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RESEARCH ARTICLE

Age-dependent pulmonary reactivity to house dust mite allergen: a model of adult-onset asthma?

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Mack S, Shin J, Ahn Y, Castaneda AR, Peake J, Fulgar C, Zhang J, Cho YH, Pinkerton KE. Age-dependent pulmonary reactivity to house dust mite allergen: a model of adult-onset asthma? *Am J Physiol Lung Cell Mol Physiol* 316: L757–L763, 2019. First published March 6, 2019; doi:10.1152/ajplung.00468.2018.—Asthma is a heterogeneous disease differentiated by factors like allergen sensitivity, inflammation, sex, and age at onset. The mouse model is widely used to study the early-life development of allergic asthma. However, age-dependent allergen responses later in life remain relatively understudied and lack a widely accepted model. To differentiate age-dependent responses to the ubiquitous house dust mite (HDM), 3- and 9-mo-old female C57BL/6 mice were randomized into two groups each and exposed to HDM or phosphate-buffered saline (control) via intranasal instillation for sensitization and challenge phases. At 24 h after challenge, all mice underwent pulmonary function testing and methacholine challenge. Bronchoalveolar lavage fluid (BALF) was collected for assessment of cell differentials, and right lung lobes were fixed, sectioned, and stained for histopathology and immunohistochemistry. Both age groups demonstrated strong inflammatory/allergic responses to HDM exposure. However, only 9-mo-old HDM-exposed mice demonstrated significant airway hyperresponsiveness compared with age-matched controls. These HDM-exposed mice also had *1*) statistically significant increases in tissue bronchiolitis, perivasculitis, and BALF neutrophilia relative to their younger counterparts and *2*) significantly increased extent of immunostaining compared with all other groups. This study presents a potential model for adult-onset asthma, focusing specifically on the atopic, perimenopausal female phenotype. Our findings suggest that lung function declines with age and that the inflammatory profile of this adult subgroup is a mixed, rather than a simple, atopic, Th2 response. This model may enhance our understanding of how age influences the development of asthmic-like symptoms in older subgroups.

animal model; atopic; eosinophils; neutrophils; pulmonary function

INTRODUCTION

Asthma is a serious chronic respiratory disease that affects more than 300 million people worldwide, including 18.7 million adults and 6.8 million children in the United States alone (2, 3, 23). Although asthma is known to be a heterogeneous disease, its many phenotypes are not clearly characterized. Generally, the main factors differentiating asthma phenotypes are atopic or nonatopic, Th2 or non-Th2 inflammation, symptom severity, sex, and age at disease onset (26).

The majority of known adult-onset asthma phenotypes are grouped as a non-Th2, or mixed immune, response in perimenopausal women or associated with smoking or neutrophilia (10, 13, 15, 25, 27). There is a need to tease out these currently identified adult-onset phenotypes to understand the mechanisms that drive them. This study proposes a model for mixed (eosinophilic/neutrophilic) inflammation in human adult asthma that could be used for future mechanistic studies. Specifically, this female-only mouse study could serve as a model for an atopic, perimenopausal phenotype and help improve the understanding of age-related differences in the adult asthmatic response.

The mouse is the most widely used animal model for the study of allergic asthma, mainly because of its availability, short life cycle, and reproducibility in creating a consistent model (18, 29). C57BL/6 mice primarily exhibit non-Th2-dominated immune responses but have the capacity to develop classic asthma-like responses, such as allergen-specific IgE, airway hyperresponsiveness (AHR), and eosinophilic airway inflammation (21), and have been used successfully in allergen challenge studies (12, 16, 18, 24). For the phenotype of interest, use of C57BL/6 mice is advantageous because of the availability of conditional mutants (21), but more importantly, C57BL/6 mice are not predisposed to Th2-related eosinophilic inflammation and, thus, have potential to present a non-Th2, or mixed, profile.

House dust mite (HDM) is a human-relevant, ubiquitous allergen and among the most common triggers of allergic asthma in humans and animals (1, 5, 11). Proteins in the excrement of HDM, such as *Dermatophagoides farinae* (Der f) 1 protease, act as the allergen (22). In addition, the natural gut flora of HDM includes gram-negative bacteria with endotoxin, which induces a strong inflammatory reaction and is thought to act as an adjuvant (17, 19, 28).

Flurkey et al. suggest that a 3-mo-old C57BL/6 mouse is the age equivalent of a 20-yr-old human (9); 3-mo-old C57BL/6 mice can be considered young adults: mature, yet not affected by aging. Nine-month-old mice can be considered middle-aged, the human age equivalent of late 30s to early 40s, when some age-related changes, such as decline in lung function, may be exhibited. In this study, subsets of 3- and 9-mo-old female C57BL/6 mice, representing young-adult and middle-aged groups, respectively, were made allergic or maintained as controls via sensitization and challenge to intranasally instilled

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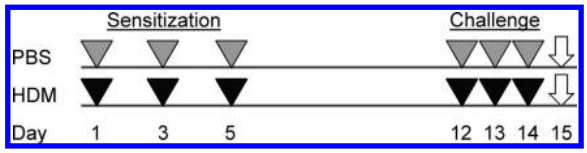

Fig. 1. Schedule for sensitization and challenge. Sensitization and challenge exposures are shown on the *left* (*days 1, 3*, and 5) and *right* (*days 12–14*), respectively. Open arrows represent *day 15* necropsy. Instillation of PBS (gray triangles) and house dust mite allergen solution (HDM; black triangles) was the same for 3- and 9-mo-old mice. Each triangle represents one (30-μl) instillation. For both PBS-exposed groups, $n = 5$; for both HDM-exposed groups, $n = 6$.

HDM or phosphate-buffered saline [PBS (the suspension vehicle for HDM)], respectively. Changes in pulmonary function, inflammation, histology, and immunohistochemical (IHC) staining were compared between the four exposure groups to determine differences in reactivity.

