), choline bitartrate (2 g/kg), 7.17% methionine solution (2 g/kg), limestone (10 g/kg), calcium/phosphate supplement (13 g/kg), salt (5 g/kg), mineral mix (2 g/kg) and vitamin mix (2 g/kg). During the experiment, mice were maintained in a specific pathogen-free state under a strict light cycle (lights on at 08:00 and off at 20:00) at 23±2°C and 50±10% relative humidity. The mice were housed in the Pusan National University-Laboratory Animal Resources Center accredited by the Korea FDA in accordance with the Laboratory Animal Act (Accredited Unit Number-000231) and AAALAC International according to the National Institutes of Health guidelines (Accredited Unit Number; 001525).

Young and old IL-4/Luc/CNS-1 Tg mice (n=5-6 per group) were randomly divided into two groups. In the first group (AOO, n=4-5), 100 µl of AOO (4:1 acetone:olive oil, v/v) was repeatedly spread on the dorsum of the ears three times a week for 2 weeks. In the second group (PA, n=4-5), 100 µl of 15% PA solution in AOO was repeatedly spread on the dorsum of the ears three times a week for 2 weeks. After final treatment, the animals in each group were euthanized using a chamber filled with CO_2 gas. Ear tissue samples and whole blood were then collected for further analysis.

Production and identification of IL-4/Luc/CNS-1 Tg mice

IL-4/Luc/CNS-1 Tg mice were produced using a previously described breeding method (Bae et al., 2011). Large numbers of IL-4/Luc/CNS-1 Tg mice were produced by breeding male IL-4/Luc/CNS-1 Tg and female HR1 mice. Founder mice containing the IL-4/Luc/CNS-1 transgene were then identified by DNA-PCR of tail-derived genomic DNA. For DNA-PCR, 10 pmol each of sense (5'-CTC GCA TGC CAG AGA TCC TA -3') and antisense (5'-CCA CAA CCT TCG CTT CAA AA-3') primers were added into the ge-

nomic DNA template mixture. Amplification was then conducted in a T100 thermal cycler (BioRad Laboratories Inc., Hercules, CA, USA) by subjecting the samples to 35 cycles of 1 min at 94°C, 1 min at 62°C and 1 min at 72°C. The amplified PCR products were subsequently separated by 1% agarose gel electrophoresis, after which the band patterns were detected using a UV-transilluminator (ATTO, Tokyo, Japan).

Observation of ear morphology and measurement of ear thickness

Changes in ear color, ear vein and other morphological characteristics were analyzed by evaluation of photographs. Additionally, ear thickness was measured to determine the degree of skin inflammation induced by AOO and PA topical application using a thickness gauge (Digimatic Indicator, Matsutoyo Co., Tokyo, Japan).

Measurement of body and organ weight

The body weights of all animals in the subset groups were measured using an electronic balance (Mettler Toledo, Greifensee, Switzerland) throughout the experimental period. In addition, the weight of the lungs, kidneys, spleen, heart, ML, thymus and pancreas was determined using the same method.

Bioluminescence imaging analysis

In vivo imaging analysis was conducted using an IVIS imaging system (Xenogen, Oakland, CA, USA) as previously described (Kwak et al., 2013). Briefly, IL-4/Luc/CNS-1 Tg mice were anesthetized with Zoletil 50 (Virbac, Carros, France) and then injected intraperitoneally with 150 mg/kg D-luciferin (Sigma-Aldrich, St. Louis, MO, USA). Ten min after D-luciferin injection, whole body and organ images of mice were taken for 3 min using an IVIS imaging system, after which the photons emitted from specific regions were quantified using the Living Image software (Xenogen). The *in vivo* luciferase activity was then expressed in photons per second.

