

Analysis of pulmonary allergic vasculitis with eosinophil infiltration in asthma model of mice

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ABSTRACT

Here the authors report pulmonary allergic vasculitis with eosinophil infiltration in an asthma model of mice and investigated its pathogenesis. C57BL/6 and BALB/c mice were sensitized with ovalbumin (OVA). After the inhalation of OVA, the authors measured the cell number and cytokine concentration in the blood and bronchoalveolar lavage fluid (BALF). The authors also examined the histological changes of the pulmonary. The number of eosinophils increased in the blood and BALF in both strains; however, the number in C57BL/6 in BALF was significantly higher than that in BALB/c. Histological analysis demonstrated severe vasculitis of the pulmonary arteries with derangement of the muscle layer and smooth muscle cell hyperplasia in C57BL/6. Semiquantitative analysis of the severity of vasculitis in the pulmonary arteries revealed that the internal vascular space was highly reduced by smooth muscle hyperplasia in C57BL/6 compared to BALB/c mice. The concentrations of interleukin (IL)-4, IL-5, and interferon (IFN)- γ in BALF of C57BL/6 were significantly high compared to those of BALB/c. C57BL/6 mice exhibited severe allergic vasculitis in the pulmonary arteries compared to BALB/c mice. The high concentrations of IL-4, IL-5, and IFN- γ in the lung may play a critical role in the pathogenesis of allergic vasculitis in C57BL/6 mice.

KEYWORDS allergic vasculitis, C57BL/6, eosinophil, IL-4, IL-5

Pulmonary vasculitis occurs often in various collagen diseases, including systemic lupus erythematosus (SLE), Wegener's granulomatosis (WG), ANCA (antineutrophil cytoplasmic antibody)-related vasculitis, etc.

Allergic granulomatous angitis (AGA) is characterized by bronchial asthma, eosinophilia, and systemic necrotizing vasculitis involving medium- and small-sized vessels with or without granulomas [1–3].

The mechanism of AGA is not completely understood. Eosinophils are the most dominant cells in blood and extravascular tissues in AGA, and are known to release cytotoxic products such as major basic protein, eosinophil-derived neurotoxin, and oxygen radicals [4, 5]. In this regard, endothelial cell injury triggered by eosinophils has been considered to be the initial step toward the vasculitis of AGA [5].

In the current study, we found severe pulmonary vasculitis with extravascular granuloma and eosinophil infiltration in sensitized C57BL/6 after antigen challenge, but the allergic vasculitis was not observed in BALB/c under the same treatment as C57BL/6. To investigate the pathogenesis of the allergic eosinophilic vasculitis in pulmonary arteries, we evaluated the difference between C57BL/6 and BALB/c by analyzing the cell differentials and cytokine concentration in serum and bronchoalveolar lavage fluid (BALF).

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METHODS

Animals

Female C57/BL6 and BALB/c mice (6 to 8 weeks old) were purchased from Japan SLC (Shizuoka, Japan). The mice were housed under specific pathogen-free conditions following a 12-hour light-dark cycle and fed a standard laboratory diet and given water *ad libitum*. All experiments described in this study were performed according to the guidelines for the care and use of experimental animals as determined by the Japanese Association for Laboratory Animals Science in 1987.

Immunization and aerosolization protocol

The mice were sensitized according to the methods described in a previous paper [6]. In brief, the mice were sensitized at days 0 and 5 of the protocol by an intraperitoneal injection of 0.5 mL aluminum hydroxide-precipitated antigen containing 8 μ g ovalbumin (OVA) (Sigma Chemical, St. Louis, MO) adsorbed overnight at 4°C to 4 mg of aluminium hydroxide (Wako Chemical, Tokyo, Japan) in phosphate-buffered saline (PBS). Twelve days after the second immunization, the mice were divided into 2 groups that consisted of 6 animals each. The C57/BL6 and BALB/c mice of the first group were placed in a plastic chamber (10 cm $\times$ 15 cm $\times$ 25 cm) and exposed to aerosolized OVA (5 mg/mL in 0.9% saline) for 1 hour. The C57/BL6 and BALB/c mice of the second group were exposed to 0.9% saline only. The aerosolized OVA was produced by a Pulmo-Aide Compressor/Nebulizer (Devilbiss) (Sunrise Medical HHG, Somerset, PA) at a flow rate of 5 to 7 L/min.

We performed this experiment 3 times. Therefore, a total of 18 mice in each group were examined in this study.

Collection and measurement of specimens

After being exposed to aerosolized saline or OVA every day over 1 week, the mice of both groups were killed by cutting the femoral artery on the 1st, 3rd, and 7th days, 24 hours after the final inhalation, and the blood, BALF, and lung tissue were collected. To collect BALF, the lungs were dissected and the trachea was cannulated with a polyethylene tube (Becton Dickinson, Sparks, MD). The lungs were lavaged twice with 0.5 mL PBS, and $\sim$ 0.8 mL of the instilled fluid were consistently recovered. The recovered fluid was centrifuged (300 $\times$ *g* for 6 minutes) and the cells were resuspended in 0.5 mL PBS. The total number of cells was counted using

an improved Neubauer hemocytometer chamber. An air-dried slide preparation was made of each sample containing 10,000 cells by cytopspin (Cytocentrifuge, Sakura Seiki, Tokyo, Japan) and stained with May-Grunwald-Giemsa stain. Differential counts of at least 500 cells were made according to standard morphologic criteria. The numbers of cells recovered per mouse were then expressed as the mean and standard error of the mean (SEM) for each treatment group.

