

# IL-6, VEGF, KC and RANTES Are a Major Cause of a High Irritant Dermatitis to Phthalic Anhydride in C57BL/6 Inbred Mice

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## ABSTRACT

**Background:** In previous studies, several strains of mice were used as chemical-induced skin irritation models to identify immunological hazards and elucidate the molecular and cellular mechanisms by which irritant dermatitis disease occur. BALB/c and C57BL/6 mice have been used for most of these experiments. Although there are some differences in the immune response to chemical allergens between these strains, few studies have been conducted to determine what regulatory factors contribute to these variations.

**Methods:** To investigate the cause of high responses to skin irritation in C57BL/6 mice that are widely used to study atopic dermatitis, changes in various immune-related factors such as ear thickness, myeloperoxidase activity, lymph node weight, IgE concentration and cytokine concentration were measured in C57BL/6 and BALB/c mice following phthalic anhydride (PA) treatment.

**Results:** Based on analysis of the skin irritation, C57BL/6 mice showed a greater skin irritation to PA than BALB/c mice, although the IgE concentration and auricular lymph node weight did not contribute to this difference in the response. However, the concentration of several cytokines and chemokines (interleukin [IL]-6 and vascular endothelial growth factor [VEGF], keratinocyte-derived chemokine [KC] and regulated on activation normal T cell expressed and secreted [RANTES]) were significantly higher in C57BL/6 mice than BALB/c mice following treatment with PA.

**Conclusions:** Our results suggest that several of the cytokines and chemokines secreted from irritant site could contribute to the regulation mechanism responsible for the difference in the skin irritation among various strains of mice following exposure to PA.

## KEY WORDS

allergy, chemokine, cytokine, phthalic anhydride, skin irritation

## INTRODUCTION

Phthalic anhydride is the organic compound and an important industrial chemical, especially for the large-scale production of plasticizers for plastics. Also, phthalic anhydride is known to provoke a strong irritant dermatitis associated with skin inflammation.<sup>1</sup> Allergic diseases, which are defined by the production of IgE in response to environmental antigens, can

lead to asthma, rhinitis, atopic dermatitis and contact dermatitis. These disorders affect 10-15% of western populations, and their prevalence has doubled in the last 10-15 years.<sup>2</sup> This increase has included allergic skin disorders such as atopic or contact dermatitis accompanied by irritant dermatitis in the site at which contact with an allergen has occurred.<sup>3</sup> Such inflammatory responses are characterized by infiltration by specific cells, including inflammatory cells such as T

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lymphocytes, leukocytes, neutrophils, eosinophils, macrophages, mast cells and regulatory and natural killer T cells.<sup>4,5</sup> Furthermore, activation of inflammatory cells by this infiltration is mediated by various cytokines and chemokines at the site of injury. For example, interleukin (IL)-6 is produced at the site of inflammation and plays a key role in the skin inflammation by increasing the mononuclear cell infiltrate.<sup>4</sup> Additionally, chemokines such as keratinocyte-derived chemokine (KC) and regulated on activation normal T cell expressed and secreted (RANTES) were recently found to be the most potent molecules involved in the regulation of selective recruitment of inflammatory cells. Moreover, these molecules were found to direct the migration of inflammatory cells into inflammatory foci.<sup>6</sup> In the serum, the concentration of RANTES has been shown to increase in atopic dermatitis patients when compared with normal controls, and this increase has been shown to augment the production of RANTES that are correlated with irritant dermatitis associated with atopic dermatitis.<sup>7</sup>

In previous studies, several strains of mice were used as chemical-induced skin irritation models to identify immunological hazards and elucidate the molecular and cellular mechanisms by which irritant dermatitis disease occur. BALB/c and C57BL/6 mice have been used for most of these experiments<sup>8</sup>; however, there are some differences in the immune response to chemical allergens between these models. In addition, it has been demonstrated that BALB/c mice are immunoglobulin (Ig) E high responders, while C57BL/6 mice are IgE low responders.<sup>5,9</sup> Despite the differences in this model of inbred mice, few studies have been conducted to determine what regulatory factors contribute to these variations. In this study, PA treatment was found to induce skin irritation response in both C57BL/6 and BALB/c mice, but the response was greater in C57BL/6 mice than in BALB/c mice. To investigate the mechanism of these responses, the IgE concentration and lymph node weight in both groups of mice was measured following PA treatment; however, these factors did not contribute to different skin irritation response between the two strains. Therefore, the levels of several cytokines and chemokines were evaluated. The results revealed that there were significantly higher levels of these cytokines in C57BL/6 mice than BALB/c mice following PA treatment. Considering our results, we concluded that skin irritation response in C57BL/6 mice is more sensitive than that of BALB/c mice, while allergic response in BALB/c mice is greater than that of C57BL/6 mice. Taken together, the cytokines and chemokines identified in this study may be useful in the development of methods to treat atopic and contact dermatitis.

