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Effect of ageing on pulmonary inflammation, airway hyperresponsiveness and T and B cell responses in antigen-sensitized and -challenged mice

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Clinical and Experimental Allergy

Summary

Background The effect of ageing on several pathologic features of allergic asthma (pulmonary inflammation, eosinophilia, mucus hypersecretion), and their relationship with airway hyperresponsiveness (AHR) is not well characterized.

Objective To evaluate lung inflammation, mucus metaplasia and AHR in relationship with age in murine models of allergic asthma comparing young and older mice.

Methods Young (6 weeks) and older (6, 12, 18 months) BALB/c mice were sensitized and challenged with ovalbumin (OVA). AHR and bronchoalveolar fluid (BALF), total inflammatory cell count and differential were measured. To evaluate mucus metaplasia, quantitative PCR for the major airway mucin-associated gene, MUC-5AC, from lung tissue was measured, and lung tissue sections stained with periodic acid-Schiff (PAS) for goblet-cell enumeration. Lung tissue cytokine gene expression was determined by quantitative PCR, and systemic cytokine protein levels by ELISA from spleen-cell cultures. Antigen-specific serum IgE was determined by ELISA.

Results AHR developed in both aged and young OVA-sensitized/challenged mice (OVA mice), and was more significantly increased in young OVA mice than in aged OVA mice. However, BALF eosinophil numbers were significantly higher, and lung histology showed greater inflammation in aged OVA mice than in young OVA mice. MUC-5AC expression and numbers of PAS+ staining bronchial epithelial cells were significantly increased in the aged OVA mice. All aged OVA mice had increased IL-5 and IFN- γ mRNA expression in the lung and IL-5 and IFN- γ protein levels from spleen cell cultures compared with young OVA mice. OVA-IgE was elevated to a greater extent in aged OVA mice.

Conclusions Although pulmonary inflammation and mucus metaplasia after antigen sensitization/challenge occurred to a greater degree in older mice, the increase in AHR was significantly less compared with younger OVA mice. Antigen treatment produced a unique cytokine profile in older mice (elevated IFN- γ and IL-5) compared with young mice (elevated IL-4 and IL-13). Thus, the airway response to inflammation is lessened in ageing animals, and may represent age-associated events leading to different phenotypes in response to antigen provocation.

Keywords ageing, airway hyperresponsiveness, asthma, eosinophil, inflammation, murine

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Introduction

Asthma is frequently thought of as a chronic inflammatory disease that develops in childhood; however, it has been reported that as many as 25% of patients develop symptoms after the age of 40 [1]. This number may be an

underestimate as there is evidence that in older individuals new onset asthma is underdiagnosed as it may be confused with underlying cardiac disease [2]. Patients with late-onset asthma may have more severe airway disease than patients with earlier onset asthma [3, 4], and hospitalization and mortality rates are the highest among

patients with asthma >65 years [5, 6]. Despite these observations, the effect of ageing on allergic airway inflammation in humans or in antigen-sensitized aged mice is not well characterized.

For many years, the dogma was that late-onset asthma was a non-atopic phenotype. However, several groups have demonstrated that elevated IgE levels and antigen-specific IgE can develop later in life in humans, and in one report, was present in up to 72% of older patients with newly diagnosed asthma [2, 5, 7, 8]. Furthermore, although it is fairly well established that there is a T-helper type 2 (Th2) cytokine bias early in life [9–11], reports on cytokine expression later in life are conflicting. Several studies investigating the cytokine response to non-specific stimulants in both aged rodents and elderly adults without atopic diseases have demonstrated a bias towards a Th1 effector response [12–14], whereas other studies have suggested that ageing is associated with decreased IFN- γ production and higher IL-4, IL-5 and IL-10 responses (a Th2 shift [15–17]). The effect of ageing on cytokine profiles, pulmonary inflammation [including histology and bronchoalveolar lavage fluid (BALF) differential] and airway hyperresponsiveness (AHR) after antigen sensitization and challenge is even less well characterized. Sensitization and challenge of 10- and 12-month-old BALB/c mice resulted in a decreased Th2-like profile and significantly decreased BALF total cell count and eosinophil numbers compared with antigen-sensitized younger mice (age ranges 1 week to 5 months) [18, 19]; airway histology was not evaluated.

This study was designed to evaluate the age-dependent inflammatory airway responses in antigen-sensitized and -challenged mice. Young (6 weeks) and older (6, 12 and 18 months old) mice were sensitized and chronically challenged with ovalbumin (OVA), with the goal to evaluate BALF, lung tissue inflammation and mucus cell metaplasia in relationship with AHR.

Methods

Mice and reagents

Young (6 weeks old) female BALB/c mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Aged female BALB/c mice (6, 12 and 18 months old) were obtained from the National Institutes of Ageing (NIA, Bethesda, MD, USA). The aged groups of mice represent 20.1, 40.2 and 60.3 human years, respectively, based upon the 24-month life span of BALB/c mice and the life expectancy of 80.4 years in a human female (source National Center for Health Statistics, www.cdc.gov/nchs). The ages were chosen to represent early, mid and later adulthood. (Preliminary data on lung histology, lung cytokine expression and airway function revealed no statistical differences between antigen-sensitized and

-challenged mice purchased from Jackson Laboratories who were allowed to age in our facilities, and similarly treated and aged mice obtained from the NIA). All mice were maintained in the animal facility at the Mount Sinai School of Medicine. Standard guidelines for laboratory animal care were followed ('Guide for the Care and Use of Laboratory Animals,' National Institutes of Health publication no. 86-23) [20] and institutional permission for animal handling approved through our animal facilities.