After centrifugation, supernatants were stored at -80°C for measurement of the cytokines. After harvesting BALF, lungs were fixed with 10% neutral-buffered formalin and were embedded in paraffin. In addition, hearts, livers, kidneys, and spleens were also fixed with 10% neutral-buffered formalin. These 3- μ m-thick sections were stained with hematoxylin-eosin (H&E) and elastica Masson's trichrome (EM). Total white blood cells were counted by flow cytometry and the cell differentials were determined under microscopy with Giemsa staining.

Measurement of IgE

Serum immunoglobulin E (IgE) levels were determined with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Yamasa, Chiba, Japan).

Antibodies and immunohistochemical staining

We adopted the biotin-streptavidin system using a Histofine Kit (Nichirei, Tokyo, Japan) for the immunohistochemical staining. The sections were deparaffinized and treated with 0.3% hydrogen peroxide in methanol for 15 minutes to block endogenous peroxidase activity. The sections were incubated with 10% normal rabbit serum for 30 minutes at room temperature to block the nonspecific antibody reaction. We used anti- α -smooth muscle actin antibody (Spring Bioscience, Fremont, CA). The sections were incubated overnight at 4°C with the primary antibody followed by the biotin-streptavidine system, then 3,3'-diaminobenzidine (DAB) was used as the chromogenic substrate.

Semiquantitative analysis of pathological changes in the pulmonary arteries

The extent of histological changes in pulmonary arteries (diameter 10 to 50 μ m) was assessed. Lungs were fixed in periodate-lysine-paraformaldehyde (PLP) for 2 hours at 4°C and embedded in paraffin. The tissue was cut into sections 3 m thick, stained

with hematoxylin-eosin, and evaluated by light microscopy. A histological score for small pulmonary arteries in each lung was determined according to the following criteria: 0 = no abnormality; 1 = minimum; 2 = mild: shedding of endothelial cells, no change of the vascular smooth muscle layer and mild perivascular cell infiltration were observed; 3 = moderate: shedding of endothelial cells, thickening of the vascular smooth muscle layer and moderate perivascular cell infiltration were observed; and 4 = severe: disruption of internal elastic laminae, proliferation of mesenchymal cells in the intraluminal space in pulmonary arteries, and moderate to severe perivascular cell infiltration. The severity was judged by the extent of endothelial injury, vascular smooth muscle cell proliferation, loss of vascular wall integrity, and perivascular cell infiltration. We scored 5 vessels whose diameters ranged from 20 to 50 μm in each of the lung tissue sections and its average was determined as the histological index of one mouse.

Cytokine measurement

Serum and BALF were used for the measurement of interleukin (IL)-2, IL-4, IL-5, tumor necrosis factor (TNF)- α , and interferon (IFN)- γ using a cytokine bead kit (Becton Dickinson).

Statistical analysis

Mann-Whitney *U* test was used in the analysis of the results. All values are expressed as means $\pm$ SEM. Values of $P < .05$ were considered statistically significant.

RESULTS

Eosinophil number in blood

After repetitive exposure of OVA, the total white blood cell number increased to 1.6-fold of the control level on the 7th day in both C57/BL6 and BALB/c (Figure 1A). The basal number of white blood cells in BALB/c was higher than that in C57BL/6. The blood eosinophil number increased along with the time course of OVA inhalation. The blood eosinophil numbers in BALB/c were significantly higher during the 7-day period than those in C57BL/6 (Figure 1B).

Increase of eosinophils in BALF after exposure to OVA

After exposure to OVA, the total cell number in BALF increased on the first day and it peaked on the 7th day

in both C57/BL6 and BALB/c (Figure 2A, B). Analysis of the cell differentials in BALF revealed that the increase of the absolute number of eosinophils was remarkable in both C57/BL6 and BALB/c (Figure 2A, B). The number of eosinophils in BALF of C57BL/6 was significantly higher than that of BALB/c (Figure 2C).

Histologic changes in pulmonary arteries

Pulmonary arteries (diameter 10 to 50 μm) showed drastic changes histologically from the 1st day of exposure to OVA. Eosinophils adhered to endothelial cells in small arteries and spaces appeared between endothelial cells and the basement membrane in C57BL/6 (Figure 3A,b, B). On the 3rd day, intimal thickening with spindle cells was prominent and almost all small pulmonary arteries were occluded (Figure 3A.c, C.b). Extravascular granuloma with eosinophil infiltration was observed (Figure 3C.a). Pulmonary arteries were highly occluded on the 3rd and 7th days of OVA exposure. On the 7th day, small pulmonary arteries were occluded with the cellular component and collagen deposition was observed in the lumen of arteries in C57BL/6 (Figure 3A.d, 3C.b). In contrast, in BALB/c, the histological changes in pulmonary arteries were mild (Figure 3A.f, A.g) and mild hypertrophy of the smooth muscle cell layer in the vessel lumen was observed (Figure 3A.h).

