

Th2-Biased Immune Responses Are Important in a Murine Model of Chronic Hypersensitivity Pneumonitis

Keiko Mitaka Yasunari Miyazaki Makito Yasui Masashi Furuie Shuji Miyake
Naohiko Inase Yasuyuki Yoshizawa

Department of Integrated Pulmonology, Tokyo Medical and Dental University, Tokyo, Japan

Key Words

Chronic hypersensitivity pneumonitis • Th2 • Fibrogenesis • A/J mouse

Abstract

Background: Chronic hypersensitivity pneumonitis (HP) can lead to irreversible pulmonary fibrosis. A good animal model is essential to elucidate the mechanisms of this disease. We previously reported that a Th2 predominance may play an important role in the fibrogenesis in chronic HP patients. A study was undertaken to evaluate whether Th2-biased immune responses were crucial during the processes of lung fibrosis in a murine model of chronic HP. **Methods:** Instillation of pigeon dropping extracts (PDE) was conducted 3 days a week for 6 or 12 weeks in C57BL/6, BALB/c and A/J mice to establish models of chronic HP. We evaluated the histopathological features, immunohistochemistry, collagen content, bronchoalveolar lavage fluid (BALF) profiles and Th1/Th2 cytokines in BALF or lung tissue with RT-PCR and ELISA. **Results:** Thickening of the alveolar walls and structural alterations were observed only in the A/J mice after 12 weeks of exposure to PDE. The fibrosis scores were significantly increased in 12-week A/J mice compared to those in the other strains. Immunohistochemistry evaluation showed that PDE was engulfed by alveolar macrophages that were incorporated into the alveolar septa of 12-week A/J

mice. Interleukin (IL)-4 mRNA increased significantly in 6- and 12-week A/J mice. IL-13 mRNA showed a significant increase in 12-week A/J mice compared with 6-week A/J mice. TGF- β 1 mRNA at 12 weeks was significantly increased in A/J mice compared with the other groups. **Conclusion:** Th2-biased genetic backgrounds may play an important role in fibrosing processes in the present chronic HP model.

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Introduction

Chronic hypersensitivity pneumonitis (HP) is a chronic interstitial lung disease caused by the repeated inhalation of various antigens contained in organic dusts. Bird fancier's lung (BFL) is a form of HP resulting from exposure to airborne avian antigens [1, 2]. Patients with BFL sometimes present with acute HP but more frequently present with 'chronic' HP because exposure to birds tends to involve chronic exposure to small amounts of antigens [3]. The histopathological patterns of chronic BFL, according to the 2002 American Thoracic Society/European Respiratory Society consensus classification of idiopathic interstitial pneumonias, are significantly correlated with the clinical course of the disease, including responsiveness to treatment and prognosis [4, 5].

Although exposure to the various antigens that cause HP is widespread, the number of individuals who develop the disease is relatively low, suggesting that host genetic factors are critical in the development of HP. Several recent studies using animal models of acute HP, which are characterized by granulomatous and inflammatory bronchioloalveolitis, have suggested that acute HP is mediated by Th1 immune responses [6, 7]. Th1-prone C57BL/6 mice are more susceptible to HP than Th2-prone DBA/2 mice after exposure to *Saccharopolyspora rectivirgula*, the causative agent in farmer's lung [8]. However, these are models of acute HP without fibrosis, as fibrosis is not seen in clinical cases of acute HP. Recently, a Th2-immune polarization was reported to play an important role in the fibrogenesis of idiopathic pulmonary fibrosis. Th2 cytokines, such as interleukin (IL)-4 and IL-13, enhance fibrotic processes by activating fibroblast proliferation and collagen production, whereas interferon (IFN)- γ , a Th1 cytokine, inhibits these processes [9–14]. We recently reported that Th2-biased immune responses of chemokines were observed in the usual interstitial pneumonia-like lesions of chronic HP patients, as already observed in idiopathic pulmonary fibrosis [15]. Barrera et al. [16] also reported that T cell profiles from patients with chronic HP displayed a Th2-like phenotype with increased CCR4 (a Th2 chemokine receptor) expression and decreased CXCR3 (a Th1 chemokine receptor) expression, higher levels of IL-4 and lower levels of IFN- γ in bronchoalveolar lavage fluid (BALF).

In the present study, we found that strain differences in inbred mice strongly influenced the histopathological changes induced by sensitization with a Th2-dominant adjuvant followed by the instillation of pigeon antigens. This chronic HP model could be a useful tool for examining the role of specific immune responses, especially the balance of Th1 and Th2 immune responses in the development of lung fibrosis.

Materials and Methods

Animals

Specific-pathogen-free 8-week-old female C57BL/6, BALB/c and A/J mice were purchased from Sankyo Medical Animal Supply (Sankyo Lab. Co., Tokyo, Japan). Mice weighing 18–24 g were housed in plastic cages at the animal facility of the Tokyo Medical and Dental University and provided with standard mice food and water. The animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee.

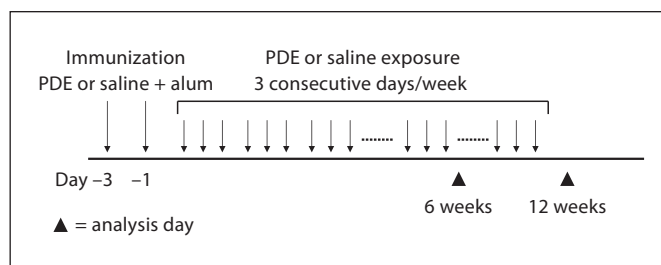


Fig. 1. Protocols for immunization with PDE-alum and subsequent PDE exposure by instillation 3 days a week for 6 weeks and 12 weeks. Mice were sacrificed 48 h after the last PDE challenge.

Preparation of Pigeon Dropping Extracts

Pigeon dropping extracts (PDE) were obtained according to a previously described method [17]. Briefly, fresh pigeon droppings were stirred with a 20-fold volume of phosphate-buffered saline solution (PBS; pH 7.4) for 24 h, followed by dialysis against distilled water. The extracts were then sterilized by filtration (Millex-GV, Millipore, Bedford, Mass., USA) and lyophilized.

