

Similarities and differences in the effects of sensitisation and challenge with *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* extracts in a murine asthma surrogate

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ABSTRACT

Patients with atopic asthma may become sensitised to the grain storage mite *Dermatophagoides farinae* (*Der f*), the house dust mite *Dermatophagoides pteronyssinus* (*Der p*) or both, but thus far little attention has been paid to date to possible variation in their pathophysiological effects. Here we present a side by side comparison of the effects of extracts of these two dust mites in a murine surrogate of atopic asthma. Compared with the *Der p*-challenged mice, however, the mice-challenged with *Der f* had favour changes in lung tissue elasticity and expression in matrix metalloproteinases in lung tissue, while the mice challenged with *Der p* showed more neutrophils infiltrating around the airway and stronger expression of steroid-resistant related cytokines in the lung tissue. Our data suggest that different dust mite crude extracts might lead different pathological characteristics, at least in murine models of asthma.

1. Introduction

Asthma is a worldwide epidemic disease affecting 1–18% of the populations of different countries [1], characterised by chronic airways inflammation, airways hyperresponsiveness, increased mucus secretion and airways remodelling [2]. At present, the pathogenesis of asthma has not been fully elucidated, and no drugs can completely cure asthma [2]. Animal surrogates of human asthma remain useful tools for the study of asthma pathogenesis and drug development [3]. The “classical” animal surrogate of asthma employs repeated exposure of the airways of mice previously sensitised to ovalbumin (OVA) by intraperitoneal injection to OVA delivered by nebulisation or pernasal inhalation. This surrogate reproduces some of the characteristic changes observed in human asthma such as elevated airways obstruction in response to bronchoconstrictors (“hyperresponsiveness”) and local inflammation. Ovalbumin is not however a respiratory allergen, so it remains controversial how far its effects reflect the pathogenesis of

human asthma [4].

The risk of development of asthma is likely influenced by many factors, including heredity, environment and others [5]. IgE-mediated sensitisation to allergens is a feature of asthma in some patients, and dust mites, principally the house dust mite *Dermatophagoides pteronyssinus* (*Der p*) and the grain storage mite *Dermatophagoides farinae* (*Der f*) are a prominent source of these sensitising allergens in atopic asthmatic patients in the clinic [6,7]. According to data derived from European Community Health Respiratory Survey, the prevalence of sensitisation to *Der p* was 21.7% underwent skin tests in adults aged 20–44 years [8]. Another study showed that, among Latina women in New York, the prevalence of sensitisation to *Der p* and *Der f* was 37% and 34% respectively [9]. Li and colleagues found that *Der f* and *Der p* were the two most common sensitising allergens in asthmatic patients in China [10]. Despite this, the hypothesis that different allergens induce distinguishably variable airways inflammation has never been addressed, and indeed it would be a challenge to do so in human

Abbreviations: AHR, airways hyperresponsiveness; *Der f*, *Dermatophagoides farinae*; *Der p*, *Dermatophagoides pteronyssinus*; OVA, ovalbumin; Mch, methacholine; BALF, bronchoalveolar lavage fluid; PBS, phosphate buffered solution; H&E, aematoxylin and eosin; PAS, periodic acid–Schiff; IHC, immunohistochemistry; MMP, matrix metalloproteinases; MCPT-1, mast cell protease 1

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subjects in the clinic. This hypothesis is more amenable to study using animal surrogates of asthma, but to our knowledge no such studies presently exist. In the present study, we compared, in parallel, differences and similarities in airways pathophysiology in experimental mice sensitised and challenged identically with crude extracts of these two dust mites. We hypothesised that these individual but related allergens induce distinguishable pathophysiological changes in the airways.

2. Materials and methods

2.1. Animals and reagents

Female, wild-type BALB/c (Vital River Laboratory, Beijing, China) mice were kept in a pathogen-free environment in the Department of Laboratory Animal Sciences, Capital Medical University, Beijing, China. All experiments were approved by the Institutional Animal Care and Use Committee of the Capital Medical University, Beijing, China. The crude dust mite extracts were purchased from Cosmo Bio Co., Japan. Aluminium hydroxide [Al(OH)₃] gel was purchased from the Thermo Fisher Company, USA.

2.2. Preparation of murine surrogates

The murine surrogates of asthma based on *Der f* or *Der p* sensitisation and challenge were established using previously described protocols with minor modifications [11]. Briefly, BALB/c mice were sensitised by intraperitoneal injection with the crude extracts of *Der f* or *Der p* (100 µg/100 µL/per mouse/per dose) emulsified in aluminium hydroxide gel on days 0 and 12, respectively, followed by daily, per nasal challenge with a solution containing the same extracts of *Der f* or *Der p* (25 µg in 50 µL normal saline per dose) on days 18 to 23 (6 consecutive days). On days 25 to 35, the mice were further challenged per nasally on every other day with the same amount of solution (25 µg/50 µL per dose). Following measurement of lung function under anaesthesia (see below), the animals were finally euthanised and examined on day 36. Concentrations of lipopolysaccharide (LPS) in *Der f* and *Der p* extracts were 0.596 EU/µg (in *Der f*) and 0.575 EU/µg (in *Der p*), respectively, measured with a commercial kit (Jinshanchuan, Beijing, China).

2.3. Measurement of airways resistance

Airways resistance was measured as previously described [12]. Briefly, following a full, general anaesthetic the trachea of each animal was exposed and intubated with a needle connected to a small animal lung function meter (FlexiVent, Canada) with the animal in a supine position and ventilated at a rate of 150 breaths/min, a tidal volume of 10 mL/kg and 2 cmH₂O of positive end-expiratory pressure (PEEP). The FlexiVent apparatus was used to measure pulmonary mechanical parameters following inhalation of normal saline (NS) and serially increasing concentrations of methacholine (Mch) solution (6, 12 and 24 mg/mL). Concentration-response curves of methacholine-induced changes in airways resistance were recorded.

2.4. Total cellular and differential counts in bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid (BALF) was collected by washing the airways of the mice twice with 0.8 mL of phosphate buffered solution (PBS). After centrifugation at 1500 rpm for 10 min, the supernatants were removed and the cells resuspended in 0.5 mL PBS. The total cells were counted using an automatic counter. Smear slides were prepared using the cell suspension, and differential cell counts were performed following staining with Congo red [12].