MATERIALS AND METHODS

Mice. Female C57BL/6 mice were obtained from Harlan Laboratories (Livermore, CA). The 3- and 9-mo-old mice were randomized into two groups each. The 9-mo-old mice had not been bred for ≥3 mo before the inception of our study. Control groups were exposed to PBS [$n = 5$ per age group (3 and 9 mo old)], and treatment groups were exposed to HDM [$n = 6$ per age group (3 and 9 mo old)]. The 3- and 9-mo-old mice were 14 and 37 wk old, respectively, at the start of sensitization, with mean body weights of 21.3 g (range 20–22 g) and 26.5 g (range 23–31 g), respectively. Experiments and animal protocols were approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

HDM exposure. Lyophilized HDM extract from whole bodies of *D. farinae* were obtained from Greer Laboratories (Lenoir, NC). HDM concentrations were calculated based on the average weight per age group. For the 3-mo-old mice, 20 μg of HDM protein were reconstituted in 30 μl of PBS (total dose 5.63 μg/g body wt). For the 9-mo-old group, each HDM dose contained 25 μg of HDM protein reconstituted in 30 μl of sterile PBS (total dose 5.66 μg/g body wt). The content of the major allergen, Der f 1, was 193 ng/μg of HDM protein. The endotoxin level was 25.6 endotoxin units/μg of HDM protein.

An allergic response was induced via intranasal instillation while mice were sedated to level 2 anesthesia by isoflurane gas in an exposure chamber. The mice were sensitized on *days 1, 3*, and *5* and challenged on *days 12, 13*, and *14* (Fig. 1). During both phases, each mouse received 30 μl of HDM suspension or PBS per day. This 14-day sensitization and challenge schedule, in addition to being highly efficient, was repeatedly found to be a highly reproducible method in our laboratory, as it allows sufficient time for development of an adaptive immune response upon allergen challenge (7).

Pulmonary function testing. On *day 15* (24 h after the final challenge), mice were deeply anesthetized with tiletamine-zolazepam (50 mg/kg ip) and dexmedetomidine (0.7 mg/kg ip) and paralyzed with succinylcholine (1 mg im). The 1-mg dose ensured a safe, sufficient, and standardized level of anesthesia for all mice, with no premature loss of animals or measurable effects on pulmonary function. After the mice were intratracheally cannulated, a flexiVent system (SCIREQ, Montreal, PQ, Canada) was used for pulmonary function testing and methacholine (MCh) challenges (0.25, 0.5, 1, 2, and 4 mg of MCh/ml). Mice were ventilated at a frequency of 150 breaths/min and a tidal volume of 10 ml/kg. Respiratory system input impedance was measured to distinguish central and peripheral lung mechanics. Resistance, compliance, and elastance were measured for the whole respiratory system. Central airway resistance, tissue hysteresis, and tissue elastance were determined after forced oscillation perturbation. All data were measured in triplicate and averaged for each animal. The effective concentration that leads to a twofold increase in resistance ($EC_{200}RL$) was calculated from the MCh challenge results to determine AHR.

Bronchoalveolar lavage fluid. Lungs were lavaged with three aliquots of 0.6 ml of Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution. Bronchoalveolar lavage fluid (BALF) was collected, and cell suspensions were centrifuged (1,500 rpm for 5 min; Shandon Cytospin, Thermo Shandon, Pittsburg, PA) onto slides (15,000 cells/slide). Slides were stained with hematoxylin and eosin (American MasterTech, Lodi, CA) and labeled with animal numbers to ensure blinded reading. For assessment of cell differentials, macrophages, monocytes, neutrophils, lymphocytes, and eosinophils were counted using light microscopy (300 cells/slide).

Lung tissue preparation and histology. Right lung lobes (cranial, middle, caudal, and accessory) were inflated and fixed at 30 cm hydrostatic pressure of 4% paraformaldehyde for 1 h and subsequently stored in 4% paraformaldehyde overnight and transferred to ethanol after 24 h. The fixed lobes were embedded in paraffin, cut into 5-μm-thick sections, and placed on seven slides. For each lung lobe, one slide was stained with hematoxylin and eosin and a coverslip was applied. A semiquantitative scoring system (Table 1) was used to assess cellular infiltrates and histological changes.

Immunohistochemistry. For each mouse, one slide per lobe was stained with hematoxylin and eosin, and six slides per lobe were stained for IHC. Each of the six slides for IHC was stained with a different primary antibody. The slides described above were deparaffinized in three changes of toluene (5, 2, and 2 min) and rehydrated in 100%, 95%, and 70% ethanol for 2 min each. For antigen retrieval, slides were submerged in ethylenediaminetetraacetic acid and heated in a high-pressure decloaker. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min. To eliminate nonspecific binding of the primary antibody, sections were incubated with protein block (Dako, Carpinteria, CA) for 10 min at room temperature. Each section was incubated for 1 h at room temperature with one of the following primary antibodies: anti-IL-4 (1:150 dilution; catalog no. ab9728, Abcam, Cambridge, MA), anti-IL-13 (1:50 dilution; catalog no. ab83162, Abcam), anti-eotaxin-1 (1:60 dilution; catalog no. ab125225, Abcam), anti-IL-17 (1:70 dilution; catalog no. ab79056, Abcam), anti-CD4 (1:100 dilution; catalog no. ab183685, Abcam), or anti-IFN-γ (1:50 dilution; catalog no. ab9657, Abcam). Negative controls were treated with PBS, instead of a primary antibody. Sections were then rinsed and incubated for 30 min at room temperature in anti-goat

Table 1. *Semiquantitative scoring rubric for histopathological analysis*

	Score Value			
Score Type	0	1	2	3
Severity	Few or no inflammatory cells visible	Slightly increased cellularity but no PMNs	Moderately increased cellularity with PMNs present	Marked influx of cells accompanied by thickened tissue
Extent	Normal tissue	About a third of lung section affected by inflammation	About half of lung section affected by inflammation	More than three-quarters of lung section affected by inflammation

This rubric was used to grade histopathology in 5-μm-thick tissue sections of the right cranial, middle, caudal, and accessory lung lobes of 3- or 9-mo-old mice. Four lobes were examined per mouse. Inflammation was evaluated throughout the parenchyma of each section. PMNs, polymorphonuclear leukocytes.

