

Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness

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Summary

Background House dust mites (HDMs) are the major source of perennial allergens causing human allergic asthma. Animal models mimicking as closely as possible the allergic features observed in human asthma are therefore interesting tools for studying the immunological and pathophysiological mechanisms involved. Especially the role of eosinophils and allergen-specific immunoglobulin (Ig) E in the pathophysiology of airway hyperresponsiveness (AHR) remains a subject of intense debate.

Objective To develop a mouse model of allergic airway inflammation and hyperresponsiveness based on the use of purified house dust mite allergen (Der p 1) as clinically relevant allergen. Furthermore, we studied the effects of low dose allergen exposure on the airway eosinophilia and AHR.

Methods On day 0, C57Bl/6 mice were immunized with purified Der p 1 intraperitoneally. From day 14–20, the mice were exposed daily to a 30-min aerosol of different concentrations of house dust mite extract.

Results Mice, actively immunized with Der p 1 and subsequently exposed to HDM aerosols, developed AHR, eosinophil infiltration of the airways and allergen-specific IgE. Moreover, lowering the concentration of the HDM aerosol also induced AHR and IgE without apparent eosinophil influx into the airways. Der p 1-sensitized mice exposed to PBS produced IgE, but did not show AHR or eosinophil influx.

Conclusion This *in vivo* model of HDM-induced allergic airway changes suggests that AHR is not related to either eosinophil influx or allergen-specific serum IgE, thereby reducing the importance of these factors as essential elements for allergic AHR.

Keywords: airway hyperresponsiveness, Der p 1, eosinophil, house dust mite, immunoglobulin E

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Introduction

Exposure to inhalant allergens, such as those derived from domestic mites, is a major risk factor for the development of asthma [1–4]. House dust mites (*Dermatophagoides* species) and their faecal particles contain several groups of molecules acting as allergen. The most important is Der p 1, a cysteine protease derived from the mite's intestinal tract [5]. In order to better understand the aetiology and pathophysiology of asthma, and the exact

role of immunoglobulin (Ig) E and eosinophils, there is a continuous need for animal models mimicking as closely as possible the typical features of human asthma [6,7]. Most of the animal models developed so far have used ovalbumin [8] or parasites [9] as antigen to induce an eosinophilic inflammation, bronchial hyperresponsiveness and production of specific IgE. However, it can be argued that common aeroallergens might be more valuable. A limited number of animal models of allergy are based on aeroallergens present in daily environment, such as ragweed [10]. Very few investigators used mites or mite extracts (still containing a range of allergens) as immunizing agents [11–13].

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We report on the development of a model in C57Bl/6 mice sensitized to purified major allergen of house dust mite, Der p 1. Furthermore, we demonstrate for the first time that, using the house dust mite allergen, the induced airway hyperresponsiveness is not related to the quantity of circulating allergen-specific IgE or to the influx of eosinophils into the airways.

Methods

Mice

Specific pathogen-free male C57Bl/6 mice, weighing 20–25 g, were obtained from Harlan (Zeist, The Netherlands). The mice were between 6 and 8 weeks of age at the beginning of the experiment.

Preparation of house dust mite and Der p 1

The lyophilized house dust mite extract (HDM) and the major house dust mite allergen (Der p 1) were produced by ALK laboratories (Hørsholm, Denmark). The 25.4-kDa major allergen Der p 1 of *Dermatophagoides pteronyssinus* was purified from an aqueous extract of mite culture by physicochemical methods as described previously [14]. The measurement of Der p 1 in HDM was performed with a sandwich ELISA and compared with the values obtained from a standard curve. The results indicated a content of 8 ng Der p 1 per μg HDM.

Immunization and antigen bronchoprovocation protocol

Groups of mice ($n = 7$ – 8) were sensitized intraperitoneally with 10 μg Der p 1 adsorbed to 1 mg aluminium hydroxide ($\text{Al}(\text{OH})_3$). Fourteen days later, the mice were exposed daily to aerosolized allergen for 7 consecutive days. The mice were placed in a Plexiglas chamber ($30 \times 20 \times 15$ cm) and exposed to aerosolized HDM extract for 30 min. The aerosol was produced by an ultrasonic nebulizer (Ultraschallvernebler Sirius Nova, Heyer Medizintechnologie, Bad Ems, Germany). According to the specifications of the manufacturer, the output of the nebulizer was 0.5–5 mL/min and the mean particle size of the aerosol was 3.2 μm . Different concentrations of HDM solution were nebulized: 3, 30 and 300 $\mu\text{g}/\text{mL}$. The control animals were either immunized with Der p 1 and exposed to aerosolized phosphate-buffered saline (PBS) or were sham immunized with adjuvants but exposed to the highest concentration of aerosolized HDM (300 $\mu\text{g}/\text{mL}$).