Experimental Protocol: Induction of HP

Alhydrogel (alum; Pierce, Rockford, Ill., USA) was mixed with a predetermined quantity of PDE or saline and incubated at room temperature for 30 min. The mice were immunized and boosted by intraperitoneal injection with 8 μ g of PDE adsorbed onto alum (fig. 1). They were then placed under light anesthesia by diethyl ether inhalation, and 8 μ g of PDE dissolved in 40 μ l of 0.9% saline was applied to the tips of their noses, which they inhaled involuntarily. Instillation was conducted 3 days a week for either 6 or 12 weeks. Control mice were injected with a physiologic saline solution adsorbed onto alum, then anesthetized and given 40 μ l of physiologic saline solution in the same manner that PDE was administered to mice in the study groups.

Bronchoalveolar Lavage

A plastic cannula was inserted into the right main bronchus. Bronchoalveolar lavage samples were obtained by washing the right lung with 0.5-ml aliquots of 0.9% saline 3 times. After centrifugation, bronchoalveolar lavage cell pellets were washed and resuspended in PBS, and total cell counts were determined. Cyto-spin preparations were fixed and stained using Diff-Quick (Dade, Behring, Deerfield, Ill., USA), and differential cell counts were analyzed.

Measurement of Anti-PDE IgG in Serum

Antibody against PDE in sera was measured with an ELISA [18]. Each well of polystyrene plates (Immulon 2; Dynatech Laboratories, Alexandria, Va., USA) was coated with 100 μ l of 1 mg/ml PDE in a carbonate buffer (pH 9.6) at 4°C overnight. After washing the wells 3 times with PBS with 0.05% polysorbate-20 (Tween-20; PBS-T), each well was treated with 0.05% bovine serum albumin-PBS-T to block the free binding surface of the wells. One hundred microliters of 1:4,000 diluted samples were added to each well and incubated at 37°C for 1 h. After washing the wells with PBS-T, 100 μ l of a 1:5,000 diluted solution of rabbit anti-

mouse IgG coupled with horseradish peroxidase was added and incubated at room temperature for 1 h. After washing the wells again, the substrate was added, color was developed and optical density was measured at 490 and 620 nm.

Histopathological Analysis

The left lung was instilled with 10% buffered formaldehyde through the left main bronchus under constant pressure (10 cm water pressure) for 1 h, then fixed in 10% buffered formaldehyde. The sections were embedded in paraffin, cut into 4- μ m-thick sections and stained with hematoxylin and eosin or elastica-Van Gieson stain to detect collagen deposition. The sections were evaluated under light microscopy.

Semiquantitation of Histological Findings

For the semiquantitative histological analysis of fibrotic changes induced by PDE, a numerical Ashcroft fibrosis scale was adapted [19]. Twenty fields on each lung section were observed with a $\times 10$ objective in each successive field, and each field was assessed individually for the severity of fibrotic changes and allotted a score from 0 (normal) to 8 (total fibrosis) using a predetermined scale of severity. The mean of the scores from all fields was calculated as the fibrosis score. In order to prevent observer bias, all histological specimens were randomly numbered and interpreted in a blinded fashion. Each specimen was scored independently by two observers (K.M. and Y.M.). The means of their individual scores were considered the fibrosis scores. Kappa scores were calculated to evaluate interobserver agreement. We randomly selected 30% of the fields to use for reliability coding.

Preparation of Polyclonal Antibody to PDE

Polyclonal antiserum to PDE was prepared according to the method described by Coligan [20]. On day 1, a rabbit was injected with a 1:1 mixture of PDE and Freund's complete adjuvant (Sigma Chemicals, St. Louis, Mo., USA). On days 29 and 58, the rabbit was again injected with the PDE-Freund's complete adjuvant mixture. The rabbit was then bled on day 68, and polyclonal antiserum was stored frozen until used. The serum was purified using an IgG purification kit (ImmunoPure Protein A IgG Purification Kit; Pierce).

Immunohistochemical Analysis

We performed immunohistochemistry on the lung specimens from each strain. We examined the localization of PDE using routine immunohistochemical techniques. Paraffin-embedded 4- μ m sections were autoclaved in an antigen unmasking solution (Vector Laboratories, Burlingame, Calif., USA). Endogenous peroxidase was blocked with 3% H₂O₂. Endogenous avidin or biotin activity was blocked with an Avidin/Biotin Blocking kit (Vector Laboratories). Sections were incubated first with anti-PDE polyclonal IgG, then with biotinylated secondary antibodies (goat anti-rabbit IgG, Vector Laboratories). After incubation with an ABC kit (Vector Laboratories), DAB (Nichirei, Tokyo, Japan) was added. Tissue sections were counterstained with Mayer's hematoxylin and mounted with a cover slip. Nonimmune rabbit IgG was applied to lung sections as a negative control.

Measurement of Collagen Content of the Right Lung

Collagen content of the right lung was determined using the Sircol Collagen Assay kit (Biocolor, Belfast, Northern Ireland, UK) according to the manufacturer's instructions.

Cytokine Assay of BALF

Cytokine levels of IL-4, IL-13, TGF- β 1 and IFN- γ in supernatants from BALF were measured using ELISA kits (Biosource, Camarillo, Calif., USA). These data were adjusted to levels of total protein in BALF measured by the Bio-Rad Protein Assay Kit (Bradford method; Bio-Rad Laboratories, Tokyo, Japan).