## METHODS

### CARE AND USE OF MICE

Female C57BL/6 and BALB/c mice (6-8 weeks old), which are usually used to evaluate the response to allergens at our institution, were used in this study. All mice were supplied by the breeding center of our facility and were handled in a Korea Food and Drug Administration (FDA) accredited animal facility in accordance with the AAALAC International Animal Care policy (Accredited Unit, Korea Food and Drug Administration: Unit no. 000996). Mice were housed in cages under a strict light cycle (lights on at 06 : 00 hr and off at 18 : 00 hr) and temperature ( $23 \pm 1^\circ\text{C}$ ). In addition, all mice were provided with a standard irradiated chow diet (Purina Mills Inc., Korea) *ad libitum* and maintained in a specified pathogen-free state.

### EXPERIMENTAL DESIGN AND ALLERGIC SKIN INFLAMMATION INDUCED BY PA TREATMENT

First, 6-8 week old female C57BL/6 and BALB/c mice were randomly divided into two subgroups ( $n = 8$ ) per strain. In the first subgroup (AOO), 40  $\mu\text{l}$  of acetone-olive oil were repeatedly spread on the dorsum of the ear and the shaved back skin of C57BL/6 and BALB/c mice 3 times a week for 4 weeks. In the second subgroup (PA), 40  $\mu\text{l}$  of 5% PA solution in vehicle (4 : 1 acetone-olive oil, v/v: AOO) were repeatedly spread on the dorsum of the ear and the shaved back skin of C57BL/6 and BALB/c mice 3 times a week for 4 weeks.

### MEASUREMENT OF EAR THICKNESS AND SKIN SCORE

The ear thickness and skin scores of the mice were measured to determine the degree of irritant dermatitis induced by PA treatment. Briefly, a thickness gauge (Digimatic Indicator, Mitutoyo, Tokyo, Japan) was used to measure the thickness of the ear. The increase in ear thickness was then determined by subtracting the ear thickness prior to the first treatment from the thickness at the time of the treatment. In addition, changes in the skin on the dorsum of the ear and the back skin of mice were observed weekly using the method described in a previous study.<sup>10</sup> In this method, the degree of four characteristics, erythema/hemorrhage, edema, excoriation/erosion and dryness, were scored from 0 points (none) to 3 points (severe) depending on the severity of the dermatitis. Finally, the total skin score was calculated from the sum of the scores of the four characters.

### DETECTION OF SERUM IgE CONCENTRATION

The serum IgE concentration was measured using an enzyme-linked immunosorbent assay kit (Shibayagi, Inc., Gunma, Japan) according to the manufacturer's instructions. Briefly, capture antibodies were plated

in a Nunc C bottom immunoplate supplied in the kit and then incubated for 1 hr at room temperature. Next, the wells were washed with washing solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0) three times, after which 200  $\mu$ l of blocking solution (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0) were added to each well. After incubation for 30 min at room temperature, the wells were washed three times with washing solution. Next, the serum samples and standards diluted 20-fold with dilution solution were added to the wells, and the plate was then incubated for 1 hr. The wells were then washed with washing solution, after which horseradish peroxidase-conjugated detection antibodies were diluted 5000-fold with conjugate diluent (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) and transferred to each well. The plates were then incubated at room temperature for 1 hr, after which they were washed three times with washing solution. An enzyme reaction was then initiated by adding substrate solution and incubating the plate at room temperature in the dark for 30 min. Finally, the reaction was terminated by adding 2 M H<sub>2</sub>SO<sub>4</sub> solution, and the absorbance at 450 nm was measured.

#### HISTOLOGICAL ANALYSIS

The ear skins were removed from mice, fixed with 10% formalin, embedded in paraffin wax, routinely processed and then sectioned into 5  $\mu$ m thick slices. The skin sections were then stained with hematoxylin and eosin and examined by light microscopy for the presence of edema and inflammatory cell accumulation.

#### MYELOPEROXIDASE (MPO) ACTIVITY

The ears were homogenized in 1 ml of T-PER protein extraction reagent (Pierce, Rockford, IL, USA) containing a protease inhibitor cocktail. The homogenates were then centrifuged at 16,000  $\times$  g for 30 min, after which the MPO activity was measured using an enzyme-linked immunosorbent assay kit (Hycult Biotechnology, Uden, The Netherlands) according to the manufacturer's instructions. The protein content of the homogenates was then measured using a Bio-Rad protein assay kit (Bio-Rad Laboratories, CA, USA) according to the manufacturer's instructions, after which the MPO activity was normalized based on the protein contents.