On the 3rd and 7th days with OVA exposure, vessel lumina of small-sized pulmonary arteries were occupied by the increased cellular component. The immunohistochemistry revealed hyperplasia of intraluminal myocytes with positive α -actin in the luminal space of obstructed pulmonary arteries (Figure 3D).

Semiquantitative analysis of the histological changes in pulmonary arteries

Semiquantitative analysis revealed that the histological vascular changes started on the 1st day of OVA exposure and almost all small pulmonary arteries were highly obstructed due to the accumulation of cellular components, with the disruption of endothelial basement membrane and internal elastic laminae on the 3rd and 7th days in C57BL/6 (Figure 4). Compared with C57BL/6, the basic structure of pulmonary arteries was maintained and obstructive changes with mural destruction were mild in BALB/c (Figure 4).

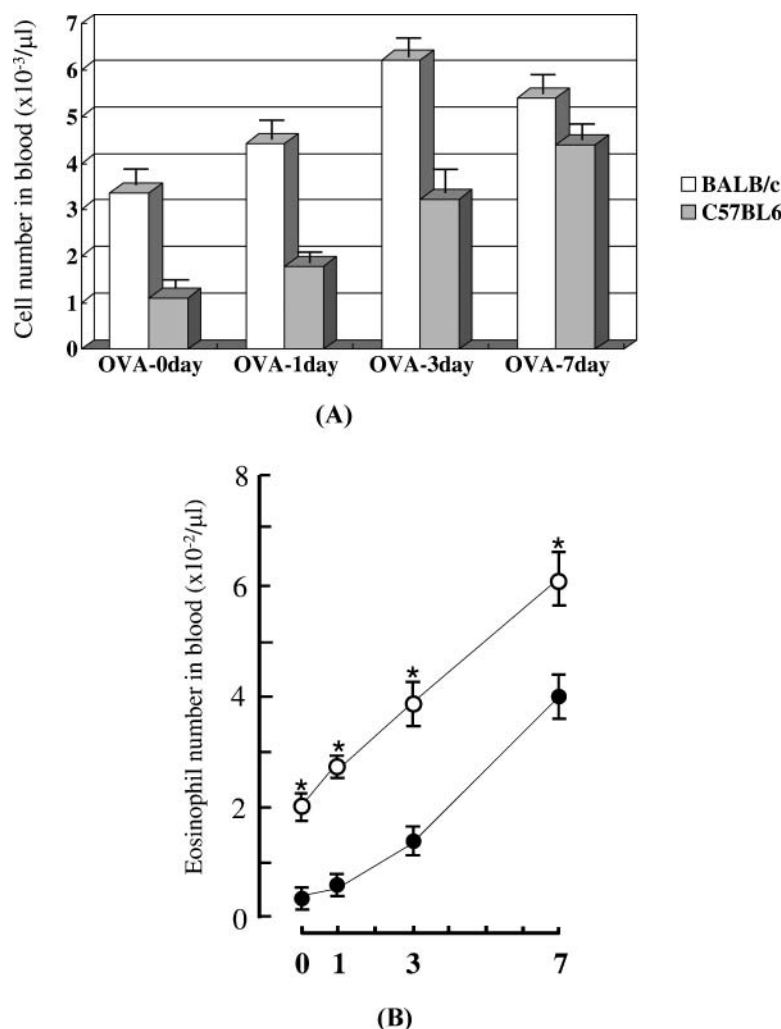


FIGURE 1 (A) Total white cell numbers in blood during the course of OVA inhalation. Open columns: BALB/c ($n = 18$); shaded columns: C57BL/6 ($n = 18$). All data are given as mean $\pm$ SEM. (B) Eosinophil numbers in blood during the course of OVA inhalation. Open circles: BALB/c ($n = 18$); closed circles: C57BL/6 ($n = 18$). All data are given as mean $\pm$ SEM. * $P < .01$ compared with C57BL/6.

Cytokines in serum and BALF after exposure to OVA

Cytokine measurement revealed that the IL-4 concentrations in the BALF of both strains had increased markedly. The IL-4 concentrations on the 3rd and 7th days in the BALF of C57BL/6 were significantly higher than those of BALB/c (Figure 5A.a). The IL-4 concentrations in sera showed a mild increase in both strains and no significant difference between these two strains (Figure 5A.b). The IL-5 concentrations in BALF of both strains had increased markedly. Especially, the IL-5 concentration on the 3rd day in the BALF of C57BL/6 was significantly higher than that of BALB/c (Figure 5B. a). The IL-5 concentrations in sera showed a mild increase on the 1st and 3rd days

in both strains and no significant difference between these 2 strains (Figure 5B.b). The IFN- γ concentrations in the BALF of both strains increased markedly. The IFN- γ concentrations on the 3rd and 7th days in the BALF of C57BL/6 were significantly higher than those of BALB/c (Figure 5C.a). In contrast, the IFN- γ concentrations in the sera of both strains did not increase (Figure 5C.b).