Enzyme-linked immunosorbent assay (ELISA) for detection of serum IgE concentration

The serum IgE concentration was measured using an ELISA kit (Shibayagi Inc., Gunma, Japan) according to the manufacturer's instructions. Briefly, wells coated with antibody were washed three times with washing solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0), after which 50 μl of serum samples and standards were added to the wells and the plate was incubated for 2 h. Following washing with the above solution, 50 μl of biotin-conjugated avidin (1000-fold dilution) was added and the samples were then incubated for 2 h. Horseradish peroxidase-conjugated detection antibodies (2000-fold dilution) were then transferred to each well. The plates were subsequently incubated at room temperature for 1 h, after which they were washed three times with washing solution. Next, an enzyme reaction was initiated by adding substrate solution and incubating the plate at room temperature for 20 min. Finally, the reaction was terminated by adding stop solution (1 M H_2SO_4 solution) and the absorbance was measured at 450 nm.

Histological analysis

Ear tissues were obtained from the IL-4/Luc/CNS-1 Tg mice and fixed in 10% formalin. Following deparaffinization and dehydration, the ear tissues were embedded in paraffin wax, routinely processed, and then sectioned into 4-μm thick slices. Next, ear sections were stained with hematoxylin and eosin (H&E), after which they were examined by light microscopy (Leica Microsystems, Wetzlar, Germany) for the presence of immune cell accumulation. The thickness of the epidermis and dermis were also measured using the Leica Application Suite (Leica Microsystems). In addition, the infiltration of mast cells into the ear tissue was detected by staining with toluidine blue as previously described (Kim et al., 2010). Following deparaffinization and dehydration, ear skin sections were stained with 0.25% toluidine blue (Sigma-Aldrich Co.) and examined by light microscopy (Leica Microsystems) for the presence of mast cells. The number of cells per

specific area was determined using the Leica Application Suite (Leica Microsystems).

Western blotting

Ear tissues collected from a subset of the groups were homogenized using a PRO-PREP™ Solution Kit (iN-tRON Biotechnology Inc., Sungnam, Korea), then centrifuged at 13000xg for 10 min. The prepared proteins were subsequently subjected to 10% SDS-PAGE, after which they were transferred to a nitrocellulose membrane (GE Healthcare, Little Chalfont, UK) for 2 h at 40 V in transfer buffer (25 mM Trizma base, 192 mM glycine and 20% methanol). Appropriate dilutions of primary antibodies including anti-IL-6 antibody (Santa Cruz Biotechnology, Santa Cruz, TX, USA), anti-VEGF antibody (Pepro Tech., Rckyhill, NJ, USA) and anti-β-actin (Sigma-Aldrich Co.) were added to the membranes and allowed to hybridize overnight at 4℃. After the antibodies were removed, the membrane was washed three times in a solution composed of 10 mM Trizma base (150 mM NaCl and 0.05% Tween-20) for 10 min. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, followed by washing as described above and developed using an enhanced chemiluminescence reagent plus kit (Amersham Biosciences). The results were quantified using the Image Analyzer System (Fluorchem FC2, Alpha Innotech, CA, USA) and expressed as the fold-increase over control values.

Statistical analysis

One-way ANOVA was used to determine whether or not significant differences existed between the PA-treated and AOO-treated groups (SPSS for Windows, Release 10.10, Standard Version, Chicago, IL, USA). Additionally, differences in response between the young and old groups were evaluated by a *post hoc* test (SPSS for Windows, Release 10.10, Standard Version) of the variance and significance levels. All values were expressed as the means±SD. A *p* value of <0.05 was considered significant.