#### MOUSE CYTOKINE ARRAY ASSAY

Mouse cytokines were analyzed in the lysate of ear tissues. The tissues were lysed in 100  $\mu$ l of protein extraction buffer (PRO-PREP, Intron, Seoul, Korea) with sonicator (Ultra-Turrax-25, Ika-Werke, USA). A lysate was collected from the sonicator mixture using centrifugation and stored at -70°C until further analyses.

Furthermore, the expression profiles of several cy-

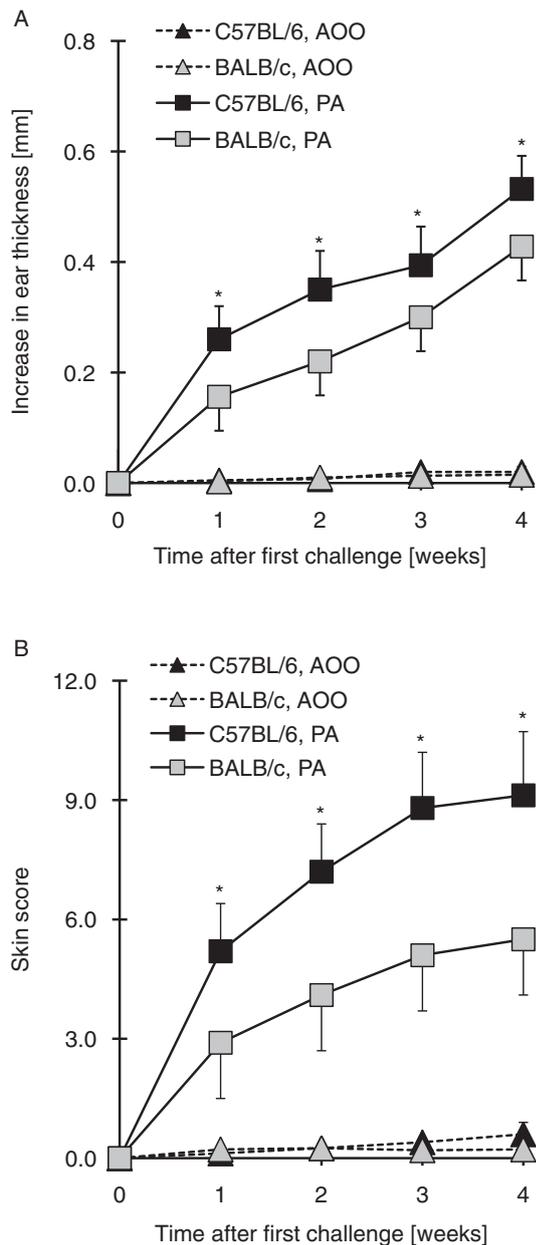
tokines derived from the lysate were then evaluated using a mouse cytokine array assay kit (RayBiotech, Norcross, GA, USA) according to the manufacturer's instructions. Briefly, the slides of the cytokine array were blocked with 5% BSA/TBS (0.01 M Tris HCl pH 7.6, 0.15 M NaCl) for 1 hr. Next, 50  $\mu$ l of the supernatants and lysates corresponding to each mouse strain were added to this slide, which was then incubated at room temperature for 30 minutes. After extensive washing with Tris buffer saline (TTBS) containing 0.1% Tween 20 (3 times, 5 min each) and TBS (2 times, 5 min each) to remove the unbound materials, the slide was incubated with a cocktail of biotin-labeled antibodies against different individual cytokines. The membranes were then washed and incubated with HRP-conjugated streptavidin (2.5  $\mu$ g/ml) for 1 hr at room temperature. Next, the unbound HRP-streptavidin was washed out with TTBS and TBS. Finally fluorescent detection was performed using the Cy3 wavelength (550 nm) of a laser scanner (GenePix 4000B, Axon Instrument, Sunnyvale, CA, USA).