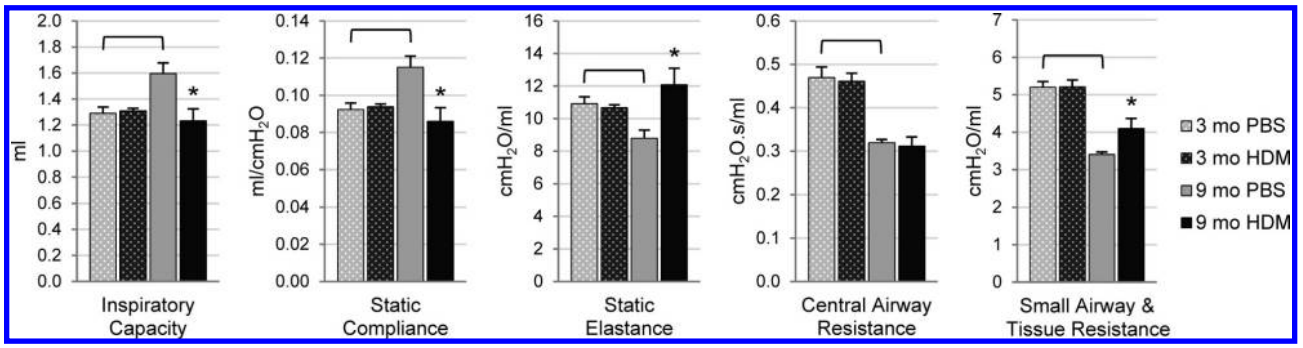

Fig. 2. Age and exposure affect pulmonary function. A flexiVent system was used to record in vivo lung function measurements: inspiratory capacity, quasistatic lung compliance, quasistatic lung elastance, central airway resistance, and small airway and tissue resistance. Values are means ± SE; $n = 5$ PBS-exposed mice and 6 house dust mite (HDM)-exposed mice. *Significant exposure-related differences from age-matched control group; brackets indicate significant age-related differences between similarly exposed groups ($P < 0.05$, by ANOVA).

(1:200 dilution; Vectastain ABC kit, catalog no. PK-4005, Vector Laboratories, Burlingame, CA) or horseradish peroxidase-labeled polymer anti-rabbit (EnVision System, catalog no. K4001, Dako) secondary antibody. Bound peroxidase activity was visualized with a 5-min incubation in a 3,3′-diaminobenzidine-positive substrate chromogen system (catalog no. K3468, Dako). Tissue sections were counterstained with hematoxylin. IHC staining was evaluated qualitatively.

Statistical analyses. Values are means ± SE. The Shapiro-Wilk test was used to assess normality of data. Appropriate logarithmic or square root transformations were performed to obtain normal distributions before analysis of variance (ANOVA). One-way ANOVAs were performed to test the effect of age or HDM exposure on pulmonary function. To determine whether age, HDM exposure, or age × HDM interaction predicted BAL cell counts, a two-way multivariate ANOVA was performed. Post hoc Tukey's honest significant difference tests were used to determine statistically significant differences between specific groups. Moreover, Pearson's correlation coefficients were obtained to determine whether BALF measurements were predictive of $EC_{200}RL$ values from pulmonary function tests. For histopathology scores, the Mann-Whitney (Wilcoxon's rank sum) test was performed to detect possible age-related differences in perivascular and alveolar inflammation following HDM exposure. This nonparametric test was chosen because semiquantitative scores obtained by multiplying two separate values produced data that were not normally distributed and could not be corrected by transformations. "Significant" differences were assessed at $P < 0.05$. Statistical analyses were performed using SAS JMP computer software (version 13, SAS Institute, Cary, NC).

RESULTS

Pulmonary function. Three-month-old mice did not demonstrate significant exposure-related differences in any pulmonary function parameters. In contrast, 9-mo-old HDM-exposed mice had significantly decreased inspiratory capacity and compliance (static and respiratory) and increased small airway/peripheral tissue resistance and elastance (static and respiratory) compared with their age-matched controls (Fig. 2; $P < 0.05$, by ANOVA). Significant differences were not detected for central airway resistance.

Significant age-related differences were noted between 3- and 9-mo-old control groups. The 9-mo-old mice exhibited significantly increased inspiratory capacity and compliance and significantly decreased elastance and resistance in both central airway and small airway/peripheral tissue compared with their younger counterparts (Fig. 2; $P < 0.05$, by ANOVA).

BALF cells. Sensitization and subsequent challenge with HDM resulted in significantly increased ($P < 0.05$, by 2-way multivariate ANOVA and Tukey's honest significant difference test) total cell counts in the BALF of both age groups compared with their respective PBS-exposed controls (Fig. 3, *left*). Moreover, inflammatory cell differentials (Fig. 3, *right*), with significantly higher counts of monocytes, neutrophils, lymphocytes, and eosinophils in both age groups after HDM than PBS exposure, suggested an allergic reaction. Between