Airway responsiveness

Airway responsiveness to carbachol was measured 24 h

after the final allergen exposure as previously described [15]. The mice were anaesthetized with pentobarbital (100 mg/kg intraperitoneally) and a tracheal cannula was inserted. The femoral artery and the jugular vein were cannulated. The animals, placed on a 37°C heated blanket, were ventilated with a Palmer respirator (Bioscience, Sheerness, UK) at 145 strokes/min (stroke volume of 0.5 mL). Neuromuscular blockade to prevent spontaneous respiration was induced by injecting pancuronium bromide (1 mg/kg) i.v. (Organon, Teknika N.V., Turnhout, Belgium). Airway resistance (R_L) was calculated from the differential pressure between the airways and the pleural cavity, tidal volume and flow. Measurements were done with a computerized pulmonary mechanical analyser (Mumed PMS800 system; Mumed Ltd, London, UK). Increasing doses of carbachol were administered (micro-infusion pump) intravenously (40, 120, 400 and 1200 $\mu\text{g}/\text{kg}$). Between each dose, the airway resistance was allowed to return to the baseline level. The provocative dose of carbachol causing a 50% increase in R_L ($\text{PD}_{50}-R_L$) was calculated from the linear interpolation on a semilogarithmic dose–response curve.

Bronchoalveolar lavage

Immediately after the assessment of airway responsiveness, 1 mL of Hank's balanced salt solution (HBSS) free of ionized calcium and magnesium but supplemented with 0.05 mmol/L sodium EDTA was instilled four times via the tracheal cannula and recovered by gentle manual aspiration. The recovered fluid was centrifuged (700 g for 10 min at 4°C). The cell pellet was washed twice and finally resuspended in 1 mL of HBSS. A total cell count was performed in a Bürker chamber and the differential cell counts on at least 400 cells were performed on cytocentrifuged preparations (Cytospin 2; Cytospin, Shandon td., Runcorn, Cheshire, UK) after staining with May-Grünwald-Giemsa using standard morphological criteria.

Histological analysis and scoring of lung inflammation

The lungs were infused via the trachea with 4% paraformaldehyde. After excision, the lungs were immersed in fresh fixative overnight. Pieces from all lung lobes were used for histological examination. Sections of 2.5- μm thickness were cut and stained with haematoxylin and eosin. A number of sections was stained with Congo Red for making the photomicrographs.

Slides were coded and graded in a blinded fashion using a reproducible scoring system described elsewhere [16]. A value from 0 to 3 per criterion was adjudged to each tissue section scored. Two criteria were scored to document the pulmonary inflammation: peribronchial inflammation and

perivascular inflammation. A value of 0 was adjudged when no inflammation was detectable, a value of 1 for occasional cuffing with inflammatory cells, a value of 2 for most bronchi or vessels surrounded by thin layer (one to five cells) of inflammatory cells and a value of 3 when most bronchi or vessels were surrounded by a thick layer (more than five cells) of inflammatory cells. As 10–15 tissue sections per mouse were scored, inflammation scores could be expressed as a mean value and could be compared between groups.

Measurement Der p 1-specific IgE

Before infusion of paraformaldehyde into the lungs, blood was drawn from the heart for measurement of serum IgE. Serum was collected and stored at -20°C until use.

Der p 1-specific IgE was measured with an ELISA. All serum samples were diluted 1:10. The microwell plates were coated with purified Der p 1. After saturation of the plates with foetal bovine serum, the test serum was added followed by addition of sheep antimouse IgE (Calbiochem, Euro Biochem, Bierge, Belgium). After an additional blocking step, peroxidase-conjugated rabbit antisheep antibody (Calbiochem, Euro Biochem, Belgium) was added. After addition of ortho-phenylenediamine-dihydrochloride (OPD), absorbance values were measured at 492 nm. As no Der p 1-specific IgE standards exist, values were expressed in units of optical density.