2.5. Lung histology

Lung sections (4 µm thickness) were cut from the

paraformaldehyde-fixed, paraffin embedded lung tissues. Peribronchial inflammation was assessed quantitatively in the lung sections as in our previously published protocols [12]. Airways were subdivided into central and peripheral according to basement membrane length. These airways greater than median calculated as 800 µm were defined as central and those less than the median were peripheral airways [13]. Objective indices of airways inflammation and mucus production were evaluated following staining of the sections with haematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) respectively. Peribronchial inflammatory cell infiltration and mucus secretion of airway epithelial cells were performed blind based on the scoring system described in previous literatures [14,15].

2.6. Immunohistochemistry

Lung immunohistochemistry was employed to quantify immunoreactivity for the most common biological markers of pulmonary fibrosis in asthma [11], including matrix metalloproteinases (MMP) 9, 12 and 13, collagen I, collagen V and fibronectin. The relevant antibodies were purchased from Abcam (Cambridge, UK). Briefly, after de-waxing and rehydration, antigen retrieval in the sections was affected using citrate or EDTA buffer [11]. Endogenous peroxidase was quenched by incubating with 3% H₂O₂. The sections were then blocked with 1% bovine serum albumin in PBS and incubated with the relevant primary antibody overnight at 4 °C. After washing, the sections were incubated with appropriate secondary antibodies and a horseradish peroxidase (HRP)-labelled third layer antibody. Signals were visualized by colour development with the HRP substrate, 3, 3'-diaminobenzidine (DAB, Solarbio). Immunoreactivity was quantified objectively using a Nikon microscope (Nikon, Tokyo, Japan) connected with a computer imaging system with proprietary software (Image-Pro Plus 6.0, Media Cybernetics, Silver Spring, USA). The data were expressed as percentages of the positively stained areas in the entire lung biopsy sections as previously described [11]. To further confirm infiltration of neutrophils in BALF, cytopins were prepared with BALF and immunocytochemistry was employed for identifying neutrophils using an antibody against neutrophil elastase (Abcam, Cambridge, UK).

2.7. Measurement of cytokine concentrations in lung homogenates

The right lungs from each animal were weighed then prepared for homogenisation as previously described [12]. Aliquots of the homogenates were stored at –80 °C until used. The concentrations of the cytokines IL-5, IL-13, IL-17A, IL-33, TSLP, MCPT-1 and eotaxin in the homogenates were measured using commercial ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions.

2.8. Measurement of total and specific serum IgE

A venous blood sample was taken from each euthanised animal via the eyeball. The blood was allowed to clot then centrifuged at 5000 rpm for 20 min at 4 °C. Serum was aspirated and stored at –80 °C pending measurement of total IgE using a commercial ELISA kit (eBioscience) according to the manufacturer's instructions. Specific serum IgE was measured as previously described [12].

2.9. Statistical analysis

All data were processed using GraphPad Prism7.0 software (GraphPad, San Diego, USA). Lung function data were analysed and compared by two-way ANOVA to determine whether there were significant differences between the groups, followed by a Tukey post hoc test for multiple comparison of the means. Differences between experimental groups were analysed using one-way ANOVA followed by a Tukey post hoc test. For all tests, *P*-values less than 0.05 were considered significant.

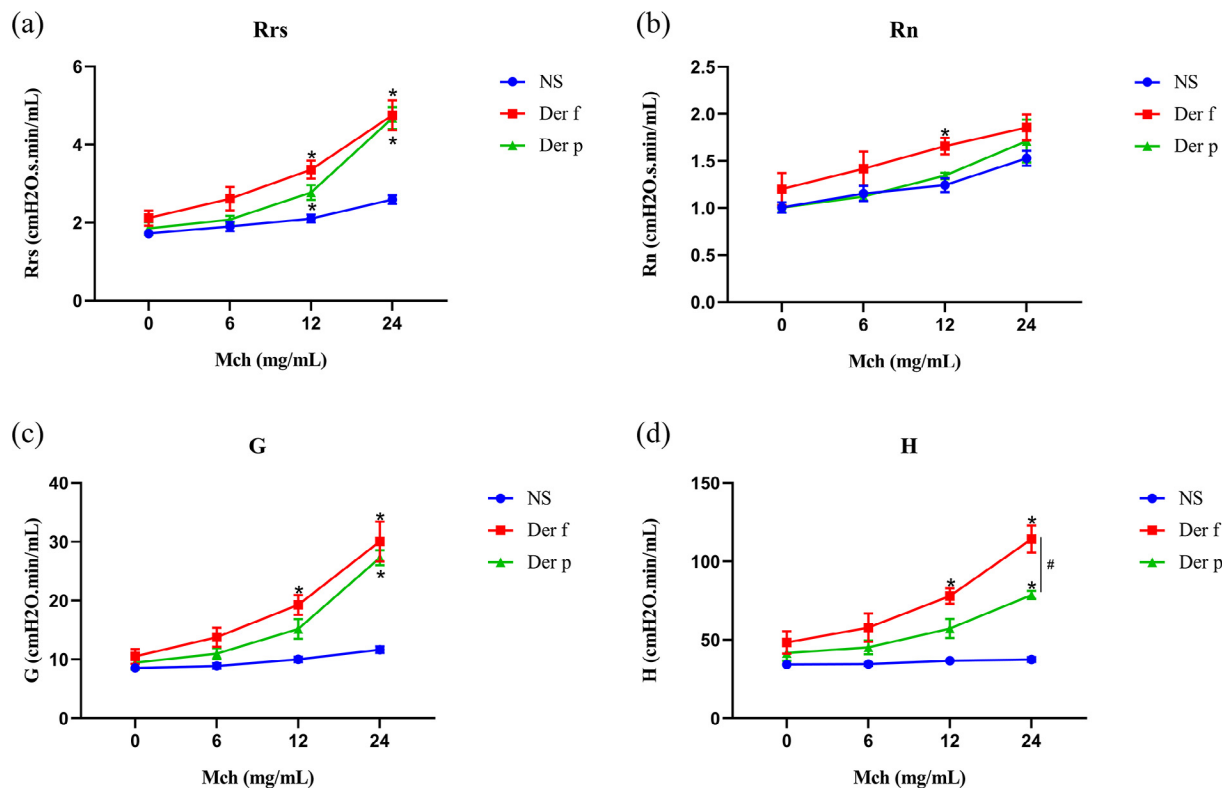


Fig. 1. Characteristics of lung function indices in a murine asthma surrogate induced by different dust mites compared with normal saline (NS) control. (a) Respiratory system resistance (Rrs); (b) Central airway resistance (Rn); (c) Tissue damping (G) and (d) Tissue elastance (H). The data are expressed as the mean $\pm$ SEM (n = 4–5 in each group). * P < 0.05 vs. NS-challenged mice. # P < 0.05 vs. *Der p*-challenged mice.