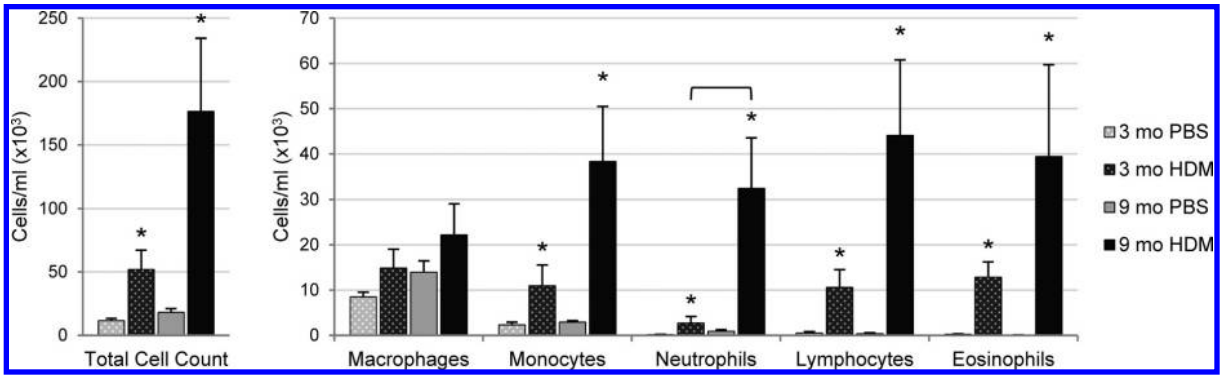

Fig. 3. Total (*left*) and differential (*right*) cell counts from bronchoalveolar lavage fluid. Total cell count was significantly higher in middle-aged (9-mo-old) than young-adult (3-mo-old) mice exposed to house dust mite (HDM). Differential cell counts showed a significant difference in number of neutrophils between HDM-exposed young-adult and middle-aged mice. Values are means ± SE; $n = 5$ PBS-exposed mice and 6 HDM-exposed mice. *Significant exposure-related differences from age-matched control group; brackets indicate significant age-related differences between similarly exposed groups ($P < 0.05$, by 2-way multivariate ANOVA and Tukey's honest significant differences test).

age groups, a significant difference in neutrophilia was seen in HDM-exposed mice, specifically, as 9-mo-old mice displayed higher counts.

Correlation between AHR and BALF cell differentials. $EC_{200}RL$ was significantly decreased in HDM-exposed, 9-mo-old (but not 3-mo-old) mice compared with their age-matched controls (Fig. 4, *left*; $P < 0.05$, by ANOVA). For these older mice, $EC_{200}RL$ most strongly correlated ($R^2 = 0.617$, $P < 0.01$) with the number of eosinophils in BALF (Fig. 4, *right*) but was also correlated, at smaller, nonsignificant magnitudes, with neutrophil, lymphocyte, and total cell counts (data not shown).

Histopathology. Semiquantitative evaluations of lung sections showed marked perivascular inflammation (vasculitis) and bronchiolitis in HDM-exposed mice compared with their age-matched controls (Table 2). Vasculitis detected in arterial and venous portions of the pulmonary circulation (Fig. 5, *A* and *B*) was significantly greater in 9-mo-old HDM-exposed mice ($P < 0.05$, by ANOVA) than in their 3-mo-old counterparts (Table 2). Alveolar or pleural inflammation was not significantly different between groups (Table 2), and no notable inflammation was observed in lung tissue sections of mice exposed to PBS alone (Fig. 5,*C* and *D*).

Immunohistochemistry. All lung sections stained positively for IL-4, IL-13, IL-17, CD4, eotaxin-1, and IFN-γ. For all target proteins, lungs of 9-mo-old HDM-exposed mice appeared to stain positively to a greater extent than lungs of all other groups (Table 3). The semiquantitative scoring guide was as follows: 0 = normal (complete lack of staining for the targeted protein of interest), 1 = mild [minimal staining of cells for the targeted protein consisting of very few immunopositive cells around airways and blood vessels where cellular infiltration of these sites is abundant (<10% of all peribronchiolar/perivascular cells)], 2 = moderate (>25% of all cells are immunopositive for the target protein in peribronchiolar and perivascular areas of significant cellular influx), and 3 = marked (>50% of all cells are immunopositive in peribronchiolar and perivascular areas of significant cellular influx). Figure 6 shows immunostaining categorized in the range of 2–3. We feel these trends in immunostaining are best illustrated using +, ++, and +++ symbols; however, a table showing numeric values scored for each target protein analyzed by IHC is included in supplemental material (Supplemental Table S1; see https://doi.org/10.5281/zenodo.2574009). Regardless of treatment, 9-mo-old mice stained positively for all antibodies except eotaxin-1. A higher percentage of stained cells was observed in the 9-mo-old HDM-exposed mice for IL-4, IL-13, IL-17A, and IFN-γ. Negative controls (data not shown) showed no staining.

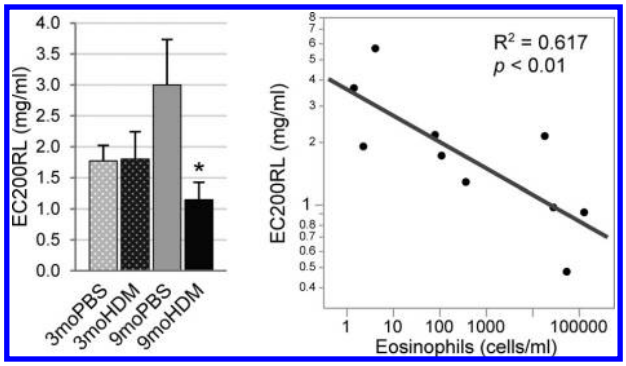

Fig. 4. Effective methacholine concentration that leads to a 2-fold increase in resistance ($EC_{200}RL$) and its correlation with bronchoalveolar lavage fluid eosinophils in middle-aged (9-mo-old) mice. A methacholine challenge was performed to determine airway hyperresponsiveness, measured as $EC_{200}RL$. $EC_{200}RL$ results (*left*) from house dust mite (HDM)-exposed middle-aged mice were tested for correlations (Pearson's) to bronchoalveolar lavage fluid eosinophilia (*right*) and neutrophilia (data not shown). Values in bar graph are means ± SE; $n = 5$ PBS-exposed mice and 6 HDM-exposed mice. *Significant exposure-related differences from age-matched control group ($P < 0.05$, by ANOVA).