Antigen sensitization and challenge

Mice (6 weeks old, $n = 5$ –8/group; 6, 12 and 18 months, $n = 10$ –15/group) were sensitized intraperitoneally (i.p.) with 200 μ g OVA (Grade V, Sigma, St Louis, MD, USA) absorbed with 2 mg alum (Pierce Biotechnology Inc., Rockford, IL, USA) in 0.4 mL phosphate-buffered saline (PBS) on days 0 and 8. Ten days after the last sensitization, mice were anaesthetized i.p. with ketamine and xylazine and challenged intratracheally (i.t.) for a total of three times on days 18, 25 and 32 with 100 μ g OVA (Table 1) in 0.05 mL PBS, determined to be optimal for distribution to both lobes of the lung [21]. (Time-points for our protocol were selected based on our previous work using young antigen-sensitized and -challenged AKR [22, 23] and BALB/c [24] mice (P. J. Busse, unpublished data) that produced significant pulmonary inflammation, AHR and antigen-specific IgE). Antigen challenge via i.t. administration was performed as described previously [25]. Control mice were age-matched naïve BALB/c mice ($n = 5$ /group). [We decided not to administer i.t. PBS to the control mice as we had previously found no significant difference in BALF cell numbers, differential cell count or airway function when comparing i.t. PBS-treated naïve mice with non-treated naïve mice (unpublished data).] Mice were euthanized at either 24–72 h after the final OVA challenge.

Measurement of late-phase airway responsiveness

Seventy-two hours following the final OVA i.t. challenge, airway responsiveness to ACh was measured as described previously [26]. AHR was defined as the time-integrated change in peak airway pressure (APT_I).

Bronchoalveolar fluid preparation and cell differential counts

The chest was opened 24–72 h after the last OVA challenge and BALF collected and total cell number and differential determined as described previously [25]. The supernatant was transferred and stored at -80°C for IL-5 and murine eotaxin-1 (CCL11) measurement by ELISA according to the manufacturer's directions (R&D Systems, Minneapolis, MN, USA).

Table 1. Experimental protocol (BALB/c mice)

Time (day)	0	8	18	25	32	33 or 35
						→
	Sensitization (i.p.)		Challenge (i.t.)			Analysis
	↑	↑	↑	↑	↑	{ AHR BALF cells Cytokines (spleen, lung) Histology Antibodies
	1st	2nd	1st	2nd	3rd	

Mice (6 weeks, 6, 12, 18 months) were sensitized intraperitoneally (i.p.) with OVA (200 µg/mouse) twice at weekly intervals and then challenged intratracheally (i.t.) on days 18, 25 and 32 with OVA (100 µg/mouse). Analysis was either performed 24 h (BALF) or 72 h after the final challenge (AHR measurement, BALF, lung histology, serum antibodies, spleen cell culture and RNA extraction from lung tissue). Naïve age-matched mice were controls.

BALF, bronchoalveolar fluid; AHR, airway hyperresponsiveness; OVA, ovalbumin.

Lung histology

The lungs were surgically removed 72 h post-OVA challenge. The left lower lobe was fixed in neutral-buffered formaldehyde, and 5 µm paraffin sections were stained with periodic acid-Schiff (PAS) for evaluation of goblet cells, and haematoxylin (H&E) and eosin for evaluation of inflammation. Slides were examined in a blinded fashion. Mucus expression was calculated adapting a previously published method [27], and assessed by determining the percentage of PAS-positive epithelial cells (number of PAS positive divided by the total cell epithelial cell number) in at least six randomly selected bronchioles per animal. Results are expressed as the mean percentage of PAS-positive cells per bronchiole for each group of mice. Perivascular and peribronchial inflammation were graded using a standard scoring method (0 = normal, 1 = mild, 2 = intermediate, 3 = severe) [28].

RNA extraction and reverse transcription

Seventy-two hours after OVA challenge, lung tissue sections were placed in TRIzol (Invitrogen, Carlsbad, CA, USA) and frozen at -80 °C until RNA extraction. To extract RNA, samples were thawed at 4 °C, homogenized and extracted according to the manufacturer's directions.

Quantitative real-time polymerase chain reaction

Real-time reverse transcription quantitative PCR (qPCR) was performed using a published SYBR green protocol using an ABI7900 HT machine (Applied Biosystems, Foster City, CA, USA) [29], with three housekeeping genes for normalization (β -actin, RPS11, α -tubulin). Primer sequences for MUC-5AC, IL-5, IL-13, RPS11, α -tubulin and β -actin have been published previously [25]. Other primer sequences were as follows: IL-4 (sense 5'-ATG-GAGCTGCAGAGACTCTT-3' and anti-sense 5'-AAAG-

CATGGTGGCTCAGTAC-3') and IFN- γ (sense 5'-GACTG-TATTGCGGGGTTGT-3' and anti-sense 5'-GGCCCGG-AGTGTAGACATCT-3').

Cell culture and quantification of cytokines

Immediately after APTI measurements, pooled spleen cells from four to five mice were isolated and cultured with either OVA (200 µg/mL), Con A (2.5 mg/mL, positive control) or medium alone for 72 h as described previously [23]. The levels of IL-4, IL-5, IL-13 and IFN- γ in spleen culture supernatants (in triplicate) were determined by ELISA (R&D Systems).

Measurement of serum ovalbumin-specific antibodies

Sera were obtained from each group of mice immediately after APTI measurements (72 h after the third OVA challenge) and stored at -80 °C. To determine OVA-specific IgE, plates were coated with OVA and blocked with 1% human serum albumin in PBS. Sera samples (diluted at 1 : 50) were added and incubated overnight at 4 °C. Sheep anti-mouse IgE (0.3 µg/mL, BD Pharmingen, San Jose, CA, USA), followed by biotinylated donkey anti-sheep IgG (0.1 µg/mL, BD Pharmingen) were added at room temperature (RT). After washing, avidin-peroxidase (Sigma, 1 : 1000) was added for 10 min at RT and the reactions were developed with 2,2'-azine-bis (3-ethylbenzthiazoline-6-sulphonic acid, KPL, Gaithersburg, MD, USA) for 20 min in the dark at RT, and read at 405 nm. For measurement of OVA-specific IgG2a and IgG1, plates were coated with OVA and then blocked and washed as above. Serum samples (1 : 50 for IgG2a, 1 : 500 for IgG1) were added to the plates in duplicate. Biotinylated rat anti-mouse IgG2a or IgG1 monoclonal antibodies (mAb) (Pharmingen) were added for 40 min (IgG2a) or 20 min (IgG1) at RT and the reactions developed and read as described above for IgE.