RESULTS

Difference in ear morphology and thickness between young and old IL-4/Luc/CNS-1 Tg mice

To investigate the effects of aging on ear phenotypes after PA application, we first evaluated changes in the ear morphology and thickness in young and old IL-4/Luc/CNS-1 Tg mice after PA treatment for 2 weeks. During morphological analysis, the ear color of IL-4/Luc/CNS-1 Tg mice changed significantly from a fleshy tint to dark brown in the PA-treated group compared to the AOO-treated group, while the outline of the ear vein became thickened. However, there were no significant differences in ear morphology between young and old IL-4/Luc/CNS-1 Tg mice (Fig. 1A). Conversely, the ear thickness was elevated in the young and old PA-treated group compared to the AOO-treated group. However, this increase (81%) in the young PA-treated group was not significantly different from that (74%) of the old PA-treated group (Fig. 1B). Thus, these results demonstrate that there is no correlation between ear morphology response to PA treatment and age in IL-4/Luc/CNS-1 Tg mice.

Difference in IL-4-derived luciferase signal between young and old IL-4/Luc/CNS-1 Tg mice

To assess the effects of aging on the IL-4-derived response to PA application, the luciferase signals were measured in seven organs from IL-4/Luc/CNS-1 Tg mice after PA treatments using Living Image software. After PA treatment, the luciferase signals were only detected in three of the seven investigated organs, the ML, thymus and pancreas. Additionally, the change in the luciferase signal derived from the IL-4 promoter was greater in the ML and pancreas of old PA-treated mice than in the young PA-treated mice, although the increase ratio was very similar between them. However, the thymus showed a similar level of luciferase in both PA-treated groups (Fig. 2). Taken together, these findings indicate that the ML and pancreas of old IL-4/Luc/CNS-1 Tg mice may be involved in the increased IL-4 activity in response to PA treatment.
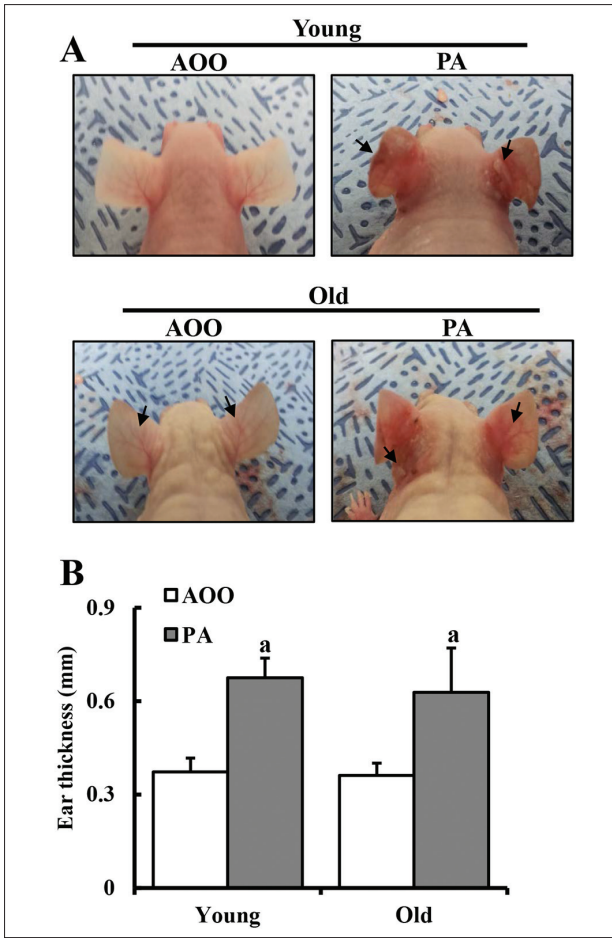
Fig. 1. Morphological analysis of the ears of IL-4/Luc/CNS-1 Tg mice. (A) Ear vein (arrow) and other morphological characteristics were analyzed in photographs of mice. (B) Ear thickness of mice in four groups was measured using a thickness gauge and phenotypes were observed as described in the Materials and Methods after the application of PA solution. Data shown are the means±SD (n=5). a, $p<0.05$ compared to the AOO-treated group.