3. Results

3.1. Characteristics of the effects of different dust mites on lung function in mice

Measurement of lung function revealed that the mean total resistance of the respiratory system (Rrs) was significantly increased in both *Der f* and *Der p* challenged mice compared with the saline-challenged controls (Fig. 1a). Similarly, mean central airways resistance (Rn), tissue damping (G) and tissue elastance (H) were also significantly elevated in both the *Der f* and *Der p* challenged mice compared with the saline-challenged controls (Fig. 1b–d). Although there were no significant differences in the mean total airways resistance, the mean central airways resistance or mean tissue damping between the two models, the mean tissue elastance of the *Der f*-challenged group was significantly higher than that of the *Der p*-challenged group (Fig. 1d).

3.2. Characteristics of the effects of different dust mites on airways inflammation and mucus production

The numbers of total cells, eosinophils, neutrophils, and lymphocytes were significantly increased in the BAL fluid of the *Der f* and *Der p* challenged mice compared with the saline challenged controls (Fig. 2a, b, c, e). In contrast, the mean numbers of macrophages, while small, were significantly reduced in both the *Der f* and *Der p* challenged mice compared with the saline-challenged controls (Fig. 2d). The mean number of BAL fluid neutrophils was significantly greater in the *Der p*, compared with the *Der f* challenged animals (Fig. 2c), while there were no significant differences in the mean numbers of the other inflammatory cells (Fig. 2a, b, d, e). The results of immunocytochemistry again showed that the numbers of elastase⁺ neutrophils were significantly elevated in the BAL fluid of the *Der f* and *Der p* challenged mice compared with the saline challenged controls, while there were

significantly more elastase⁺ neutrophils in *Der p* group compared to *Der f* group (Fig. 2f).

Haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining of the murine lung sections showed large numbers of inflammatory cells infiltrating around the airways of the mice challenged with both dust mites, accompanied by metaplasia of the airway epithelial cells and increased mucus secretion (Figs. 3 and 4). Further comparison showed that there were more infiltrating inflammatory cells around the central airways in the animals challenged with *Der p* compared with those challenged with *Der f* (Fig. 3a), but no significant differences in mucus secretion by epithelial cells in airways of various diameters in the animals challenged with *Der p* compared with those challenged with *Der f* (Fig. 4).

3.3. Characteristics of the effects of different dust mites on expression of fibrosis-associated features around the airways

Global immunohistochemical analysis revealed significantly elevated mean expression of immunoreactivity for the airways remodeling-associated proteins fibronectin and matrix metalloproteinases (MMP) 9, 12 and 13 in the lungs of both the *Der f* and the *Der p* challenged mice compared with the saline-challenged controls (Figs. 5c, 6). The mean global immunoreactivity for MMP9 and MMP12, but not MMP13 was significantly elevated in the airways of the *Der f*, compared with the *Der p* challenged mice (Fig. 6a, b). In contrast, there were no significant differences in the mean immunoreactivity for collagens I and V in the lungs of the mice challenged with both mite extracts and the saline challenged controls (Fig. 5a, b), which was consistent with the results of q-PCR (data not shown).