#### WESTERN BLOT

Protein samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% acrylamide gel and then transferred to nitrocellulose membranes (Amersham, UK) for 1 h at 300 mA in transblotting buffer (0.2 M glycine-HCl, 25 mM Tris-base, and 20% (v v<sup>-1</sup>) methanol). To block non-specific antibody binding, membranes were incubated for 1 h in blocking buffer (PBS pH7.4, 0.1% Tween-20) containing 5% (w v<sup>-1</sup>) non-fat dry milk. Membranes were then incubated overnight at 4°C with the IL-6 polyclonal antibody (Santa Cruz, CA, USA) or VEGF polyclonal antibody (Peprotech, NJ, USA) used at a dilution of 1 : 1000 in blocking buffer where non-fat milk was replaced with 5% BSA. Membranes were washed with blocking buffer for 3  $\times$  5 min and incubated with 1 : 1500 dilution of alkaline phosphatase-conjugated anti-rabbit secondary antibody, washed and protein detection was carried out using CDPStar™ chemiluminescent reagent. Membranes were drained from excess developing solution and exposed to Kodak X-OMAT-S film.

#### STATISTICAL ANALYSIS

One-way ANOVA was used to determine if significant differences existed between PA treated and AOO treated mice (SPSS for Windows, Release 10.10, Standard Version, Chicago, IL, USA). In addition, differences in the responses of C57BL/6 and BALB/c mice were evaluated using a Post-Hoc test (SPSS for Windows, Release 10.10, Standard Version) of the variance and significance levels. All values are reported as the mean  $\pm$  SEM. A *p* value of <0.05 was considered significant.



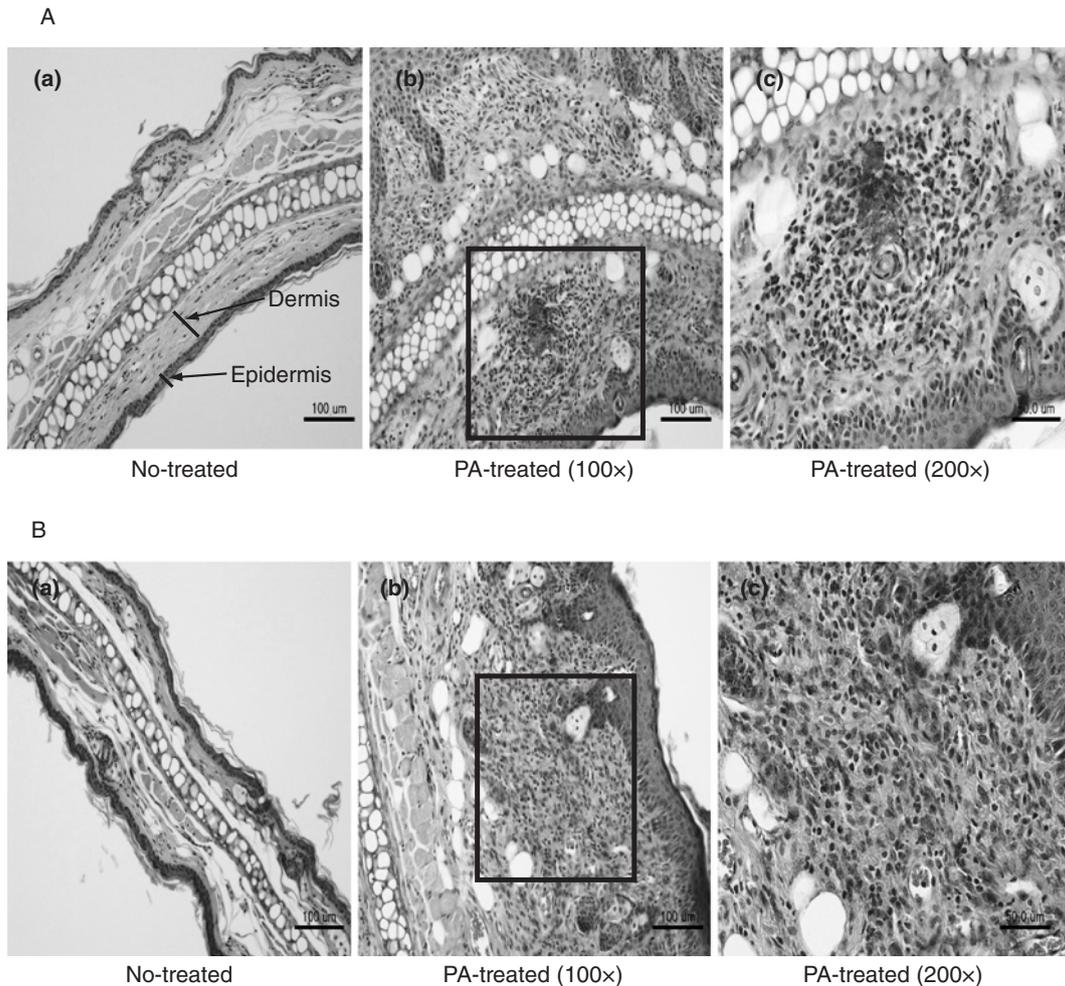
**Fig. 1** Differences in the response of C57BL/6 and BALB/c mice to PA treatment based on the ear thickness and skin score. PA solution was repeatedly applied to the dorsum of the ear and shaved back skin of C57BL/6 and BALB/c mice. After 4 weeks, the difference in the skin irritation between C57BL/6 and BALB/c mice was determined based on the ear thickness (A) and back skin score (B) using the procedure described in the Methods. Data shown are the means  $\pm$  SEM ( $n = 6-8$ ). \* $p < 0.05$  when compared with BALB/c mice (One-way ANOVA).