Fig. 2. Detection of luciferase signal in seven organs of IL-4/Luc/CNS-1 Tg mice. Mice were treated with AOO and PA for 2 weeks and imaged at 24 h after final treatment using Living Image software. The color overlay on the image represents the photons per second emitted from the organs in accordance with the pseudocolor scale shown next to the image. In this image, red indicates the highest number of photons per second, while blue indicates the lowest. L – lung; K – kidney; S – spleen; H – heart; ML – mesenteric lymph node; T – thymus; P – pancreas. a, $p<0.05$ compared to the AOO-treated group.

Difference in organ weight between young and old IL-4/Luc/CNS-1 Tg mice

To measure the difference in PA application between young and old IL-4/Luc/CNS-1 Tg mice, body and organ weight were measured in the subset groups after PA treatment for 2 weeks. The whole body weight in both groups was maintained at a constant level throughout the experimental periods (data not shown). However, only three of the seven organs investigated sho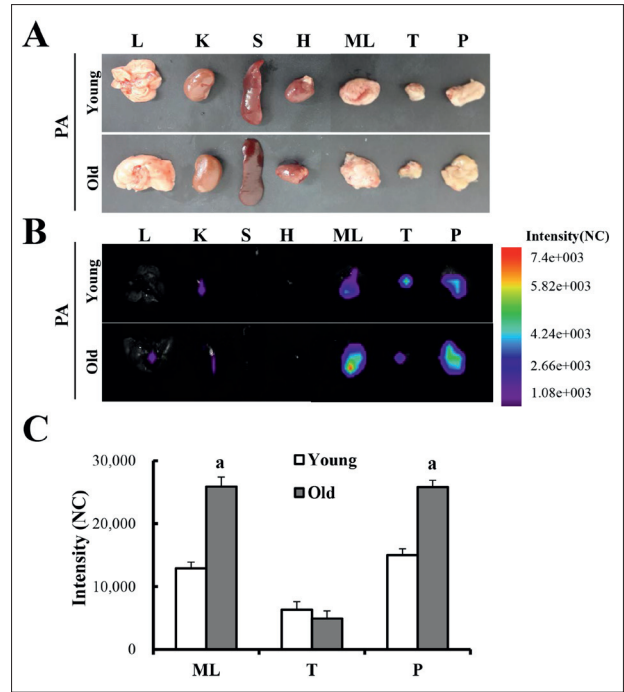wed significant alteration after PA treat-ment. The weights of the spleen and ML increased significantly by 82% and 63%, respectively, in the young PA-treated group compared to the AOO-treated group, while these levels were maintained at a constant level in old Tg mice (Table 1). However, the weight of the thymus in the young and old groups was decreased in the PA-treated group compared to the AOO-treated group, although the decrease was slightly greater in the young group (57%) than in the old group (50%). Taken together, the results of the present study indicate that the increase in spleen and ML weight may be more sensitive to PA topical application in young Tg mice than in old mice. In addition, the above results suggest that PA application can induce a large decrease in the thymus weight in young and old IL-4/Luc/CNS-1 Tg mice.

Table 1. Comparison of organ weights between young and old group

Categories	Weight of young mice (g)		Weight of old mice (g)	
	AOO	PA	AOO	PA
Lung	0.21±0.028	0.18±0.007	0.26±0.031	0.24±0.035
Kidney	0.23±0.057	0.29±0.099	0.37±0.116	0.39±0.244
Spleen	0.11±0.028	0.20±0.023*	0.20±0.057	0.21±0.014
Heart	0.16±0.007	0.17±0.007	0.20±0.322	0.21±0.055
Mesenteric lymph node	0.19±0.028	0.31±0.021*	0.32±0.102	0.27±0.020
Thymus	0.07±0.000	0.03±0.007*	0.06±0.007	0.03±0.014*
Pancreas	0.20±0.028	0.17±0.050	0.29±0.023	0.25±0.080

*, $p<0.05$ compared to the AOO-treated group.