Analysis of mRNA Expression by Real-Time Quantitative Polymerase Chain Reaction

The expression levels of IL-4, IL-13, TGF- β 1, TNF- α and IFN- γ genes were determined by a quantitative real-time reverse transcription-polymerase chain reaction (PCR). For real-time PCR, total RNA was extracted from the right lung with TRIzol (Invitrogen, Carlsbad, Calif., USA). Genomic DNA was removed with the Qiagen RNeasy kit (Qiagen Inc., Mississauga, Ont., Canada). RNA was reverse-transcribed with the SuperScript III First-Strand Synthesis Supermix kit (Invitrogen), using oligo-dT as a primer. For IL-4, the forward primer was 5'-GAATGTACCAGG-AGCCATATC-3' and the reverse primer was 5'-CTCAGTACT-ACGAGTAATCCA-3'. For IL-13, the forward primer was 5'-GAGCGAATGGAGTGAAGAGG-3' and the reverse primer was 5'-GCTCAATGTGGGTTCAGGTT-3'. For TGF- β 1, the forward primer was 5'-AGCCCGAAGCGGACTACTAT-3' and the reverse primer was 5'-TCCACATGTTGCTCCACACT-3'. For TNF- α , the forward primer was 5'-CCTCCAGAAAAGACACC-ATGA-3' and the reverse primer was 5'-GGTCTGGGCCATAG-AACTGAT-3'. For IFN- γ , the forward primer was 5'-TGGC-TGTTTCTGGCTGTTACT-3' and the reverse primer was 5'-CTGATGGCCTGATTGTCTTTC-3'. The PCR was performed in the Mini Opticon (Bio-Rad, USA), operated by MJ Opticon Monitor version 3.1 analysis software (Bio-Rad), using ready-made fluorogenic probes (SYBR green; Bio-Rad). Gene expression was quantified relative to the expression of GAPDH, using the $\Delta\Delta$ CT method, according to the manufacturer's protocols.

Statistical Analysis

The results were expressed as means $\pm$ SD, and differences between the groups were analyzed using analysis of variance, followed by Tukey's test. Data lacking a normal distribution were reported as median and range, and differences were evaluated using the nonparametric Kruskal-Wallis test, followed by Dunn's multiple comparison test. Statistical significance was set at $p < 0.05$ bimarginally.

Results

Assessment of Histology in Three Murine Strains

Lung histopathology revealed that PDE exposure was associated with a marked infiltration of inflammatory cells in the peribronchiolar and perivascular areas and lymphoid follicles in all 3 strains after a 6-week exposure. Intra-alveolar accumulations of macrophages were observed, similar to the desquamative interstitial pneumonia pattern (fig. 2B1, C1, D1). Granuloma formations and multinucleated giant cells were present in A/J and BALB/c mice, but not in C57BL/6 mice, after a 6-week exposure