IgE level in serum after exposure to OVA

IgE levels in sera increased until the 7th day during daily exposure of OVA to both C57BL/6 and BALB/c. There was no significant difference observed between the 2 strains (Figure 6).

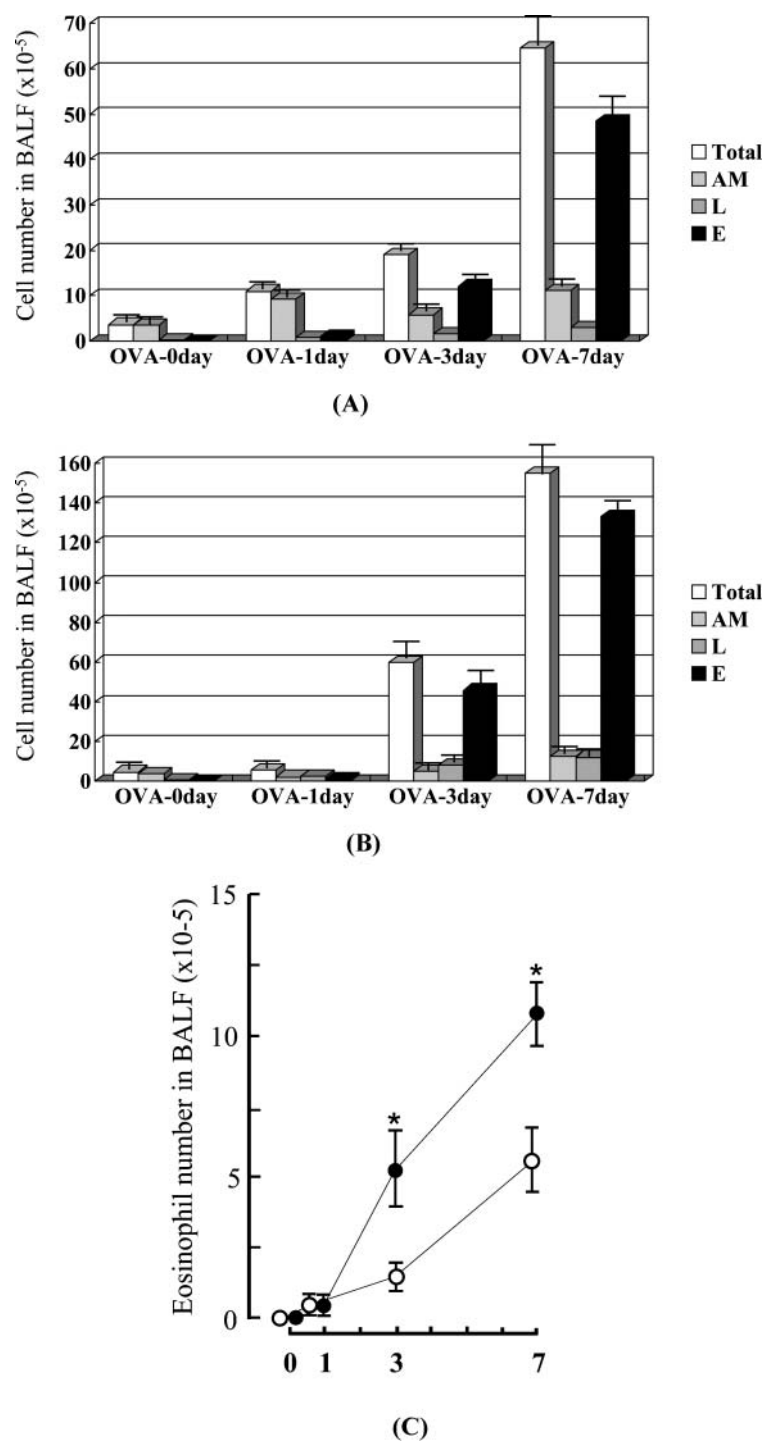


FIGURE 2 (A) Cell differentials and numbers in the BALF of BALB/c ($n = 18$) during the course of OVA inhalation. Open columns: total cells (Total); dotted columns: alveolar macrophages (AM); hatched columns: lymphocytes (L); closed columns: eosinophils (E). All data are given as mean $\pm$ SEM. (B) Cell differentials and numbers in the BALF of C57BL/6 ($n = 18$) during the course of OVA inhalation. Open columns: total cells; dotted columns: alveolar macrophages (AM); hatched columns: lymphocytes (L); closed columns: eosinophils (E). All data are given as mean $\pm$ SEM. (C) Eosinophil number in the BALF during the course of OVA inhalation. Open circles: BALB/c ($n = 18$); closed circles: C57BL/6 ($n = 18$). All data are given as mean $\pm$ SEM. * $P < .01$ compared with BALB/c.