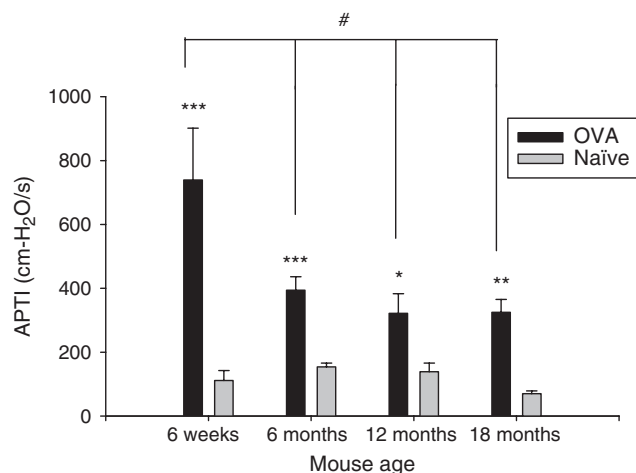


Fig. 1. Effect of age on airway hyperresponsiveness (AHR). Peak airway pressure (APTI) after acetylcholine significantly increased in all ovalbumin (OVA) mice (greatest in 6-week-old OVA mice) compared with naïve age-matched controls, indicating AHR in aged mice. Data are mean $\pm$ SEM for six to nine OVA mice or five naïve mice per groups. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with age-matched naïve controls; # $P < 0.05$ compared with 6-week-old OVA mice.

Statistical analysis

Statistical analyses were performed using one-way ANOVA pairwise and multiple comparison testing between groups as well as Mann–Whitney rank sum test. A P -value < 0.05 was considered to be statistically significant. All statistical analyses were performed using SigmaPlot/Stat software (Systat Software Inc., Point Richmond, CA, USA).

Results

Induction of airway hyperresponsiveness in aged ovalbumin mice

Upon sensitization and challenge with OVA, APTI levels were significantly increased in all age groups of mice compared with their naïve age-matched controls (Fig. 1). APTI levels were significantly greater ($P < 0.05$) in 6-week-old OVA mice (739.2 ± 162.0 cm H₂O/s) compared with each group of aged OVA mice (394.02 ± 42.1 , 321.5 ± 61.8 , 324.8 ± 40.5 cm H₂O/s, 6, 12, 18 months, respectively) (Fig. 1). There were no significant differences in APTI levels between naïve control groups.

Induction of airway inflammation in aged ovalbumin mice

To evaluate the basis for the difference in APTI as well as the effect of ageing on pulmonary inflammation after antigen sensitization and challenge, we first determined the total numbers of cells in BALF immediately following APTI measurement. The mean total number of leucocytes and eosinophils were increased significantly in the aged

OVA mice compared with the 6-week OVA mice (Fig. 2a). There was no significant difference in the total cell count between all naïve groups, and no eosinophils were detected. The mean eosinophil percentage was elevated (significantly in the 12- and 18-month-old mice) in aged OVA mice compared with the 6-week-old OVA mice (Fig. 2b). There were no significant differences in BALF differential counts between all age groups of naïve mice. There was no correlation between total eosinophil numbers and AHR in aged OVA mice. The Pearson's product correlation coefficient between APTI and absolute eosinophil number was 0.884, -0.543 , 0.168 and 0.299 for the 6-week, 6-, 12- and 18-month-old OVA mice, respectively.

To correlate BALF total cell counts with lung tissue inflammation, H&E staining of lung sections collected 72 h after OVA challenge was performed. All OVA mice demonstrated peribronchial (PB) and perivascular (PV) inflammation, which was not seen in the naïve control mice (Fig. 2c). Inflammation grades were the greatest in aged OVA mice [2.091 ± 0.19 (PB, $P < 0.01$), 2.182 ± 0.14 (PV, $P < 0.001$), 6 months; 1.750 ± 0.25 (PB, NS), 1.875 ± 0.13 (PV, $P < 0.05$), 12 months; 2.071 ± 0.23 (PB, $P < 0.001$), 2.286 ± 0.21 (PV, $P < 0.001$), 18-month-old mice] compared with the 6-week-old OVA mice [1.375 ± 0.082 (PB), 0.938 ± 0.22 (PV)]. Representative examples of histologic findings are shown in Fig. 2d. These results demonstrate that i.t. antigen challenge of sensitized aged mice produces increased BALF leucocyte and eosinophil counts and pulmonary inflammation in the aged OVA mice as compared with young OVA mice. However, despite increased inflammation, aged OVA mice exhibited less AHR.

Lung and systemic cytokine production

In an attempt to understand the mechanisms of increased lung inflammation and its dichotomy with AHR, we studied the induction of cytokines in this model. Cytokine expression following OVA sensitization/challenge was measured in lung tissue using qPCR. (Results are expressed in fold increase in mRNA copy number from OVA mice compared with mRNA copy number from age-matched naïve mice.) In the aged OVA mice, the fold increase in IL-5 mRNA expression in lung tissue was significantly increased compared with young 6-week-old OVA mice, suggesting a potential mechanism for the increased airway eosinophils in the aged mice (Fig. 3a). The fold expression of mRNA for IL-4 and IL-13 was the greatest in the young OVA mice (Fig. 3a). However, the fold increase in lung mRNA for IFN- γ increased with increasing age in the OVA mice (Fig. 3a). To examine cytokine protein levels, we stimulated pooled spleen cells with OVA. The expression of IL-5 significantly increased ($P < 0.05$) with age in the OVA mice groups (Fig. 3b). IL-4, IL-13 and IFN- γ protein also correlated with lung tissue