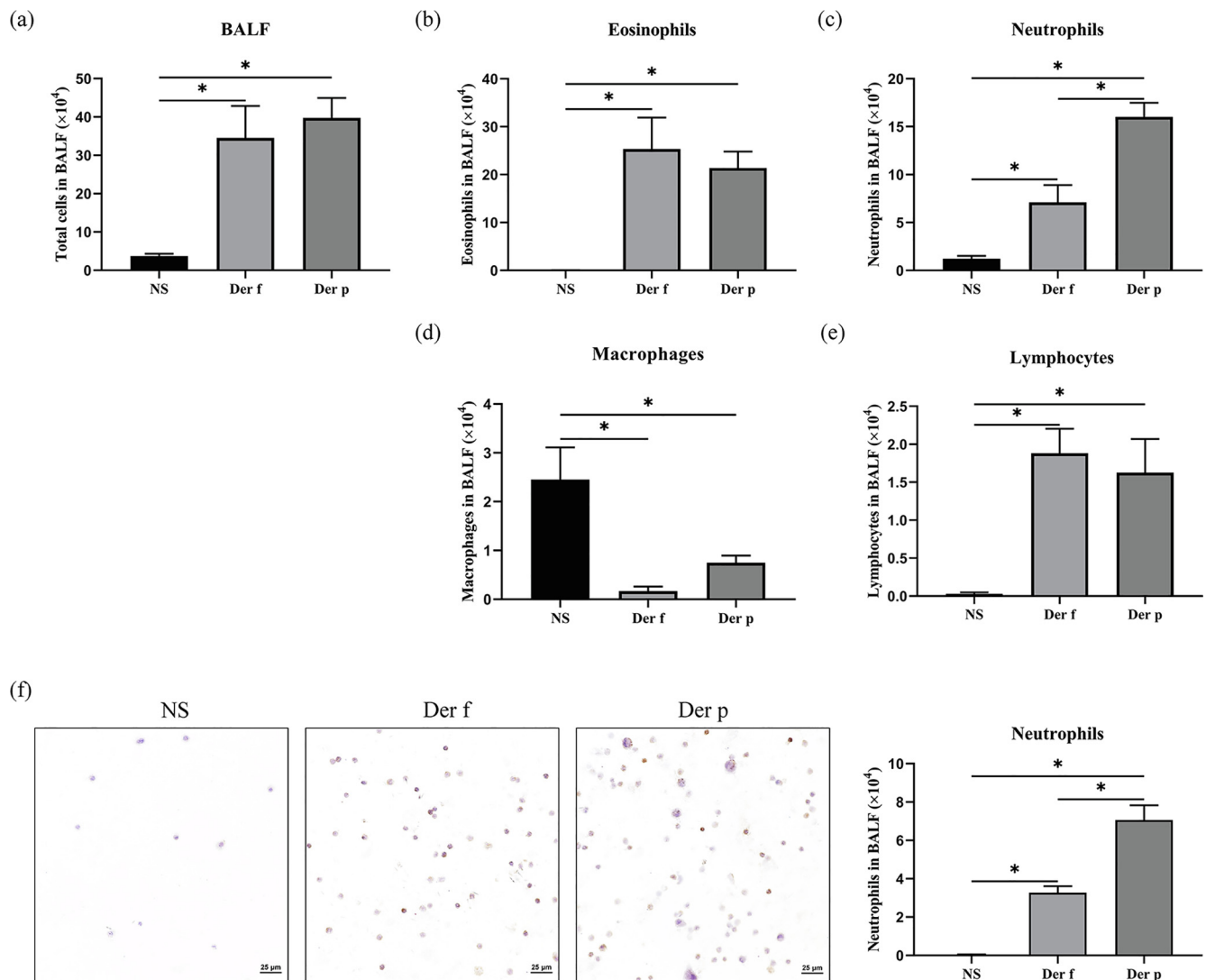


Fig. 2. Total cells (a), eosinophils (b), neutrophils (c), macrophages (d) and lymphocytes (e) in BAL fluid of mice challenged with different dust mites compared with normal saline (NS) controls. (f) showing typical microphotographs and the numbers of elastase⁺ neutrophils in BALF (brown) of mice challenged with NS, *Der f* and *Der p* extracts, stained by immunocytochemistry. Original magnification ×400. The data are expressed as the mean ± SEM (n = 3–5 in each group). *P < 0.05.

3.4. Effects of different dust mite extracts on concentrations of inflammatory mediators in lung tissues and of IgE in the serum

ELISA analysis revealed that, compared with the mice challenged with saline control, the mean concentrations of the Th2 cytokine IL-13 and the chemokine eotaxin were significantly elevated in the lung homogenates of the mice challenged with both mite extracts, with no significant difference in the mean concentrations following challenge with each extract (Fig. 7). Similarly, the mean concentrations of the epithelial-derived alarmin cytokine IL-33 were also elevated in the lung homogenates of the mice challenged with both dust mite extracts compared with those challenged with saline control, but there was no difference between the two groups (Fig. 7). In addition, concentrations of TSLP and IL-17A were only elevated in the lung homogenate of *Der p*-challenged mice (Fig. 7).

Concomitantly, the mean serum concentrations of total and antigen-specific IgE were significantly elevated following sensitisation and challenge of the animals with both dust mite extracts compared with those challenged with saline control, again with no significant difference between the groups challenge with each extract (Fig. 7). Finally, the mean concentrations of MCPT-1 (mast cell protease 1) were significantly increased in the lung homogenates of mice challenged with

both mites compared with those challenged with saline control, while the mean concentration of MCPT-1 was significantly higher in the animals challenged with *Der p* compared with those challenged with *Der f* (Fig. 7).

4. Discussion

Although it is well known that both dust and storage mite sensitisation frequently accompany asthma and that the protein sequences of the major allergens of *Der f* and *Der p* are highly similar, the differences in their composition and geographical distribution suggest that the asthma associated with exposure and sensitisation to the different dust mites might have distinct features. At present, there remains a paucity of research in this area, either clinically or experimentally. In the present study, we believe for the first time, we have systematically compared the similarities and differences in changes in airways physiology, inflammation, mucus secretion and remodelling in a murine surrogate of asthma in which the animals are challenged in parallel with the dust mites *Der f* and *Der p*. Previous studies have established that both *Der f* and *Der p* can cause asthma-like changes in mice, including increased airways resistance, inflammatory cellular infiltration and elevated cytokine expression [16,17], findings which are confirmed by our present