Table 2. *Semiquantitative scores to characterize histopathological inflammation*

	Inflammation Score			
Treatment Group	Bronchiolar	Perivascular	Alveolar	Pleural
3 mo PBS	0.2 ± 0.2	0	0.2 ± 0.2	0
3 mo HDM	1.8 ± 0.4*	3.0 ± 0.5*	0.7 ± 0.4	0.2 ± 0.2
9 mo PBS	0	0.2 ± 0.2	0.2 ± 0.2	0
9 mo HDM	3.3 ± 0.4*‡	6.5 ± 0.5*‡	2.2 ± 0.7	0

Values are means ± SE; $n = 5$ PBS-exposed (control) mice and 6 house dust mite (HDM)-exposed mice. Semiquantitative scores of bronchiolitis, perivasculitis, alveolitis, and pleuritis were calculated for each animal by multiplying severity and extent scores (see Table 1 for associated rubrics). *Significant exposure-related difference from age-matched control group; ‡significant age-related difference between similarly exposed groups ($P < 0.05$, by Wilcoxon's rank sum test).

DISCUSSION

Age-dependent differences in the asthmatic response are consistently observed in humans but are understudied and lack an animal model. Allergic asthma primarily begins in childhood and will either resolve or persist into adulthood. Asthma that begins in adulthood is grouped separately from persistent early-onset asthma, as it has several distinct phenotypes, including a more severe presentation clinically due to increased comorbidities (8). The age-related subgroups suggest effects of age on susceptibility to allergic asthma and the subsequent inflammatory response. One major phenotype presents as a perimenopausal change in females; this female-only study could serve as a unique model for that particular phenotype. The model clearly shows a distinction between the asthmatic response in young-adult (3-mo-old) and middle-aged (9-mo-old) mice, but the specific endotypes and how they are mechanistically different require further investigation.

Pulmonary function testing demonstrated a significant hyperreactive airway response to HDM exposure in only the middle-aged mice. Resistance was increased only in the small airways and tissues, suggesting a change in the more distal airways, rather than in the larger bronchi. The decrease in inspiratory capacity and compliance and the increase in elastance are consistent with an asthmatic response. Additionally, significant differences were observed between the middle-aged and young-adult control mice in all pulmonary function testing measurements. This suggests that there are age-related effects on pulmonary function, even in the absence of an allergen. It is not clear whether such significant differences would be found between healthy young-adult and middle-aged humans. Human lungs mature by age 20–25, and lung function begins to decline at around age 35, whereas mouse lung function generally does not begin to decline at the equivalent age (14, 20).