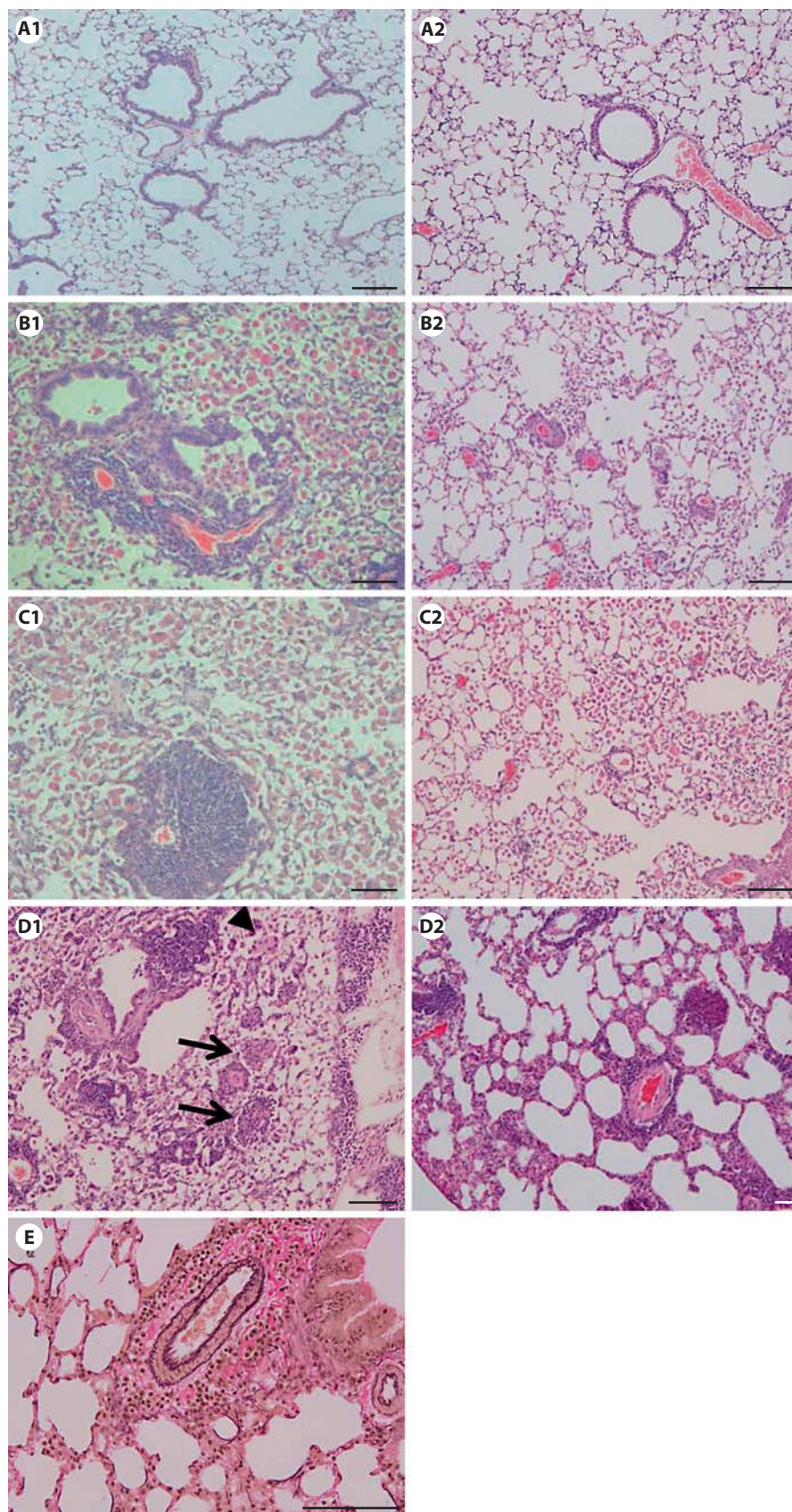


Fig. 2. Representative photos of the lungs. Intranasal PDE caused chronic persistent inflammation, granuloma formation and pulmonary fibrosis in A/J mice. The lungs from the A/J mice were treated with normal saline for 6 weeks (**A1**) or normal saline for 12 weeks (**A2**), then with PDE for 6 weeks (**D1**) or PDE for 12 weeks (**D2**). BL/6 mice were treated with PDE for 6 weeks (**B1**) or PDE for 12 weeks (**B2**), and BALB/c mice were treated with PDE for 6 weeks (**C1**) or PDE for 12 weeks (**C2**). **B2, C2** Large macrophages accumulated in the air spaces, but no significant structural changes were observed in C57BL/6 and BALB/c mice after 12 weeks. **D1** Dense collections of mononuclear cells, granulomas (arrows) and multinucleated giant cells (arrowhead) are present. **D2** Granulomas disappeared and marked thickening of alveolar walls and remodeling of lung architecture were observed in 12-week A/J mice. **E** Collagen deposition was observed in the peribronchial and perivascular areas and alveolar walls of A/J mice. All scale bars = 100 μ m. **A–D** Hematoxylin and eosin stain. Magnification $\times 100$. **E** Elastic-Van Gieson stain. Magnification $\times 200$.

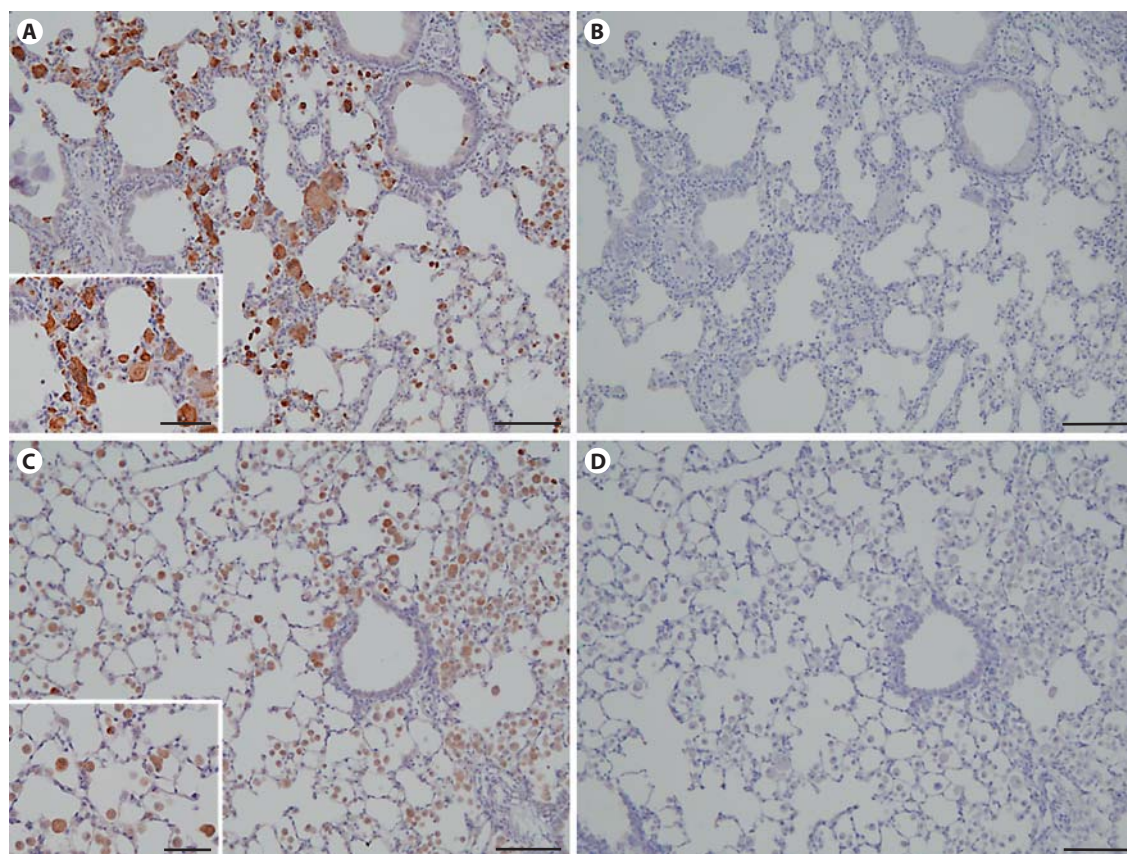


Fig. 3. Representative pictures of immunohistochemistry staining with anti-PDE antibody. Instilled PDE was immunostained with a polyclonal anti-PDE antibody. **A** PDE was observed in alveolar macrophages with robust staining (brown). These macrophages were incorporated into the alveolar septa and seemed to contribute to the development of cellular alveolitis and fibrosis. Inset: high-magnification image. **B** PDE was observed in alveolar mac-

rophages, with weaker staining only in the alveolar lumens. Inset: high-magnification image. **A, B** The lungs from A/J mice treated with PDE for 12 weeks. **C, D** The lungs from C57BL/6 mice treated with PDE for 12 weeks. **A, C** Staining with an anti-PDE antibody. **B, D** Staining with nonimmune rabbit IgG. Scale bars = 100 μ m; 50 μ m in inset images.

(fig. 2B1, C1, D1). After 12 weeks, only the A/J mice showed structural alteration of the alveoli and thickening of the alveolar walls, which are similarly observed in the nonspecific interstitial pneumonia pattern in humans (fig. 2D2). After a 12-week exposure, granulomas disappeared in the BALB/c and A/J mice (fig. 2C2, D2). Lung fibrogenesis was found only in the A/J mice with elastica-Van Gieson staining (fig. 2E). In the immunohistochemical analysis, PDE was observed in the alveolar macrophages of each mouse strain. In 12-week A/J mice, these macrophages were incorporated into the alveolar septa and seemed to contribute to the development of cellular alveolitis and fibrosis (fig. 3A). On the other hand, in the C57BL/6 mice, although PDE was engulfed by alveolar macrophages with weaker staining, it resided only in the

alveolar lumens, not in the parenchyma (fig. 3C). The extent of pulmonary histological abnormalities was quantitatively assessed under a light microscope. The fibrosis scores were significantly increased in the 12-week A/J mice compared with those in the C57BL/6 and BALB/c mice (A/J vs. C57BL/6: 2.00 ± 0.60 vs. 0.70 ± 0.14 , $p < 0.01$; A/J vs. BALB/c: 2.00 ± 0.60 vs. 0.40 ± 0.16 , $p < 0.01$; fig. 4A). There was good agreement between the two observers (Kappa = 0.687, 95% CI = 0.634–0.725). The collagen contents of the right lung after 12 weeks versus 6 weeks of exposure were significantly increased only in the A/J group (A/J: 6 weeks, 3.50 ± 0.20 mg, 12 weeks, 5.50 ± 0.48 mg, $p < 0.01$; C57BL/6: 6 weeks, 3.50 ± 0.30 mg, 12 weeks, 4.10 ± 0.30 mg, $p > 0.10$; BALB/c: 6 weeks, 3.51 ± 0.30 mg, 12 weeks, 3.08 ± 0.13 mg, $p > 0.10$; fig. 4B).