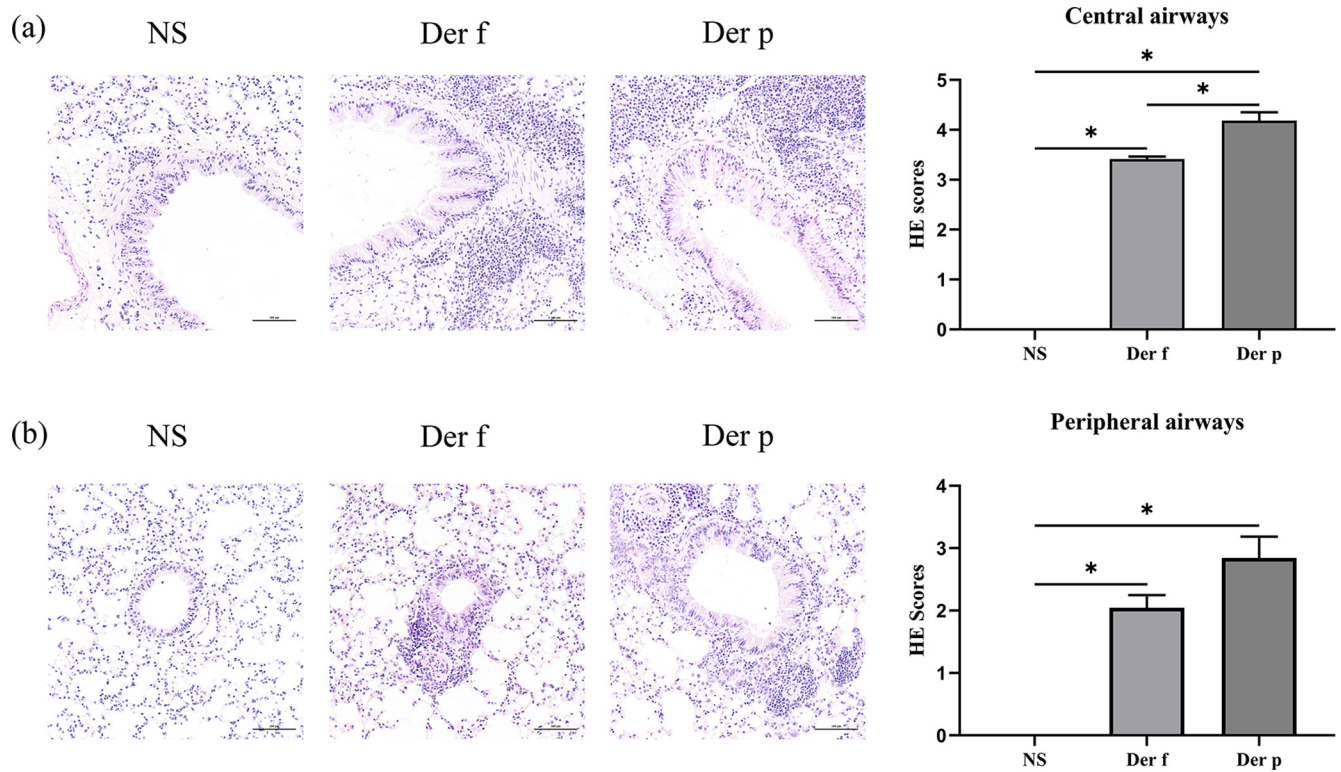


Fig. 3. Haematoxylin and eosin (H&E) staining of inflammatory cells infiltrating around central (a) and peripheral (b) airways in lung sections of mice challenged with different dust mite extracts compared with normal saline (NS) control. Representative photomicrographs and quantitative analysis of the inflammation scores from H&E stained lung sections. Original magnification $\times 200$. The data are expressed as the mean $\pm$ SEM ($n = 4-5$ in each group). $*P < 0.05$.

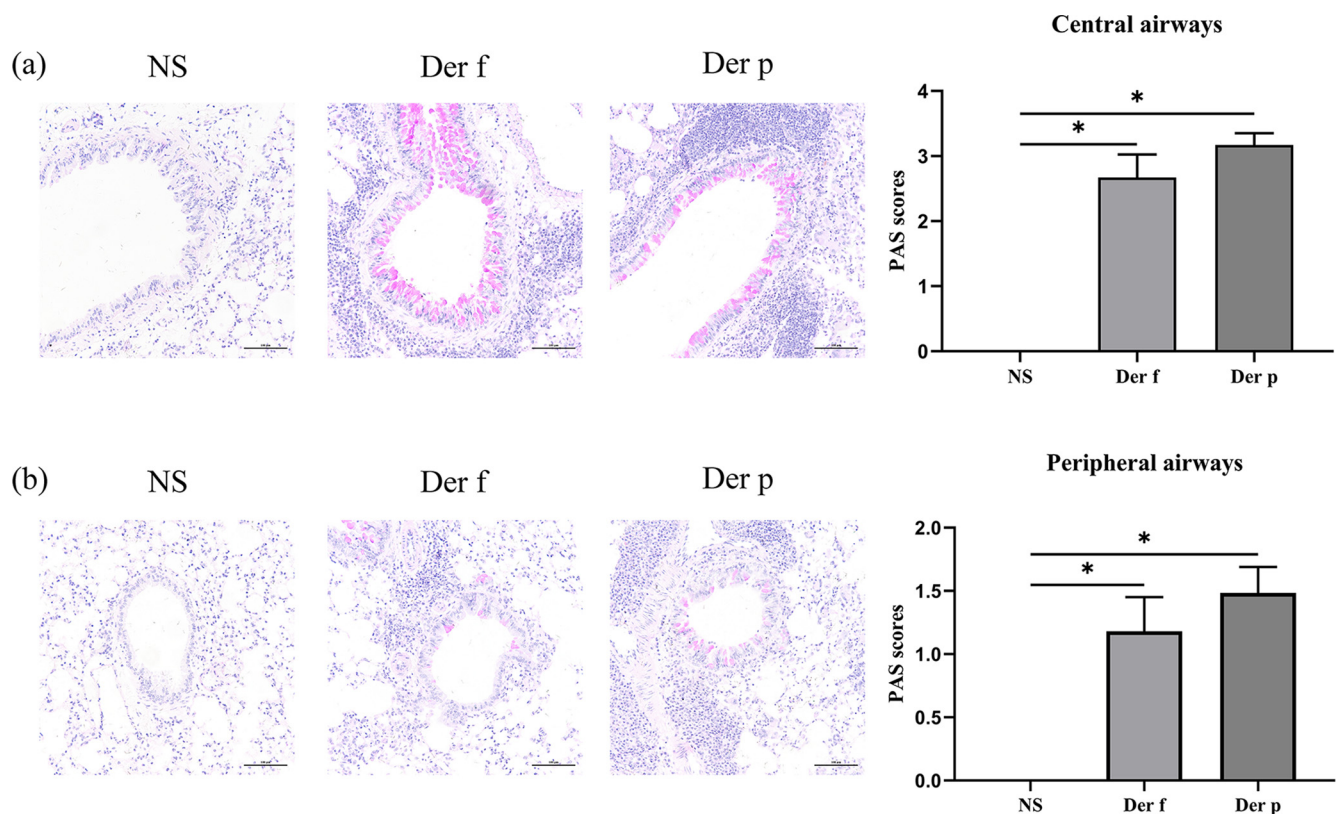


Fig. 4. Periodic acid-Schiff (PAS) staining of mucus in central (a) and peripheral (b) airways in lung sections of mice challenged with different dust mite extracts compared with normal saline (NS) control. Representative photomicrographs and quantitative analysis of the mucus scores from PAS stained lung sections. Original magnification $\times 200$. The data are expressed as the mean $\pm$ SEM ($n = 4-5$ in each group). $*P < 0.05$.