Statistical analysis

Data were analysed with the statistical packet SPSS 6.1.2. (SPSS Inc.; Chicago, IL, USA). All results are expressed as mean $\pm$ standard error of the mean (SEM). ANOVA tests were applied to compare groups, and *post hoc* analysis with Bonferroni's conservative correction was performed when ANOVA tests were significant at $P < 0.05$.

Results

Bronchoalveolar lavage fluid

Sensitization with Der p 1 and subsequent exposure to aerosolized house dust mite ($300\text{ }\mu\text{g/mL}$) induced significantly (ANOVA, $P < 0.05$) higher bronchoalveolar (BAL) cell numbers (Table 1). Lower concentrations of house dust mite extract had no significant effect on total cell numbers. Similarly, differential cell counts showed that only the sensitized group exposed to house dust mite aerosol of $300\text{ }\mu\text{g/mL}$ developed an important airway eosinophilia in the BAL fluid (ANOVA, $P < 0.001$).

Histopathology of lung

Figures 1 and 2 represent the results of the histopathological scoring in the different groups of mice. Both peribronchial and perivascular inflammation was detectable in sensitized animals exposed to $300\text{ }\mu\text{g/mL}$ HDM aerosol whereas exposure to $30\text{ }\mu\text{g/mL}$ resulted in clearly detectable inflammation in the peribronchial regions but scarcely in the perivascular regions. Detachment of bronchial epithelial cells was also observed in these groups. Control animals as well as animals exposed to the lowest dose of house dust mite ($3\text{ }\mu\text{g/mL}$) had no histological evidence of peribronchial inflammation.

Airway responsiveness

Figure 3 shows the dose–response curve of the *in vivo* airway responsiveness to carbachol. The sensitized mice challenged with any of the three concentrations of HDM aerosol developed a significant hyperresponsiveness compared with the control animals (ANOVA, $P < 0.001$). No statistically significant difference was observed between the three Der p 1-sensitized and HDM-exposed groups ($P = 0.2$), nor between the two control populations ($P = 0.2$). In line with these observations, the PD_{50}R_L in control animals

Table 1. Characterization of the bronchoalveolar lavage fluid of nonimmunized and Der p 1-immunized mice exposed to different concentrations of house dust mite aerosol. Values are expressed as means $\pm$ SEM. ANOVA, * $P < 0.001$ vs other groups, ** $P < 0.05$ vs other groups

Imm. day 0	HDM challenge day 14–20	Total cells ($\times 10^3/\text{mL}$)	Macrophages ($\times 10^3/\text{mL}$)	Lymphocytes ($\times 10^3/\text{mL}$)	Neutrophils ($\times 10^3/\text{mL}$)	Eosinophils ($\times 10^3/\text{mL}$)
Sham	$300\text{ }\mu\text{g/mL}$	145.0 ± 16.3	143.4 ± 16.2	0.9 ± 0.3	0.5 ± 0.2	0.1 ± 0.1
Der p 1	PBS	142.5 ± 23.4	140.1 ± 23.1	1.3 ± 0.4	0.8 ± 0.3	0.3 ± 0.2
Der p 1	$3\text{ }\mu\text{g/mL}$	132.0 ± 22.3	129.9 ± 22.0	0.8 ± 0.3	1.3 ± 0.3	0.0 ± 0.0
Der p 1	$30\text{ }\mu\text{g/mL}$	128.6 ± 15.9	123.3 ± 16.3	2.7 ± 0.9	2.4 ± 1.2	0.2 ± 0.2
Der p 1	$300\text{ }\mu\text{g/mL}$	$228.0 \pm 38.5^{**}$	166.7 ± 29.6	$6.8 \pm 2.0^{**}$	$6.5 \pm 2.2^{**}$	$48.0 \pm 16.3^*$