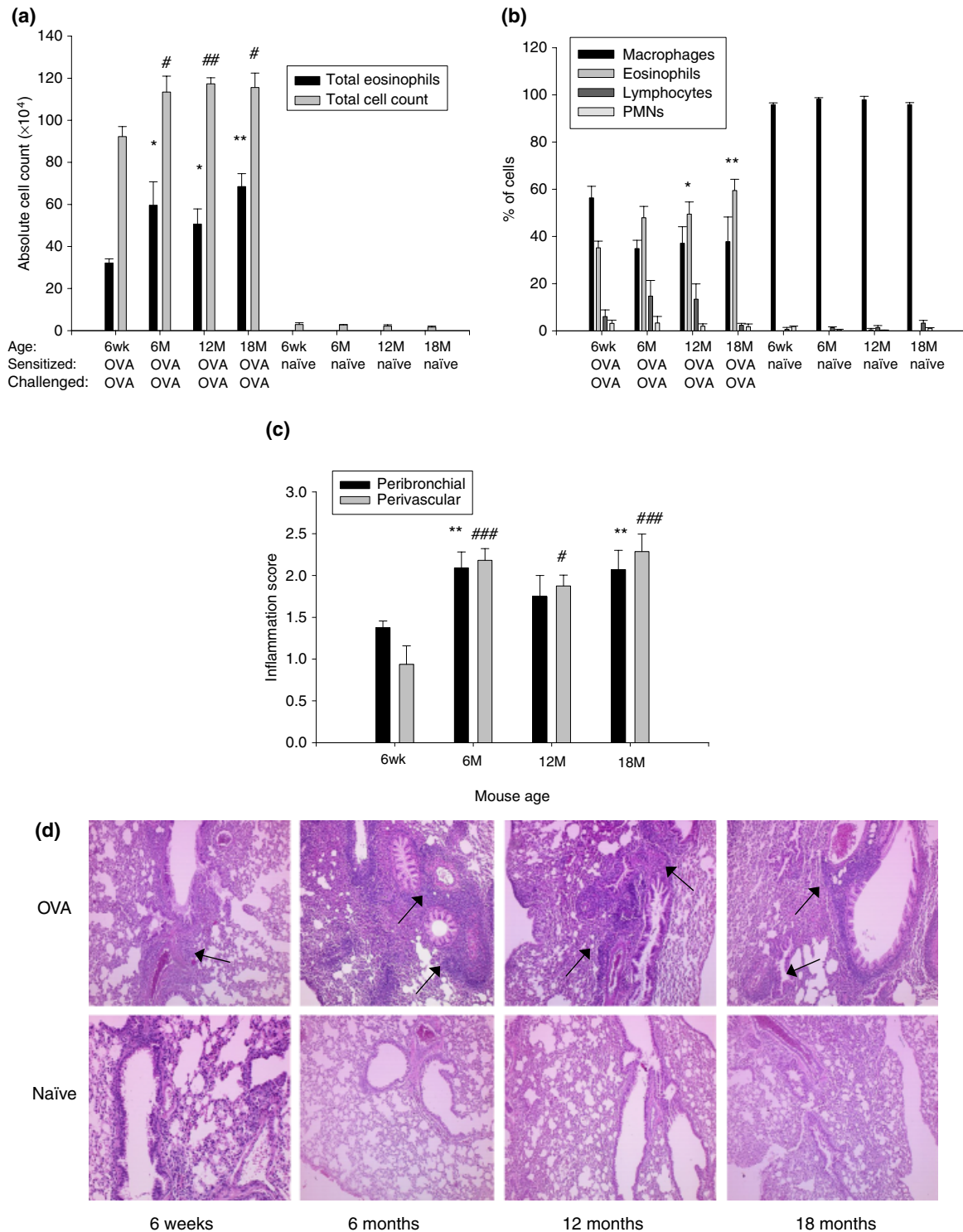


Fig. 2. Pulmonary inflammation with ageing. (a) Increased total cell and eosinophil counts and (b) eosinophil percentage in aged ovalbumin (OVA) mice compared with 6-week-old OVA mice. (c) Inflammation grades for peribronchial and perivascular inflammation in OVA mice. (d) Haematoxylin staining (arrows marking increased inflammation) in OVA mice (10 $\times$). One representative histologic sample per group. Data in mean $\pm$ SEM for six to nine OVA mice or five naïve mice per groups. *,# $P < 0.05$; **,## $P < 0.01$; ### $P < 0.001$ compared with 6-week-old OVA mice. wk, week; M, month.