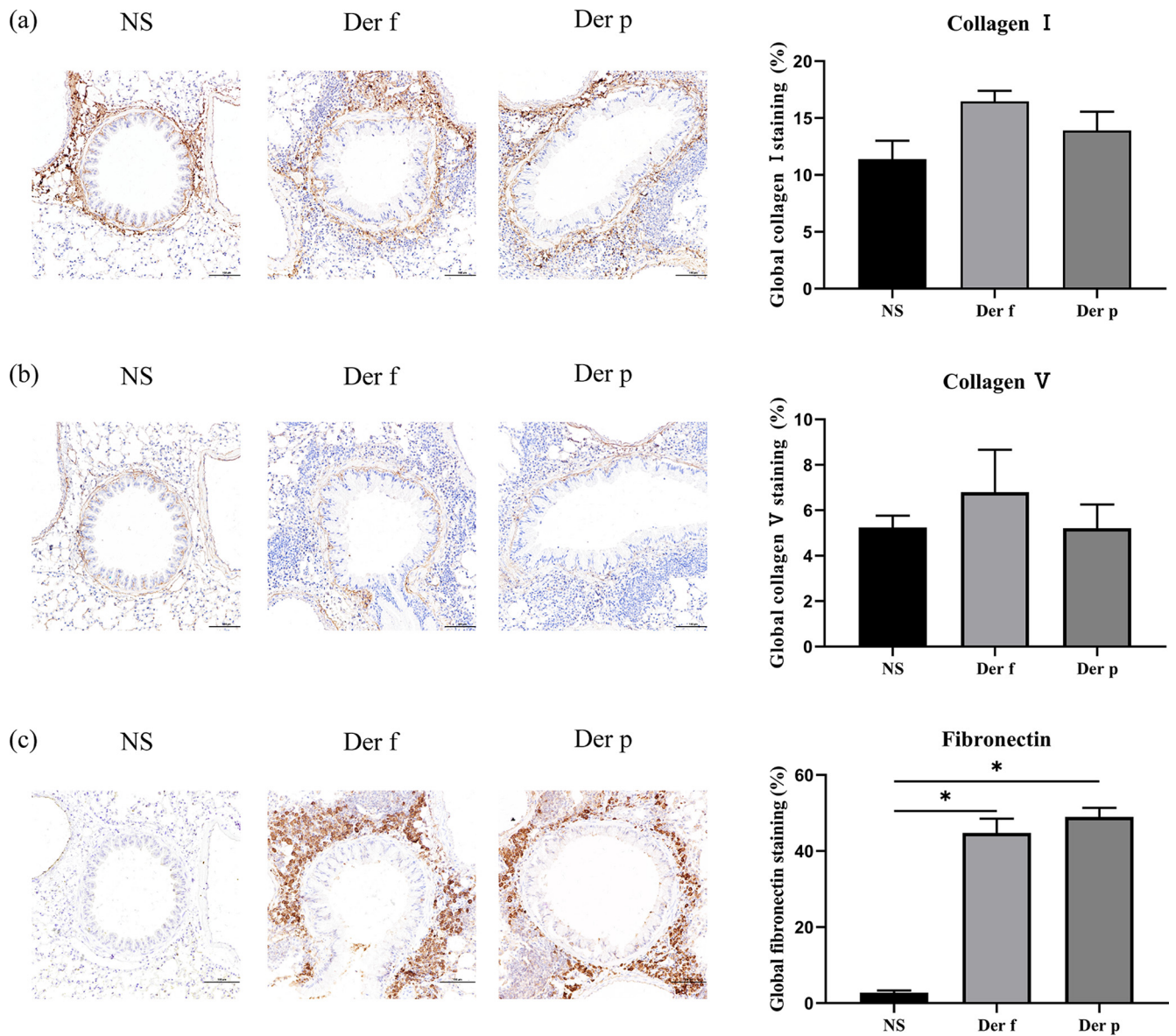


Fig. 5. Global immunoreactivity for collagens I and V and fibronectin in the lung tissue sections of mice challenged with different dust mite extracts compared with normal saline (NS) control. Representative photomicrographs and quantitative analysis of collagen I (a) and V (b) and fibronectin (c) immunoreactivity in lung sections. Original magnification $\times 200$. The data are expressed as the mean $\pm$ SEM ($n = 4-5$ in each group). * $P < 0.05$.

data. Our data further show that mice sensitised and challenged with *Der f* favoured increases in lung tissue elasticity, and in the expression of MMP9 and MMP12 in lung tissue. Compared with mice challenged with *Der f*, challenged with *Der p* induced a significantly more predominant infiltration of the airways lumen with neutrophils and elevated expression of IL-17A and MCPT-1 in lung homogenates. Concomitantly, H&E staining showed that *Der p* exposure induced significantly more prominent inflammatory cellular infiltration into the central airways, with a trend for greater mucus production in these regions of the airways. On the other hand, expression of some of the remodelling proteins such as MMP9 and MMP12 was significantly less pronounced. Taken together, these data are consistent with the hypothesis that *Der p* is more likely to induce airways inflammation, with a relative prominence of neutrophils, whereas *Der f* is more likely to induce permanent remodelling changes. Although these details, of course, require further clarification, our findings provide a paradigm for a deeper analysis and understanding of the contribution of different dust mites, and indeed all allergens to the pathophysiology and immunopathology of asthma.

It is well known that Th17 cells play a role in the development of some endotypes of asthma, in particular a corticosteroid-resistant endotype, mainly secondary to their production of the cytokine IL-17A and IL-17-mediated accumulation of neutrophils in the lungs [18–20]. These mechanisms are corticosteroid-resistant: adoptive transfer of Th17 cells in mice induces asthma with predominantly neutrophilic airways infiltration and resistance to treatment with dexamethasone [18–20]. Our data here show that the neutrophil exudation into the BAL fluid of the mice challenged with *Der p* was significantly greater than that of the mice challenged with *Der f*. Correspondingly, the mean concentration of IL-17A was also significantly elevated in the lung tissue homogenates of the *Der p* group. Furthermore, the epithelial alarmin cytokines IL-33 and TSLP have also been shown to be closely involved in the generation of corticosteroid-resistant endotypes of asthma, where they act on ILC2 cells to induce release of Th2 cytokines which in turn promote Th2 cell differentiation [21,22]. In the present study, both *Der p* and *Der f* exposure significantly increased the local expression of IL-33, whereas *Der p* exposure in addition significantly increased TSLP expression (there was a trend for a similar effect of *Der*