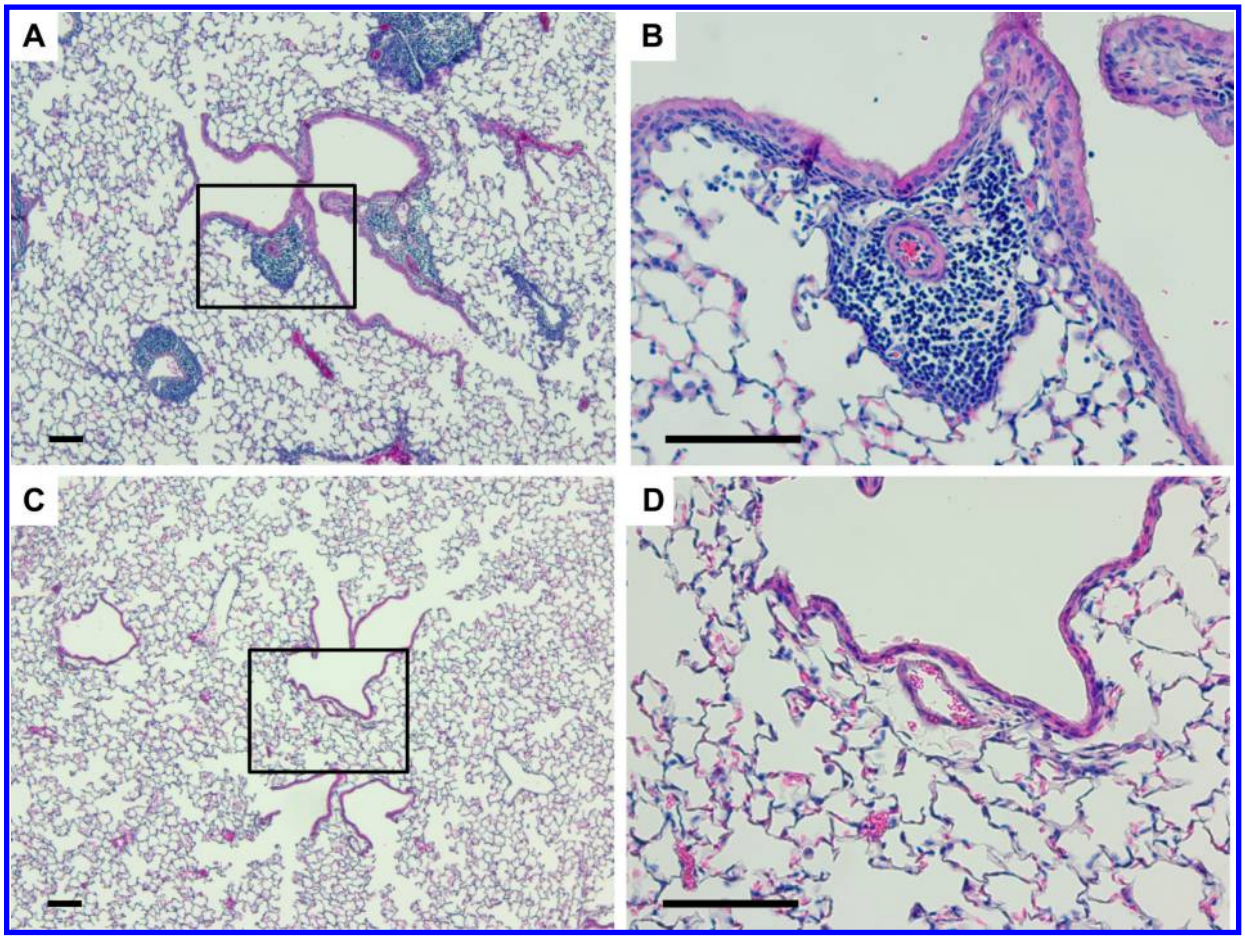

Fig. 5. Hematoxylin-and-eosin staining of right lung sections for histopathological scoring and evaluation. Bright-field microscopy images are shown of observed responses in right lung sections from 9-mo-old mice. Sections were stained with hematoxylin and eosin (H&E). Images show two magnifications of middle-aged PBS-treated (*A* and *B*) and middle-aged house dust mite (HDM)-treated (*C* and *D*) mice. $n = 5$ for PBS-exposed groups and 6 for HDM-exposed groups. Scale bars = 100 μm.

However, as our results depict a significant age-based difference, it is possible that this functional decline led to the hypersensitivity in the presence of an allergen.

Both age groups exposed to HDM showed significant allergic and inflammatory responses in the BALF compared with their age-matched PBS-exposed controls. However, the overall cellular response was more than threefold higher in the 9- than 3-mo-old mice exposed to HDM. Increased neutrophilia was the only statistically significant observation between the 3- and 9-mo-old HDM-exposed groups. This adds merit to human studies that show severe asthmatic symptoms connected to airway neutrophilia, rather than the more classic eosinophilic inflammation, in perimenopausal women. At least one study showed a greater Th17-neutrophilic airway response in 15- than 3-mo-old mice (4). In the present study, although much of the cell differential results did not appear to be affected by age, lymphocytes, eosinophils, and monocytes were significantly increased in BALF of 9-mo-old HDM-exposed mice compared with 9-mo-old PBS-exposed mice. More than a simple non-Th2 response, this result indicates that the inflammatory response is mixed, which is how late-onset asthma is defined in a recent review by Carr et al. (6).

The cytokine panel revealed by IHC staining supports a mixed immune response. The presence of IL-4 and IL-17A in the

Table 3. *Qualitative assessment of immunohistochemical staining*

	Treatment Group			
Target Protein	3 mo PBS	3 mo HDM	9 mo PBS	9 mo HDM
CD4	+	++	++	++
IL-4	+	++	++	+++
IL-13	+	+	+	++
IL-17A	+	++	++	+++
IFN-γ	−	−	−	++
Eotaxin-1	−	−	+	+

Immunostaining for each target protein was evaluated in all 4 lobes of the right lung and scored on a scale from 0 to 3. 0 = complete lack of staining for targeted protein of interest, 1 = minimal staining for targeted protein [very few immunopositive cells around airways and blood vessels where cellular infiltration of these sites is abundant (<10% of all peribronchiolar/perivascular cells)], 2 = greater than 25% of all cells are immunopositive for target protein in peribronchiolar and perivascular areas of significant cellular influx, 3 = greater than 50% of all cells are immunopositive in peribronchiolar and perivascular areas of significant cellular influx. Average scores for each treatment group were transformed to the following scales: 0 = normal (−), 0.1–1 = mild (+), 1.1–2 = moderate (++), 2.1–3 = marked (+++). For each target protein, all treatment groups were immunostained at the same time. For PBS-exposed mice, $n = 5$; for house dust mite (HDM)-exposed mice, $n = 6$.