## RESULTS

### INDUCTION OF AN ALLERGIC SKIN INFLAMMATION BY PA TREATMENT

It has previously been reported that PA induced a skin irritation in various strains of mice.<sup>1,11</sup> Therefore to investigate the difference in the skin irritation response between BALB/c and C57BL/6 mice, indicators of skin irritation including ear thickness, skin score and myeloperoxidase (MPO) activity were measured in both strains of mice following topical application of PA solution for 4 weeks. First, the ear thickness was measured in the ears of both C57BL/6 and BALB/c mice using a thickness gauge. As shown in Figure 1A, the ear thickness of both mice increased steadily in the PA-treatment group with time, while it did not change in the AOO-treatment group. In addition, the rate of the increase in ear thickness was greater in C57BL/6 mice than in BALB/c mice in the PA-treatment group. Second, the skin score was used to determine the degree of the erythema/hemorrhage, edema, excoriation/erosion and dryness on the back skin of both C57BL/6 and BALB/c mice. Following PA treatment, the skin scores increased significantly in both strains of mice; however, the rate of increase was higher in C57BL/6 mice than in BALB/c mice (Fig. 1B).

Next, to identify the induction of the skin irritation, histological analysis of the ear tissue of C57BL/6 and BALB/c mice was performed. Potent inflammatory changes were observed in the PA treatment group, but not in the AOO treatment group. Furthermore, in the PA treatment group, the edema, epidermal hyperplasia and a large number of inflammatory cells were detected in both strains of mice (Fig. 2). However, enumeration of the infiltration of inflammatory leukocytes into the dermis revealed that these changes were more apparent in C57BL/6 mice than in BALB/c mice. Finally, neutrophils infiltration was detected in the ear homogenates of both mice, which indicates that neutrophils infiltration was involved in the inflammatory process. In this process, neutrophils destroy infectious pathogens via the specific enzyme, MPO, which catalyzes the reaction of hydrogen peroxide with chloride ions to produce the strongly antiseptic hypochlorite ion. Therefore, the magnitude of neutrophil infiltration was indirectly detected as the measured quantity of the MPO activity.<sup>12</sup> In the present study, MPO activity increased in the PA treatment group, but was unchanged in the AOO treatment group. Furthermore, the increase in MPO activity that was observed in the PA treated group was greater in C57BL/6 mice than in BALB/c mice (Fig. 3). Taken together, these results suggest that PA treatment led to a significant increase in skin irritation in both strains of mice. However, these responses were more sensitive in C57BL/6 mice than in the BALB/c mice.



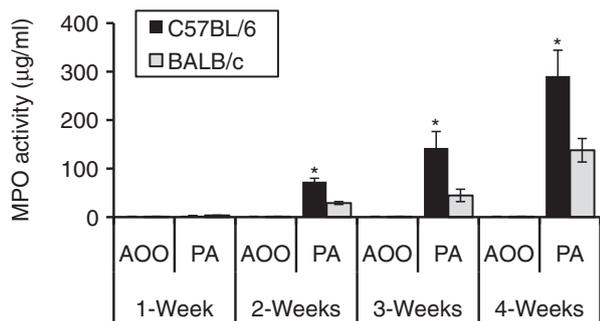
**Fig. 2** Differences in the response of C57BL/6 and BALB/c mice to PA treatment based on the histopathology. PA solution was repeatedly applied to the dorsum of the ear and the shaved back of C57BL/6 (**A**) and BALB/c (**B**) mice. After 4 weeks, histological changes were determined as described in the Methods. The slide sections of ear tissue were stained with hematoxylin & eosin and observed at the original magnification  $\times 100$  or  $\times 200$ . In addition, the rectangle in **A-b** and **B-b** indicated the area of **A-c** and **B-c** showing the enlargement. The scale bar in **A-a**, **A-b**, **B-a** and **B-b** indicate 100  $\mu\text{m}$ , while it indicates 50  $\mu\text{m}$  in **A-c** and **B-c**.