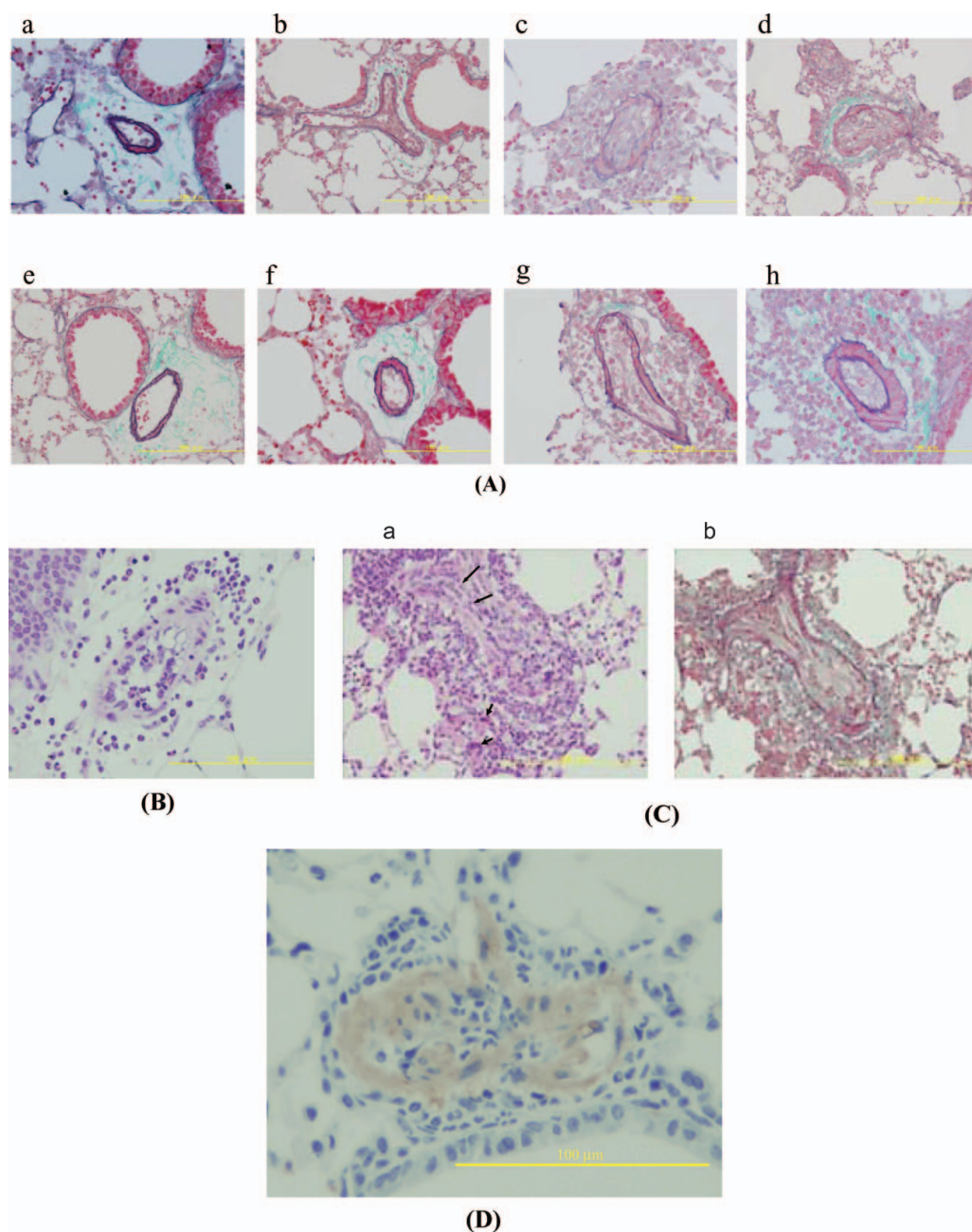


FIGURE 3 (A) Histological changes in pulmonary arteries during the course of OVA inhalation. (a and e) Before OVA inhalation; (b and f): 1 day after OVA inhalation; (c and g): 3 days OVA inhalation; (d and h): 7 days OVA inhalation. (a–d) C57BL/6; (e, f) BALB/c. Tissue sections were stained with Elastica-Masson. Yellow bars indicate 100 μm or 200 μm . (B) Endothelial damage on the 1st day of OVA inhalation in C57BL/6. Adherence of eosinophils to endothelial cells and edema beneath the endothelial cells were observed (H&E staining). Yellow bar indicates 100 μm . (C) (a) Obliteration of pulmonary artery by mesenchymal cells. Spindle cells occupy the lumen of pulmonary artery (arrows). Infiltration of mononuclear cells and eosinophils, and glaucoma (arrow heads) with multinuclear giant cells were observed around the artery (H&E staining). (b) Spindle cells and green-colored collagen fibers and disrupted internal elastic layer were observed in the lumen of an artery (EM staining). Yellow bars indicate 200 μm . (D) α -Actin-positive cells in the lumen of an artery. Spindle cells in the vessel lumen were stained with anti- α -actin. The yellow bar indicates 100 μm .