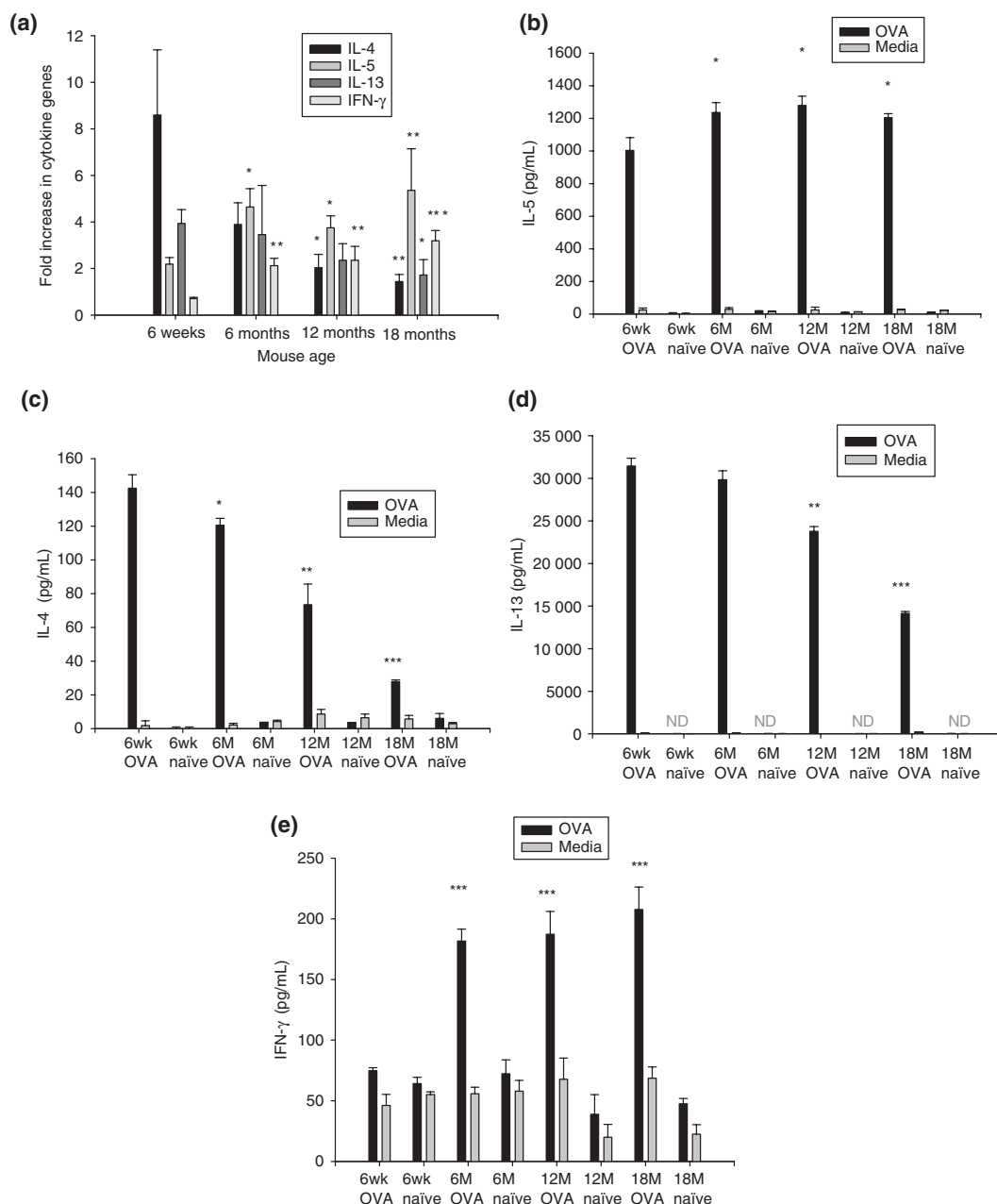


Fig. 3. Pulmonary tissue and spleen-cell culture cytokines. (a) Quantitative PCR for IL-4, IL-5, IL-13, IFN- γ , expressed as fold increase in copy number. Each ovalbumin (OVA) mice group = 6–9 mice. Spleen cells cultured with OVA or medium alone, supernatant ELISA for (b) IL-5, (c) IL-4, (d) IL-13, (e) IFN- γ . Culture results from pooled data from three experiments are presented as mean $\pm$ SEM. * P < 0.05; ** P < 0.01; *** P < 0.001 compared with 6-week-old OVA mice. ND, non-detectable; wk, week; M, month.

mRNA (Figs 3c–e). Con A stimulation (positive control) of splenocyte cultures produced increased expression of all cytokines (data not shown).

Modulation of lung eosinophilia by eotaxin and interleukin-5

To evaluate the mechanism underlying the differences in lung eosinophilic inflammation between young and aged

OVA mice, we measured the eosinophil chemotactic factors eotaxin and IL-5 in BALF. Both eotaxin and IL-5 expression was greater at 24 h than at 72 h (Figs 4a and b), as was found previously in young OVA mice [30]. BALF eotaxin was significantly elevated in 6-week-old OVA mice at 24 h (135.5 ± 22.86 pg/mL) compared with the aged OVA mice (83.93 ± 13.94 , 32.67 ± 3.66 , 47.85 ± 14.75 pg/mL, 6, 12, 18 months, respectively). By 72 h, BALF eotaxin returned to baseline (similar to that of naïve mice) in all the aged

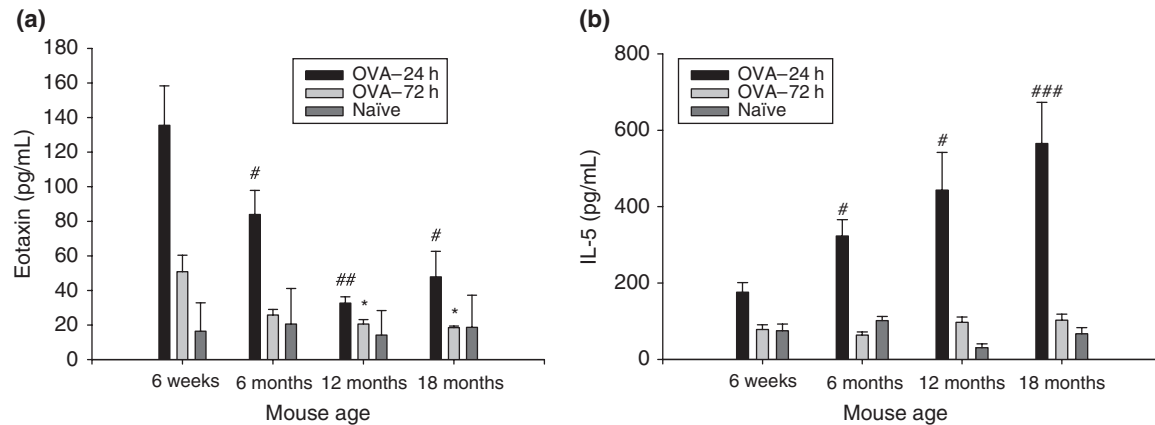


Fig. 4. Eosinophil chemoattractant factors. Bronchoalveolar fluid supernatant collected 24 or 72 h after ovalbumin (OVA) challenge; eotaxin and IL-5 protein expression determined by ELISA. (a) Eotaxin protein decreases in aged OVA mice compared to young OVA mice. (b) IL-5 protein increases in aged OVA mice. Data are mean $\pm$ SEM using five to nine mice per group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with 6-week-old OVA mice.