### CHANGES IN THE WEIGHT OF THE AURICULAR LYMPH NODE AND SERUM IgE CONCENTRATION FOLLOWING PA TREATMENT

Next, we investigated whether the cause of differences in the skin irritation between C57BL/6 and BALB/c mice is different allergic response. To accomplish this, we evaluated the auricular lymph node weight and serum IgE concentration in both strains of mice. It is well known that the weight of the local lymph node increases in response to topical application of agents that have allergenic or sensitizing potential.<sup>13</sup> In this study, PA treatment was found to induce an increase in the auricular lymph node weight in both strains of mice; however, no change in weight was observed in response to AOO treatment. Addi-

tionally, the weight of the auricular lymph node was greater in BALB/c mice than in C57BL/6 mice (Fig. 4A).

It is well known that the hyperproduction of IgE is one of the characteristic features of allergic hypersensitivity<sup>14</sup> and an indicator of the magnitude of allergic immune response.<sup>9</sup> Therefore, the serum IgE concentration was measured in both strains of mice at different time points to determine if it contributed to the difference in their response to PA treatment. As shown in Figure 4B, repeated topical application of PA solution induced a significant increase in serum IgE concentrations in both C57BL/6 and BALB/c mice after PA treatment. However, no changes in the IgE concentration were observed in the AOO treatment group. Moreover, the BALB/c mice in the PA



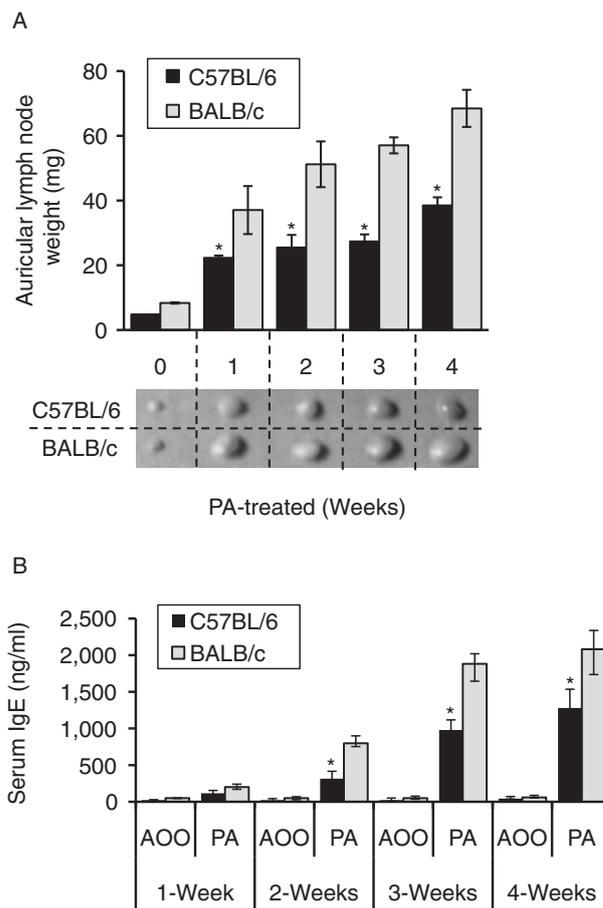
**Fig. 3** Differences in the response of C57BL/6 and BALB/c mice to PA treatment based on the MPO activity. PA solution was repeatedly applied to the dorsum of the ear and the shaved back skin of C57BL/6 and BALB/c mice. After 4 weeks, to measure the MPO activity, the ear tissue was excised from the mice and homogenized, after which the MPO activity was determined as described in the Methods. Data shown are the means  $\pm$  SEM ( $n = 6-8$ ). \* $p < 0.05$  when compared with BALB/c mice (One-way ANOVA).

treatment group were found to have a higher IgE concentration than the C57BL/6 mice (Fig. 4B).

Taken together, these results suggest that the increase in auricular lymph node weight and IgE concentration did not contribute to the skin irritation that was observed in C57BL/6 mice in response to PA treatment.