To quantify the presence of inflammatory cells in BALF after the different periods of exposure, the numbers of total cells, macrophages, lymphocytes, neutrophils and eosinophils were counted. PDE exposure resulted in a significant increase in total cells, macrophages, lymphocytes and neutrophils in all three strains compared with controls. The total number of bronchoalveolar lavage cells from A/J mice was increased after a 6-week exposure and further increased after a 12-week exposure (6-week vs. 12-week A/J: $9.6 \pm 4.6 \times 10^4$ vs. $27.2 \pm 8.8 \times 10^4$ cells; $p < 0.01$). In contrast, C57BL/6 and BALB/c mice showed no persistent increases in total cell numbers after a 12-week exposure, despite being challenged using the same protocol (fig. 5A). We found that more severe and persistent lymphocytic and neutrophilic inflammation was induced in A/J mice after a 12-week exposure compared with a 6-week exposure (fig. 5B, C). Small numbers of eosinophils were detected in A/J BALF at 12 weeks (fig. 5D).

Quantification of Anti-PDE Antibody

We observed a significant production of anti-PDE antibodies in all three strains compared with normal saline controls after a 6-week and a 12-week PDE exposure (6-week A/J vs. controls: 1.37 ± 0.10 vs. 0.03 ± 0.04 , $p < 0.01$; 6-week BALB/c vs. controls: 1.10 ± 0.10 vs. 0.04 ± 0.04 , $p < 0.01$; 6-week C57BL/6 vs. controls: 1.30 ± 0.08 vs. 0.03 ± 0.04 , $p < 0.01$; 12-week A/J vs. controls: 1.30 ± 0.10 vs. 0.04 ± 0.05 , $p < 0.01$; 12-week BALB/c vs. controls: 1.06 ± 0.10 vs. 0.04 ± 0.03 , $p < 0.01$; 12-week C57BL/6 vs. controls: 1.30 ± 0.10 vs. 0.03 ± 0.04 , $p < 0.01$). However, there were no significant differences among the three strains.

Expression of Th1/Th2 Cytokines in the Lung

The expression of IL-4 mRNA was significantly up-regulated in 6- and 12-week A/J mice compared with the other strains (fig. 7; 6-week A/J vs. controls: 21.30 ± 18.70 vs. 0.60 ± 0.15 , $p < 0.05$; 12-week A/J vs. controls: 26.50 ± 20.50 vs. 1.30 ± 2.07 , $p < 0.01$), although there was no significant difference between the 6- and 12-week A/J groups. The kinetics of mRNA expression of IL-13, another Th2 cytokine, showed a 15.5-fold increase at the 12-week instillation compared with the control group, and IL-13 mRNA in the 12-week A/J group was significantly increased compared with the 6-week A/J group (fig. 7). There were also significant differences between A/J mice and the two other strains after a 12-week instillation (A/J, 20.7 ± 11.9 vs. C57BL/6, 2.1 ± 1.5 , $p < 0.01$, vs. BALB/c, 7.1 ± 5.6 , $p < 0.01$). The expression of TGF- β 1 mRNA af-

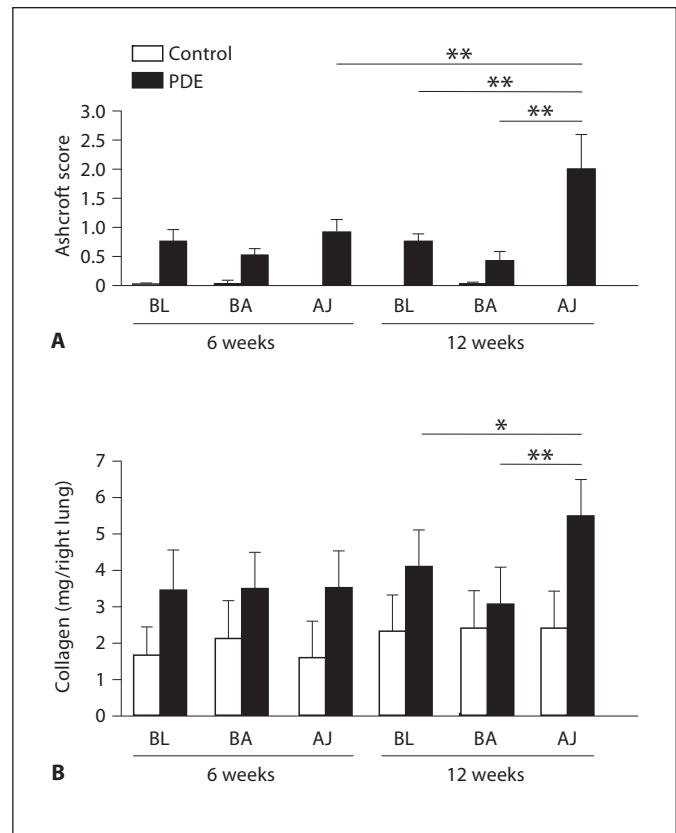


Fig. 4. Evaluation of fibrotic changes in the lung by numerical fibrosis score and measurement of collagen content in lungs treated with PDE and normal saline. **A** The fibrosis score was obtained using a continuous numerical scale for determining the degree of fibrotic changes in the lung. PDE instillation induced minimal alveolitis in both C57BL/6 and BALB/c mice. The average score for the A/J mice treated with PDE was higher than those of the C57BL/6 mice and BALB/c mice after 12 weeks. There was a significant difference among these three groups after 12 weeks ($p < 0.01$). **B** Measurement of collagen content in the right lung after 12 weeks of exposure to PDE. Significant increases in lung collagen content were demonstrated only in the A/J mice. Data are presented as means $\pm$ SD for each group ($n = 5$). BL = C57BL/6 mice; BA = BALB/c mice; AJ = A/J mice. * $p < 0.05$, ** $p < 0.01$.