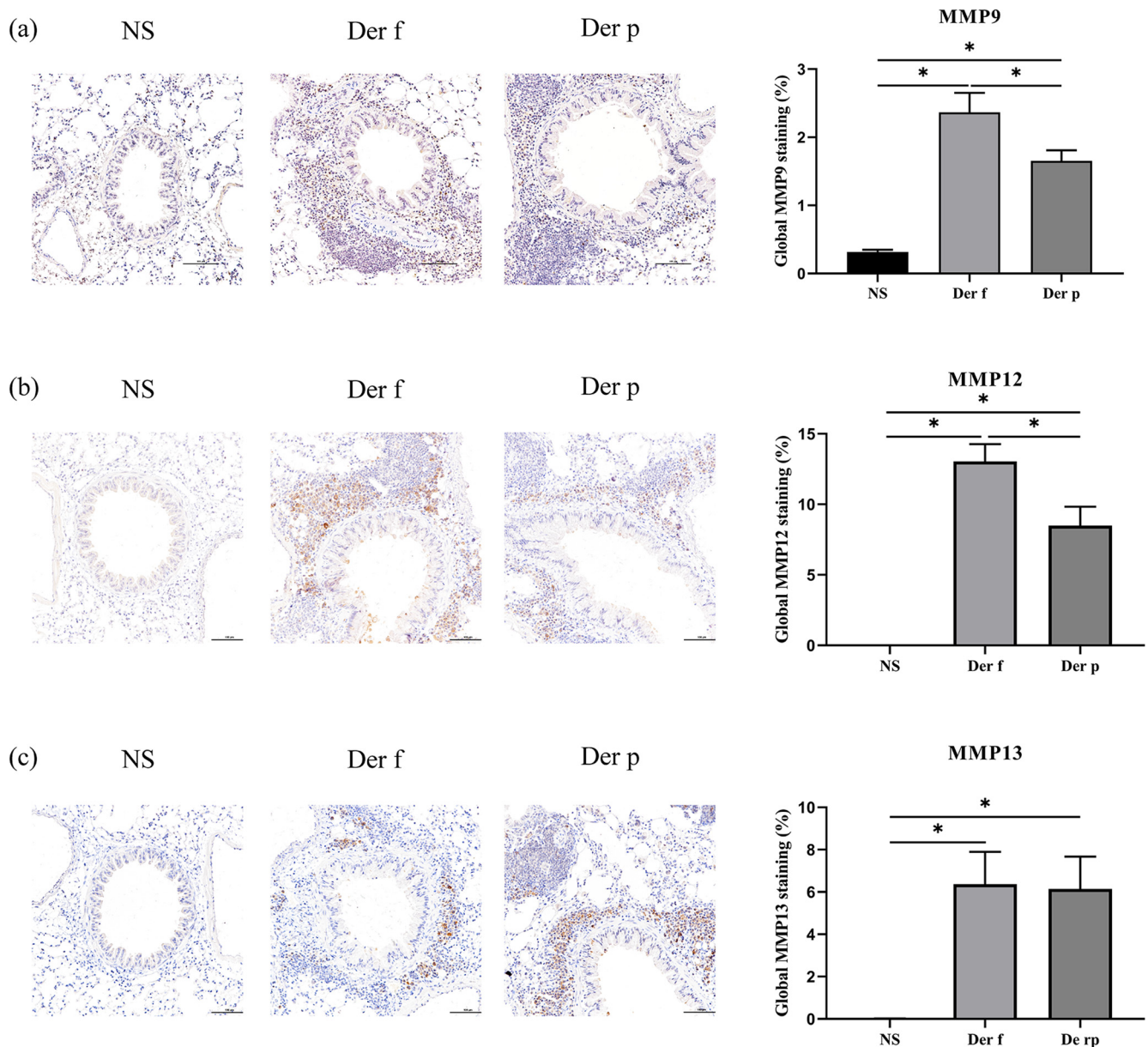


Fig. 6. Global immunoreactivity for MMP9, MMP12 and MMP13 in the lung tissue sections of mice challenged with different dust mite extracts compared with normal saline (NS) control. Representative photomicrographs and quantitative analysis of MMP9 (a), MMP12 (b) and MMP13 (c) immunoreactivity in lung sections. Original magnification $\times 200$. The data are expressed as the mean $\pm$ SEM ($n = 4-5$ in each group). $*P < 0.05$.

f). These results again support the hypothesis that asthma induced by *Der p* exposure is more likely to be corticosteroid-resistant. Since macrolides can inhibit the migration of neutrophils to the lungs [23–25], the question whether patients with *Der p* sensitivity are more likely to respond clinically to macrolide than corticosteroid therapy requires further, controlled studies.

MCPT-1 is an enzyme produced mainly by activated mast cells, and is thus considered a mast cell activation marker. Activated mast cells may release a variety of additional inflammatory mediators, such as cysteinyl leukotrienes, prostaglandin D₂, histamine and others which promote eosinophil and neutrophil accumulation around the airways in the pathogenesis of asthma, and can trigger airways oedema and other lesions [26]. Our present data show that the mean concentration of MCPT-1 in the murine lung homogenates induced by *Der p* was significantly higher than that induced by *Der f*, suggesting variability in the capacity of different allergens to activate mast cells. Whether this effect is in whole or part mediated by cross-linking of mast cell-bound

allergen-specific IgE remains to be further investigated. In addition, MCPT-1 is able to stimulate the release of TGF- β and can convert IL-1 β into an active form [26] critical for the survival and activation of granulocytes. This might provide another explanation for our observed ability of *Der p* to induce greater inflammatory cellular infiltration around the airways.

While in our present experiments challenge with both *Der p* and *Der f* resulted in significantly elevated expression in the airways of a range of matrix metalloproteinases implicated in tissue destruction and remodelling, *Der f* was significantly more effective than *Der p* in this regard. Correspondingly, our present data showed that the mice challenged with *Der f* showed significantly poorer lung tissue elasticity (higher H measurement, which might represent the stiffness of alveolar tissue and small airway lesions as well). This observation is consistent with the hypothesis that different allergens, along with other environmental substances inhaled into the airways may differ in their capacity to promote airways remodelling changes and irreversible obstruction.