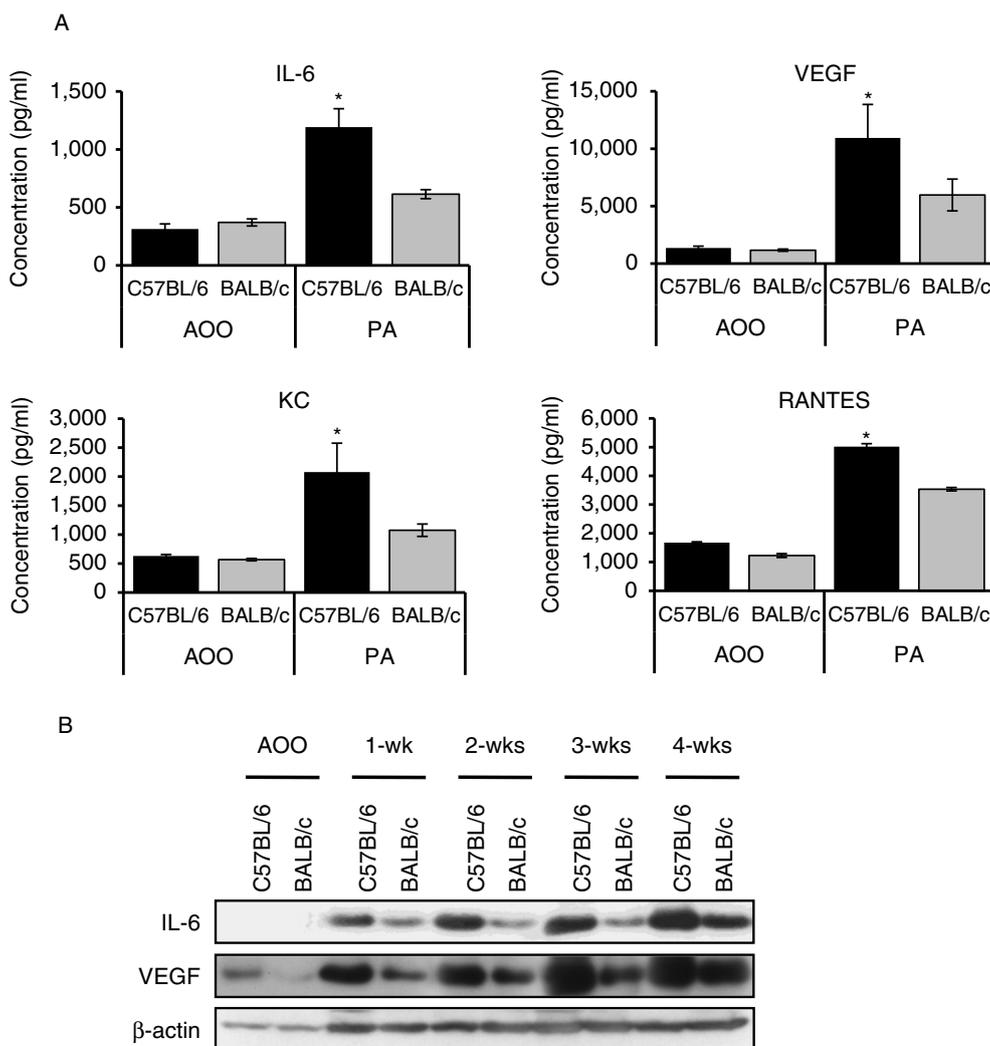
### CHANGES IN THE EXPRESSION OF CYTOKINES AND CHEMOKINES FOLLOWING PA TREATMENT

It has been reported that several cytokines and chemokines could regulate the irritant dermatitis.<sup>15</sup> Therefore, to investigate another cause of the difference in the skin irritation response between BALB/c and C57BL/6 mice, the expression levels of cytokines and chemokines were analyzed in the lysate of ear tissue in C57BL/6 and BALB/c mice using cytokine array assay. After PA treatment, the level of several cytokines and chemokines were significantly increased in both C57BL/6 and BALB/c mice, while others decreased in both strains. However, AOO treatment did not induce any alteration of many cytokines and chemokines (data not shown). Especially, four cytokines and chemokines including IL-6, VEGF, KC and RANTES in all analyzed factors were selected a target for investigating a clue of differential response between C57BL/6 and BALB/c mice. The expression level of these four members was greatly increased in both strains of mice after PA treatment. But, the rate of increase of these four members in response to PA treatment was greater in C57BL/6 mice than in BALB/c mice (Fig. 5A). Furthermore, to make sure these alterations, the protein level of IL-6 and VEGF were directly confirm with the specific antibody for IL-6 and VEGF. Western blot analysis showed that



**Fig. 4** Changes in the auricular lymph node weight and serum IgE concentration in C57BL/6 and BALB/c mice following PA treatment. After PA treatment, the mice were sacrificed under anesthesia. The auricular lymph nodes (A) were then harvested from the neck region of the mice using a microscissor, after which they were weighed using a chemical balance. The serum used to measure the IgE concentration (B) was prepared from blood samples collected from the abdominal vein of the mice. The IgE concentration in serum was quantified by an enzyme-linked immunosorbent assay. Data shown are the means  $\pm$  SEM ( $n = 6-8$ ). \* $p < 0.05$  when compared with BALB/c mice (One-way ANOVA).

the expression level of these two proteins were higher in C57BL/6 mice than that of BALB/c mice (Fig. 5B). As time goes on, the expression level of two proteins were markedly increased. The highest level of these proteins was detected at 4-weeks after PA treatment. Taken together, these results suggest that four cytokines and chemokines may contribute to the difference in the level of skin irritation that is observed in C57BL/6 mice when compared to BALB/c mice following PA treatment.