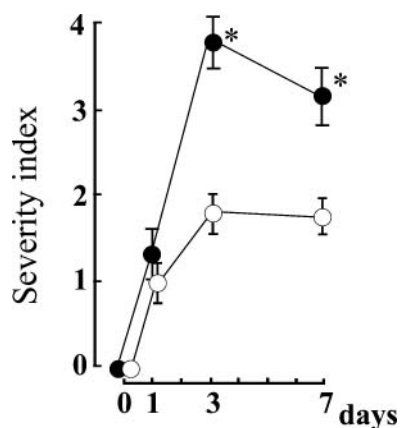


FIGURE 4 Comparison of severity of vascular changes between BALB/c and C57BL/6. Open circles: BALB/c ($n = 18$); closed circles: C57BL/6 ($n = 18$). All data are given as mean $\pm$ SEM. * $P < .01$ compared with BALB/c.

DISCUSSION

We demonstrated drastic obstructive remodeling of small-sized pulmonary arteries in an OVA-sensitized murine model with repetitive OVA inhalation. Besides the obstructive changes, we also found extravascular eosinophil infiltration and granuloma. These pathological changes had some similarity to those of AGA. We believe the data provided by this murine model could contribute to the understanding of the vasculitis in AGA.

To date, there have been relatively few reports concerning experimental models of AGA. Alwie et al. reported a murine model with allergic granulomatous vasculitis by injection of *Ascaris suum* antigen to BALB/c CrSl c mice [7]. They demonstrated fibrinoid vasculitis and marked infiltration of eosinophils in the sites of injection and granulomatous vasculitis in the small pulmonary arteries after 12 weeks. However, the vascular smooth muscle cell proliferation and loss of the vascular structure in their model were not as severe as those observed in our experiment. The strain difference might influence the severity of the vasculitis.

We performed OVA sensitization and repetitive OVA exposure to mice by inhalation. This is an established method for murine models of allergic asthma and BALB/c mice have been widely used for this model due to their Th2 tendency. Whereas we found drastic pathological changes in small pulmonary arteries in C57BL/6 mice during the course of daily inhalation of OVA, in contrast, minimal changes were observed in the arteries of BALB/c

mice. An eosinophilic inflammatory response in pulmonary vessels has been observed in sensitized mice with repetitive allergen inhalation. Tormanen et al. reported the remodeling of pulmonary vessels in OVA exposed BALB/c mice sensitized with OVA [8]. They revealed an increase of the smooth muscle mass in pulmonary arterial walls caused by the cell proliferation. We also confirmed an increase of the smooth muscle cell layer and perivascular eosinophil infiltration in the BALB/c mice in our experiment, in which the protocol of OVA inhalation was similar to that in their paper. However, severe vasculitis was not demonstrated and exaggerated smooth muscle proliferation was not observed in BALB/c mice despite the same experimental protocol used for C57BL/6 mice. These results indicated these two strains of mice showed qualitatively different vascular responses to allergic stimuli.

Strain differences in the biological responses to various stimuli have been reported in experiments with mice. In particular, differences in the airway responses to allergen among mouse strains have been reported [9–11]. BALB/c mice are widely used to study induced airway hyperresponsiveness (AHR) because they generate high titers of OVA-specific IgE and IgG1, eosinophilia in the BAL fluid, and AHR in response to intravenous or inhaled methacholine (MCh) and/or acetylcholine [11–13]. Takeda et al. reported an interesting result in a comparison of airway hyperresponsiveness and eosinophil localization in the lungs between BALB/c and C57BL/6 mice [14]. Despite the larger number of eosinophils in the BALF of C57BL/6 mice, airway hyperresponsiveness increased more in BALB/c mice than in C57BL/6 mice. To elucidate the reason, they measured the eosinophil distribution in the lung tissue and revealed that eosinophils accumulated in both the peribronchial and the peripheral tissue in BALB/c mice; in contrast, their distribution was in the peripheral tissue in C57BL/6. Consistent with those findings, the present study showed that the eosinophil number in the BALF of C57BL/6 mice was significantly higher than that in BALB/c mice, and it also revealed severe cell infiltration including mainly eosinophils in the perivascular space. These results suggested that the strength of the inflammatory reaction and tissue remodeling differs not only depending on the strain of mice but also depending on the tissue and organ. The increased number of accumulated eosinophils in the perivascular space may have contributed to the severity of the vascular wall remodeling.