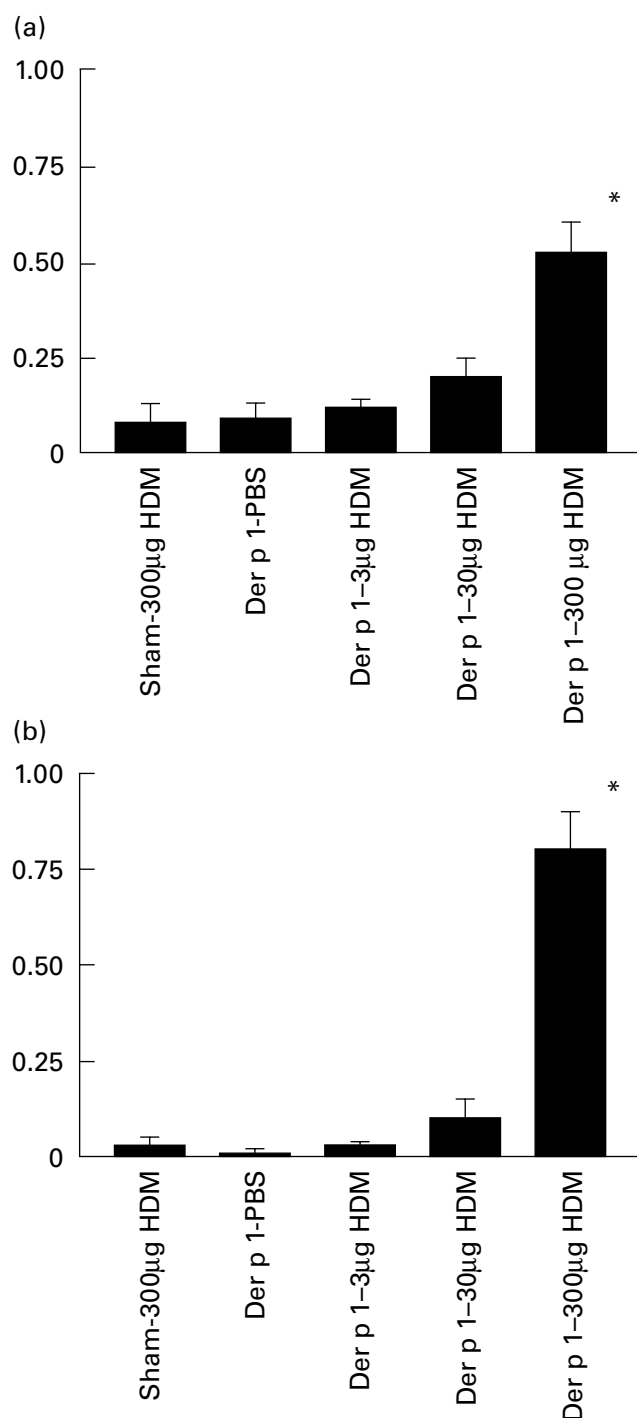


Fig. 1. Results of histological scoring for peribronchial (a) and perivascular (b) inflammatory changes of lung sections from different groups of mice. A score of 0–3 was adjudged to the tissue sections of the mice (see Methods). Bars represent means $\pm$ SEM. ANOVA, * $P < 0.05$ vs other groups.

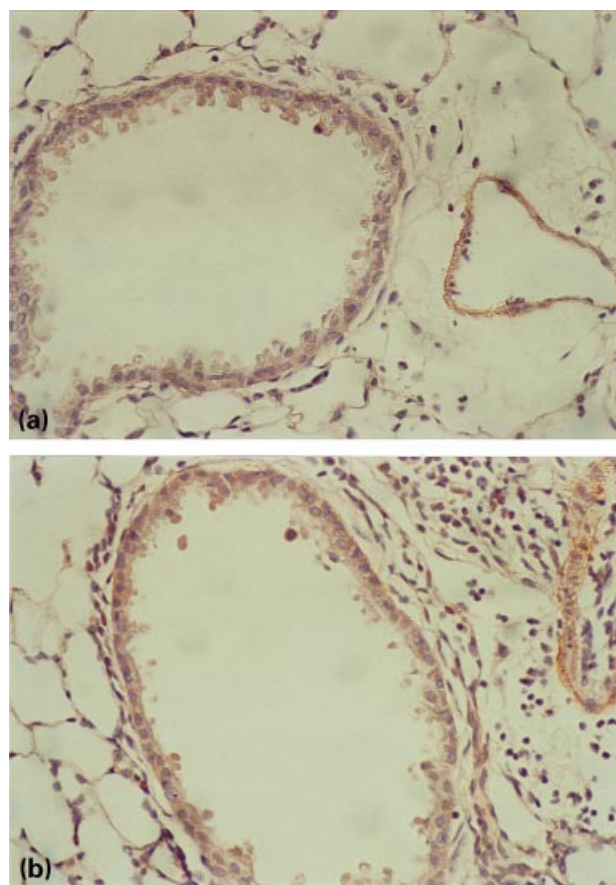


Fig. 2. Photomicrographs of the allergic airway changes in the Der p 1-sensitized mice. Mice were exposed to 3 µg HDM/mL (a) or to 300 µg HDM/mL (b). Magnification $\times 400$, Congo Red — Haematoxylin staining.