**Fig. 5** Differences in the response of cytokine expression in ear tissue between C57BL/6 and BALB/c mice following PA treatment. Ear tissues were lysed in 500  $\mu$ L of protein extraction buffer by sonication. The lysates were then collected from the total sonicated-extract and used for a mouse cytokine array assay (**A**) and western blot (**B**) as described in the Methods. \* $p < 0.05$  when compared with BALB/c mice (One-way ANOVA).

## DISCUSSION

Irritant dermatitis occurs as a result of xenobiotic chemicals penetrating the skin and chemically reacting with self proteins, which results in a hapten-specific immune response.<sup>16</sup> In the present study, we observed a difference in the level of skin irritation between C57BL/6 mice and BALB/c mice. Specifically, the ear thickness and back skin score revealed that C57BL/6 mice were more sensitive to PA treatment than BALB/c. We also found that the difference in the skin irritation between C57BL/6 mice and BALB/c mice originated from differences in the expression of several cytokines. To evaluate the magnitude of the local immune reaction of mice in response to topical application of PA, we evaluated the increase in

auricular lymph node weight and serum IgE levels. Allergic response to chemical allergens is generally acknowledged to be dependent on IgE-mediated mechanisms, and the hyper-production of IgE is one of the most characteristic features of allergic response<sup>14</sup>; however, there are differences among species. Because the auricular lymph node weight and serum IgE levels in BALB/c mice were higher than the levels in C57BL/6 following PA treatment, we concluded that the magnitude of the local allergic reaction did not affect the severity of the skin irritation.

Irritant dermatitis is typically characterized by the infiltration of inflammatory cells.<sup>4</sup> Several cytokines and chemokines mediate this infiltration and activation of these inflammatory cells at the site of injury.<sup>15</sup> As seen in Figure 5, the expression of VEGF, KC, and

RANTES in the ear tissue of C57BL/6 and BALB/c mice increased following treatment with PA solution. However, a greater increase in these cytokines and chemokines was observed in C57BL/6 mice than in BALB/c mice. Also, IL-6 increased in only C57BL/6 mice following PA treatment. These findings indicate that these cytokines and chemokines play a role in the degree of skin irritation.

IL-6 induces the infiltration of mononuclear cell and is believed to play an important role in ongoing chronic skin irritation.<sup>4,17</sup> VEGF is a potent mediator of angiogenesis that stimulates the migration and proliferation of endothelial cells, increases vascular permeability and stimulates the expression of intercellular adhesion molecules (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 on endothelial cells.<sup>18-20</sup> Moreover, it has been reported that overexpression of VEGF in the skin of K14/VEGF transgenic mice induces an increase in ear thickness, epidermal thickness, angiogenesis and infiltration of T cells.<sup>21</sup> KC contributes to neutrophil chemotaxis and activation in rodent model of acute inflammation.<sup>22</sup> RANTES belongs to the CC-chemokine family, which induce eosinophil migration into local tissue.<sup>23</sup> These findings explain the observation that the infiltration of inflammatory leukocyte into the dermis was greater in C57BL/6 mice than in BALB/c mice following treatment with PA (Fig. 2).

The results of previous studies have demonstrated that both environmental and genetic factors play a role in the pathogenesis of chronic irritant dermatitis and inflammation diseases such as asthma and atopic dermatitis.<sup>24,25</sup> Furthermore, polymorphisms and mutations of the genes encoding several cytokines are known to influence the progression of irritant dermatitis and inflammation diseases. Moreover, mutations that affect the function of the promoter region of the RANTES and type 2 helper T cell cytokines have been identified in patients with Alzheimer's disease.<sup>26</sup> Based on these findings and the results of the present study, we suggest that such genetic variations may result in differences in the level of cytokine expression in C57BL/6 and BALB/c mice, which could subsequently lead to differences in the skin irritation response.

In conclusion, the results of this study suggest that the difference in skin irritation response between C57BL/6 mice and BALB/c mice did not occur due to differences in the magnitude of local allergic response, but that they did occur in response to differences in the expression of several cytokines and chemokines. Additionally, these biased expressions of cytokines among strains of mice suggest that modulation of these factors may have therapeutic potential for the treatment of chronic skin disease.

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