In the present study, we focused on the pulmonary vasculitis in the 2 strains of mice. They were sensitized with and exposed to OVA according to methods widely used for murine asthma models [6]. Although

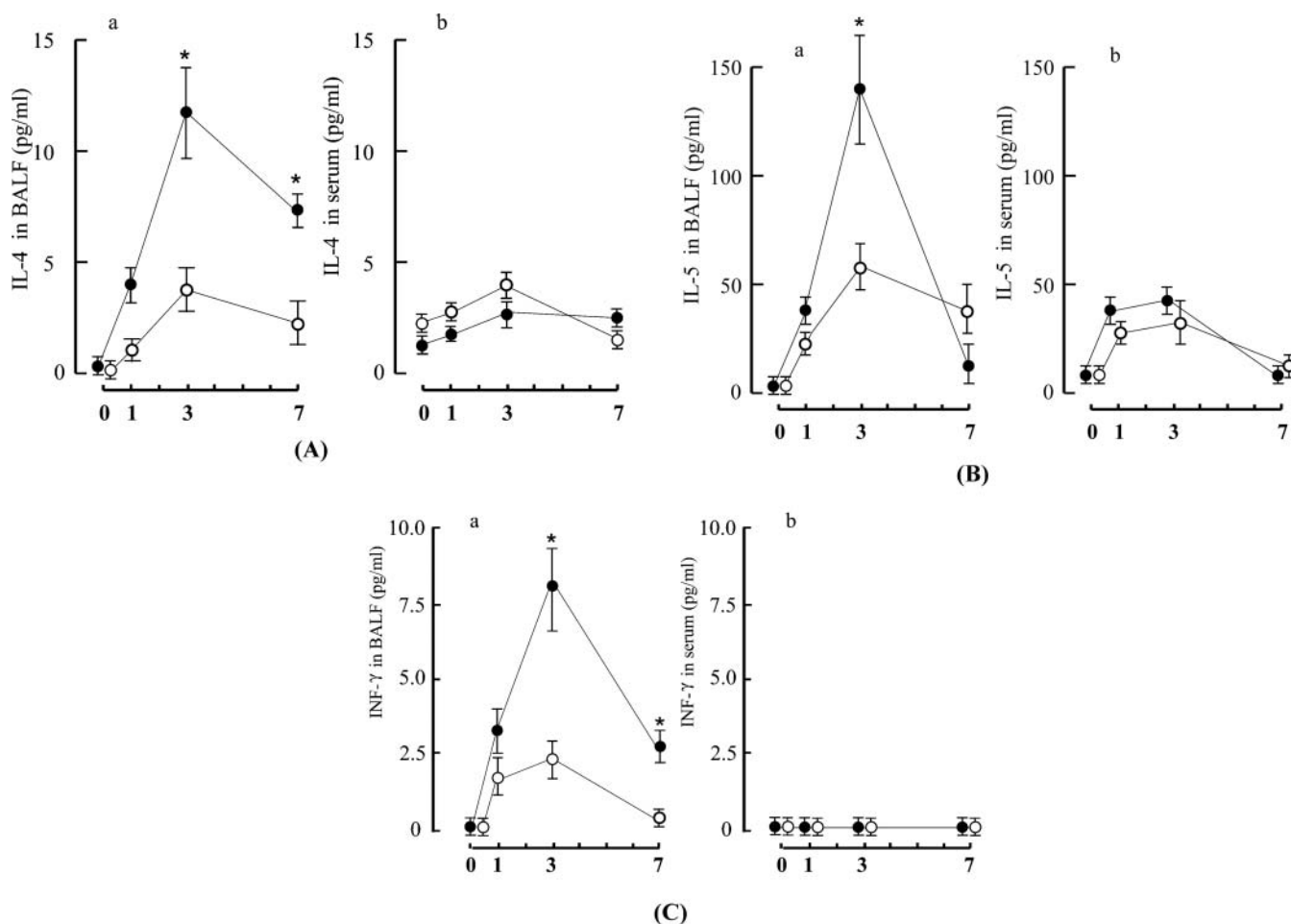


FIGURE 5 (A) IL-4 concentration in the BALF and serum during the course of OVA inhalation. (a) IL-4 concentration in the BALF. (b) IL-4 concentration in serum. Open circles: BALB/c ($n = 18$); closed circles: C57BL/6 ($n = 18$). All data are given as mean $\pm$ SEM. * $P < .01$ compared with BALB/c respectively. (B) IL-5 concentration in the BALF and serum during the course of OVA inhalation. (a) IL-5 concentration in the BALF. (b) IL-5 concentration in serum. Open circles: BALB/c ($n = 18$); closed circles: C57BL/6 ($n = 18$). All data are given as mean $\pm$ SEM. * $P < .01$ compared between the 2 strains. (C) IFN- γ concentration in the BALF and serum during the course of OVA inhalation. (a) IFN- γ concentration in the BALF. (b) IFN- γ concentration in serum. Open circles: BALB/c ($n = 18$); closed circles: C57BL/6 ($n = 18$). All data are given as mean $\pm$ SEM. * $P < .01$ compared between the 2 strains.

we did not investigate the airway responses in this study, asthmatic airway responses were thought to have occurred in both strains of mice as reported previously [6, 14].