Difference in histological structure of young and old Tg mice

Differences in the histological structure of ear tissue between young and old PA-treated Tg mice were evaluated by histological analysis of ear tissue of IL-4/Luc/CNS-1 Tg mice after PA treatment for 2 weeks. The epidermis and dermis of the ear tissue in the mice was thicker in the PA-treated group than in the AOO-treated group. However, in the PA-treated group, the increase in epidermal and dermal thickness was greater; increased thickness (120-125%) was observed in old Tg mice as compared to young Tg mice (Fig. 3). Taken together, these results showed that age may be closely correlated with increasing epidermal and dermal thickness in IL-4/Luc/CNS-1 Tg mice after PA treatment.

Mast cells play important roles in asthma, eczema, itch, allergic rhinitis, allergic conjunctivitis and skin inflammation (Prussin et al., 2003). Therefore, ear skin sections were stained with toluidine blue and mast cells were measured to investigate the effects of aging on the infiltration of mast cells after PA treatment. The total number of mast cells in the dermis region increased significantly in the PA-treated group compared to the AOO-treated group. However, the fold change in the total number of mast cells was significantly greater, with 25.1% more observed in old Tg mice than in young Tg mice after PA application
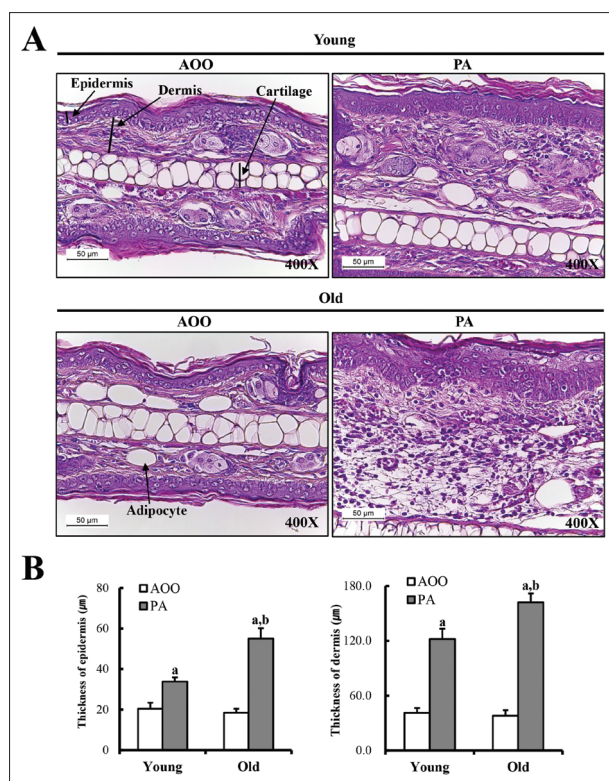

Fig. 3. Histological analysis of ear tissue. PA solution was repeatedly applied to the dorsum of the ear of IL-4/Luc/CNS-1 Tg mice. After 2 weeks, ear tissues were collected from AOO- and PA-treated IL-4/Luc/CNS-1 Tg mice. Histological changes in the slide sections of ear tissue were identified by staining with H&E followed by observation at 400x magnification. Data shown are the means±SD (n=5). a, $p<0.05$ compared to the AOO-treated group. b, $p<0.05$ compared to the young Tg mice.

(Figs. 4A and B). These results suggest that aging can affect the levels of PA-induced mast cells infiltration in the dermis of IL-4/Luc/CNS-1 Tg mice.