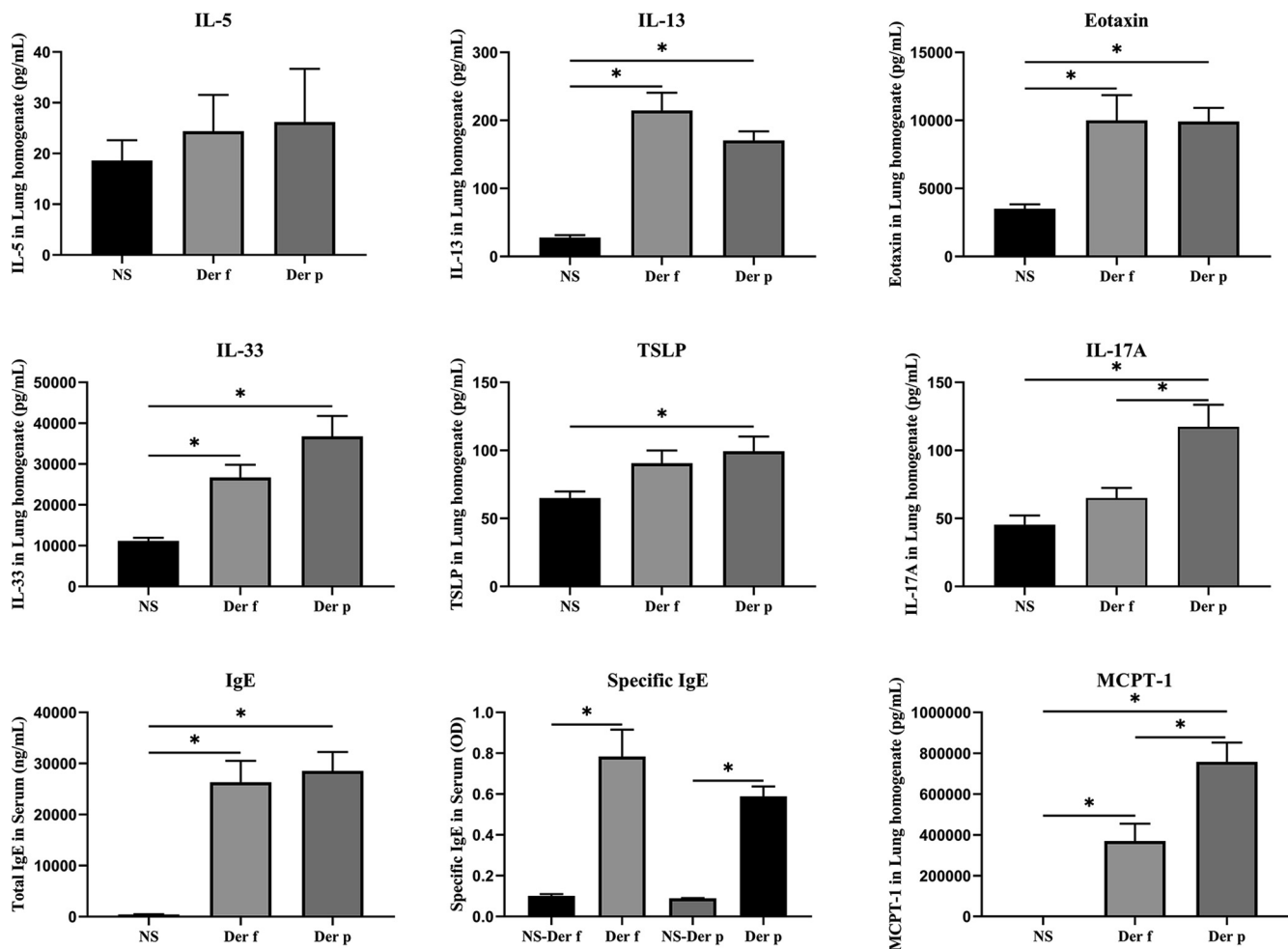


Fig. 7. Concentrations of cytokines and other mediators in lung homogenates, and total and specific IgE in serum of mice challenged with different dust mite extracts compared with normal saline (NS) control. The data are expressed as the mean $\pm$ SEM (n = 4–6 in each group). * P < 0.05.

Finally, our data raise questions about the possible role of IgE in our observed phenomena. While challenge with both *Der f* and *Der p* induced a marked increase in serum concentrations of total and antigen-specific IgE in the experimental animals, a well-recognised feature of this animal surrogate, with the exception of the evidence of mast cell activation discussed above, a possible role for IgE in the other effects of the inhaled dust mite extracts observed in our study is open to question: while exposure to allergens may acutely exacerbate asthma symptoms in suitably sensitised, atopic asthmatic subjects by inducing the release of histamine and other mediators from IgE-sensitised mast cells in the airways, it is possible to hypothesise that many of the airways inflammatory, remodelling and physiological changes observed in our experiments do not depend on the presence of allergen-specific IgE. Although there are reports in the literature of negative correlations between serum *Der f*-specific IgE concentrations and lung function in patients with atopic asthma [27], it is possible to hypothesize that this IgE production is epiphenomenal and simply reflects the capacity of certain allergens to induce alarmin and Th2 type responses in the airways epithelium and submucosa. This again is amenable to further investigation in future studies.

At present, therapy for human asthma, even arguably with the advent of biological therapies in addition to the well-established oral and inhaled drugs, remains largely generic, while disease prophylaxis remains a pipe dream. Understanding the reactivity of individual subjects to individual proteins, including allergens and other environmental substances inhaled into the airways may allow delineation of endotypes of disease susceptibility and predict the characteristics of the airways

inflammation in individuals prospectively, so that therapy and above all prophylaxis may eventually be individualised [28]. We believe that the present study offers a small but important step towards achieving this aim.

Disclosures

The authors declare no conflicts of interest.

CRediT authorship contribution statement

Wenjun Wang: Conceptualization, Methodology, Investigation, Formal analysis, Data curation, Writing - original draft. **Gao An:** Investigation, Data curation. **Yan Li:** Supervision, Resources. **Chris J. Corrigan:** Conceptualization, Writing - review & editing. **Wei Wang:** Conceptualization, Supervision, Resources, Writing - review & editing. **Sun Ying:** Conceptualization, Methodology, Supervision, Resources, Writing - review & editing. **Kewu Huang:** Conceptualization, Methodology, Supervision, Writing - review & editing, Project administration.

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