was significantly higher (0.63 ± 0.05 and 0.61 ± 0.24 mg carbachol/kg for sham-sensitized and PBS-challenged animals, respectively) when compared with the Der p 1 sensitized and HDM-challenged mice (0.16 ± 0.05 , 0.19 ± 0.05 and 0.18 ± 0.04 mg/kg for the mice exposed to 3, 30 and 300 µg HDM/mL aerosol, respectively) (ANOVA, $P = 0.009$).

Der p 1-specific IgE

Elevated serum concentrations of Der p 1-specific IgE antibodies were observed in all sensitized animals regardless the concentration used for the HDM exposure (ANOVA, $P < 0.001$) (Table 2).

Discussion

In the present study, we describe an *in vivo* mouse model of house dust mite-induced airway changes. In addition, we demonstrate that the allergen-induced airway hyperresponsiveness is not related either to the quantity of allergen-specific serum IgE, or to the influx of eosinophils into the airways.

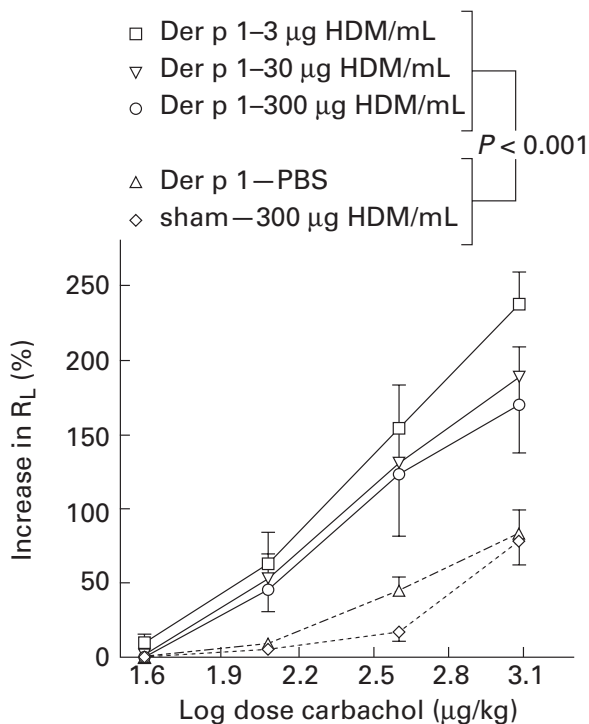


Fig. 3. Effect of exposure to various concentrations of house dust mite extract on airway responsiveness to carbachol. ANOVA, $P < 0.001$ compared with sham-sensitized but HDM-exposed or Der p 1-sensitized but PBS-exposed mice.

In vivo animal models, their limitations notwithstanding, can provide valuable information for a better understanding of the pathophysiology and treatment of asthma. Most of the animal models developed so far are based on antigens that are not readily involved in human asthma, one of the most frequently used being ovalbumin [8]. The nature of the antigen is, however, one of the determining factors in the ensuing immunological response. It is therefore important to

Table 2. Der p 1-specific serum IgE levels in nonimmunized and Der p 1-immunized mice exposed to different concentrations of house dust mite aerosol. All samples were diluted 1 : 10 and values are expressed as units of optical density (OD) measured at 450 nm. Values are expressed as means $\pm$ SEM. ANOVA, $*P < 0.001$ vs sham-immunized mice