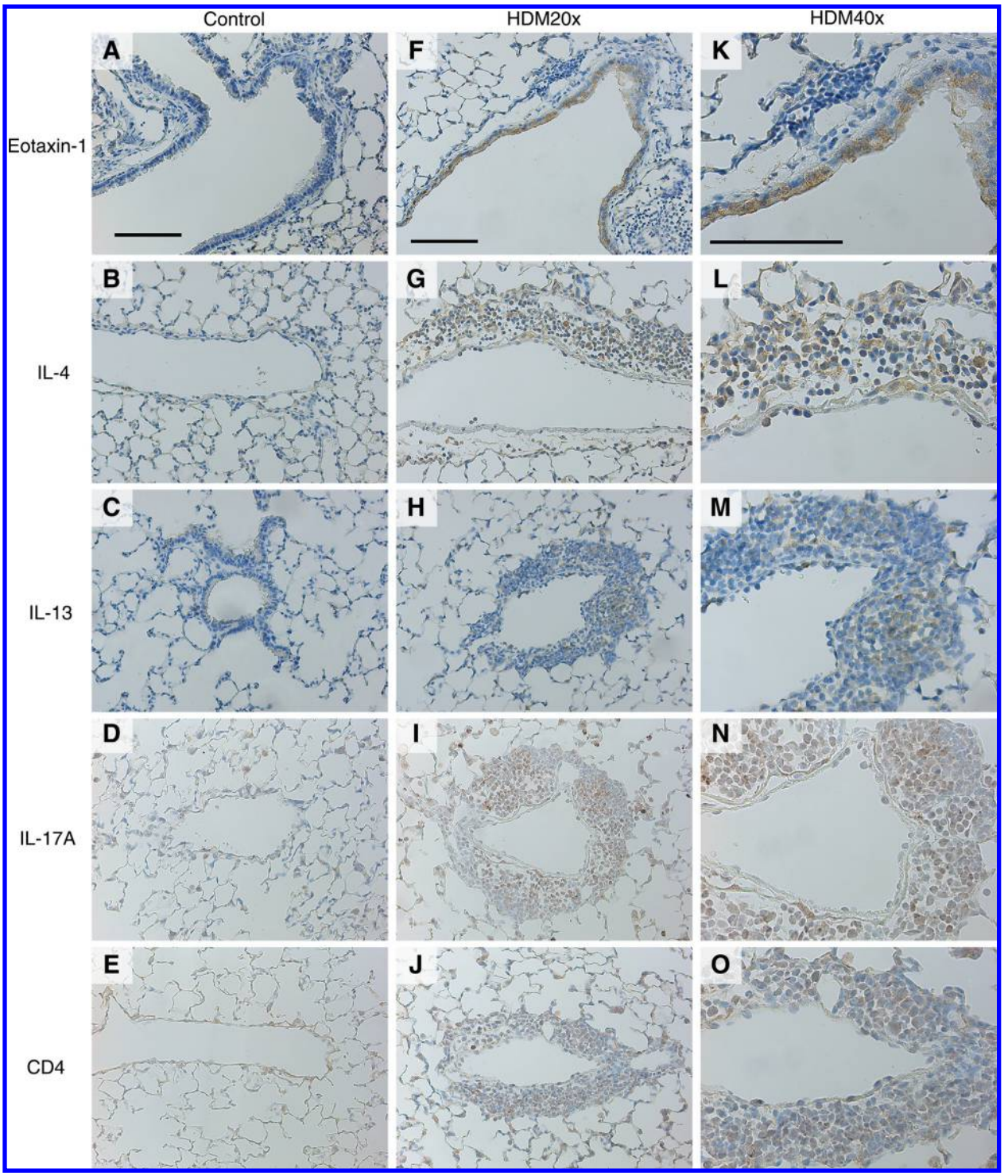

Fig. 6. Immunohistochemical staining for eotaxin-1, IL-4, IL-13, IL-17A, and CD4 proteins. Bright-field microscopy images are shown of specific immunohistochemical responses to PBS or house dust mite (HDM) exposure in middle-aged mice. Images show lung tissue sections from the former (*A–E*) and the latter (*F–O*) at two different magnifications. Tissues were stained with antibodies for eotaxin-1 (*A*, *F*, *K*), IL-4 (*B*, *G*, *L*), IL-13 (*C*, *H*, *M*), IL-17A (*D*, *I*, *N*), and CD4 (*E*, *J*, *O*). Scale bars: 100 μm.

lymphocytes, combined with the presence of CD4 and IFN-γ, does not allow us to distinguish between the two responses. This separates this endotype from the obviously Th2-mediated, young-adult allergic response. Extensive investigation is needed to understand this mixed immune response and the greater expression of IL-4, IL-17, and CD4 in the lungs of middle-aged mice following HDM sensitization and challenge. More studies utilizing this model could help elucidate this mechanism.

It is important to reiterate that this study involved only female mice and that results may not be comparable for male mice. Thus it is possible that the results of this study are dependent on sex in conjunction with age. To validate this,

future studies are needed with male C57BL/6 mice. However, as previously stated, because adult-onset asthma in humans often presents as a perimenopausal change in females, this female-only study could serve as a model for that particular phenotype.

As an acute asthma model, changes associated with chronic asthma, such as airway remodeling and chronic inflammation of the airways, were not observed. However, significant differences in lung function, as well as in the inflammatory profile, were observed between the HDM-exposed age groups, demonstrating an age-related difference in the acute response. This observation warrants future mechanistic studies. Further characterization of this model may lead to a better understanding of the effect of age on late-onset asthma and differences between Th2 and mixed phenotypes in humans.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Y.A. and K.E.P. conceived and designed research; S.M., J.S., Y.A., A.R.C., J.P., C.F., J.Z., and Y.H.C. performed experiments; S.M., J.S., and Y.H.C. analyzed data; S.M., A.R.C., and K.E.P. interpreted results of experiments; S.M. prepared figures; S.M. and J.S. drafted manuscript; S.M. and K.E.P. edited and revised manuscript; S.M., J.S., Y.A., A.R.C., J.P., C.F., J.Z., Y.H.C., and K.E.P. approved final version of manuscript.