ter a 12-week exposure was significantly increased in A/J mice compared with the C57BL/6 and BALB/c groups and the 6-week A/J group (12-week A/J, 7.67 ± 3.36 vs. 12-week C57BL/6, 1.79 ± 0.52 , $p < 0.01$, vs. 12-week BALB/c, 3.62 ± 0.48 , $p < 0.01$, vs. 6-week A/J, 3.19 ± 0.64 , $p < 0.01$; fig. 7). The mRNA expression of IFN- γ showed no significant differences among the three strains (fig. 7). After a 12-week exposure, TNF- α mRNA was significantly increased in C57BL/6 mice and BALB/c mice compared with control mice, but in A/J mice there was no increase

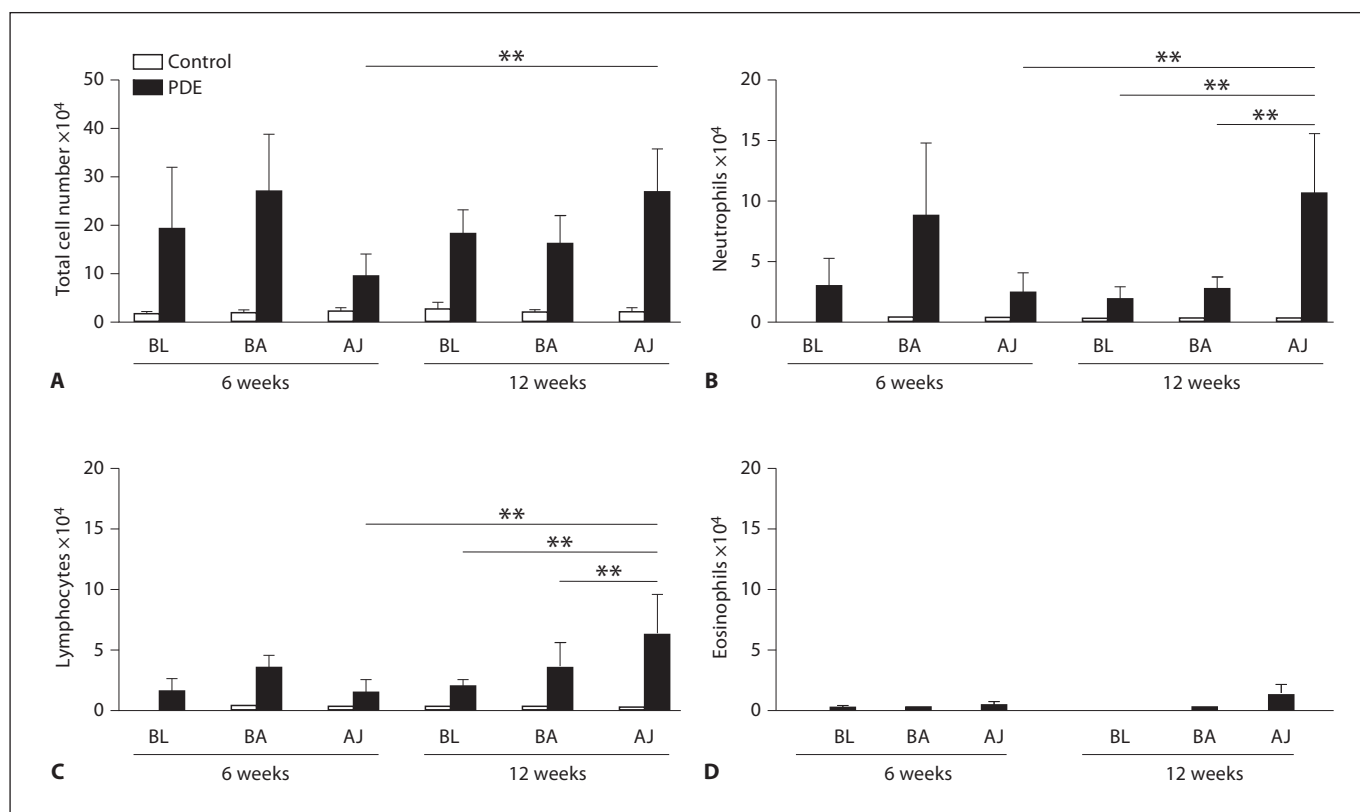


Fig. 5. Total cell number (A) and cell differentials [neutrophils (B); lymphocytes (C); eosinophils (D)] in the BALF of mice after repeated exposure to normal saline and PDE. Data are presented as means $\pm$ SD for each group (n = 5). BL = C57BL/6 mice; BA = BALB/c mice; AJ = A/J mice. ** p < 0.01.

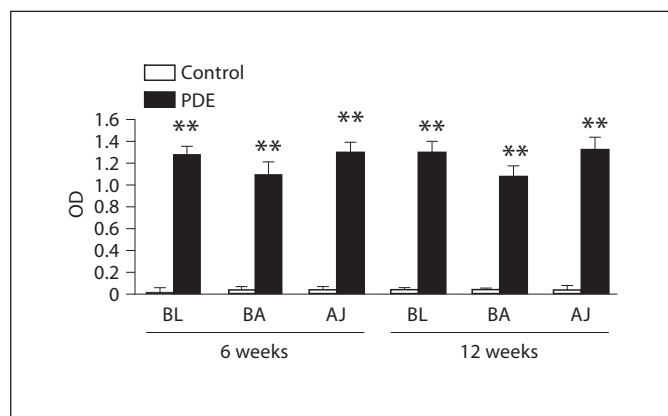


Fig. 6. Increased serum anti-PDE IgG after intranasal PDE. Increased production of serum anti-PDE IgG was noted in all three strains, but there were no significant differences among the three strains. Data are presented as means $\pm$ SD for each group (n = 5). BL = C57BL/6 mice; BA = BALB/c mice; AJ = A/J mice. ** p < 0.01 compared to the same-strain normal saline control for the same exposure period.