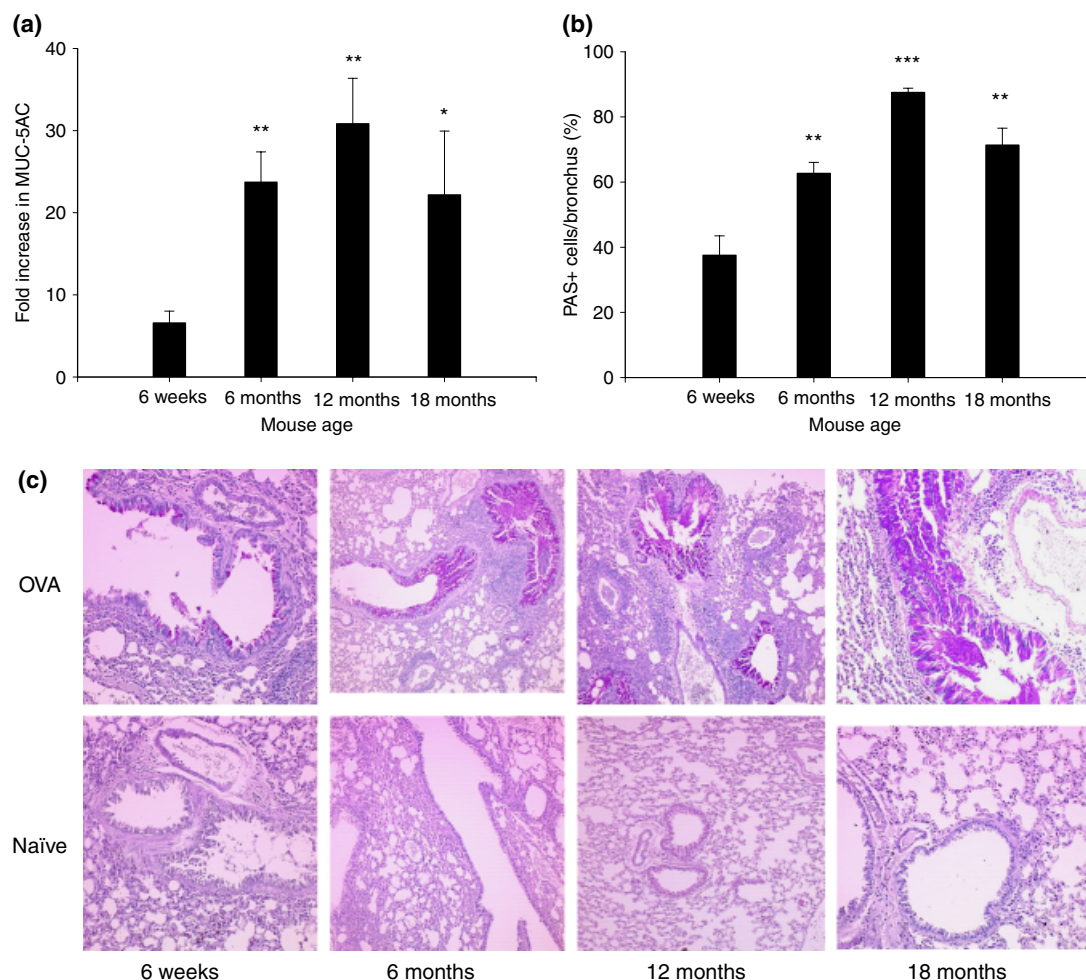


Fig. 5. Airway mucus metaplasia. (a) Quantitative PCR for MUC-5AC expressed as fold increase in copy number; $n = 5-8$ mice/group. (b) Periodic acid-Schiff (PAS) grading. (c) PAS-positive (deep purple) staining demonstrating mucus metaplasia in 6-week- (10 $\times$), 6-month- (10 $\times$), 12-month- (10 $\times$), 18-month-old (20 $\times$) ovalbumin (OVA) mice; none in age-matched naïve mice. One representative histologic sample per group shown. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ compared with 6-week-old OVA mice.

mice, but was still elevated in 6-week-old mice. In contrast, the mean IL-5 protein levels in BALF collected 24 h after the final OVA challenge significantly increased with increasing age (176.2 ± 24.92 , 323.2 ± 42.82 , 443.1 ± 98.89 , 565.2 ± 107.59 , 6 weeks, 6, 12 and 18 months, respectively). BALF IL-5 returned to baseline at 72 h. Thus, the increase in BALF eosinophils in aged mice correlates with increased IL-5, but not eotaxin.

Mucus cell metaplasia increased with age in ovalbumin mice

As mucus cell metaplasia has been associated with increased inflammation and eosinophilia [31, 32], we performed real-time PCR for the major airway mucus-producing gene, MUC-5AC [33], performed on lung tissue. Results are expressed as the fold change in mRNA copy number of MUC-5AC in the OVA mice compared with their age-matched naïve controls. MUC-5AC mRNA expression was significantly increased in aged OVA mice compared with the young OVA mice (Fig. 5a). To correlate the increase in mRNA for MUC-5AC with histologic changes, lung tissue sections were stained for PAS. The number of positively staining PAS cells in bronchioles were significantly increased in aged OVA mice, consistent with the pattern of the mRNA MUC-5AC expression (Fig. 5b). There were no PAS-positive airway epithelial cells in naïve mice. Figure 5c shows the representative histology of OVA-sensitized/challenged and naïve mice, demonstrating increased PAS staining in the lungs of the aged OVA groups.

Development of antigen-specific immunoglobulin in aged mice

To assess the degree of sensitization of aged mice, we measured OVA-specific serum IgE. All mice developed significantly elevated serum-specific IgE to OVA in response to OVA sensitization and challenge (Fig. 6a). Aged OVA mice exhibited higher levels of OVA-specific serum IgE than 6-week-old OVA mice, reaching statistical significance in the 12- and 18-month-old groups. OVA-specific IgE was virtually undetectable in all aged naïve mice. Serum OVA-specific IgG₁ levels were not significantly different in young and aged groups of OVA mice, but were significantly increased compared with naïve age-matched control mice, where OVA-specific IgG₁ was nearly undetectable (Fig. 6b). Similar to IgG₁, all OVA mice had significantly increased OVA-specific IgG_{2a} compared with their age-matched controls (Fig. 6c). OVA-specific serum IgG_{2a} was significantly ($P < 0.01$) elevated in 6-week-old OVA mice compared with 18-month-old OVA mice, but not significantly different from the 6- or 12-month-old OVA mice. OVA-specific IgG_{2a} was nearly undetectable in all naïve mice.