REFERENCES

1. **Ahluwalia SK, Matsui EC.** The indoor environment and its effects on childhood asthma. *Curr Opin Allergy Clin Immunol* 11: 137–143, 2011. doi:10.1097/ACI.0b013e3283445921.
2. **Blackwell DL, Lucas JW, Clarke TC.** Summary health statistics for US adults: National Health Interview Survey, 2012. *Vital Health Stat* 260: 1–161, 2014.
3. **Bloom B, Jones LI, Freeman G.** Summary health statistics for US children: National Health Interview Survey, 2012. *Vital Health Stat* 258: 1–81, 2013.
4. **Brandenberger C, Li N, Jackson-Humbles DN, Rockwell CE, Wagner JG, Harkema JR.** Enhanced allergic airway disease in old mice is associated with a Th17 response. *Clin Exp Allergy* 44: 1282–1292, 2014. doi:10.1111/cea.12388.
5. **Calderón MA, Linneberg A, Kleine-Tebbe J, De Blay F, Hernandez Fernandez de Rojas D, Virchow JC, Demoly P.** Respiratory allergy caused by house dust mites: what do we really know? *J Allergy Clin Immunol* 136: 38–48, 2015. doi:10.1016/j.jaci.2014.10.012.
6. **Carr TF, Zeki AA, Kraft M.** Eosinophilic and noneosinophilic asthma. *Am J Respir Crit Care Med* 197: 22–37, 2018. doi:10.1164/rccm.201611-2232PP.
7. **Castaneda AR, Pinkerton KE.** Investigating the effects of particulate matter on house dust mite and ovalbumin allergic airway inflammation in mice. *Curr Protoc Toxicol* 68: 18.18.1–18.18.18, 2016. doi:10.1002/cptx.5.
8. **Dunn RM, Busse PJ, Wechsler ME.** Asthma in the elderly and late-onset adult asthma. *Allergy* 73: 284–294, 2018. doi:10.1111/all.13258.
9. **Flurkey K, Currer J, Harrison DE.** Mouse models in aging research. In: *The Mouse in Biomedical Research* (2nd ed.), edited by Fox JG, Davisson MT, Quimby FW, Barthold SW, Newcomer CE, Smith AL. Burlington, VT: Academic, 2007, chapt. 20, p. 637–672.
10. **Haldar P, Pavord ID, Shaw DE, Berry MA, Thomas M, Brightling CE, Wardlaw AJ, Green RH.** Cluster analysis and clinical asthma phenotypes. *Am J Respir Crit Care Med* 178: 218–224, 2008. doi:10.1164/rccm.200711-1754OC.
11. **Johnson JR, Wiley RE, Fattouh R, Swirski FK, Gajewska BU, Coyle AJ, Gutierrez-Ramos JC, Ellis R, Inman MD, Jordana M.** Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling. *Am J Respir Crit Care Med* 169: 378–385, 2004. doi:10.1164/rccm.200308-1094OC.
12. **Kumar RK, Herbert C, Foster PS.** The "classical" ovalbumin challenge model of asthma in mice. *Curr Drug Targets* 9: 485–494, 2008. doi:10.2174/138945008784533561.
13. **Miranda C, Busacker A, Balzar S, Trudeau J, Wenzel SE.** Distinguishing severe asthma phenotypes: role of age at onset and eosinophilic inflammation. *J Allergy Clin Immunol* 113: 101–108, 2004. doi:10.1016/j.jaci.2003.10.041.
14. **Mohr U.** *Pathobiology of the Aging Mouse*. Washington, DC: ILSI, 1996.
15. **Moore WC, Meyers DA, Wenzel SE, Teague WG, Li H, Li X, D'Agostino R Jr, Castro M, Curran-Everett D, Fitzpatrick AM, Gaston B, Jarjour NN, Sorkness R, Calhoun WJ, Chung KF, Comhair SA, Dweik RA, Israel E, Peters SP, Busse WW, Erzurum SC, Bleecker ER; National Heart, Lung, and Blood Institute's Severe Asthma Research Program.** Identification of asthma phenotypes using cluster analysis in the Severe Asthma Research Program. *Am J Respir Crit Care Med* 181: 315–323, 2010. doi:10.1164/rccm.200906-0896OC.
16. **Morokata T, Ishikawa J, Ida K, Yamada T.** C57BL/6 mice are more susceptible to antigen-induced pulmonary eosinophilia than BALB/c mice, irrespective of systemic T helper 1/T helper 2 responses. *Immunology* 98: 345–351, 1999. doi:10.1046/j.1365-2567.1999.00890.x.
17. **Murphy K, Travers P, Walport M, Janeway C.** *Janeway's Immunology*. New York: Garland Science, 2012.
18. **Nials AT, Uddin S.** Mouse models of allergic asthma: acute and chronic allergen challenge. *Dis Model Mech* 1: 213–220, 2008. doi:10.1242/dmm.000323.
19. **Platts-Mills TA, Woodfolk JA.** Allergens and their role in the allergic immune response. *Immunol Rev* 242: 51–68, 2011. doi:10.1111/j.1600-065X.2011.01021.x.
20. **Sharma G, Goodwin J.** Effect of aging on respiratory system physiology and immunology. *Clin Interv Aging* 1: 253–260, 2006. doi:10.2147/ciia.2006.1.3.253.
21. **Shin YS, Takeda K, Gelfand EW.** Understanding asthma using animal models. *Allergy Asthma Immunol Res* 1: 10–18, 2009. doi:10.4168/aair.2009.1.1.10.
22. **Thomas WR, Hales BJ, Smith WA.** House dust mite allergens in asthma and allergy. *Trends Mol Med* 16: 321–328, 2010. doi:10.1016/j.molmed.2010.04.008.
23. **To T, Stanojevic S, Moores G, Gershon AS, Bateman ED, Cruz AA, Boulet LP.** Global asthma prevalence in adults: findings from the cross-sectional world health survey. *BMC Public Health* 12: 204, 2012. doi:10.1186/1471-2458-12-204.
24. **Tournoy KG, Kips JC, Schou C, Pauwels RA.** Airway eosinophilia is not a requirement for allergen-induced airway hyperresponsiveness. *Clin Exp Allergy* 30: 79–85, 2000. doi:10.1038/nm.2678.
25. **Wenzel SE.** Asthma phenotypes: the evolution from clinical to molecular approaches. *Nat Med* 18: 716–725, 2012. doi:10.1038/nm.2678.
26. **Wenzel SE.** Emergence of biomolecular pathways to define novel asthma phenotypes. type-2 immunity and beyond. *Am J Respir Cell Mol Biol* 55: 1–4, 2016. doi:10.1165/rcmb.2016-0141PS.
27. **Wu W, Bleecker E, Moore W, Busse WW, Castro M, Chung KF, Calhoun WJ, Erzurum S, Gaston B, Israel E, Curran-Everett D, Wenzel SE.** Unsupervised phenotyping of Severe Asthma Research Program participants using expanded lung data. *J Allergy Clin Immunol* 133: 1280–1288, 2014. doi:10.1016/j.jaci.2013.11.042.
28. **Zhu Z, Oh SY, Zheng T, Kim YK.** Immunomodulating effects of endotoxin in mouse models of allergic asthma. *Clin Exp Allergy* 40: 536–546, 2010. doi:10.1111/j.1365-2222.2010.03477.x.
29. **Zosky GR, Sly PD.** Animal models of asthma. *Clin Exp Allergy* 37: 973–988, 2007. doi:10.1111/j.1365-2222.2007.02740.x.