compared with controls (C57BL/6 vs. controls: 1.76 ± 2.70 vs. 0.38 ± 0.18 , $p < 0.01$; BALB/c vs. controls: 2.13 ± 2.43 vs. 0.49 ± 0.12 ; $p < 0.01$; A/J vs. controls: 0.97 ± 1.02 vs. 0.45 ± 0.35 ; fig. 7). We then evaluated the protein levels of cytokines in the supernatants of BALF. No cytokines were detected in BALF except for IL-13. A/J mice showed significantly higher levels of IL-13 compared with C57BL/6 mice after a 12-week instillation (A/J vs. C57BL/6: 0.4212 ± 0.0809 vs. 0.0980 ± 0.0006 pg/ μ g, $p < 0.01$), but no significant difference was observed between A/J and BALB/c mice (A/J vs. BALB/c: 0.4212 ± 0.0809 vs. 0.1855 ± 0.0040 pg/ μ g; fig. 8).

Discussion

The present study has shown that A/J mice developed chronic HP, based on the fibrosis score in histology and an increased collagen content resulting from a Th2 adjuvant and a 12-week instillation of PDE.

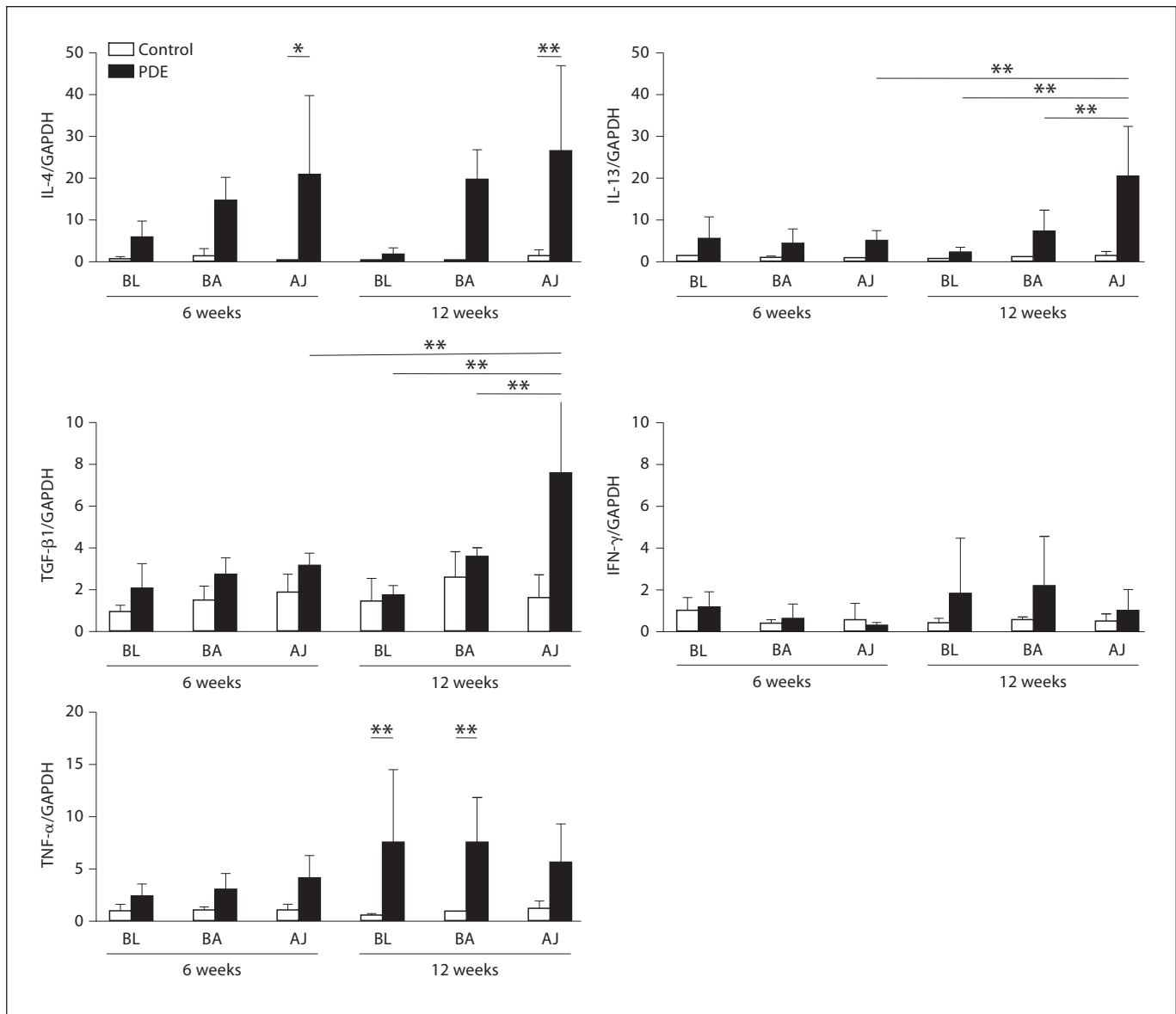


Fig. 7. The presence of mRNA for IL-4, IL-13, TGF-β1, IFN-γ and TNF-α or GAPDH (internal control) in each lung sample was detected with real-time PCR using SYBR green. Each sample was run in triplicate. The data represent the means $\pm$ SD of each mRNA level (fold induction compared to saline-exposed C57BL/6 mice) in the lung samples (n = 5). BL = C57BL/6 mice; BA = BALB/c mice; AJ = A/J mice. * p < 0.05, ** p < 0.01.