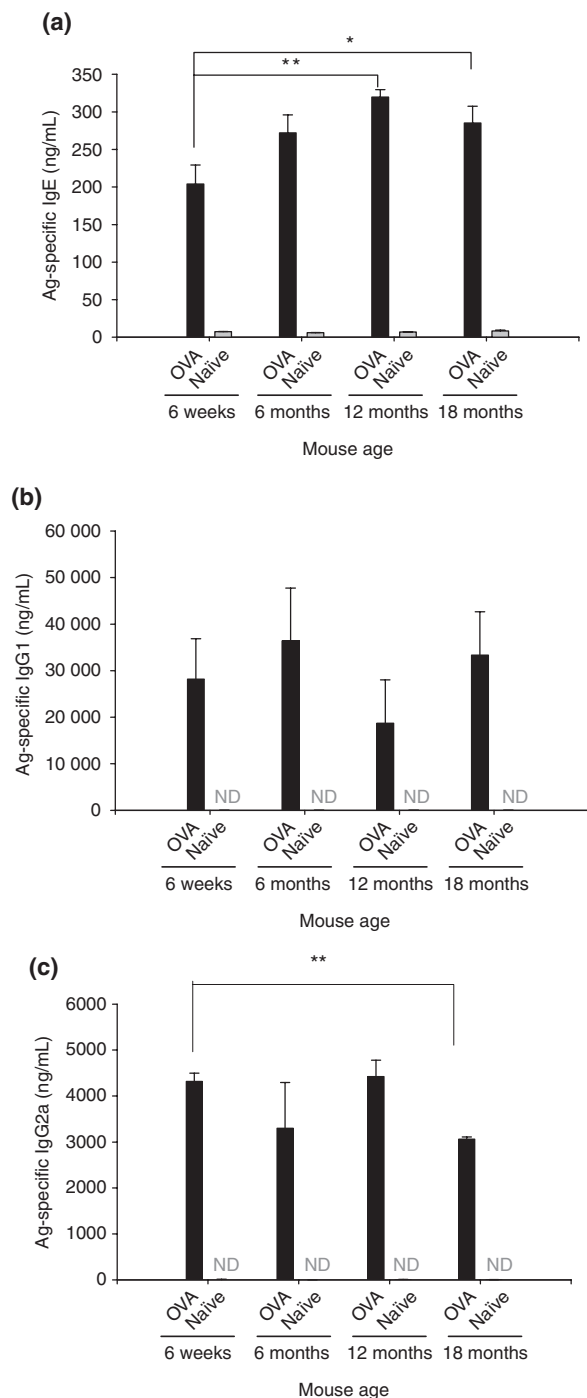


Fig. 6. Serum ovalbumin (OVA)-specific Ig. OVA-specific (a) IgE, (b) IgG₁ and (c) IgG_{2a} determined by ELISA from sera collected 72 h after the final OVA challenge. OVA-specific IgG₁, IgG_{2a} were undetectable for naïve mice, labelled as ND (non-detectable). Data expressed in mean $\pm$ SEM for using five to nine mice per group. * $P < 0.05$; ** $P < 0.01$ compared with 6-week-old OVA mice.

Discussion

Asthma is a heterogeneous disease with several phenotypes including childhood onset, late onset, allergen-induced, nocturnal and exercise-induced. For many years,

Table 2. Features of airway inflammation in different ages of antigen-treated mice

Group	Tissue inflammation*	BALF eosinophils	Mucus metaplasia [†]	Airway cytokine				Spleen cell culture				AHR	OVA-IgE [‡]
				IL-5	IL-4	IL-13	INF- γ	IL-5	IL-4	IL-13	INF- γ		
6 weeks	+	+	+	+	+++	++	—	++	+++	+++	+	+++	+
6 months	++	++	++	++	++	++	+	+++	++	+++	++	++	++
12 months	++	++	+++	++	+	+	+	+++	+	++	++	++	+++
18 months	++	+++	++	++	+	+	+	+++	+	+	+++	++	++

*Determined by histology and BALF total cell count.

[†]Determined by PAS staining and MUC-5AC mRNA expression.

[‡]Serum levels.

BALF, bronchoalveolar fluid; AHR, airway hyperresponsiveness; OVA, ovalbumin; PAS, periodic acid-Schiff.

the dogma was that late-onset asthma is rare and not atopic. However, several reports in humans have suggested otherwise [2, 5, 7, 8]. As part of the Normative Ageing Study, the presence of an IgE antibody to dust mite antedated the development of new-onset wheezing in late adulthood [8], and in a Japanese cohort of asthmatics, the proportion of skin-test-positive patients among late-onset asthmatics was significantly larger than that seen in a younger-onset group [34]. Despite the occurrence of late-onset allergic asthma, the effect of ageing on allergic airway inflammation in humans or in aged murine asthma models is not well characterized.

In the present study, we sensitized and i.t. challenged young (6 weeks) and older (6, 12, 18 months) mice with OVA and demonstrated that aged mice develop a different pulmonary pathology with greater inflammation, notably increased BALF eosinophils and increased mucus cell metaplasia compared with the younger mice. Furthermore, the aged antigen-treated mice also developed a distinct cytokine pattern (elevated IL-5 and INF- γ) in their lung tissue and spleen-cell cultures, whereas similarly treated younger mice responded with a Th2 pattern alone. Despite this, antigen sensitization and challenge produced significantly lower AHR in the aged OVA mice compared with the young OVA mice. The difference in AHR between older and young mice was antigen specific because there was no difference in airway responses (APTI) to Ach provocation between aged and young naïve mice. These age-related inflammatory changes in response to antigen sensitization and challenge are illustrated in Table 2.

There are only a few reports in the literature investigating airway inflammation in aged antigen-sensitized and -challenged rodents: one in aged Brown Norway rats (100–120 weeks) [35] and two in BALB/c mice (10 or 12 months) [18, 19]. These studies measured airway inflammation by BALF collection and histology (in one study), and found increased total leucocyte cell count and eosinophil percentage in the young, but not in the aged antigen-sensitized rodents. Our protocol using BALB/c mice differed slightly in that we used a more rigorous and chronic antigen challenge protocol (over 3 weeks)

compared with 2 or 3 consecutive days and we used several groups of ageing mice that included older groups (12 and 18 months). Additionally, the mice were antigen challenged intratracheally, which directly introduces the antigen into the airways and allows for less gastric deposition of antigen [21], whereas the previous two studies in BALB/c mice used aerosol challenges.

Although our findings of greater lung eosinophilia and inflammation in antigen-sensitized and -challenged aged mice differ from previous aged rodent studies, it is similar to a human study that compared the characteristics of adult-onset with childhood-onset asthma. Miranda et al. [4] found that there was significantly greater airway tissue and lavage fluid eosinophilia in patients who presented with asthma at a mean age of 27 ± 1.3 years than in asthmatics who developed airway disease at a mean age of 2.6 ± 1.0 years. (However, it should be noted that our aged mice represent a relatively older age group.)

To further define eosinophil recruitment to the lungs of our aged antigen-sensitized mice, we measured two eosinophil chemoattractants, IL-5 and eotaxin, in the BALF supernatant. We found that the IL-5 levels were higher and the eotaxin levels were lower in the lungs of aged OVA mice than young OVA mice. Our data suggest that IL-5 may be more important than eotaxin in airway eosinophil recruitment in aged OVA mice. One potential explanation for a decrease in eotaxin production in the aged OVA mice is that fibroblasts, which are one source of airway eotaxin [36], decrease with ageing [37].