C57BL/6 mice are generally recognized as hyporesponders based on their poor IgE response to OVA and low response to bronchoconstrictors [11, 12]; this strain also appears to produce fewer Th2-like cytokines [11, 15]. However, the serum IgE concentration increase was the same in the BALB/c and C57BL/6 mice. Both the IL-4 and IL-5 concentrations in the BALF of C57BL/6 were significantly higher than those of BALB/c in our experiment. On the other hand, there was no difference in these cytokine concentrations in the serum between C57BL/6 and BALB/c. These results suggested that the high levels of IL-4 and IL-5 produced in the lung might

play a role in the exaggerated eosinophil infiltration in the perivascular tissue observed in C57BL/6 mice. In addition, the IFN- γ concentration in the BALF of C57BL/6 mice was also significantly higher than that found in BALB/c mice. These results indicated that both Th1 and Th2 responses were activated in the lungs of C57BL/6 mice in the present study. Consistent with the clinical observation, the peripheral blood mononuclear cells (PBMCs) from patients with AGA secreted significantly increased amounts of IFN- γ in response to T cell-specific stimuli compared to healthy controls [16]. IFN- γ has been known as an important Th1 cytokine involved in the formation of granulomatous lesions [17]. However, it is difficult to conclude that the high concentration of IFN- γ may play a critical role in the pathogenesis of AGA. There is a possibility that the

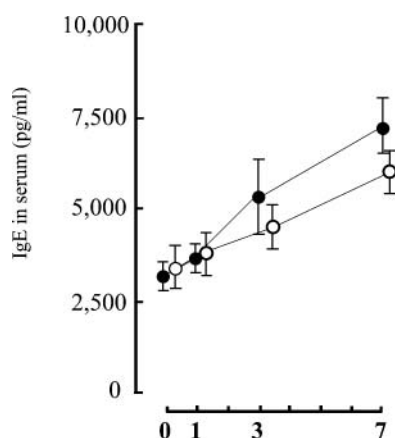


FIGURE 6 IgE concentration in serum. Open circles: BALB/c ($n = 18$); closed circles: C57BL/6 ($n = 18$). All data are given as mean $\pm$ SEM.

increased production of IFN- γ in the lungs of C57BL/6 mice was due to a feedback mechanism involving IL-4 [18]. Further infusion of IFN- γ to C57BL/6 mice in our experiment would likely have led to a reduction of the eosinophil number in the lungs. As a matter of fact, IFN- α administration reduced the eosinophil number in the blood and led to a clinical improvement of the patients with AGA [19–21]. We need to elucidate further the role of IFN- γ in our model with infusion of IFN- γ or using IFN- γ knockout mice.

Because myeloperoxidase (MPO)-specific antineutrophil cytoplasmic autoantibodies (MPO-ANCAs) are known to be elevated in some patients with AGA, MPO-ANCA has been thought to be a causal factor of vasculitis in some cases of AGA [22, 23]. To detect MPO-ANCA in the sera of the C57BL/6 mice with severe pulmonary eosinophilic vasculitis, we made a recombinant murine myeloperoxidase according the previous report [24] and tried to detect a precipitating antibody in serum with that recombinant protein. However, we could not detect an antibody against the recombinant murine myeloperoxidase in the serum of the mice (data not shown). Further study to determine the existence of MPO-ANCA in mice with pulmonary eosinophilic vasculitis, as shown in current study, is needed.

Although the pathological changes in pulmonary arteries, including eosinophil infiltration, vasculitis, and granuloma formation, in this study were similar to those found in AGA, there are differences in the findings between the allergic vasculitis in the present study and AGA. Firstly, the allergic vasculitis occurred only in the pulmonary arteries in our model. Because the mice were exposed to allergen by

inhalation, the allergen was thought to have accumulated mainly in the airways. In this regard, we think that the accumulated allergen in the lung may have caused the immune response in the pulmonary vessels. In another experiment, we administered OVA to sensitized mice systemically via intraperitoneal injection. However, vasculitis was not found in the organs, including the lung, the heart, the liver, the spleen, and the kidney (data not shown). In future experiments, it will be necessary to change the methods of allergen administration and to examine the occurrence of allergic granulomatous vasculitis in other organs. Secondly, the present study revealed that the blood eosinophil counts were not as high as seen in AGA. In addition, the levels of Th2 cytokines such as IL-4 and IL-5 were also not high in the blood. In contrast, we observed increased numbers of eosinophils and increased levels of IL-4 and IL-5 in the BALF. The localization of high concentrations of IL-4 and IL-5 might have contributed to allergic vasculitis that was limited to the lung in our study. Despite some differences between AGA and allergic vasculitis in our experimental model, this model will be useful for understanding the pathogenesis of AGA through comparisons of various factors between C57BL/6 and in BALB/c mice.

In summary, allergic vasculitis with eosinophilic infiltration in pulmonary arteries occurred in C57BL/6 mice sensitized with OVA followed by repetitive inhalative exposure to OVA. The high concentrations of IL-4, IL-5, and IFN- γ in the BALF might have influenced the pathogenesis of this allergic vasculitis.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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