Granulomas Tended to Subside during Long-Term Exposure to PDE

Previous studies have suggested that a Th1-type immune response is crucial in inducing HP in mice following *S. rectivirgula* exposure [6–8]. Th1-biased C57BL/6 mice are susceptible to experimental HP, whereas Th2-biased DBA/2 mice are resistant to the development of acute HP [8]. Although these *S. rectivirgula* models are

characterized by diffuse bronchioloalveolitis and granuloma formation in the lung [8], no mice developed the lung fibrosis commonly observed in chronic HP in humans. Severe alveolitis and granuloma formation in the lung are common in acute HP, whereas chronic HP lacks these findings [4]. Pathology studies of end-stage chronic HP have shown that the alveoli were almost completely replaced with connective tissue and honeycombing, al-

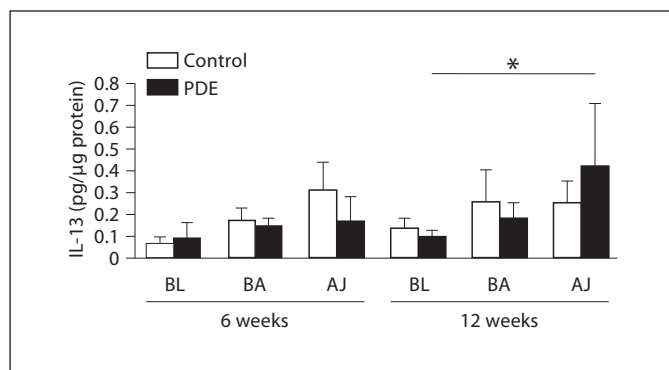


Fig. 8. The IL-13 levels in BALF obtained from the three strains of mice (control or PDE exposure). A/J mice showed significantly higher levels of IL-13 compared with C57BL/6 mice after a 12-week instillation. The data represent the means $\pm$ SD for each group (n = 5). BL = C57BL/6 mice; BA = BALB/c mice; AJ = A/J mice. * p < 0.05.

though no granulomas were found [21–24]. In a previous study, our group reported that in 26 patients with chronic BFL, only 19.2% showed granulomas upon surgical lung biopsy [4]. Our murine model showed that granulomas were observed in both BALB/c and A/J mice, which are Th2-biased strains, but not in C57BL/6 mice, a Th1-biased strain, after a 6-week challenge. However, granulomas tended to subside after a 12-week instillation, even in the BALB/c and A/J groups, suggesting that a change from an acute to a chronic phase occurred between 6 and 12 weeks.

A Th1/Th2 Immune Response Associated with Pulmonary Fibrosis

The properties of an antigen may tend to elicit a Th2 immune response independent of the subject's genetic background. C57BL/6 mice, which are Th1 prone, develop a Th2 immune response when they are immunized with ovalbumin [25]. In previous studies, hypersensitivity granuloma formation induced by *Schistosoma mansoni* antigen or *Aspergillus fumigatus* was greatly influenced by exogenous IL-4 and IL-13, Th2 cytokines, although the periods of exposure were short [26–28]. This cytokine balance differed from that of the *S. rectivirgula* model, which is mediated by Th1 immune responses [8]. It is well known that the Th2 cytokines IL-4 and IL-13 enhance the fibrotic process by inducing fibroblast proliferation and collagen production [10, 29], whereas IFN- γ , a Th1 cytokine, inhibits these processes [9]. Fibrosis occurs in chronic inflammatory disorders when the cytokine balance shifts in favor of Th2 [30, 31].

Genetic factors probably contribute to chronic disease. There were no differences among Th1 strains in previous models of acute HP [32]. In a previous asthma model, which was representative of Th2-mediated disease, Th2 cytokine levels in A/J mice were increased compared with those in BALB/c mice [25]. In this asthma model, continually challenged mice usually developed antigen tolerance, which led to the reduction/loss of eosinophilic inflammation and airway hyperresponsiveness. However, in A/J mice, Th2 cytokine production was increased relative to BALB/c mice, and lasting eosinophilic inflammation was observed [25]. Since animal models of HP with prolonged, repeated exposure to an antigen also generally lead to attenuation of pulmonary histological changes [33, 34], quenching T cell tolerance may be necessary to ensure ongoing fibrosis. We speculated that a strain possessing genetic susceptibility to chronic HP would exist. The present study shows that A/J mice had a tendency to develop chronic HP as a strong and persistent Th2-biased strain. In BALB/c mice, this susceptibility was weaker than in A/J mice. BALB/c mice have the same H-2d haplotype as DBA/2 mice, which are known as an acute HP-resistant strain.

In immunohistochemistry, our data showed that only in A/J mice, not in C57BL/6 mice, were PDE engulfed by alveolar macrophages and the macrophages were effectively incorporated into the alveolar walls with alteration of lung structure. The different strains responded differently to PDE, suggesting that their genetic backgrounds affected their immune responses. In our model, collagen contents of the lung associated with upregulation of mRNA expression of IL-4, IL-13 and TGF- β 1 were significantly increased only in A/J mice. In contrast, the mRNA expression of TNF- α and IFN- γ was not increased in A/J mice. Furthermore, in 12-week A/J mice, IL-4 mRNA was not significantly increased compared with the 6-week exposure, whereas the mRNA expression of IL-13 and TGF- β 1 was significantly increased with the 12-week exposure compared to the 6-week exposure. IL-13 levels in BALF were increased only in the 12-week A/J mice, although IL-4 and TGF- β 1 levels in BALF were not detected. IL-13 plays a greater part in the pathogenesis of fibrosis than IL-4, as discussed in a previous study [35] as well as in the present study. IL-13 is the key mediator of the fibrotic response in the pathogenesis of a variety of other fibrotic disorders, including asthma [29, 36–38], systemic sclerosis [39] and hepatic fibrosis [40–43]. It was previously demonstrated that IL-13 induced tissue fibrosis via its ability to stimulate and activate TGF- β 1 [44]. These findings are closely related to the histological changes

leading to fibrogenesis in the 12-week A/J mice. Consistent with these findings, the present study also demonstrated the critical role of IL-13 and TGF- β 1 in fibrogenesis. Many lymphocytes accumulated around the alveolar macrophages with PDE in the A/J mice. In a future study, we will examine whether these cells include Th2 cells and play a role in fibrogenesis along with Th2 cytokines.

In conclusion, the present study emphasizes that a Th2-biased genetic background is important to establish fibrosis in a chronic HP model. Currently, the most suitable murine chronic HP model for investigating lung fi-

brosis seems to be intranasal instillation of a PDE antigen in A/J mice after an initial immunization with alum. Our murine model could provide scientific insights into the mechanisms leading to fibrogenesis in chronic HP.

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