Difference in IgE concentration between young and old Tg mice

Serum IgE concentration was measured in the subset groups to determine if age can contribute to the stimulation of IgE secretion associated with skin inflammation induced by PA treatment. In the AOO-treated group, the serum IgE concentrations were 728% higher in old Tg mice than in young Tg mice. Although the level became similar (1200 ng/mL) after
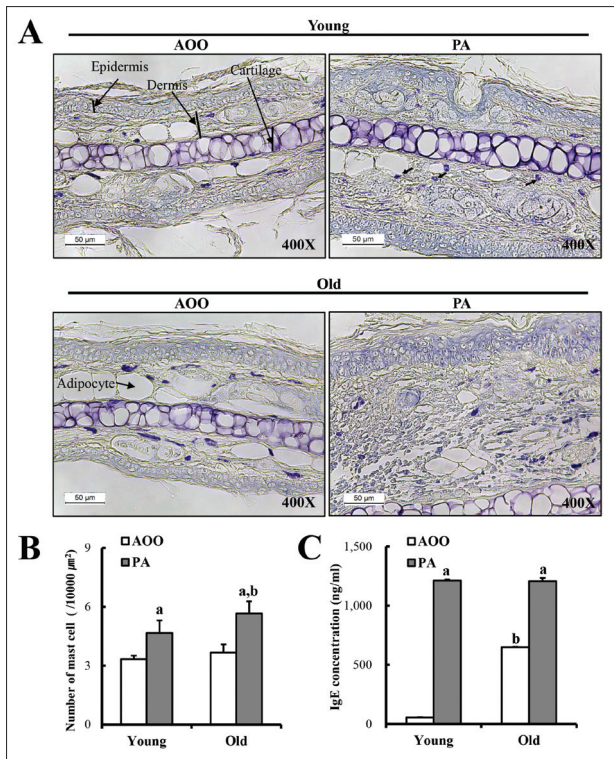
Fig. 4. Analysis of mast cells and IgE concentration. (A and B) Infiltration of mast cells in the slide sections of ear tissue was identified by staining with toluidine blue followed by observation at 400x magnification. Arrows indicate the infiltrated mast cells in the dermis of the ear tissue. (C) The serum used to measure the IgE concentration was prepared from blood samples collected from the abdominal veins of mice. The serum IgE concentration was quantified by an enzyme-linked immunosorbent assay. Data shown are the means±SD (n=5). a, $p<0.05$ compared to the AOO-treated group. b, $p<0.05$ compared to the young Tg mice.

Fig. 5. Analysis of cytokine expression in ear tissues. The levels of IL-6 and VEGF in the ear tissue of IL-4/Luc/CNS-1 Tg mice from subset groups were detected by Western blot analysis using specific antibodies. The intensity of each band was determined using an imaging densitometer and the relative level of each protein was calculated based on the intensity of actin protein as an endogenous control. Data shown are the means±SD (n=5). a, $p<0.05$ compared to the AOO-treated group. b, $p<0.05$ compared to the young Tg mice.

PA treatment, the change in this level was greater in the young PA-treated group (1100%) than in the old PA-treated group (85%) (Fig. 4C). These results suggest that IgE concentration may be more sensitive to PA topical application in young IL-4/Luc/CNS-1 Tg mice.