Imm. day 0	HDM challenge days 14–20	Der p 1-specific serum IgE (OD)
Sham	300 µg/mL	0.06 $\pm$ 0.00
Der p 1	PBS	0.47 $\pm$ 0.05*
Der p 1	3 µg/mL	0.49 $\pm$ 0.04*
Der p 1	30 µg/mL	0.50 $\pm$ 0.03*
Der p 1	300 µg/mL	0.47 $\pm$ 0.08*

develop animal models using the main aeroallergens encountered by human asthmatics. The far most frequent allergen in perennial asthma is house dust mite. The association of Der p 1 exposure and allergy with the later development of asthma is striking as in some areas about 80% of the asthmatic children are allergic to house dust mite [17]. In other words, dust-mite sensitization is a risk factor for the development of asthma [3]. Airway hyperresponsiveness is a key feature of asthma, and airway inflammation is thought to underlay this altered airway behaviour. However, the precise role of various types of inflammatory cells and mediators involved in the pathophysiology of airway hyperresponsiveness remains to be fully determined. The current paradigm is that sensitization leads to the preferential development of TH2 cells which will then induce IgE synthesis through the release of interleukin (IL) -4 and eosinophil recruitment through IL-5 synthesis. The relative role of the IgE-mast cell pathway as opposed to the IL-5-eosinophil pathway in the pathophysiology of altered airway responsiveness is an area of intense debate.

In the present study, Der p 1 sensitization induced a production of IgE, which was not further enhanced by repeated exposure to house dust mite extract. This contrasts with previous findings, using ovalbumin as allergen, and points towards differences linked to the antigen used. We selected for the development of this animal model the C57Bl/6 inbred mouse. Previous investigators have shown that this C57Bl/6 mouse (H-2b), albeit a poor IgE responder [18], is capable of recognizing the purified Der p 1 allergen with the production of allergen-specific IgE [19,20]. On the contrary, the BALB/c mouse (H-2d), known as a strong IgE responder for ovalbumin [18], responded very poorly (as measured with a passive cutaneous reaction) or not at all (as measured with Der p 1-specific IgE) to purified Der p 1 allergens (nor after primary sensitization, nor after a secondary booster) [19,20, own observations]. Identical findings were described for Der f 1 from *Dermatophagoides farinae* [21]. The fact that the C57Bl/6 strain is generally a poor IgE responder could at least partially explain why no secondary increase in IgE was observed after allergen exposure.

No bronchial hyperresponsiveness was observed in the Der p 1-sensitized but PBS-exposed animals, dissociating circulating allergen-specific IgE from bronchial hyperresponsiveness. This finding is in agreement with other observations [22–24]. Importantly, most of the studies that support the link between IgE and airway hyperresponsiveness were performed in BALB/c mice, using other sensitization and exposure schemes [25–27]. This could at least explain the differences observed with the present results observed in C57Bl/6 mice [28].

We also observed a discrepancy between airway hyperresponsiveness and eosinophil influx into the airways. Exposure in the Der p 1-sensitized mice to the highest

concentration of HDM aerosol induced eosinophilic airway inflammation whereas exposure to 10- or 100-fold lower concentrations did not. Nevertheless, the latter animals developed an increased airway responsiveness. It is unlikely that the airway hyperresponsiveness observed in the mice with the low dose allergen exposure just resulted from an increased vascular permeability caused by the house dust mite aerosol since it was demonstrated that T cell-mediated induction of airway hyperresponsiveness in mice is independent of changes in the pulmonary vascular permeability [29]. Increasing evidence accumulates indicating that the eosinophil is not a crucial link in the induction of allergen-induced AHR [25,30–35] although some investigators stressed the importance of the eosinophil [36]. Again, differences in the strains and in experimental conditions could be a contributing factor. Despite the fact that the genetic background has a striking and selective effect on the phenotype of the murine allergic pulmonary response [18,37], it seems that the T-cell activation plays a pivotal role in the pathogenesis of airway hyperresponsiveness, probably through the release of a cytokine cocktail that might vary under different conditions in different strains [38].

Whether eosinophil influx and IgE are unrelated epiphenomena, or whether they play an enhancer role or whether they are crucial mediators of the T cell-induced changes in airway responsiveness certainly needs to be further investigated before extrapolations can be made towards the human condition. However, our findings provide, in agreement with other observations, new insights that eosinophils and IgE are not the essential mediators for the induction of house dust mite-induced allergic airway hyperresponsiveness.

In conclusion, we report on the development of a mouse model of house dust mite-induced allergic airway changes and we demonstrate that airway hyperresponsiveness is not related either to the quantity of allergen-specific serum IgE or to the influx of eosinophils into the airways.

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