Despite increased pulmonary inflammation and eosinophilia in the aged OVA mice, AHR was more pronounced in the young OVA mice. However, unlike previous murine studies [19, 38], we found that antigen sensitization and challenge of aged mice produced AHR. The relationship between airway inflammation, specifically eosinophilia, and AHR in asthma is not entirely clear, and although a causal relationship has been proposed, this hypothesis is controversial [39–41]. Furthermore, the role of the eosinophil in AHR of aged patients or in animal models of asthma may differ. In the Miranda et al. [4] study, patients with later-onset disease showed a weaker association

between asthma symptoms and eosinophilia compared with those with early-onset disease. In addition, the presence or absence of eosinophils in the late-onset group was not associated with significant differences in pulmonary function. Our results potentially argue against a direct relationship between pulmonary inflammation and eosinophils in AHR in aged antigen-challenged sensitized animals, although further studies are necessary.

The mechanisms of AHR are complex, and other factors besides airway inflammation and eosinophilia are involved in its induction, including direct cytokine modulation. We demonstrated that antigen sensitization and challenge of older mice produced a unique cytokine profile as determined by gene expression in lung tissue and protein levels in spleen cell cultures. The aged OVA mice all developed increased IL-4, IL-5, IL-13 when compared with their age-matched naïve controls, suggesting that antigen sensitization stimulates a Th2-like response in aged mice. However, the young OVA mice had a greater fold change in mRNA gene expression of IL-4 and IL-13, but not for IL-5. IL-5 protein levels from lung tissue and spleen cell cultures in the young OVA mice paralleled lung tissue mRNA expression. The aged OVA mice also produced more IFN- γ than the younger OVA mice. One previous study of antigen-sensitized/challenged aged BALB/c mice examined cytokine profiles in BALF by ELISA and found both IL-4 and IL-5 to be significantly decreased compared with the young antigen-sensitized mice [19]. Another study of aged mice demonstrated decreased IL-4, IL-5, IL-13 cytokine expression in purified naïve CD4⁺ T cell by splenocytes of ageing OVA-specific TCR $\alpha\beta$ transgenic mice stimulated with OVA peptide and irradiated APCs from young BALB/c mice [18], a protocol that may not reflect cytokine changes in lungs following antigen sensitization and challenge. These studies also found increased IFN- γ in the aged OVA mice compared with the young OVA mice, similar to our data.

The distinct cytokine pattern in the antigen-sensitized and -challenged aged mice could explain why the AHR in these groups was not as significantly elevated when compared with that of the young OVA mice. IL-13 can induce AHR independent of eosinophilia [42], and like IL-4, directly stimulates airway smooth muscle cells [41, 43]. Therefore, the lower IL-4 and IL-13 might explain a decreased AHR independent of inflammation in the aged OVA mice. Furthermore, the effects of IL-4 and IL-13 on airway smooth muscle may be important as its function and response may change with ageing. Additionally, IFN- γ , elevated in both the lung tissue and spleen cell culture in the aged OVA mice, may offer protection from AHR [44, 45]. A recent study demonstrated that overexpression of IFN- γ in the airways of antigen-sensitized and -challenged mice increased airway inflammation, but decreased AHR compared with wild-type mice [46].

Decreased gastric mucus production with ageing has been reported [47], but mucus production has not been evaluated in the airway of aged rodents. None of the previous studies using aged antigen-sensitized rodents have evaluated mucus cell metaplasia. The mRNA expression of the airway mucin-producing gene, MUC-5AC, was increased in the aged OVA mice compared with the younger OVA mice. Quantification of positive PAS airway staining paralleled the MUC-5AC mRNA expression. IL-13 and IL-5 have been suggested to be important cytokines inducing mucus metaplasia and MUC-5AC expression in the airways [31, 48]; the role of IL-4 in mucus hypersecretion is less well established [49]. However, in aged mice, IL-13 and IL-4 may not be as critical for mucus induction, and IL-5 may be more important; this requires further investigation. Several other cytokines including IL-6, IL-9, TNF- α , IL-1 β and factors including oxidant stress, proteases and histamine have also been shown to induce airway mucus cell metaplasia/hyperplasia and mucin-gene expression [25, 48], and may be more relevant factors for mediating these effects during ageing. Furthermore, the eosinophil may induce mucus cell metaplasia [31, 32, 50], offering an explanation in our model. Finally, in the aged OVA mice, mucus hypersecretion did not appear to correlate directly with AHR. The effect of mucus hypersecretion on AHR is not clearly established [51, 52] (unpublished data), and may differ in an aged population.

Finally, we demonstrated that antigen-sensitized and chronically challenged aged mice develop Ag-specific IgE. A previous study showed that OVA-specific IgE was detected in sensitized aged rats, but was decreased compared with younger rats [38]. One of the studies using BALB/c mice found a lower total serum IgE in the aged OVA-treated mice compared with the younger mice [19]. They did not, however, measure OVA-specific IgE. In our study, the aged OVA mice produced more OVA-specific IgE than younger mice, suggesting that although sensitization can occur later in life, other factors besides, or in conjunction with, IgE regulate AHR in aged rodents. The mechanism of increased OVA-specific IgE production in the aged OVA mice despite lower lung tissue mRNA IL-4 expression and IL-4 in splenocyte culture is more difficult to explain. However, it may be that the level of IL-4 protein expression was sufficient to induce class switching towards IgE in the plasma cells of the aged OVA mice.

In conclusion, this is the first study to demonstrate increased pulmonary eosinophilia, inflammation and increased mucus cell metaplasia in antigen-sensitized and -challenged older mice. In addition, antigen sensitization/challenge of aged mice produced an altered cytokine pattern. Contrary to previous reports, we demonstrated that aged mice are able to develop AHR. Although increased AHR was found in the aged antigen-sensitized/challenged mice, it was not as great as younger antigen-sensitized/challenged mice, suggesting discordance

between airway inflammation and AHR with ageing. This study is important because it suggests that antigen sensitization and challenge of aged mice produces a distinct pattern of pulmonary inflammation and cytokine expression and offers a murine model of late-onset allergic asthma.

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