Cytokine expression in ear tissue

To understand the mechanism by which ear thickness and cytokine expression increased, we investigated the ear tissue of the subset groups. VEGF is known to be a multifunctional proinflammatory cytokine responsible for erythema and edema in atopic der-
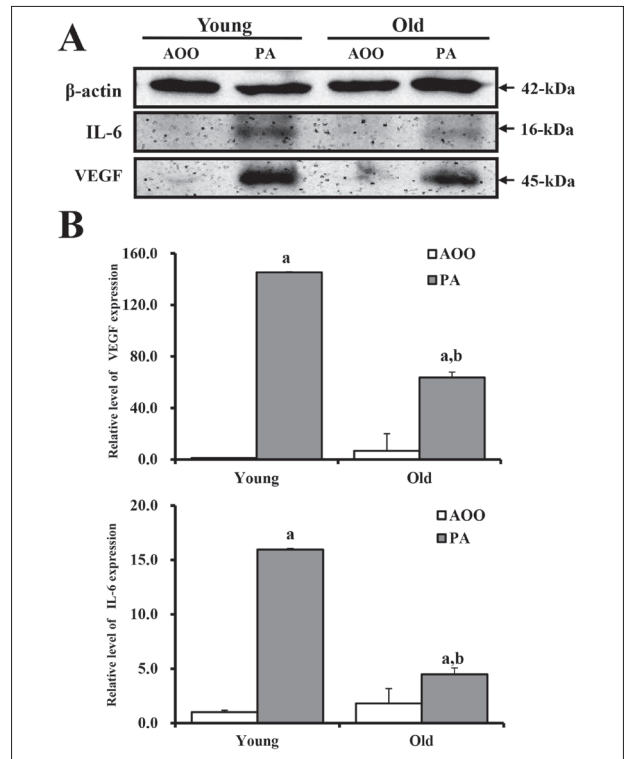
matitis (Kasperska-Zaiac and Koczy-Baron, 2011). Moreover, IL-6 is widely known as an important proinflammatory mediator in allergic inflammation and atopic dermatitis (Zhao et al., 2005). The expression of VEGF and IL-6 was significantly enhanced in the PA-treated group compared to the AOO-treated group. However, the increase in VEGF and IL-6 expression was greater in the young PA-treated group (1650% and 1500%, respectively) than the old-PA treated group (362% and 125%, respectively) (Fig. 5). Overall, the above results indicate that the increase in IL-6 and VEGF expression in the skin inflammation induced by PA treatment may be suppressed by aging in IL-4/Luc/CNS-1 Tg mice.

DISCUSSION

The changes in the immune system associated with age include a significant reduction in responsiveness, dysregulation of immune effector cells and remodeling of the cytokine network (Albright et al., 2004; Linton and Dorshkind, 2004). However, there is still a poor correlation between immunosenescence and the clinical outcome of many diseases that preferentially affect elderly individuals (Shurin et al., 2007). Therefore, in the present study, we investigated the age-related alterations in skin inflammation of IL-4/Luc/CNS-1 Tg mice during PA application. The results demonstrated that aging can affect several pathological phenotypes, as well as the luciferase signal derived from the IL-4 promoter in response to PA topical application.

Th2 cytokines including IL-4, IL-5, IL-10 and IL-13 mediate immune responses against a large number of pathogens and regulate allergic inflammation through the stimulation of IgE class switching and eosinophil activation (Romagnani, 2001). Among these cytokines, IL-4 stimulation of B cells to produce IgE is primarily responsible for allergic inflammatory and atopic dermatitis responses (Khodoum et al., 2004). However, it is not clear if the IL-4 response against respiratory sensitizers is affected by age. Indeed, several studies have shown opposite results regarding age-related changes in IL-4 response to some allergens. The IL-4 concentration in the supernatants of peripheral mononuclear cells after 48 h of stimulation with phytohemagglutinin did not differ significantly between two groups of young (20-64 years) and old humans (70-93 years) (Di Lorenzo et al., 2003). However, semiquantitative RT-PCR analysis of bronchoalveolar lavage (BAL) cells revealed that cells from young Brown Norway rats (8- to 10-wk-old) sensitized with ovalbumin preferentially expressed the mRNA of Th2-type cytokines, including IL-4 and IL-5 (Ide et al., 1999). Juvenile mice infected with the parasitic nematode *Nippostrongylus brasiliensis* showed significantly reduced IL-4 expression in the early stages of infection (day 5 and 9) relative to adult mice (6-8-wk-old) (Hendrik et al., 2011). In this study, old Tg mice showed a greater increase in IL-4-derived luciferase

than young Tg mice in the PA-treated group (Fig. 2). The results of the present study are in agreement with those of several previous studies of juvenile mice, although the levels of change vary. However, our results differ from those of previous studies that showed IL-4 expression was higher in young Brown Norway rats sensitized with OVA. This difference was likely due to differences in the properties of the sensitizing chemicals and the specificity of the target organs of the inflammatory response.

It is well known that the hyperproduction of IgE is characteristic of type 1 hypersensitivity and an indicator of the magnitude of allergic immune response (Gao et al., 2004; Dearman et al., 2003). However, there are conflicting results regarding the effects of aging on serum total IgE concentration after allergen sensitization. In 20- to 93-year-old humans, serum total IgE concentration did not differ significantly between the young and old (Di Lorenzo et al., 2003). However, a significantly lower concentration of total IgE was measured in juvenile mice infected with *N. brasiliensis* than in adult mice (Hendrik et al., 2011). In the present study, a high fold change of IgE concentration was observed in young Tg mice after PA topical application. These findings differ from those of previous reports where IgE was preferentially suppressed in a young group after allergen infection. Therefore, the results presented herein provide additional evidence that aging affects IgE production in PA-treated IL-4/Luc/CNS-1 Tg mice.

Mast cells are widely distributed through several mammalian tissues including blood vessels, the central nerve system (CNS), epithelium and smooth muscle (Busse and Mathur, 2010). These cells have been shown to regulate the protection of immune responses to some parasites and bacteria, as well as IgE-mediated immediate hypersensitivity and allergic disorders (Kawakami et al., 2009; Galli, 2000). Similarly, numbers of mast cell were detected in young (6 weeks) and old (3-12 months) C57BL/6 mice (Nguyen et al., 2005). Although the effects of aging on mast cell degranulation are unclear, mast cell degranulation in ear tissue after PGE injection was significantly enhanced in old (>6 months) mice relative to young (2

Table 2. Quantitative comparison of allergic phenotypes between the young and old groups.

Categories	Response magnitude against PA (change rate to vehicle group)		Difference between young and old group
	Young	Old	
Ear thickness	+0.302	+0.267	11.6% lower in old group
Luciferase signal			
Mesenteric lymph node	$+1.29 \times 10^4$	$+2.59 \times 10^4$	101% higher in old group
Thymus	$+6.32 \times 10^3$	$+4.94 \times 10^3$	21.8% lower in old group
Pancreas	$+1.50 \times 10^4$	$+2.58 \times 10^4$	72% higher in old group
Thymus weight (g)	- 0.04	- 0.03	25% lower in old group
IgE concentration (ng/mL)	+1,157.30	+558.96	51.8% lower in old group
Epidermal thickness (μm)	+6.39	+36.64	473.4% higher in old group
Dermal thickness (μm)	+80.82	+123.79	53.8% higher in old group
Mast cells number (ea)	+1.33	+2	50.4% higher in old group
Relative level of VEGF expression	+144.20	+56.87	61.1% lower in old group
Relative level of IL-6 expression	+14.95	+2.67	82.1% lower in old group

"+" represents "increase", "–" represents "decrease".

months) mice. However, they showed similar mast cell degranulation in the ear tissue after IgE-dependent passive sensitization (Nguyen et al., 2005). In this study, alterations in the number of mast cells in the ears of IL-4/Luc/CNS-1 Tg mice were investigated in young and old Tg mice after PA treatment. As shown in Fig. 4, the total number of mast cells that infiltrated the dermis was significantly higher in the PA-treated group than in the AOO-treated group, although the fold change was greater in old Tg mice. These results are not directly comparable to those of previous studies because of differences in the type of sensitizing chemical and the specificity of analysis factor of the inflammatory response. However, our results provide novel scientific evidence that the infiltration of mast cells into ear tissue after PA treatment may be affected by aging in IL-4/Luc/CNS-1 Tg mice.

Overall, we investigated the relationship between aging and allergic phenotypes including IL-4 response against PA application using IL-4/Luc/CNS-1 Tg mice. Differences in the IL-4-derived luciferase signal as well as general phenotypes of skin inflammation induced by PA application was detected in both young and old Tg mice as shown Table 2. The results presented herein suggest that animal age should be considered when using IL-4/Luc/CNS-1 Tg mice for the investigation of PA-induced skin inflammatory responses.

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